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Actinobacteria

Basics and Biotechnological Applications

Edited by Dharumadurai Dhanasekaran and Yi Jiang

ACTINOBACTERIA - BASICS AND BIOTECHNOLOGICAL APPLICATIONS

Edited by **Dharumadurai Dhanasekaran** and **Yi Jiang**

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Meet the editors

Dr. Dharumadurai Dhanasekaran is working as an Assistant Professor, at Department of Microbiology, School of Life Sciences, Bharathidasan University, Tiruchirappalli, India. He has experience in fields of actinobacteriology and mycology. Dr. Dhanasekaran's current research focus is on actinobacteria, microalgae, fungi and mushroom for animal and human health improvement. He has deposited around 64 nucleotide sequences in GenBank, 5 bioactive compounds in Pubchem, published 89 research and review articles, and books; guided 7 PhD candidates and organized several national level symposia, conference and workshop programs.

Dr. Yi Jiang, PhD of Kiel University, Germany; and Associate Professor of Yunnan Institute of Microbiology, Yunnan University. She is a specialist in taxonomy, ecology and resources of actinomycetes. Dr. Yiang published 93 academic articles and 3 books, obtained 9 patents, and awarded a first prize of Natural Science of Yunnan and Second prize of Kunming City.

Contents

Foreword

December 2, 2015

Actinobacteria species are the primary microbial biomass in the majority of soil and water biomes around the globe. To date, they have been recovered from even the most inhospitable locations on the planet including arid deserts, alkaline soils, salty aquatic environments, caves and even the gut flora of animals. Their role in these locations is as diverse as their habitats and includes the ability to generate hydrolytic enzymes able to digest cellulose and chitin as well as synthesize a staggering variety of antimicrobial compounds.

Clearly, just from the synopsis above, this large and fascinating group of microorganisms is more than worthy of its own book. This field is a continuously expanding one and in "Actinobacteria - Basics and Biotechnological Applications" you are exposed to a state of the art analysis of this group as seen through the eyes of experts in the field. Actinobacteria are first discussed in five chapters, which serve to provide taxonomic divisions and culture methodologies with especial reference to rare and extremophile species. Actinobacteral classification is attacked via three strategies namely:

- Isolation and cultivation methodologies, including the usage of novel media, "starvation" techniques to force the production of enzymes and antimicrobials
- Colonial appearance on culture
- Chemotaxonomy with reference to the biochemistry of their cellular envelope
- Molecular phylogeny through G+C and DNA-DNA hybridization.

Under the section "Bioprospecting", three chapters of the book delve into the importance of Actinobacteria in terms of their ability to synthesize antimicrobial agents, important enzymes and the utility of extremophile halophile species in treating infectious diseases including cancer. Benefits of Actinobacteria include their ability to:

- Generate diverse classes of antimicrobial compounds; 20-50% of the thousands of species in the Actinobacteria group are believed to have the capability to generate anti biotic, anti- malarial and anti-HIV agents, this would suggest the microbes have an almost limitless potential in the treatment of infectious disease
- Novel anti-cancer drugs from the unique extremophile halophilic species
- Respond to redox stress in contaminated aquatic environments.

The final section is one often overlooked in texts covering Actinobacteria and makes this book a valuable adjunct to current knowledge of the Actinobacteria. Many parts of our world are inhospitable for human habitation with poor soil quality and inconsistent access to potable water. Additionally we face an energy crisis which will require a shift into new sources of fuel. In section "Agricultural and Industrial Utility", seven chapters explore the diverse and unique capacities of Actinobacteria to help solve some of these issues in that they can:

- Secrete oxidative enzymes able to be used in bioremediation and removal of waste products including heavy metals and pesticides
- Improve soil fertility, through enhancing plant growth; Actinobacteria, for example, are able to assist the colonization of plant species in inhospitable soils through the stimulation of local fungal species (mycorrhizae)
- Act as "biocatalysts" to improve and accelerate industrially important processes
- Degrade toxic hydrocarbons in heavily contaminated soils; a unique property of Frankia species
- Generate enzymes able to manufacture biofuels and degrade household waste
- Synthesize nanoparticles, which have widespread health applications including im proved targeting of radiation therapy and non-toxic delivery vehicles for various pharmaceuticals.

As a whole "Actinobacteria - Basics and Biotechnological Applications" provides an excellent overview of a group of microbes able to survive in all areas of our planet. A useful reference work for student and researcher alike, this book will provide its reader with the information needed to either begin research in the field or continue in a new direction. Crammed with useful tips and techniques, not always available in classic research articles, it is a "go to" for anyone in this area.

Best regards,

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Preface

The Actinomycetes or Actinobacteria are a group of Gram-positive bacteria with high guanine and cytosine content in their DNA. They are morphologically diverse, ranging from coccoid, fragmenting hyphal forms to those with a highly differentiated branched mycelium. Many of these bacteria produce external spores and are widely distributed in terrestrial (*Streptomyces* spp.) and aquatic (freshwater – *Micromonospora* sp. and *Nocardia* sp. and marine – *Micropolyspora* sp.) ecosystems, where they play a crucial role in decomposition of organic materials, thereby contributing in organic matter turnover and carbon cycle. This replenishes the supply of nutrients in the soil and is an important part of the humus formation. Actinobacteria also inhabit a vast array of plants (commensals – *Leifsonia* sp.; nitrogen-fixing symbionts – *Frankia* sp.) and animals (gastrointestinal tract resident – *Rhodococcus* sp. and *Bifidobacterium* sp.). Furthermore, several pathogenic species (*Mycobacterium* sp., *Nocardia* sp., *Tropheryma* sp., *Corynebacterium* sp., and *Propionibacterium* sp.) are often encountered among the phylum of Actinobacteria.

Actinobacteria hold a prominent position as targets in screening programs due to their diversity and their proven ability to produce novel metabolites. They are universally renowned as secondary metabolite producers and hence are of high pharmacological and commercial interest. Approximately, 23,000 microbial secondary metabolites with tremendous economic importance have been identified so far, in which 12,000 compounds are produced by Actinobacteria. Interestingly, 7600 bioactive compounds are reported from the members of the genus Streptomyces with antibacterial, antifungal, antiviral, antiprotozoan, antihelminthic, antialgal, antimalarial, anticancer, anti-inflammatory, antithrombotic, and neuritogenic activities. In addition, they also employed as insecticides, herbicides, and fungicides and as growth promotants for certain plants and food animals (probiotics). Furthermore, the ability of Actinobacteria in biodegradation of agricultural wastes and their extensive distribution in soil, compost, water, and elsewhere in the environment make them very important to the agricultural industries.

This book presents an introductory overview of Actinobacteria with three main divisions: taxonomic principles, bioprospecting, and agriculture and industrial utility, which covers isolation, cultivation methods, and identification of Actinobacteria and production and biotechnological potential of antibacterial compounds and enzymes from Actinobacteria. Moreover, this book also provides a comprehensive account on plant growth-promoting (PGP) and pollutant degrading ability of Actinobacteria and the exploitation of Actinobacteria as ecofriendly nanofactories for biosynthesis of nanoparticles, such as gold and silver. This book will be beneficial for the graduate students, teachers, researchers, biotechnologists, and other professionals, who are interested to fortify and expand their knowledge about Actino-

bacteria in the field of Microbiology, Biotechnology, Biomedical Science, Plant Science, Agriculture, Plant pathology, Environmental Science, etc.

The book comprises a total of 16 chapters from multiple contributors around the world, including Brazil, South Africa, Saudi Arabia, Colombia, India, China, Mexico, and the United States. We are grateful to all the contributors and leading experts for the submission of their stimulating and inclusive chapters in the preparation of the edited volume to bring the book on Actinobacteria basics and biotechnological applications. We offer our special thanks and appreciation to Ms. Ana Pantar and Ms. Sandra Bakic, Publishing Process Manager, for their encouragement and help in bringing out the book in the present form.

We express our heartfelt gratitude to Prof. Cheng-Lin Jiang, Key Laboratory for Microbial Resources of Ministry of Education, Yunnan Institute of Microbiology, Yunnan University, Kunming, Yunnan 650091, P.R. China, for his valuable conceptual suggestions and proof comments during the review process of this book. Appreciations are due to my research scholars, Ms. S. Latha, Ms A. Ranjani, and Ms. G. Vinothini, for their sincere efforts and diligence toward this book. We are also indebted to InTech-Open Science, Bharathidasan University, and Yunnan University for their concern, efforts, and support in the task of publishing this volume.

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Section 1

Taxonomic Principles

Chapter 1

An Introduction to Actinobacteria

Ranjani Anandan, Dhanasekaran Dharumadurai and Gopinath Ponnusamy Manogaran

Additional information is available at the end of the chapter

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Abstract

Actinobacteria, which share the characteristics of both bacteria and fungi, are widely distributed in both terrestrial and aquatic ecosystems, mainly in soil, where they play an essential role in recycling refractory biomaterials by decomposing complex mixtures of polymers in dead plants and animals and fungal materials. They are considered as the biotechnologically valuable bacteria that are exploited for its secondary metabolite production. Approximately, 10,000 bioactive metabolites are produced by Actinobacteria, which is 45% of all bioactive microbial metabolites discovered. Especially *Streptomyces* species produce industrially important microorganisms as they are a rich source of several useful bioactive natural products with potential applications. Though it has various applications, some Actinobacteria have its own negative effect against plants, animals, and humans. On this context, this chapter summarizes the general characteristics of Actinobacteria, its habitat, systematic classification, various biotechnological applications, and negative impact on plants and animals.

Keywords: Actinobacteria, Characteristics, Habitat, Types, Secondary metabolites, Applications, Pathogens

1. Introduction

Actinobacteria are a group of Gram-positive bacteria with high guanine and cytosine content in their DNA, which can be terrestrial or aquatic. Though they are unicellular like bacteria, they do not have distinct cell wall, but they produce a mycelium that is nonseptate and more slender. Actinobacteria include some of the most common soil, freshwater, and marine type, playing an important role in decomposition of organic materials, such as cellulose and chitin, thereby playing a vital part in organic matter turnover and carbon cycle, replenishing the supply of nutrients in the soil, and is an important part of humus formation. Actinobacterial

colonies show powdery consistency and stick firmly to agar surface, producing hyphae and conidia/sporangia-like fungi in culture media.

Actinobacteria produce a variety of secondary metabolites with high pharmacological and commercial interest. With the discovery of actinomycin, a number of antibiotics have been discovered from Actinobacteria, especially from the genus *Streptomyces*. They are widely distributed in soil with high sensitivity to acid and low pH. Actinobacteria have a number of important functions, including degradation/decomposition of all sorts of organic substances such as cellulose, polysaccharides, protein fats, organic acids, and so on. They are also responsible for subsequent decomposition of humus (resistant material) in soil and for the earthy smell of freshly ploughed soils, producing a number of antibiotics like streptomycin, terramycin, aureomycin, and so on. This chapter gives an overview about the types of Actinobacteria, their habitat, systematic classification and various biotechnological applications, and their ill effects on plants and animals.

2. Habitat of Actinobacteria

2.1. Terrestrial environment

Soil remains the most important habitat for Actinobacteria with streptomycetes existing as a major component of its population. According to numerous reports, *Streptomyces* was encountered to be the most abundant genus isolated in each of the study. Terrestrial Actinobacteria have various interesting antimicrobial potentials. Oskay et al [1] isolated Actinobacteria that had capability of producing novel antibiotics with high antibacterial activity. In anoxic mangrove rhizosphere, Actinobacterial species such as *Streptomyces, Micromonospora*, and *Nocardioform* were found to be abundant, which is 1000 to 10000 times smaller than arable lands because of tidal influence [2]. Similarly, *Nocardia* isolated from mangrove soil produced new cytotoxic metabolites that strongly inhibited human cell lines, such as gastric adenocarcinoma [3]. Dessert soil is also considered as an extreme terrestrial environment where only certain species, especially wherein Actinobacteria, often use *Microcoleus* as a source of food. There are several reports showing the distribution of Actinobacteria in various locations, such as sandy soil (Cario, Egypt; Falmouth, MA), black alkaline soil (Karnataka, India), sandy loam soil (Keffi Metropolis, Nigeria; Presque Isle, PA), alkaline dessert soil (Wadi El Natrun, Egypt; Wadi Araba, Egypt), and subtropical dessert soil (Thar, Rajasthan), where *Streptomyces* sp. were dominant followed by the other organisms, such as *Nocardia, Nocardiopsis,* and Actinomycetes [4]. In the study of Nithya et al [5], 134 morphologically distinguished culturable Actinobacteria were isolated from 10 different desert soil samples, and the isolates were found to have varying level of antibacterial activity against bacterial pathogens. Equally, Actinobacteria play a major part in rhizosphere microbial community in the turnover of recalcitrant plant organic matter, and thus the rhizosphere region is considered as one of the best habitats for isolation of these microorganisms. Priyadharsini et al [6] in her study isolated 45 morphologically distinct colonies from 12 different paddy field soils and observed their ability to inhibit the growth of *Cyperus rotundus*. The isolates include *Streptomyces* sp., *Streptoverticillium* sp.,

Actinomadura sp., *Kitasatosporia* sp., *Nocardiopsis* sp., *Pseudonocardia* sp., and *Kibdelosporangium* sp.

2.2. Aquatic environment

Actinobacteria are widely distributed in aquatic habitats, which may sometimes be washed in from surrounding terrestrial habitats. It is vitally important that the numbers and kinds of Actinobacteria are interpreted in the light of information on organisms, such as *Thermoactinomyces* and *Rhodococcus coprophilus*, which are known to be good indicators of the terrestrial component of Actinobacterial propagules in water and sediments. The resistant endospores of *Thermoactinomyces* are produced in self-heating composts, overheated fodders, and surface soil, but they can be washed into aquatic habitats where they are deposited in muds and sediments. It has been assumed that these thermophiles are unable to grow at ambient temperatures in most aquatic habitats. Similarly, the resting coccal stage of *R. coprophilus* passes into freshwater and marine habitats, where it can survive but probably does not grow.

2.2.1. Freshwater

Cross [7] in his study evidenced that Actinobacteria can readily be isolated from freshwater sites. Some of the major type of Actinobacteria dwelling in freshwater include *Actinoplanes, Micromonospora, Rhodococcus, Streptomyces*, and the endospore-forming *Thermoactinomyces*. *Actinoplanes* are commonly found in soils, rivers, and lakes, and the spore vesicles of these organisms have the ability to withstand prolonged desiccation, but they release their motile spores for dispersal when rehydrated [8]. The zoospores are motile by means of a tuft of flagella exhibiting chemotaxis and require an exogenous energy source. *Micromonospora* are also considered to be a common freshwater Actinobacteria and found to be indigenous to such habitats where they turnover cellulose, chitin, and lignin. Numerous reports confirmed the presence of *Micromonospora* in streams, rivers, and river sediments and considered them to be an integral part of the aquatic microflora. Johnston & Cross [9] found that streptomycetes failed to grow in a various lakes, notably in the deeper mud layers where micromonosporae were found to be predominant, whereas another study of AI-Diwany et al [10] showed a significant correlation between micromonosporae and thermoactinomycetes isolated from the River Wharfe in West Yorkshire, where increased number of micromonosporae was found in the adjoining soil. A study revealed that *Micromonospora* spores were washed into freshwater habitats where they can remain dormant for several years [7]. Though streptomycetes spores are also continually washed into freshwater and marine habitats, there is only little evidence that they can be active in such environments. The existence of aquatic streptomycetes has been claimed, but AI-Diwany et al [10] found a high degree of correlation between counts of streptomycetes, fecal *Streptococci*, and *Rhodococci*. Other inhabitants of freshwater include *Actinomadura madurae*, *Mycobacterium kansasii*, and *Arthrobacter, Corynebacterium,* and *Nocardia* species. The concentration of hydrophobic spores and hyphae at the water/air interface can increase the number of streptomycetes, micromonosporae, and *Rhodococci* in foam on river water. Evidence clearly

shows that Actinobacteria can become active in freshwater ecosystem in the presence of suitable substrates and conditions for growth rather than specifically adapting themselves to live in such environment.

2.2.2. Marine

When comparing the Actinobacterial diversity in terrestrial environment, the greatest biodiversity lies in the oceans. The marine environment is an untapped source of novel Actinobacteria diversity and thus of new metabolites. Marine Actinobacteria dwelling in extremely different environment produce different types of bioactive compounds compared with terrestrial ones. Marine Actinobacteria had to adapt from extremely high pressure and anaerobic conditions at temperatures just below 0- 8 °C on the deep sea floor to high acidic conditions at temperatures of over 8- 100°C near hydrothermal vents at the mid-ocean ridges. *Rhodococcus marinonascene*, the first marine Actinomycete species to be characterized, supports the existence of marine Actinobacteria. Members of the genera *Dietzia*, *Rhodococcus, Streptomyces*, *Salinispora*, *Marinophilus*, *Solwaraspora*, *Salinibacterium*, *Aeromicrobium marinum*, *Williamsia maris*, and *Verrucosispora* have been designated as indigenous marine Actinobacteria [11–15]. Grossart et al [16] have illustrated that Actinobacteria account for approximately 10% of the bacteria colonizing marine organic aggregates and that their antagonistic activity might be highly significant in maintaining their presence, which affects the degradation and mineralization of organic matter. The presence of indigenous marine Actinobacteria in the oceans and the distribution of marine Actinobacteria in different marine environments and habitats are confirmed by various recent researches. Innagi et al [17] isolated various marine Actinobacteria, such as *Dietzia maris*, *Rhodococcus erythropolis,* and *Kocuria erythromyxa,* from a subseafloor sediment core collected at a depth of 1225 meters off Hokkaido. Jensen et al [14] isolated five new actinomycete phylotypes from marine sediments collected around the island of Guam. Similarly, Actinobacteria were also isolated from samples collected at the deepest abyss, the Challenger Deep off the Marianas, at a depth of 10,923 meters [15]. Unusual Actinobacteria, belonging to *Micrococceae, Dermatophilaceae,* and *Gordoniaceae* have been isolated from sponges. Dhanasekaran et al [18] isolated 17 Actinobacteria from soil samples belonging to the saltpan regions of Cuddalore, Parangipettai, and screened for primary antibacterial activity among which *Streptomyces* spp. and *Saccharomonospora* sp. collected showed promising antimicrobial activity against different bacteria. An antibacterial methyl-substituted β-lactam compound was isolated and characterized from *Streptomyces noursei* DPTD21 in saltpan soil of Parangipettai Porto Novo in Cuddalore district, Tamil Nadu, by Dhanasekaran et al [19]. In another study, soil and sediment samples were collected from different locations in Muthupet mangrove region, and Actinobacteria were isolated viz *Streptomyces* sp. CC17 and SM13, *Streptosporangium* sp. SH15, and *Micropolyspora* sp. S22, which showed highest larvicidal activity against *Anopheles* mosquito larvae [20]. All the above-stated Actinobacteria isolated from marine environments, such as the deep sea floor, marine invertebrates and marine snow, sea shore soil, and deep sea sediments, represent unique ecosystems that cannot be found anywhere else in the world. Equally, these isolates produce various novel metabolites, which are listed in Table 1. Even with the limited screening efforts that have been dedicated to date to marine Actinobacteria, the discovery rate of novel secondary metabolites

from marine Actinobacteria has been recently exceeded terrestrial counterparts, as evident by the isolation of many new chemical entities from marine Actinobacteria.

3. General characteristics of Actinobacteria

Actinobacteria comprises a group of branching unicellular microorganisms, most of which are aerobic-forming mycelium known as substrate and aerial. They reproduce by binary fission or by producing spores or conidia, and sporulation of Actinobacteria is through fragmentation and segmentation or conidia formation. The morphological appearance of Actinobacteria (Figure 1) is compact, often leathery, giving a conical appearance with a dry surface on culture media and are frequently covered with aerial mycelium.

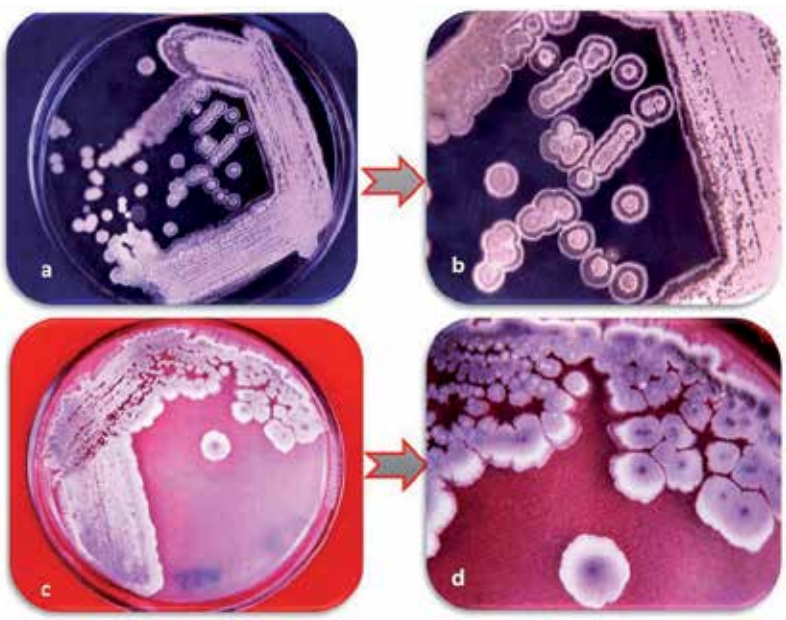

Figure 1. Appearance of Actinobacteria isolates on Starch casein agar plate. a, c Plate view of the Actinobacterial isolates. b, d Morphology of individual colonies.

3.1. Aerial mycelium

The aerial mycelium is usually thicker than the substrate mycelium (Figure 2a). The aerial mycelium shows sufficient differentiation that a miscellaneous assortment of isolates can be segregated into a number of groups having similar morphological characteristics under fixed condition. This is designated as one of the most important criteria for the classification of the genus *Streptomyces* into species, comprising structure (cottony, velvety, or powdery), formation of rings or concentric zones, and pigmentation.

3.2. Substrate mycelium

The substrate mycelium of Actinobacteria varies in size, shape, and thickness (Figure 2b). Its color ranges from white or virtually colorless to yellow, brown, red, pink, orange, green, or black.

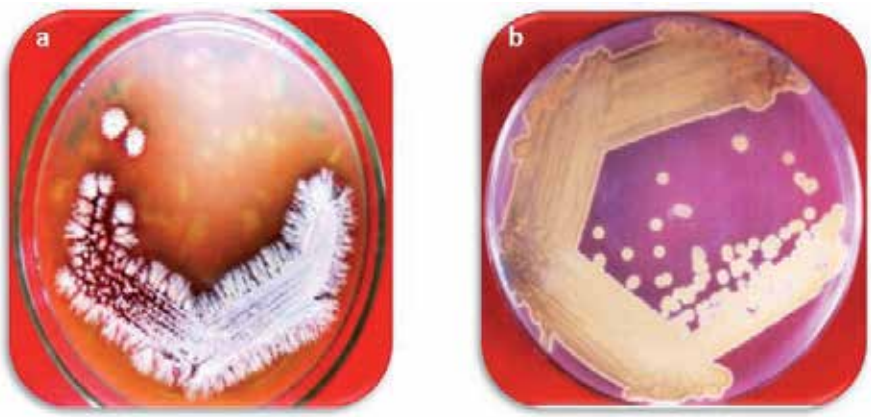

Figure 2. Abundant growth of Actinobacterial isolate on starch casein agar medium. a. Aerial mycelium. b. Reverse side of plate showing substrate mycelium.

3.3. Morphological appearance

Morphology has been an important characteristic to identify Actinobacteria isolates, which was used in the first descriptions of *Streptomyces* species (Figure 3). This is made using various standard culture media, including International *Streptomyces* Project (ISP). For nonstreptomycetes or rare Actinobacteria, strains maintained on ATCC Medium No.172 (NZ-amine glucose starch agar) (American Type Culture Collection, 1982) were used. Various morphological observations, including germination of spores, elongation and branching of vegetative mycelium, formation of aerial mycelium, color of aerial and substrate mycelium, and pigment production, have been used to identify Actinobacteria [21]. Light microscopy was used to study the formation of aerial mycelium and substrate mycelium, and scanning electron microscopy (Figure 4) was used to study the spores, the spore surface, and spore structure.

4. Systematics of Actinobacteria

In the Bergey's Manual of Determinative Bacteriology, Actinobacteria are included in several sections of volume four. All Actinobacteria are included under the order Actinomycetales. The order Actinomycetales is divided into four families—Streptomycetaceae, Actinomycetaceae, Actinoplanaceae, and Mycobacteriaceae [23]. The "Bergey's Manual of Systematic Bacteriology—2nd edition" for Actinobacteria classification has five volumes, which contain interna-

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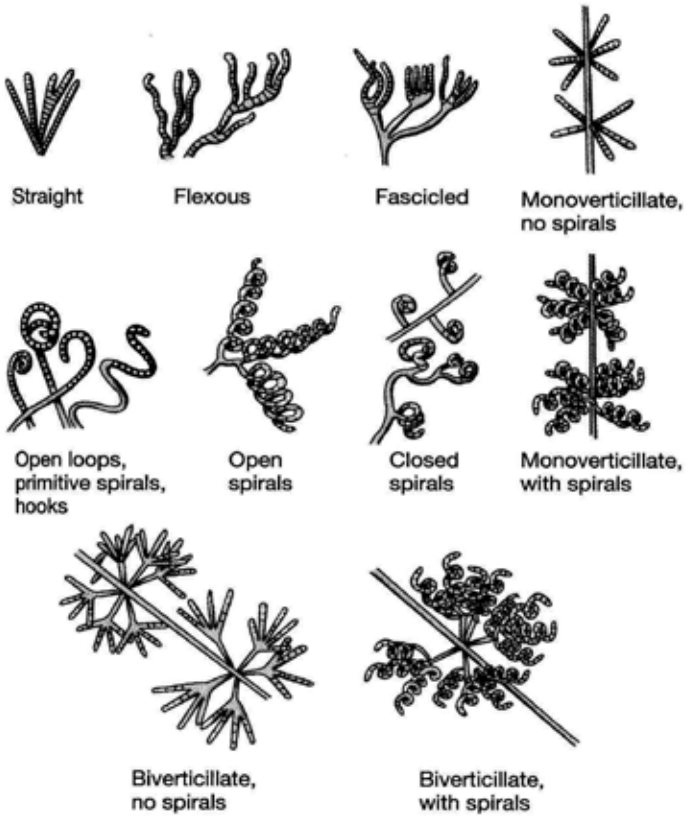

Figure 3. Type of spore-bearing structure in streptomycetes [22].

tionally recognized names and descriptions of bacterial species. Classification of Actinobacteria has been rearranged as follows:

The Archaea and the Deeply Branching and Phototrophic Bacteria	**Volume 1**
The Proteobacteria	**Volume 2**
The Firmicutes	**Volume 3**
The Bacteroidetes, Spirochaetes, Tenericutes (Mollicutes), Acidobacteria, Fibrobacteres, Fusobacteria	**Volume 4**
The Actinobacteria	**Volume 5**

In Volume 5, the phylum Actinobacteria is divided into six classes, namely Actinobacteria, Acidimicrobiia, Coriobacteriia, Nitriliruptoria, Rubrobacteria, and Thermoleophilia. The class Actinobacteria is further divided into 16 orders that are Actinomycetales, Actinopolysporales, Bifidobacteriales, Catenulisporales, Corynebacteriales, Frankiales, Glycomycetales, Jiangel-

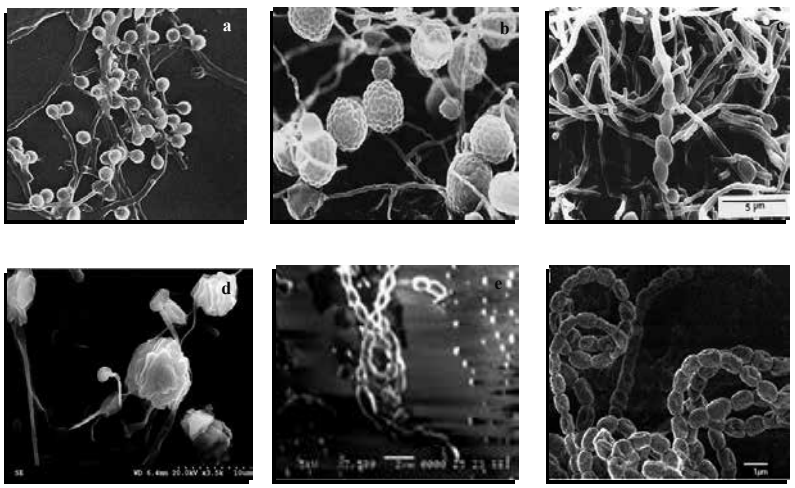

Figure 4. Scanning electron photographs of various Actinobacterial isolates. a. *Micromonospora* sp. b. *Streptosporangium* sp. c. *Saccharopolyspora* sp. d. *Actinosynnema* sp. e. *S. noursei* DPTD21. f. *Streptomyces* sp. JD9.

lales, Kineosporiales, Micrococcales, Micromonosporales, Propionibacteriales, Pseudonocardiales, Streptomycetales, Streptosporangiales, and Incertae sedis. In the order of abundance in soils, the common genera of Actinobacteria are *Streptomyces* (nearly 70%), *Nocardia*, and *Micromonospora*, although *Actinoplanes*, *Micromonospora*, and *Streptosporangium* are also generally encountered. At present, the molecular identification is based on 16S rDNA sequences, which is most significant for Actinobacteria identification [24].

5. Types of Actinobacteria

5.1. Thermophilic Actinobacteria

Number of studies has been carried out by the researchers to confirm the existence of extremophilic and extreme tolerant soil Actinobacteria (acid tolerant and alkali tolerant, psychrotolerant and thermotolerant, and halotolerant and haloalkalitolerant or xerophilic). Mesophilic Actinobacteria can grow at an optimal temperature from 20°C to 42°C, among which thermotolerant species exist, which can survive at 50°C. Moderately thermophilic Actinobacteria have an optimum growth at 45°C–55°C [29], whereas strictly thermophilic Actinobacteria grow at 37°C–65°C with the optimum temperature at 55°C–60°C [25]. Incubation temperatures of 28°C, 37°C, and 45°C are considered optimal for isolation of soil mesophilic, thermotolerant, and moderately thermophilic Actinobacteria. *Thermoactinomyces*, which is presently excluded from the order Actinomycetales, are described as thermophilic forms depending on its phenotypic and molecular genetic characteristics, as well as among some species of *Thermomonospora, Microbispora, Saccharopolyspora, Saccharomonospora,* and *Streptomyces*.

5.2. Acidophilic Actinobacteria

Acidophilic Actinobacteria, which are common in terrestrial habitats such as acidic forest and mine drainage soil, grow in the pH range from about 3.5 to 6.5, with optimum rates at pH 4.5 to 5.5 [26, 27]. It has been shown that acidophilic Actinobacteria consistently form two distinct aggregate taxa (namely, the neutrotolerant acidophilic and strictly acidophilic cluster groups) based on numerical phenetic data; members of the two groups share common morphological and chemotaxonomic properties [26]. Also some members of the strictly acidophilic group form a distinct taxon, such as the genus *Streptacidiphilus*, which has been assigned to the revised family Streptomycetaceae, together with the genera *Kitasatospora* and *Streptomyces*.

5.3. Halophilic Actinobacteria

Halophilic Actinobacteria are categorized into different types based on their growth in media containing different concentrations of salt. Extreme halophiles grow best in media containing 2.5–5.2 M salt, whereas borderline extreme halophiles grow best in media containing 1.5–4.0 M salt, moderate halophiles grow best in media containing 0.5–2.5 M salt, and finally halotolerants that do not show an absolute requirement to salt for growth but grow well up to often very high salt concentrations and tolerate 100 g/l salt (equivalent to 1.7 M NaCl) at least. Seawater, saline soils, salt lakes, brines, and alkaline saline habitats are considered as the best habitats for isolating halophilic Actinobacteria. Generally, most of the halophilic Actinobacteria have been isolated from saline soils. Halophilic Actinobacteria isolated from marine environments are assigned to a few genera, including *Micromonospora, Rhodococcus,* and *Streptomyces* [28]. The other group includes *Dietzia, Salinispora, Marinophilus, Solwaraspora, Salinibacterium, Aeromicrobium, Gordonia, Microbacterium, Mycobacterium, Nocardiopsis, Pseudonocardia, Actinomadura, Saccharopolyspora, Streptosporangium, Nonomuraea, Williamsia,* and *Verrucosispora* [28–31].

5.4. Endophytic Actinobacteria

Endophytic Actinobacteria are defined as those that inhabit the internal part of plants, causing apparently no visible changes to their hosts. These Actinobacteria play specific roles, for instance, protecting the host plants against insects and diseases. Endophytic Actinobacteria constitute a large part of the rhizosphere, which are also found inside plants in which the extensively studied species are from the genus *Frankia*, nitrogen-fixing bacteria of nonleguminous plants [32], and a few species of the genus *Streptomyces* that are phytopathogens. Generally, the endophytic Actinobacteria include *Streptomyces,* but the genera *Streptoverticillium*, *Nocardia*, *Micromonospora, Kitasatospora, Pseudonocardia, Microbispora*, *Kibdelosporangium, Actinopolyspora, Nocardioides, Brevibacterium, Actinomadura, Glycomyces Plantactinospora, Polymorphospora, Promicromonospora*, and *Streptosporangium* are also found in the plants, such as *Palicourea longifolia, Calycophyllum acreanum, Monstera spruceana, Croton lechleri, Cantua buxifolia, Siparuna crassifolia*, and *Eucharis cyaneosperma.*

5.5. Symbiotic Actinobacteria

About 15% of the world's nitrogen is fixed naturally by the symbiotic relationships between various species of the *Frankia* belonging to the family of Actinobacteria. The plants that form symbiotic relationships with *Frankia* are called actinorhizal plants. Researchers have found over 160 plants that have Actinobacteria as their host, including alders, Russian olive, bayberry, sweet fern, bitterbrush, and cliff rose. The *Frankia* have the ability to provide most or all of the host plant's nitrogen needs. Numerous *Frankia* species including *Casuarina* isolates form nitrogen-reducing (NIR) vesicles *in vitro* and in planta [33]. These nitrogen-fixing bacteria and their host plants are often pioneer species on young nitrogen-deficient and disturbed soils such as moraines, volcanic flows, and sand dunes.

5.6. Endosymbiontic Actinobacteria

An endosymbiont is any organism that lives within the body or cells of another organism. Endosymbiosis process is sometimes obligate, that is, either the endosymbiont or the host cannot survive without the other. Members of the phylum Actinobacteria have been identified as abundant members of sponge-associated microbial communities. *Mycobacterium* along with *Micrococcus, Micromonospora, Microbacterium, Brevibacterium, Kocuria, Corynebacterium, Rhodococcus, Brachybacterium, Rubrobacter, Streptomyces, Dietzia, Salinispora, Actinokineospora, Gordonia, Arthrobacter, Nocardiopsis, and Rothia* species were found to live as endosymbionts in marine sponges *Callyspongia aff. Implexa, Aplysina aerophoba, Spheciospongia vagabunda, Hemimycale culumella, Hyrtios erecta, Dysidea tupha, Callyspongia* sp., *Dysidea avara, Amphimedon* sp., and *Negombata magnifica*. However, the Actinobacterial endosymbionts have also been reported in other group of animals, such as *Hylobates hoolock, Rhinopithecus roxellanae, Rhinopithecus bieti, Panthera tigris altaica, Panthera tigris tigris, Panthera tigris amoyensis, Ailurus fulgens, Cavnlvara zlrsidae, Ursus thibetanus, Cervus elaphus, Elaphurus davidianus,* and *Vicugna pacos.*

5.7. Gut Actinobacteria

Though Actinobacteria are found in various diverse habitats, some are also known to form intimate associations with invertebrates and vertebrates. Symbiotic interactions are essential mainly for the survival and reproduction because they play a crucial role in nutrition, detoxification of certain compounds, growth performance, and protection against pathogenic bacteria. Many studies have shown that some symbiotic Actinobacterial species, that is probiotics, control bacterial diseases in livestock, poultry, and aquaculture. They also take part in host health by converting the feedstuffs into microbial biomass and fermentation end products that can be utilized by the animal host. Tan et al [34] isolated *Streptomyces*, *Nocardiopsis*, and *Oerskovia* from healthy goat feces. Similarly, Latha and Dhanasekaran [35] isolated 87 Actinobacterial cultures from different feces of goat and chicken collected from various locations in Pudukkottai and Tiruchirappalli Districts, Tamil Nadu, among which 45 isolates were selected for the screening of antibacterial activity and extracellular digestive enzyme production. The ability of the probiont *Streptomyces* sp. JD9 from gut of chicken possesses all the characteristics needed to satisfy the indigenous Actinobacterial probiont for enhanced broiler production [36].

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6. Applications of Actinobacteria

Actinobacteria are well recognized for their production of primary and secondary metabolites that have important applications in various fields. They are also a promising source of wide range of important enzymes, which are produced on an industrial scale. A large fraction of antibiotics in the market is obtained from Actinobacteria. They produce enzyme inhibitors useful for cancer treatment and immunomodifiers that enhance immune response. They have the ability to degrade a wide range of hydrocarbons, pesticides, and aliphatic and aromatic compounds. They perform microbial transformations of organic compounds, a field of great commercial value. Members of many genera of Actinobacteria can be potentially used in the bioconversion of underutilized agricultural and urban wastes into high-value chemical products. Actinobacteria are also important in plant biotechnology as strains with antagonistic activity against plant pathogens are useful in biocontrol. Their metabolic potential offers a strong area for research. Here, we have a brief description about important applications of Actinobacteria (Figure 5).

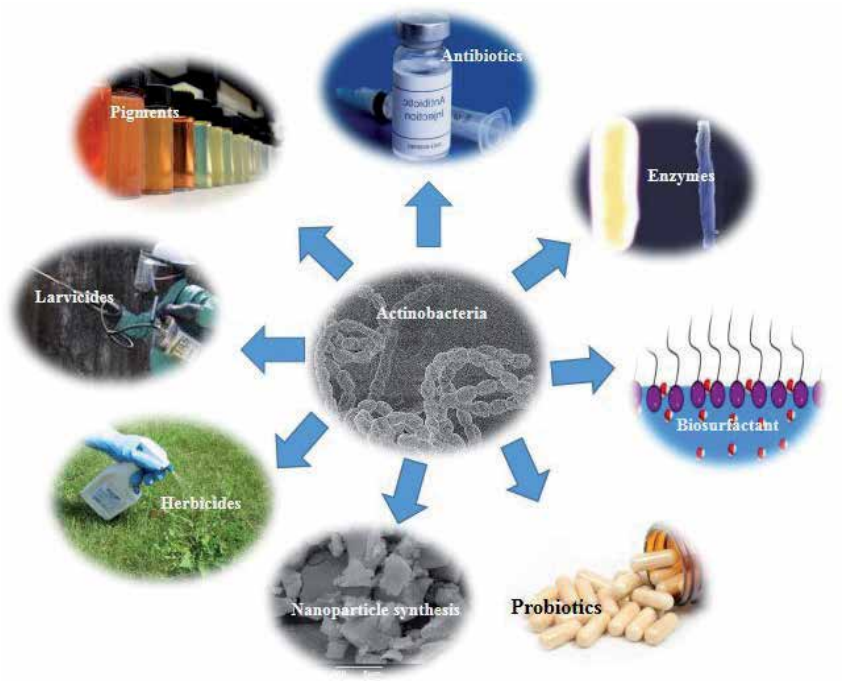

Figure 5. Biotechnological applications of Actinobacteria.

6.1. Antimicrobials

Actinobacteria hold a significant role in producing variety of drugs that are extremely important to our health and nutrition. Recently, diseases due to multidrug-resistant patho-

genic bacteria are sturdily increasing, and thus search for new antibiotics is effective against the multidrug-resistant pathogens. Natural products having novel structures have been observed to possess useful biological activities [37]. Nature always remains the richest and the most versatile propitious source for new antibiotics, though there is considerable progress within the fields of chemical synthesis and engineered biosynthesis of antibacterial compounds. Toxic nature of certain antibiotics led to their limited usage although thousands of antibiotics have been discovered till date. To get through this problem, search of new antibiotics that are more effective and that do not have any toxic side effects is in progress. As already mentioned, one of the major healthcare problems is the antibiotic resistance. One approach to solve this problem is to search for new antibiotics with new mechanism of action. Figure 6 shows that a majority of antibiotics are derived from microorganisms, especially from the species Actinobacteria. Almost 80% of the world's antibiotics are known to be derived from Actinobacteria, mostly from the genera *Streptomyces* and *Micromonospora* [38, 39].

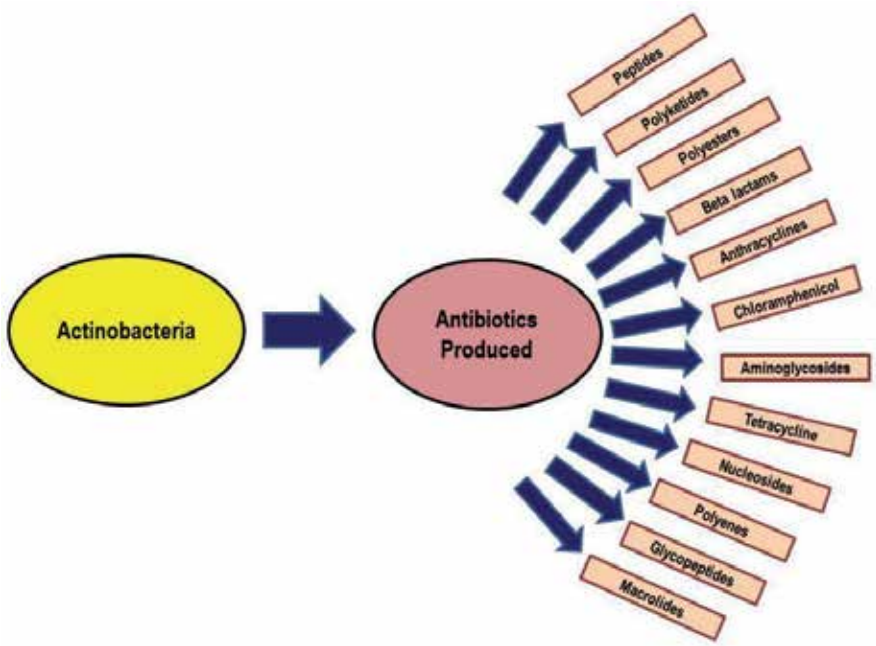

Figure 6. Antibiotics from Actinobacteria.

Particularly, *Streptomyces* species produce around 7600 compounds, many of which are secondary metabolites that are potent antibiotics, which has made streptomycetes the primary antibiotic-producing organisms exploited by the pharmaceutical industry [40, 41]. The reason behind the ability of the genus *Streptomyces* to produce commercially significant compounds remains supreme because of the extra large DNA complement of these bacteria [42]. It has been estimated that the last five decades have seen the discovery of more than 12,000 antibiotics, out of which the Actinobacteria yielded about 70% of them and the remaining 30% are products

of filamentous fungi and nonActinobacteria. The antibiotics from Actinobacteria are differentiated into several major structural classes, such as aminoglycosides (e.g., streptomycin and kanamycin), ansamycins (e.g., rifampin), anthracyclines (e.g., doxorubicin), β-lactam (cephalosporins), macrolides (e.g., erythromycin), and tetracycline. *Streptomyces* strains have produced many of the antibiotics known to humans, which appears that these organisms produce antibiotics to kill off potential competitors [43]. One of the first antibiotics used is streptomycin produced by *Streptomyces griseus* [44]. Indeed, different *Streptomyces* species produce about 75% of commercially and medically useful antibiotics. In the course of screening for new antibiotics, several studies are oriented toward isolation of streptomycetes from different habitats. Different conditions of nutrition and culturing may affect the ability of *Streptomyces* cultures to form antibiotics, and hence the medium constitution together with the metabolic capacity of the producing organism greatly affects antibiotic biosynthesis.

Antagonistic Actinobacteria produce a variety of antibiotics that vary in chemical nature, in antimicrobial action, in toxicity to animals, and in their chemotherapeutic potentialities. Some of the antibiotics that have been isolated so far from Actinobacteria are crude preparations, whereas others have been crystallized, and considerable information has been gained concerning their chemical nature, which includes lysozyme, actinomycin, micromonosporin, streptothricin, streptomycin, and mycetin. Some Actinobacteria produce more than one antibiotic substance (e.g., *S. griseus*), as well as the same antibiotic may be produced by different species of Actinobacteria (e.g, actinomycin, streptothricin). A given antibiotic may, therefore, be identical, even when produced by different Actinobacteria, as shown by its chemical composition and antibiotic spectrum. Table 1 represents the list of antibiotics produced by various Actinobacteria with excellent antimicrobial application (Table 1).

Antibiotic compound	**Application**	**Actinobacteria**
1,8-Dihydroxy-2-ethyl-3 methylanthraquinone	Antitumor	*Streptomyces* sp.
1-Hydroxy-1-norresistomycin	Antibacterial; anticancer	*Schisandra chinensis*
2-Allyloxyphenol	Antimicrobial; food preservative; oral disinfectant	*Streptomyces* sp.
Anthracyclines	Antitumor	*S. galileus*
Arenicolides A–C	Mild cytotoxicity	*Salinispora arenicola*
Arenimycin	Antibacterial; anticancer	*S. arenicola*
Avermectin	Antiparasitic	*Streptomyces avermitilis*
Bafilomycin	ATPase inhibitor of microorganisms, plant and animal cells	*S. griseus, Streptomyces halstedii*
Bisanthraquinone	Antibacterial	*Streptomyces* sp.
Butenolides	Antitumor	*Streptoverticillium luteoverticillatum*
Carboxamycin	Antibacterial; anticancer	*Streptomyces* sp.
Chinikomycins	Anticancer	*Streptomyces* sp.

Antibiotic compound	Application	Actinobacteria
Chloramphenicol	Antibacterial, inhibitor of protein biosynthesis	*Streptomyces venezuelae*
Cyanospraside A	Unknown	*Solieria pacifica*
Daryamides	Antifungal; anticancer	*Streptomyces* sp.
Frigocyclinone	Antibacterial	*S. griseus*
Glaciapyrroles	Antibacterial	*Streptomyces* sp.
Hygromycin	Antimicrobial, immunosuppressive	*Streptomyces hygroscopicus*
Lajollamycin	Antibacterial	*Streptomyces Nodosus*
Lincomycin	Antibacterial, inhibitor of protein biosynthesis	*Streptomyces lincolnensis*
Marinomycins A–D	Antimicrobial; anticancer	*Marinispora*
Mechercharmycins	Anticancer	*Thermoactinomyces* sp.
Mitomycin C	Antitumor, binds to double-stranded DNA	*Streptomyces lavendulae*
Pacificanones A & B	Antibacterial	*S. pacifica*
Piericidins	Antitumor	*Streptomyces* sp.
Proximicins	Antibacterial; anticancer	*Verrucosispora* sp.
Rapamycin	Immunosuppressive, antifungal	*S. hygroscopicus*
Resistoflavin methyl ether	Antibacterial; antioxidative	*Streptomyces* sp.
Saliniketal	Cancer chemoprevention	*S. arenicola*
Salinispyrone	Unknown	*S. pacifica*
Salinispyrone A & B	Mild cytotoxicity	*S. pacifica*
Salinosporamide A	Anticancer; antimalarial	*Salinispora tropica*
Salinosporamide B & C	Cytotoxicity	*S. tropica*
Sesquiterpene	Unknown	*Streptomyces* sp.
Staurosporinone	Antitumor; phycotoxicity	*Streptomyces* sp.
Streptokordin	Antitumor	*Streptomyces* sp.
Streptomycin	Antimicrobial	*S. griseus*
Streptozotocin	Diabetogenic	*S. achromogenes*
Tetracyclines	Antimicrobial	*Streptomyces achromogenesStreptomyces rimosus*
Tirandamycins	Antibacterial	*Streptomyces* sp.
Valinomycin	Ionophor, toxic for prokaryotes and eukaryotes	*S. griseus*
ZHD-0501	Anticancer	*Actinomadura* sp.
Elaiomycins B and C	Antitumor	*Streptomyces* sp. BK 190
N-[2-hydroxyphenyl)-2-phenazinamine (NHP),	Anticancer; antifungus	*Nocardia dassonvillei*
Chromomycin B, A2, A3	Antitumor	*Streptomyces coelicolor*

Antibiotic compound	Application	Actinobacteria
1,4-dihydroxy-2-(3-hydroxybutyl)-9, 10-anthraquinone 9, 10-anthrac	Antibacterial	*Streptomyces* sp. RAUACT-1

Table 1. List of antibiotics produced from Actinobacteria

6.2. Enzymes

A wide variety of biologically active enzymes are produced by both marine and terrestrial Actinobacteria (Figure 7; Table 2). They secrete amylases to the outside of the cells, which helps them to carry out extracellular digestion. This enzyme is of great significance in biotechnological applications such as food industry, fermentation, and textile to paper industries because of their ability to degrade starch [45]. Another important aspect of Actinobacteria is the production of cellulases, which are a collection of hydrolytic enzymes that hydrolyze the glucosidic bonds of cellulose and related cello-digosaccharide derivatives. Lipase is produced from various Actinobacteria, bacteria, and fungi and is used in detergent industries, foodstuff, oleochemical, diagnostic settings, and also in industries of pharmaceutical fields [46]. Many Actinobacteria have been isolated from various natural sources, as well as in plant tissues and rhizospheric soil. Biological functions of Actinobacteria mainly depend on sources from which the bacteria are isolated. Actinobacteria, particularly streptomycetes, are known to secrete multiple proteases in the culture medium [47]. Similarly, Actinobacteria have been revealed to be an excellent resource for L-asparaginase, which is produced by a range of Actinobacteria, mainly those isolated from soils, such as *S. griseus, Streptomyces karnatakensis, Streptomyces albidoflavus,* and *Nocardia* sp. [48, 49]. The roots and rhizomes of several Thai medicinal plants such as lemon grass (*Cymbopogon citratus*) and ginger (*Zingiber officinale*) have long been used in Thai traditional medicine for stomach ache and asthma treatment. Rhizosphere soil of these plants may be an attractive Actinobacterial source, which has the ability to produce novel secondary metabolites. Enzymes such as catalase, chitinase, and urease are also produced from Actinobacteria. Interestingly, keratinase, an enzyme that degrades the poultry chicken feather, has been successfully produced from *Nocradiopsis* sp. SD5 isolated from feather waste in Tamil Nadu, India [50]. Similarly, Actinobacteria isolated from chicken and goat gut showed the presence of various enzymes such as amylase, protease, phytase, and lipase [35].

Figure 7. Different types of enzymes produced by Actinobacteria. a. Amylase. b. Protease. c. Lipase. The zone of inhibition around the inoculated Actinobacteria confirms the production of particular enzyme.

Enzyme	Actinobacteria	Use	Industry of application
Protease	*Thermoactinomyces* sp., *Nocardiopsis* sp., *Streptomyces pactum, Streptomyces thermoviolaceus, Streptomyces* sp.	Detergents	Detergent
		Cheese making	Food
		Clarification- low calorie beer	Brewing
		Dehiding	Leather
		Treatment of blood clot	Medicine
Cellulase	*Streptomyces* sp., *Thermobifida halotolerans* , *Streptomyces* sp., *Thermomonospora* sp., *Streptomyces ruber*	Removal of stains	Detergent
		Denim finishing, softening of cotton	Textile
		Deinking, modification of fibers	Paper and pulp
Lipase	*Streptomyces griseus*	Removal of stains	Detergent
		Stability of dough and conditioning	Baking
		Cheese flavoring	Dairy
		Deinking, cleaning	Textile
Xylanase	*Actinomadura* sp., *Streptomyces* spp.	Conditioning of dough	Baking
		Digestibility	Animal feed
		Bleach boosting	Paper and pulp
Pectinase	*Streptomyces lydicus*	Clarification, mashing	Beverage
		Scouring	Textile
Amylase	*Streptomyces* sp., *Streptomyces erumpens,Nocardiopsis* sp. ,*Thermobifida fusca, Nocardiopsis* sp.	Removal of stains	Detergent
		Softness of bread softness and volume	Baking
		Deinking, drainage improvement	Paper and pulp
		Production of glucose and fructose syrups	Starch industry
		Removal of starch from woven fabrics	Textile
Glucose oxidase	*Streptomyces coelicolor*	Strengthening of dough	Baking
Keratinase	*Nocradiopsis* sp. SD5	Feather degradation	Animal feed
Phytase	*Streptomyces luteogriseus* R10	Phytate digestibility	Animal feed

Table 2. Enzymes and their industrial applications

6.3. Bioherbicides

Another interesting application of the Actinobacteria is the use of their secondary metabolites as herbicides against unwanted herbs and weeds. *Streptomyces saganonensis* produce herbicidines and herbimycins that controls monocotyledonous and dicotyledonous weeds. Anisomycin, which is produced by *Streptomyces* sp., is a type of growth inhibitor for annual grassy weeds such as barnyardness and common crabgrass and broad-leaved weeds; anisomycin can destroy the ability of the plants to synthesize chlorophyll. Similarly, bialaphos, a metabolite of *Streptomyces viridochromogenes*, is widely used to control annual and perennial grassy weeds and broad-leaved weeds by inhibiting glutamine synthesis. Anisomycin can make small seedlings of barnyardness and common crabgrass die above 50 ppm and inhibit radicle growth below 12.5 ppm. Its synthetase may accumulate ammonia and control photosynthetic phosphorylation, causing plant death [51]. *S. hygroscopicus* produce carbocyclic coformycin and hydantocidin, which can decrease synthetase of aclenylosuccinate by increasing the content of ATP and hold back the synthesis of protein [52]. In addition, phthoxazolin, hydantocidin, and homoalanosin from *Streptomyces* sp. can control several weeds [53]. Dhanasekaran et al [54] reported that *Streptomyces* sp. had the ability to inhibit the growth of *Echinochilora crusgalli*. Similarly, *Streptomyces* sp. KA1-3, KA1-4, KA1-7, and KA23A were found highly effective against *C. rotundus* [55]. Herbicidal activity of the bioactive compounds *N*-phenylpropanamide and N (naphthalene-1-yl) propanamide from *Streptomyces* sp. KA1-3 [56, 57] was also evaluated.

6.4. Probiotics

Probiotics are the live microbial adjunct that has a beneficial effect on the host by various means, such as modifying the host associated or ambient microbial community, by ensuring the improved use of the feed or enhancing its nutritional value, by enhancing the host response towards disease, or by improving the quality of its ambient environment. Despite several other important applications, marine Actinobacteria have been given its attention for their use as probiotics. The potential of Actinobacteria against shrimp pathogenic *Vibrio* spp. made marine Actinobacteria as potential probiotic strains due to their ability to degrade macromolecules, such as starch and protein, in culture pond water; the production of antimicrobial agents; and the formation of heat- and desiccation-resistant spores [58]. Recently, a few studies were made on the possible use of marine Actinobacteria in disease prevention against aquatic pathogens. Das et al [59] in their preliminary study reported the use of *Streptomyces* sp. on the growth of black tiger shrimp. An antibiotic product extracted from marine Actinobacteria was incorporated into feed to observe the *in vivo* effect on white spot syndrome virus in black tiger shrimp. Again, You et al [58] reported the activity of marine actinomycete as a potential organism against biofilms produced by *Vibrio* spp. and recommended the use of Actinobacteria to prevent the disease caused by *Vibrio* spp. In another study, Latha et al [36] screened 18 of the Actinobacteria isolated from chicken for probiotic properties, and the results revealed that *Streptomyces* sp. JD9 was the potent isolate with well-distinct probiotic properties.

6.4.1. Aggregative peptide pheromones

Aggregation is one of the most important criteria for the selection of a good probiotic candidate, which is the process of reversible accumulation of cells with one or more strains. For this aggregating process to take place, pheromone production is one of the main criteria that involves defense against predators, mate selection, and in overcoming host resistance by mass attack. In particular, sex pheromone peptides in culture supernatants have been shown to promote aggregation not only with the same species but also with related species [60–62]. Thus, the auto-aggregating ability of a probiotic is a prerequisite for colonization of the gastrointestinal tract, whereas coaggregation provides a close interaction with pathogenic bacteria. Though there are a number of studies in accordance with peptide pheromone–mediated signaling, it is lacking in the case of Actinobacteria, and thus a novel report on isolation and purification of diffusible aggregation promoting factor, that is, pheromones from potent Actinobacterial probiont *Streptomyces werraensis* LD22 isolated from the gut region of goat, were described by Muthu Selvam et al [63].The results clearly portray that Actinobacterial strain *S. werraensis* LD22 secretes a heat-stable, acidic pH–resistant, low molecular weight peptide pheromone that promotes the aggregation propensity and enhances the biofilm forming ability of other Actinobacterial isolates.

6.5. Biosurfactants

Biosurfactants are the microbially derived compounds that share hydrophilic and hydrophobic moieties that are surface active. When compared with chemically derived surfactants, biosurfactants are independent of mineral oil as a feedstock; they are readily biodegradable and can be produced at low temperatures. Biosurfactants can be applied in various areas, such as the nutrient, cosmetic, textile, varnish, pharmaceutical, mining, and oil recovery industries [64–66]. The lipopeptide antibiotic daptomycin is an Actinobacterial biosurfactant that has already entered the market and is used in the treatment of diseases caused by Gram-positive pathogens and has been marketed as Cubicin by Cubist Pharmaceuticals. Diverse types of biosurfactants or bioemulsifiers have been described to be produced within the class *Actinobacteria*. Among the best described biosurfactants are glucose-based glycolipids, most of which have a hydrophilic backbone consisting of glycosidic-linked glucose units forming a trehalose moiety.

6.6. Vitamins

Vitamin B_{12} as it exists in nature may be produced by bacteria or Actinobacteria [67]. Isolation of vitamin B_{12} from Actinobacteria fermentations [68, 69] stirred up considerable interest in possible production of vitamin by microbial fermentations. Addition of cobalt salts to the media apparently acts as a precursor for all Actinobacteria to produce vitamin. As cobalt is a rather effective bactericidal agent, this precursor must be added carefully. The fermentations producing the antibiotics streptomycin, aureomycin, grisein, and neomycin will produce some vitamin B12 as well if the medium is supplemented with cobalt without apparently affecting the yields of antibiotic substances. Several other studies suggested that some Actinobacteria

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that are non–antibiotic-producing cultures produce more of this vitamin than those producing antibiotics. Actinobacteria have been shown to produce other water soluble vitamins, with special studies on production of thiamine and the pteroylglutamic acid derivative that is active in promoting the growth of certain strains of *Leuconostoc citrovorum* and coenzyme A.

6.7. Pigments

As synthetic dyes have some limitations such as usage of hazardous chemicals for their production, creating worker safety concerns and generation of hazardous wastes, microbe-oriented pigments are of great concern. Specially, Actinobacteria are characterized by the production of various pigments on natural or synthetic media (Figure 8) and are considered as an important cultural characteristic in describing the organisms. Any phenotypic changes induced by environmental influences will help Actinobacteria as they boast distinctive colony morphologies and produce variety of pigments and aerial branching filaments called hyphae, which give them a characteristic fuzzy appearance. These pigments usually comes in various shades of blue, violet, red, rose, yellow, green, brown, and black, which may be dissolved into the medium or it may be retained in the mycelium. The pigments produced by *Streptomyces* may be either endopigments (bound to certain cell structures) or exopigments (excreated into the surrounding medium). Sometimes different antibiotics produced by the Actinobacteria are considered as pigments. Since the formation of pigment is influenced by the pH of the medium, aeration, temperature of the growth, and carbon and nitrogen sources, only a little is known about the exact chemical nature of pigments. Its formation is also linked to respiratory mechanisms, defense mechanisms, and ultraviolet protection. These microbes also have the ability to synthesize and excrete dark pigments, melanin or melanoid, which are considered to be a useful criterion for taxonomical studies. The textile industry produces and uses approximately 1.3 million tonnes of dyes, pigments, and dye precursors, valued at around $23 billion, almost all of which are manufactured synthetically. Table 3 provides a list of pigments from different Actinobacteria.

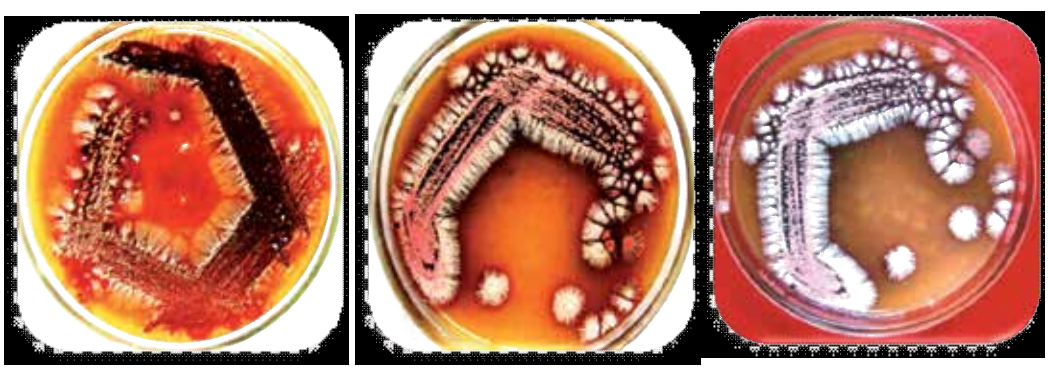

Figure 8. Diffusible pigment produced by various Actinobacteria in starch casein agar medium.

Pigment	Class	Actinobacteria
Rhodomycin	Anthracycline glycoside	*Synodontis violaceus* DSM 40704
Actinomycin	Phenoxazinone	*Streptomyces* sp.
III Undecylprodigiosin IV Metacycloprodigiosin	Prodigiosin	*Streptomyces longispororuber* DSM 40599
Granaticin	Naphthoquinone	*Streptomyces litmocidin* DSM 40164

Table 3. Pigments from Actinobacteria

6.8. Nanoparticle synthesis

Nanoparticles are of great scientific interest as they bridge the gap between bulk materials and atomic or molecular structures. Generally, the chemical methods are low cost for high volume; however, their drawbacks include contamination from precursor chemicals, use of toxic solvents, and generation of hazardous byproducts. Hence, there is an increasing need to develop high-yield, low-cost, nontoxic, and environmentally benign procedures for synthesis of metallic nanoparticles. Therefore, the biological approach for synthesis of nanoparticles becomes important. In fact, Actinobacteria are efficient producers of nanoparticles, which show a range of biological properties, namely antibacterial, antifungal, anticancer, antibiofouling, antimalarial, antiparasitic, and antioxidant. *Streptomyces* and *Arthrobacter* genera have been studied as possible "nanofactories" for the development of clean and nontoxic methods of the synthesis of silver and gold nanoparticles. A recent example of silver nanoparticle synthesis from an Actinobacteria *Streptomyces* sp. GRD was performed by Gopinath et al [70]. Ranjani et al [71] observed the diversity of silver nanoparticle synthesizing Actinobacteria from marine environment and showed that 25 isolates of 49 synthesized silver nanoparticles, the genus of which includes *Streptomyces* sp., *Nocardiopsis* sp., *Kitasatosporia* sp., *Actinopolyspora* sp., *Thermoactinomyces* sp., *Actinomadura* sp., *Kibdelosporangium* sp., *Saccharopolyspora* sp., and *Thermomonospora* sp. Table 4 shows the synthesis of nanoparticles using various genera.

Actinobacteria	Nanoparticles
Streptomyces sp., *Thermoactinomyces* sp., *Nocardiopsis* sp., *Rhodococcus* sp., *Streptomyces albidoflavus, Streptomyces hygroscopicus, Streptomyces rochei*	Silver
Streptomyces aureofaciens, Actinobacteria,Streptomyces glaucus, Streptomyces viridogens, Thermoactinomycete spp., *Thermomonospora* spp., *Nocardia farcinica, Streptomyces hygroscopicus*	Gold
Streptomyces sp.	Zinc, copper, manganese

Table 4. List of nanoparticles synthesized using Actinobacteria

6.9. Bioremediation

Actinobacteria possess many properties that make them good candidates for application in bioremediation of soils contaminated with organic pollutants. In some contaminated sites, Actinobacteria represent the dominant group among the degraders [72]. They play an

important role in the recycling of organic carbon and are able to degrade complex polymers. Sanscartier et al [73] reported that the greater use of petroleum hydrocarbons that are widely used in our daily life as chemical compounds and fuel has become one of the most common contaminants of large soil surfaces and eventually is considered as a major environmental problem. Some reports suggests that *Streptomyces* flora could play a very important role in degradation of hydrocarbons [74, 75]. Many Actinobacterial strains have the ability to solubilize lignin and degrade lignin-related compounds by producing cellulose- and hemicellulose-degrading enzymes and extracellular peroxidase [76]. Actinobacteria species have the ability to live in an oily environment and thus they can be used in bioremediation to reduce oil pollutants. *Nocradiopsis* sp. SD5 degraded feather waste by producing keratinase enzyme [50].

6.10. Control of plant diseases

The worldwide efforts in the search of natural products for the crop protection market have progressed significantly, and Actinobacteria, especially genus *Streptomyces*, appear to be good candidates in finding new approaches to control plant diseases. The agroindustry shows a marked interest for Actinobacteria as a source of agroactive compounds of plant growth–promoting rhizobacteria (PGPR) and of biocontrol tools [77, 78]. About 60% of the new insecticides and herbicides reported in the past 5 years originate from *Streptomyces* [78]. Kasugamycin is a bactericidal and fungicidal metabolite discovered in *Streptomyces kasugaensis* [79], which acts as an inhibitor of protein biosynthesis in microorganisms but not in mammals, and its toxicological properties are excellent. To market the systemically active kasugamycin for control of rice blast *Pyricularia oryzae* and bacterial *Pseudomonas* diseases in several crops, Hokko Chemical Industries developed a production process. Polyoxin B and D were isolated as metabolites of *Streptomyces cacaoi* var. *asoensis* in 1965 by Isono et al [80] as a new class of natural fungicides. The ability of the polyoxins to interfere with the fungal cell wall synthesis by specifically inhibiting chitin synthase [81] makes them acceptable with regard to environmental considerations. Polyoxin B found application against a number of fungal pathogens in fruits, vegetables, and ornamentals. Polyoxin D is marketed by several companies to control rice sheath blight caused by *Rhizoctonia solani*. The validamycin family was detected by Takeda researchers in 1968 in a greenhouse assay when screening streptomycete extracts for activity against rice sheath blight. Validamycin A was found to be a prodrug, which is converted within the fungal cell to validoxylamine A, an extremely strong inhibitor of trehalase [82]. This mode of action gives validamycin A a favorable biological selectivity because vertebrates do not depend on the hydrolysis of the disaccharide trehalose for their metabolism. Inhibition of plant pathogenic *Rhizoctonia solani* under *in vitro* condition was assessed with the culture supernatant of *Streptomyces* sp., which showed that the tested Actinobacteria had the ability to reduce damping off severity in tomato plants. Table 5 represents some of the antibiotics produced by the Actinobacteria that suppresses various plant diseases.

Disease	Actinobacteria	Antibiotic produced
Potato scab	*Streptomyces melanosporofaciens* EF-76 and FP-54	Geldanamycin
Grass seedling disease	*Streptomyces violaceusniger* YCED9	Nigericin and guanidylfungin A
Root rot of Pea	*Streptomyces hygroscopicus* var. *geldanus*	Geldanamycin
Asparagus root diseases	*Streptomyces griseus*	Faeriefungin
Rice blast disease	*Streptomyces kasugaensis*	Kasugamycin
Broad range of plant diseases	*Streptomyces griseochromogenes*	Blasticidin S
Sheath blight of rice	*Streptomyces hygroscopicus* var. *limoneus* No. T-7545	Validamycin
Brown rust of wheat	*Streptomyces hygroscopicus*	Gopalamycin
Phytophthora blight of pepper	*Streptomyces violaceusniger*	Tubercidin
Phytophthora blight of pepper	*Streptomyces humidus*	Phenylacetic Acid
Damping-off of cabbage	*Streptomyces padanus*	Fungichromin
Rice sheath blight	*Streptomyces cacaoi* var. *asoensis*	Polyoxin B and D
Powdery mildew	*Streptoverticillium rimofaciens*	Mildiomycin
Rice root disease	*Micromonospora* sp. SF-1917	Dapiramicin
Rice blast	*Micromonospora* sp. M39	2,3-dihydroxybenzoic acid, phenylacetic acid, cervinomycin A1 and A2
Blotch of wheat	*Streptomyces malaysiensis*	Malayamycin
Powdery mildew of cucumber	*Streptomyces* sp. KNF2047	Neopeptin A and B

Table 5. Plant disease suppression by antibiotics produced by Actinobacteria

6.11. Nematode control

It has been known for decades that effective control of plant-parasitic nematodes is dependent on chemical nematicides. Due to its ill effects with respect to the environmental hazards, hazardous nematicides have emphasized the need for new methods to control nematodes. Today, numerous microorganisms are recognized as antagonists of plant-parasitic nematodes. Especially, Actinobacteria have potential for use in biological control as they are known to produce antibiotics. The production of avermectins by a species of *Streptomyces* shows that soil-borne organisms can produce highly nematicidal compounds. *S. avermitilis* produces ivermectin, which has an excellent activity against *Wucheria bancroftii* [83]. Similarly various other antiparasitic compounds are produced from different *Streptomyces* sp., *Salinispora* sp., and *Marinactinospora* sp., which includes *Milbemycin, Antimycin* A9, *Fervenulin, bafilolides, Valinomycin, Salinosporamide* A, *Kalafungin, Thiamycins, and Axenomycins.*

6.12. Enhancement of plant growth

Despite the well-documented history of *Streptomyces* in biocontrol and preliminary evidence of their capacity to enhance plant growth [84], *Streptomyces* species have been poorly investigated specifically for their potential as PGPR. While the beneficial effect of some strains of PGPR on particular crops is certain, the mechanisms employed by PGPR are unclear [85]. PGPR can affect plant growth in two general ways, either directly or indirectly. Indirect promotion occurs when PGPR lessen or prevent the harmful effects of one or more deleterious microorganisms. This is chiefly attained through biocontrol or the antagonism of soil plant pathogens. Specifically, colonization or the biosynthesis of antibiotics and other secondary metabolites can prevent pathogen invasion and establishment. Direct promotion of plant growth by PGPR occurs when the plant is supplied with a compound that is synthesized by the bacteria, or when PGPR otherwise facilitates plant uptake of soil nutrients. Merriman et al [86] reported the use of *S. griseus* for seed treatment of barley, oat, wheat, and carrot to increase their growth. The isolate was originally selected for the biological control of *Rhizoctonia solani*. Though the *S. griseus* isolate did increase the average grain yield, dry foliage weight, tiller number, and advanced head emergence for both wheat and oat over controls, the differences were not statistically significant. As a seed treatment for carrot, the isolate was more successful. Marketable yields were increased over controls by 17% and 15% in two separate field trials. Specifically, both trials also indicated an increased yield of large and very large grade carrots over controls [86]. El-Abyad et al [87] described the use of three *Streptomyces* spp. in the control of bacterial, *Fusarium* and *Verticillium* wilts, early blight, and bacterial canker of tomato. The isolates used were *Streptomyces pulcher*, *Streptomyces canescens*, and *Streptomyces citreofluorescens*. In addition, tomato growth was observed to be significantly improved with the antagonistic *Streptomyces* spp. as a seed coating. An increased availability of growth regulators produced by the inoculum was the reason proposed for the improvement in tomato growth, although this was not formally tested. The information available on streptomycetes as plant growth promoters is limited, so is the information describing the possibility of their direct growth promotion mechanisms. Like most rhizobacteria, it seems highly probable that streptomycetes are capable of directly enhancing plant growth.

6.13. Phytohormone production

The production of the plant hormone indole-3-acetic acid (IAA) and the pathways of its synthesis by various *Streptomyces* sp., including *S. violaceus*, *Streptomyces scabies*, *S. griseus*, *Streptomyces exfoliatus*, *S. coelicolor*, and *S. lividans,* were described by Manulis et al [88]. While prior works had reported IAA synthesis in *Streptomyces* spp., this was the first confirmation of its production using modem analytical methods, such as high-performance liquid chromatography (HPLC) and gas chromatography (GC)–mass spectrometry (MS), and Manulis et al [88] provided a detailed description of the IAA biosynthetic pathways in *Streptomyces.* Aldesuquy et al [84] studied the effect of streptomycete culture filtrates on the growth of wheat plants that showed significant increase in shoot fresh mass, dry mass, length, and diameter, displayed statistically with certain strains at varying sample times. *Streptomyces olivaceoviridis* had a pronounced effect on yield components (spikelet number, spike length, and fresh

and dry mass of the developing grain) of wheat plants. This activity may be due to, at least in part, an increase in bioavailable phytohormones that are PGPR produced since ail PGPR strains (*Streptomyces rimosus*, *Streptomyces rochei* and *S. olivaceoviridis*) produced substantial amounts of exogenous auxins (IAA), as well as gibberellins and cytokinins.

6.14. Biolarvicides

Extensive use of chemical insecticides for controlling malaria, filaria, dengue, chickungunya, Japanese encephalitis, and other mosquitoes have resulted in hazards to the environment and caused development of resistance in vector mosquitoes. Accordingly, various biological control agents have gained importance with innumerable advantages over the chemical insecticides. At very low doses, these biolarvicides are highly effective against mosquito larvae and are completely safe to other nontarget organisms, environment, man, and wild life. Several varieties of microorganisms, including fungi, bacteria, and nematodes have been reported as strategies to biologically control the vectors. Specifically, Actinobacteria produce many important bioactive compounds of high commercial value and continue to be routinely screened for new bioactive substances. In a study made by Vijayan and Balaraman [89], extracellular secondary metabolites were produced from 35 different Actinobacterial isolates that showed high larvicidal activity against *Culex* and *Anopheles* mosquitoes. Dhanasekaran et al [20] reported that the isolates *Streptomyces* sp., *Streptosporangium* sp., and *Micropolyspora* sp. had high larvicidal activity against *Anopheles* mosquito larvae. Rajesh et al [90] synthesized silver nanoparticles from *Streptomyces* sp. GRD cell filtrate and observed larvicidal activity against *Aedes* and *Culex* vectors, which are responsible for the transmission of dengue and filariasis. Yet again, Rajesh et al [91] studied the larvicidal effect of Actinobacterial extracts against *Culex* larvae and found that 1000 ppm concentration of the isolate *Streptomyces* sp. KA13-3 showed 100% mortality and *Streptomyces* sp. KA25-A showed 90% mortality. Variety of other secondary metabolites from Actinobacteria, namely tetranectin [92], avermectins [93], macrotetrolides [94], and flavonoids [95] were found to be toxic to mosquitoes.

6.15. Odor and flavor compounds production

Actinomycetes have long been associated with musty odors in water but their actual contribution to odor in freshwater was unknown. But in late 1960s, secondary metabolites, geosmin and 2-methylisoborneol (MIB), were identified from actinomycete cultures [96] after which actinomycetes have gained considerable importance throughout the water industry as major sources of drinking water taste and odor. Gaines and Collins [97] studied the metabolites of *Streptomyces odorifer* and concluded that the earthy odor might be due to the production of a combination of trivial compounds, such as acetic acid, acetaldehyde, ethyl alcohol, isobutyl alcohol, isobutyl acetate, and ammonia. They emphasized that the other constituents contributing to the odor might also be produced. A number of odor-producing compounds have been identified from Actinobacteria (Table 6). Earthy odors in adequately treated water supplies cause concern among consumers, who may think water with these odors is unsafe to drink. These odors are the second most common cause of odor problems recorded by water utilities, behind chlorine.

Actinobacteria	Secondary metabolite	Odor type
Streptomyces sp.	Trans-1,10-dimethyl-trans-9-decalol (Geosmin)	Earthy
	1,2,7,7-tetramethyl-2-norbornanol	Musty
	6-ethyl-3-isobutyl-2-pyrone (mucidone)	Potato like
	2-isobutyl-3-methoxypyrazine or 2-isopropyl-3-methoxypyrazine	
Actinomadura sp.	(2-methylisoborneol)	Musty
Thermoactinomyces sp.	6-methyl-5-hepten-2-one	Potato like
Pseudonocardia sp. *Saccharomonospora* sp. *Thermoactinomyces* sp. *Thermomonospora* sp.	Dimethyl trisulfide	Potato like

Table 6. Odor-producing compounds from Actinobacteria

7. Harmful effects

7.1. Actinobacterial plant diseases

A number of significant plant diseases are caused by Actinobacteria. Actinobacteria currently assigned to the genus *Corynebacterium* [98] cause a variety of diseases (Table 7). Serodiagnosis has been used to detect and identify *Corynebacterium insidiosum* and *Corynebacterium sepedonicum*, but most of these Corynebacteria are still identified by inoculation tests in host plants. Most phytopathogenic Corynebacteria are considered to produce their antagonistic effects by the production of hormones, polysaccharides, and toxins, whereas the potential of some strains to form biosurfactants may help in attachment to the host. The taxonomy of the phytopathogenic Corynebacteria is unsettled, though some prefer to retain these organisms in the genus *Corynebacterium* [99]. Others accept that there is an overwhelming case for restricting *Corynebacterium* for animal pathogenic Corynebacteria and related strains and a consequent need to reclassify the plant pathogens. *Corynebacterium betae, Corynebacterium oortii,* and *Corynebacterium poinsettiae* are genetically identified with *Corynebacterium flaccumfaciens* [100] and have many properties in common with the genus *Curtobacterium* [100], which would serve as a suitable niche for them. *C. insidiosum* and *Corynebacterium michiganense*, also fell into a single genospecies, were recovered in a loose DNA homology group with *Corynebacterium nebraskense* and *Corynebacterium sepedonicum* and together with the latter probably from the nucleus of a new genus.

7.2. Actinobacterial human and animal diseases

Actinobacteria have proved to be the causal agents of many human and animal infections, which include a number of common and intensively studied diseases, such as diphtheria, tuberculosis, and leprosy. There is also a wide range of infections that are less well known;

Arthrobacter ilicis	B light of holly (flex opaca)
C. betae	Wilt and leaf spot of red beet (*Beta vulgaris*)
C. flaccumfaciens	Wilt of bean (*Phaseolus vulgaris*)
C. insidiosum	Wilt and stunting of alfalfa (*Medicago sativum*)
C. michiganense	Canker of tomato (*Lycopersicon esculentum*) and some other solanaceous plants
C. nebraskense	Wilt and blight of corn
C. oortii	Spot of tulip leaves and bulbs
Corynebacterium Poinselliae	Stem canker and leaf spot of poinsettia (*Euphorbia pulcherrima*)
Corynebacterium rathayi	Gumming of cereals
C. sepedonicum	Wilt and tuber rot of potato (*Solanum tuberosum)*
Nocardia vaccinii	Galls and bud proliferation in blueberry plants (*Vaccinium*)
Rhodococcus fascians	Leaf gall in many plants, fasciation of sweet pea
S. aureofaciens S. flaveolus. S. griseus	Common scab of potato
S. ipomoeae	Sweet potato scab
S. scabies, Streptomyces sp.	Common and russet scab of potatoes, sugar beet, etc

Table 7. Plant diseases caused by Actinobacteria

some, like actinomycosis and nocardiosis, are proving to be more clinically significant than previously thought. In addition, it is becoming increasingly evident that *Actinomyces* play a role in the etiology of caries and periodontal disease. Following are the human and animal diseases caused by Actinobacteria (Table 8).

Disease	**Actinobacteria**	**Site of infection**
Actinomycetoma	*A. madurae, pellerieri, Nocardia asteroides, Nippostrongylus brasiliensis, N. otitidiscaviarum, Streptomyces somaliensis*	Feet, legs, upper extremities, and other sites
Actinomycosis	*Actinomyces bovis, Actinomyces israelii, Arachnia propionica*	Cervicofacial, thoracic, abdominal, and uterine regions
Bacterial kidney disease	*Renibacterium salmoninarium*	Kidney, liver, spleen, and other internal organs
Bovine farcy	*Mycobacterium farcinogenes, Mycobacterium senegalense*	Lymphatic system
Dermatophilosis & streptothricosis	*Dermatophilis congolensis*	Skin
Diphtheria	*Corynebacterium diphtheriae*	Throat, occasionally wounds
Endocarditis	*Oerskovia turbata, Rothia dentocariosa*	Endocardium

Disease	Actinobacteria	Site of infection
Equine pneumonia	*Rhodococcus (Corynebacterium) equi*	Lung
Hypersensitivity pneumonitis	*Micropolyspora jaeni, Saccharomonospora viridis, Thermoactinomyces vulgaris*	Lung
Leprosy	*Mycobacterium leprae*	Skin
Mycobacterioses	Several *Mycobacterium* Species	Lungs, lymph nodes, and skin
Pulmonary nocardiosis	*Nocardia asteroides, rarely N. brasiliensis, possibly Nocardiopsis dassonvillei*	Lung
Systemic nocardiosis	*Nocardia asteroides, rarely N. brasiliensis*	Lung, central nervous system, kidney, muscle, and other tissues
Superficial nocardiosis	*Nocardia asteroides, N. brasiliensis, possibly Nocardiopsis dassonvillei*	Any part of body surface, especially the extremities
Purulent infections including abscesses	*Actinomyces,* (*Corynebacterium*) *pyogenes, Corynebacterium pseudotuberculosis*	Abscess formation in various organs (brain, spinal cord, and joints)
Pyelonephritis in cattle	*Corynebacterium renale*	Kidney
Tuberculosis	*Mycobacterium tuberculosis*	Lung

Table 8. Human and animal diseases caused by Actinobacteria

8. Conclusion

Actinobacteria is one of the dominant groups of microorganisms that produce industrially important secondary metabolites. A wide range of antibiotics in the market is obtained from Actinobacteria. Products such as enzymes, herbicides, vitamins, pigments, larvicides, phytohormones, and surfactants are produced by these several genera of Actinobacteria, which are of great commercial value. They are capable of degrading a wide range of hydrocarbons, pesticides, and feather waste, and their metabolic potential offers a strong area for research. However, many of the rare genera of Actinobacteria have been neither discovered from unexplored locations nor employed for their biotechnological and industrial potential. Thus, studies on unique ecological environments could yield molecules that could become future harbingers of green technology.

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Chapter 2

Isolation and Cultivation Methods of Actinobacteria

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Additional information is available at the end of the chapter

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Abstract

Actinobacteria (actinomycetes) have been received much attention, as these bacteria produce a variety of natural drugs and other bioactive metabolites. The distribution of actinomycetes in various natural habitats, including soil, ocean, extreme environments, plant, lichens and animals, is described. The collection and pretreatment of test samples from different sources, design principle of selective isolation media, selection of inhibitors, selective isolation procedures of special actinomycetes, and cultivation methods are introduced and discussed.

Keywords: Actinobacteria, isolation, cultivation

1. Introduction

Actinomycetes (actinobacteria) have been received much attention, as these bacteria produce a variety of natural drugs and other bioactive metabolites, including antibiotics, enzyme inhibitors, and enzymes. More than 22,000 bioactive secondary metabolites (including antibiotics) from microorganisms have been identified and published in the scientific and patent literature, and about a half of these compounds are produced by actinomycetes. Currently, approximately 160 antibiotics have been used in human therapy and agriculture, and 100–120 of these compounds, including streptomycin, erythromycin, gentamicin, vancomycin, vermectin, etc., are produced by actinomycetes [1, 2]. However, the use of general approaches to develop new drugs from actinomycetes is more and more difficult [3, 4]. Although a large number of microorganisms have been identified, described, screened, and used, more than 90% of all microorganisms remain uncultivable [5–8]. These uncultivable microbes might offer a new hope for the development of new drugs.

To overcome the challenges of drug development from microbes, new concepts based on genomics have been described, i.e., "new habitats, new methods, new species, new gene

clusters, new products and new uses" [4, 9]. In other words, novel microbes should contain new gene clusters synthesizing novel secondary metabolites. Many laboratories and companies have focused on new actinomycete sources from new habitats, such as oceans [10–15], extreme environments [16, 17], plants [18–20], faeces of animals [21-23] and lichens [24, 25], for the development of new drugs. So Baltz proposed a "renaissance in antibacterial discovery from actinomycetes"[26].

Becoming the uncultured to cultivable actinomycetes and providing new sources for the discovery of new drug leads are the tasks of this chapter.

Dispersion and differential centrifugation (DDC) and high-throughput methods (HTM) [27, 28] can be used for the isolation of actinomycetes. However, the dilution plate method as a key procedure for the isolation of actinomycetes will be described and discussed in this chapter.

2. Distribution of actinomycetes in nature habitats

More than thousands of test samples were collected from western China and Batic Sea, and the diversity of cultural actinomycetes was studied in our laboratories in recent decades. A part of the results is summarized in Table 1. Twenty-nine genera of actinomycetes were isolated and identified in soil samples collected from tropical rain forest in Xishuangbanna (Fig. 1D) and 19 genera from primeval forest in Grand Shangri-La. In contrast, only 13 genera were isolated from secondary growth forest in the Sichuan. The results showed that diversities of actinomycetes in primeval forest soil are more complex than secondary forest, and the diversities in tropical rain forest soil are remarkably complex than frigid forest. It is worth emphasizing extreme environments that have extreme acidity, alkalinity, salt, radioactivity, heat (hot springs), or cold (Polar Regions and snowy mountains); we found many unique microorganisms living in these environments [38]. Members of 21 actinobacteria were isolated from hypersaline soil in Qinghai and Gansu Province. *Haloactinopolyspora*, *Haloglycomyces*, *Jiangella*, *Myceligererans*, *Salinimicrobium*, *Streptomonospora*, *Yania*, and *Zhihengliuella* are novel genera published by our colleagues.

Rock Gypsum-Salt Forest (Gaolin) is located in Yuanjiang, Yunnan, China, only 3 km^2 and a special geological wonder. It is formed by various factors in a long term, and calcium sulfate is a main constituent part. The test samples were collected from there. Actinomycetes were isolated and identified. Twenty-five genera of actinobacteria were identified. It shows that the actinomycete community is very diverse (Fig. 1C).

Test samples were collected from 90 species of medical plant in Yunnan. Thirty-four genera of pure cultural actinobacteria were identified. Like this, 28 genera actinobacteria were identified from only three species of lichens (Fig. 1B).

Fifty-one genera of actinomycetes were identified in feces samples collected from 42 species of animals (Fig. 1A). One new genus, *Enteractinococcus*, was described and published. More than 250 compounds were found from animal fecal actinomycetes. These results unfolded a bright prospect.

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Habitats	Diversity of cultured actinomycetes	References
Subtropical every-green forest in Sichuan	*Actinomadura*, *Actinopolymorpha*, *Micromonospora*, *Mycobacterium*, *Nocardia*, *Nocardioides*, *Nonomurae*, *Promicromonospora*, *Pseudonocardia*, *Rhodococcus*, *Saccharomonospora*, *Streptomyces*, *Verrucosispora*	[29]
Primeval frigid forest in Grand Shangri-La	*Actinomadura*, *Actinopolymorpha*, *Agromyces*, *Allokutzneria*, *Arthrobacter*, *Dactylosporangium*, *Georgenia*, *Kocuria*, *Lentzea*, *Mycetocola*, *Nocardia*, *Nocardioides*, *Oerskovia*, *Promicromonospora*, *Pseudonocardia*, *Rhodococcus*, *Streptomyces*, *Streptosporangium*, *Tsukamurella*	[30]
Primeval tropical rain forest in Xishuangbanna	*Actinomadura*, *Actinoplanes*, *Actinopolymorpha*, *Actinomycetospora*, *Agrococcus*, *Agromyces*, *Arthrobacter*, *Citricoccus*, *Dactylosporangium*, *Friedmanniella*, *Kribbella*, *Lentzea*, *Microbacterium*, *Microlunatus*, *Micromonospora*, *Mycobacterium*, *Nocardia*, *Nocardioides*, *Nonomuraea*, *Oerskovia*, *Planosporangium*, *Promicromonospora*, *Pseudonocardia*, *Rhodococcus*, *Saccharopolyspora*, *Sphaerisporangium*, *Stackebrandtia*, *Streptomyces*, *Streptosporangium*	[31]
Hyper saline soil in Qinghai	*Actinopolyspora*, *Citricoccus*, *Corynebacterium*, *Haloactinopolyspora*, *Haloglycomyces*, *Isoptericola*, *Jiangella*, *Marinococcus*, *Microbulbifer*, *Myceligererans*, *Nesterenkonia*, *Nocardiopsis*, *Prauserella*, *Rhodococcus*, *Saccharomonospora*, *Salinimicrobium*, *Streptomonospora*, *Streptomyces*, *Thermobifida*, *Yania*, *Zhihengliuella*	[32]
Rock Gypsum-Salt Forest in Yuanjiang	*Actinoplanes*, *Actinokineospora*, *Aerococcus*, *Amycolatopsis*, *Arthrobacter*, *Brevibacterium*, *Cellulomonas*, *Glycomyces*, *Kineococcus*, *Kocuria*, *Kribbella*, *Microbacterium*, *Micrococcus*, *Micromonospora*, *Neisseria*, *Nocardia*, *Nocardioides*, *Nocardiopsis*, *Prauserella*, *Promicromonospora*, *Pseudonocardia*, *Rhodococcus*, *Saccharothrix*, *Streptomyces*	Qinyuan Li et al. unpublished
Baltic Sea in Kiel Bay in Germany	*Actinomadura*, *Actinoplanes*, *Amycolatopsis*, *Arthrobacter*, *Cellulomonas*,*Isoptericola*, *Kocuria*, *Microbacterium*, *Micromonospora*, *Myceligererans*, *Mycobacterium*, *Nocardiopsis*, *Promicromonospora*, *Rhodococcus*, *Streptomyces*	[33]
90 species of medicinal plant in Yunnan	*Actinocorallia*, *Actinomadura*, *Amycolatopsis*, *Arthrobacter*, *Blastococcus*, *Catellatospora*, *Dactylosporangium*, *Dietzia*, *Delftia*, *Glycomyces*, *Gordonia*, *Herbidospora*, *Janibacter*, *Jiangella*, *Kineococcus*, *Kineosporia*, *Lentzea*, *Microbacterium*, *Micrococcus*, *Micromonospora*, *Mycobacterium*, *Nocardia*, *Nocardiopsis*, *Nonomurae*, *Oerskovia*, *Phytomonospora*, *Plantactinospora*, *Plantactinospora*, *Pseudonocardia*, *Rhodococcus*, *Saccharopolyspora*, *Streptomyces*, *Streptosporangium*, *Tsukamurella*	[34]
3 species of lichens in Yunnan	*Actinomadura*, *Actinoplanes*, *Amnibacterium*, *Arthrobacter*, *Candidatus*, *Cellulomonas*, *Cellulosimicrobium*, *Curtobacterium*, *Corynebacterium*, *Friedmanniella*, *Kineococcus*, *Kocuria*, *Kribbella*, *Microbacterium*,	Yi Jiang et al., unpublished

Habitats	Diversity of cultured actinomycetes	References
	Micrococcus, Microlunatus, Micromonospora, Mycobacterium, Nocardia, Oerskovia, Pseudonocardia, Pseudosporangium, Rhodococcus, Saccharopolyspora, Saccharothrix, Streptomyces, Streptosporangium, Williamsia	
42 species of animal feces in Yunnan, China	*Actinocorallia, Actinotalea, Agrococcus, Arthrobacter, Blastococcus, Brachybacterium, Brevibacterium, Cellulomonas, Cellulosimicrobium, Citricoccus, Corynebacterium, Curtobacterium, Dietzia, Enteractinococcus, Gordonia, Gulosibacter, Isoptericola, Janibacter, Jiangella, Kineococcus, Kocuria, Labedella, Leucobacter, Luteococcus, Microbacterium, Micrococcus, Microlunatus, Micromonospora, Mobilicoccus, Mycobacterium, Nocardia, Nocardiopsis, Oerskovia, Patulibacter, Plantibacter, Promicromonospora, Pseudoclavibacter, Pseudonocardia, Rhodococcus, Saccharomonospora, Saccharopolyspora, Salinibacterium, Sanguibacter, Sphaerobacter, Streptomyces, Tessaracoccu, Tsukamurella, Verrucosispora, Williamsia, Yaniella, Zimmermannella*	[22]

Table 1. Diversity of cultured actinomycetes in different habitats cited from the study results in author's laboratories

A. Panda B. lichen C. Rock Gypsum-salt Forest in Yuanjiang D. Primeval tropical rain forest in Xishuangbanna

Figure 1. Sources of a part of test samples.

3. Basic principle for the isolation of actinobacteria

In general, the isolation of actinomycetes has three targets.

First is the study on the community of actinomycetes in a special environment. In this condition, all of actinomycetes as the pure cultures should be isolated and identified. In order to manage to this target, the isolation media used should be propitious to the growth of possible more actinomycetes, and other microbes do not grow. Three to five media with different components should be used. Inhibitors against Gram-negative bacteria and fungi should be added into the media.

Second is the isolation of special actinomycetes, for example, a known species or genus, or some kind of actinomycetes with special physiological characteristics, including the resistance to antibiotics, chemicals, alkaline, acid, salts, and high and low temperatures. The isolation media should meet the requirement of target actinomycetes and inhibit the growth of unwanted microbes at the same time. For example, in order to isolate halophytic and alkalophytic actinomycetes, the salt concentration of isolation media should be 15% to 25%, and the pH level of the media should be adjusted to 10 to 12.

Third is the isolation of unknown actinomycetes. Up to now, countless actinomycetes have been isolated and identified from various habitats in the whole world. Thus, isolating unknown actinomycetes is the most difficult but most important. It requires the restraint of the growth of not only Gram-negative bacteria, some Gram-positive bacteria, and fungi but also most of the common actinomycetes.

In order to isolate as more as unknown actinomycetes, researchers should be familiar with all of the knowledge about the physiology and taxonomies of actinobacteria and other microbes and the role of each isolation factor (including components and concentration of media, pH, inhibitor, cultural temperature, etc.), and they should have rich experience. Isolation procedures should be ceaselessly renewed and improved. The isolation method of actinomycetes is on road and has no end ever.

4. Collection and pretreatment of test samples

4.1. Collection of test samples from different sources

Actinomycetes occur as saprophytes in diverse natural habitats, including soil, lake, ocean, plant, and animal. Soil remains a fruitful source of novel actinobacteria. The numbers and kinds of actinobacteria found in soil and other substrates are greatly influenced by primary ecological factors, such as nutrient, aeration, pH, temperature, salinity, and moisture and organic matter content. Indeed, the success in isolating large numbers of specific actinobacteria can be highly dependent on the choice of environmental samples. It is best to collect the soil samples from pristine area, including primeval forest, saline, alkaline soils, and desert. Soil samples in depth 5–20 cm are collected and put in sterile paper or plastic bag.

Actinomycetes are widely distributed in ocean, and a large number of natural products were found from them. Sediment in deep ocean is collected with sampler, and the samples are put in sterilized glass bottle and conserve at 4°C.

Actinomycetes exist widely in plant. Novel plant endophytic actinomycetes, especially from traditional Chinese drug, are also a promising source of antimicrobial and antitumor agents. Fresh samples of different plant tissues are collected and immediately put in sterilized container. The fresh samples should be used for the isolation of actinomycetes as soon as possible.

Recently, Mohamed et al. analyzed the biosynthetic gene cluster in human microbiome and discovered new bioactive substance, lactocillin, and considered that human microbiome is a huge molecular drug house [35]. There are uncountable species of animal in the whole world; animal feces are a huger actinomycete community, and animal microbiome should be huger molecular drug house. Discovering new drug leads from actinomycetes of animal feces is very important and tempting. In order to isolate actinomycetes, the fresh fecal samples should be put in sterilized container, conserve at 4°C, and used for the isolation of actinomycetes as soon as possible.

4.2. Pretreatment of test samples

Pretreatment is very important for the selective isolation of actinomycetes, which grow slower than other bacteria and fungi. In general, pretreatment regimes select target actinomycetes by inhibiting or eliminating unwanted microorganisms. Several chemical and physical pretreatments have been used for the isolation of actinomycetes. Actinomycete spores are more resistant to desiccation than most bacteria; hence, simply air-drying soil, sediment, lichen, and fecal samples at room temperature will eliminate most unwanted Gram-negative bacteria, which might otherwise overrun isolation plates. Air-dried soil heated or soil suspensions heated treatment can be used for selectively isolating special actinobacterial taxa (Table 2).

Sample suspensions can be treated with ultrasonic waves at 180 W for 40′. It can release the saprophytes fixed by soil granule into the suspension, increased account of actinomycetes, and reduced bacteria in the sample [36] (Fig. 2).

Based on the differential resistant ability of actinobacterial spores to withstand treatment with chemicals, such as benzethonium chloride, chlorhexidine gluconate, phenol, SDS, and various antibiotics, these different chemicals were used to isolate special actinobacterial taxa. Treatment with these agents for 30 min at 30°C can kill Gram negative cells of aerobic, endospore-forming bacilli and pseudomonads, increase frequency of actinomycete, and reduce bacteria (Table 3).

Pretreatment	Target
Air-dried soil heated at 120°C for an hour	*Microbispora* *Streptosporangium* .
Air-dried soil heated at 100°C for 15 min	*Actinomadura* spp.
Water or soil suspensions heated at 45°C or 50°C for 10 min	*Streptomyces* spp.
Water or soil suspensions heated at 60°C for 30 min	*Micromonospora* spp.
Air-dried soil heated at 120°C for an hour	*Dactylosporangium* and *Streptosporangium* spp.
Air-dried soil heated at 28°C for a week	*Herbidospora cretea*
Soil suspension heated at 110°C for an hour	*Microtetraspora glauca*

Table 2. Selective heat pretreatments for the isolation of actinobacteria.

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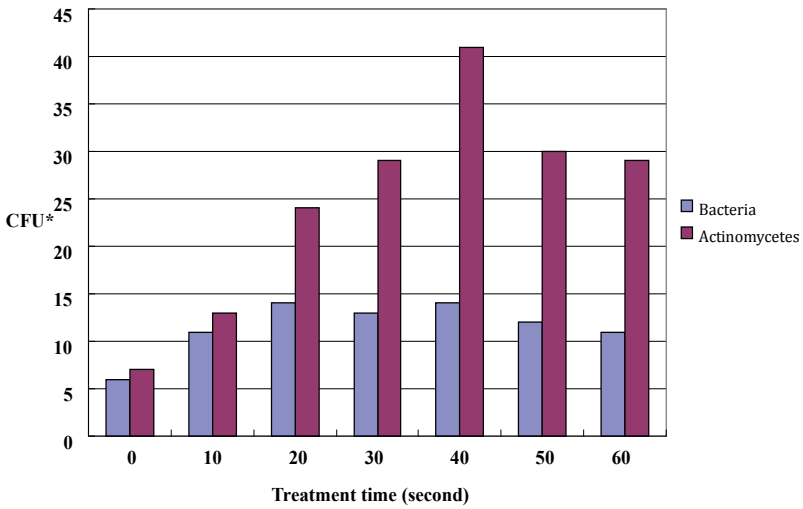

Figure 2. Influence of ultrasonic wave treatment to CFU between actinomycetes and other bacteria. *CFU = colony-forming units.

Pretreatment	CFU ($\times 10^5$/g)			
	Actinomycetes		Bacteria	
Control	117	100%	152	100%
YE, 2%	192	164%	169	110%
HA, 2%	183	156%	92	60%
CA, 1%	170	145%	165	109%
VA, 0.2%	159	136%	178	117%
ME, 0.2%	163	139%	136	87%
SDS, 0.05%	152	157%	10	7%
SDS + YE, 6%	183	157%	22	14%
SDS + HA, 1%	173	148%	9	5%
SDS + CA, 1.5%	153	131%	15	9%
SDS + VA, 0.6%	147	126%	19	12%
SDS + ME, 0.2%	158	135%	14	8%

YE = yeast extract; HA = humic acid; CA = acasein hydrolysate; VA = valine; SDS = sodium dodecyl sulfate; ME = mercaptoethanol.

Table 3. Chemical pretreatment for the isolation of actinomycetes.

5. Principle designing medium

The design of selective Isolation media needs to colligate each factor, such as isolation goals, target actinobacterial taxa, medium component, and inhibitors. The component (carbon and nitrogen sources) of selective isolation media can be formulated by using information from taxonomic databases and phenotypic databases. Appropriate inhibitors should be selected based on ability of target actinobacterial spores to withstand with antibiotics and chemicals.

6. Isolation methods of actinobacteria from different habitats

6.1. Isolation of thermophilic actinobacteria

In order to isolate thermophilic actinobacteria, the samples from hot spring or hot environments were air-dried at room temperature for 7 to 10 days, treated at 120°C for 1 h.

Isolation media (for 1000 ml distilled water):

YIM 14 improved Czapek medium: sucrose 20 g, $NaNO_3$ 2 g, K_2HPO_4 1 g, $MgSO_4$ $7H_2O$ 0.5 g, KCl 0.5 g, $FeSO_4$ $7H_2O$ 0.01 g, vitamin mixtures [37] 3.7 mg, agar 25 g, pH 7.2.

YIM 17 glycerol asparagine medium: L-asparagine 1 g, glycerol 10 g, K_2HPO_4 1 g, vitamin mixtures 3.7 mg, trace salt* 1 ml, agar 20 g, pH 7.2–7.4.

YIM 21 oatmeal medium: oatmeal 20 g (cook or steam 20 g oatmeal in 1000 ml distilled water for 20 min, filter through cheese cloth, and add distilled water to restore volume of filtrate to 1000 ml), vitamin mixture 3.7 mg, trace salts 1 ml, agar 20 g, pH 7.2.

*Trace salts solution: $FeSO_4 \cdot 7H_2O$ 0.1 g, $MnCl_2$ 0.1 g, $ZnSO_4 \cdot 7H_2O$ 0.1 g, distilled water 100 ml.

Fifty milligrams of potassium dichromate and 1 mg of penicillin are added in the isolation media.

The plate dilution method was used to isolate actinobacteria from the sample suspension. Approximately 0.1–0.2 ml of each sample (10^{-2} and 10^{-3} dilutions) was used to coat the plates and cultivated for 7 days in a moist chamber at 55°C. Single actinomycete colony is picked to inoculate an agar slant containing the same isolation medium.

6.2. Isolation of halophilic and alkalophilic actinobacteria

Media for halophilic actinobacteria (for 1000 ml distilled water):

YIM 6 Starch-casein medium: soluble starch 10 g, casein 0.3 g, KNO_3 2 g, $CaCO_3$ 0.02 g, $FeSO_4$ 10 mg, salt mixtures*, agar 25 g, pH 7.2–7.4.

YIM 17 glycerol asparagine medium (the same as above), salt mixtures*, agar 25 g, pH 7.2–7.4.

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YIM 47 soil extracts medium: soil extracts (soil 400 g, 120°C for 1 h, filter through cheese cloth, and add distilled water to restore volume of filtrate to 1000 ml), meat extracts 3 g, peptone 5 g, salt mixtures*, agar 25 g, pH 7.2–7.5.

T3 medium [38]: cellulose 10 g, casein 0.3 g, KNO_3 0.2 g, K_2HPO_4 1 g, $CaCO_3$ 0.02 g, $FeSO_4$ 10 mg, salt mixtures*, agar 25 g, pH 7.5.

Horikoshi medium [39]: glucose 10 g, yeast extracts 5 g, peptone 5 g, K_2HPO_4 1 g, $MgSO_4$ $7H_2O$ 0.2 g, salt mixtures*, agar 25, pH 7.2–7.5.

Salt mixtures (for 1 L): NaCl 100–150 g, KCl 20 g, $MgCl_2$ $6H_2O$ 30 g, $MgSO_4$ $7H_2O$ 5 g, K_2HPO_4 1 g.

Medium preparation: The salt mixtures are dissolved in a half volume of water, other components of medium are dissolved in other half volume of water, and they both have to be sterilized separately. Then the whole medium is spread into the plates after mixing the both while hot.

In order to isolate the alkalophilic actinomycetes, the five media as above can be used. However, it does not need the salt mixtures; pH should be adjusted to 10 to 11 with sterilized NaOH or Na_2CO_3 before spreading plate.

Twenty-five to 40 mg (for 1 L medium) of nalidixic acid should be added into all of media for inhibiting Gram-negative bacteria.

The growth of halophilic actinomycetes is always very slow. Thus, isolation media should be thicker, and the cultivation time of isolation plates should be lengthened to 20 to 35 days in keeping humidity. Single actinomycete colony is picked to inoculate a slant with the same isolation medium.

6.3. Isolation of acidophilic actinobacteria

Study on acidophilic actinomycetes is few worldwide, and only some report on acidophilic streptomycetes exists. The isolation of this actinomycete is difficult because of the fast growth of fungi and other bacteria in the test samples in isolation plate with lower pH. YIM 6, YIM 17, YIM 21, and YIM 47 media can be used for isolating acidophilic actinomycetes. Twenty-five grams of agar for 1 L should be used, and pH should be adjusted to 4.0 to 4.5 with sterilized HCl before spreading plate. All media are supplemented with filter-sterilized mixtures [50 mg cycloheximide + 50 mg nystatin + 20 mg nalidixic acid, or 50 mg sterilized potassium dichromate ($K_2Cr_2O_7$)].

6.4. Isolation of plant endophytic actinobacteria

Isolation media (for 1000 ml distilled water):

Water yeast extract medium [40]: yeast extract 0.25 g, K_2HPO_4 0.5 g, agar 18 g, pH 7.2.

Sodium propionate medium [41]:sodium propionate 1 g, L-asparagine 0.2 g, KH_2PO_4, 0.9 g, K_2HPO_4 0.6 g, $MgSO_4$ $7H_2O$ 0.1 g, $CaCl_2$ $2H_2O$ 0.2 g, agar 15 g, pH 7.2.

YIM 7 HV medium [37]:humic acid 1.0 g, Na_2HPO_4 0.5g, KCl 1.7 g, $MgSO_4$ $7H_2O$ 0.05 g, $FeSO_4$ $7H_2O$ 0.01 g, $CaCl_2$ 1 g, B-vitamins (0.5 mg each of thiamine–HCl, riboflavin, niacin, pyridoxin, Ca-pantothenate, inositol, *p*-aminobenzoic acid, and 0.25 mg of biotin), agar 18 g, pH 7.2.

Supplied in each medium were the following: 50–100 mg cycloheximide, 100 mg nystatin, 25 mg nalidixic acid, and 5 mg penicillin for 1000 ml.

Samples were air-dried for 48 h at room temperature and were then washed with an ultrasonic step (160 W, 15 min) to remove the surface soils and adherent epiphytes completely. After drying, the samples were subjected to a five-step surface sterilization procedure: 4- to 10-min wash in 5% NaOCl, followed by 10-min wash in 2.5% $Na_2S_2O_3$, 5-min wash in 75% ethanol, wash in sterile water, and final rinse in 10% $NaHCO_3$ for 10 min. After being thoroughly dried under sterile conditions, the surface-sterilized tissues were subjected to continuous drying at 100°C for 15 min [34]. Surface-treated tissues are aseptically crumbled into small fragments and homogenized with a glass homogenizer; 0.1 ml of the suspension at three dilutions is spread on the isolation plate. The inoculated plates were incubated at 28°C for 2 to 4 weeks.

6.5. Isolation of actinobacteria in animal feces

Isolation media (for 1000 ml distilled water):

YIM 7 HV medium

YIM 47 soil extracts medium

YIM 171 improved glycerol-asparagine medium: glycerol 10 g, asparagine 1 g, K_2HPO_4 H_2O 1 g, $MgSO_4$ $7H_2O$ 0.5 g, $CaCO_3$ 0.3 g, vitamin mixture of HV medium 3.7 mg, and agar 15 g, pH 7.2.

YIM 212 mycose-proline medium: mycose 5 g, proline 1 g, $(NH_4)_2SO_4$ 1 g, NaCl 1 g, $CaCl_2$ 2 g, K_2HPO_4 1 g, $MgSO_4$ $7H_2O$ 1 g, vitamin mixtures, agar 15 g, pH 7.2.

YIM 601 improved starch-casein medium: solution starch 10 g, casein 0.3 g, KNO_3 2 g, $MgSO_4$ $7H_2O$ 0.05 g, NaCl 2 g, K_2HPO_4 2 g, $CaCO_3$ 0.02 g, $FeSO_4$ 10 mg, vitamin mixtures, agar 15 g, pH 7.2~7.4.

Fresh fecal samples were collected. The samples were immediately transferred to sterile glass dishes and dried for 10 days at 28°C. Two grams of each dried sample was pretreated at 80°C for 1 h and subsequently dissolved in 18 ml of sterile water containing 0.1% $Na_4P_2O_5$, followed by shaking at 220 rpm/min for 60 min. The suspension is treated with ultrasound waves for 40 s at 150 W before coating. The suspension was diluted from 10^{-1} to 10^{-7}, and three dilutions, 10^{-5}, 10^{-6}, and 10^{-7}, were used for isolating actinomycetes.

The abundance of Gram-negative bacteria in animal feces presents a major challenge for the isolation of fecal actinobacteria. To eliminate Gram-negative bacteria and fungi and to obtain more unknown actinobacteria, some key points for sampling and isolation should be given attention.

First, based on the results of previous experiments, it is best to collect fresh fecal samples from wild animals living in original habitats. Second, the fresh samples should be dried at 25–28°C for 7 to 10 days. Third, the dried samples should be treated for 60 min at 80°C, and the fecal suspension should be treated with ultrasound waves for 40 s at 150 W before coating [34]. Fourth, potassium bichromate 50 mg and 5 mg penicillin or nystatin 50 mg, nalidixic acid 20 mg, and 5 mg penicillin per 1 L should be added into isolation medium to inhibit the growth of Gram-negative bacteria and fungi. Fifth, the samples should be diluted to 10^{-5}, 10^{-6}, and 10^{-7}, and the optimum dilution concentration for each animal fecal sample should be determined in advance. Sixth, YIM 212, YIM 171, and HV medium are better for the isolation of fecal actinobacteria, and these media should be improved and constantly updated with respect to different samples. Seventh, all experiments should be performed under strict sterile conditions for avoiding spread of pathogen.

6.6. Isolation of actinobacteria associated lichens

Isolation media (for 1000 ml distilled water)

YIM 6 starch-casein medium

YIM 171 improved glycerol-asparagine medium

YIM 709 Fungus polysaccharides medium: Chinese caterpillar fungus polysaccharides 1 g, $(NH_4)_2SO_4$ 2.64 g, NaCl 2 g, KCl 2 g, $MgCl_2$ $6H_2O$ 2 g, K_2HPO_4 1 g, KNO_3 0.2 g, $CaCO_3$ 0.2 g, $FeSO_4$ 10 mg, vitamin mixtures, trace salts 1 ml, agar 15 g, pH 7.5.

YIM 711 Casein Soybean peptone medium: casein 1.5 g, soybean peptone 0.5 g, K_2HPO_4 H_2O 1 g, $MgSO_4$ $7H_2O$ 0.5 g, $CaCO_3$ 0.3 g, NaCl 5 g, vitamin mixtures, agar 15 g, pH 7.5.

Inhibitors: all media were supplemented with filter-sterilized mixtures of 50 mg cycloheximide, 50 mg nystatin, and 25 mg nalidixic acid as inhibitors against fungi and Gram-negative bacteria.

The plate dilution method was used to isolate the actinobacteria. Two grams of each dried sample was grinded with a sterile glass homogenizer and dissolved in 18 ml of sterile water containing 0.1% $Na_4P_2O_5$, followed by shaking at 220 rpm/min for 60 min. The suspension was treated with ultrasound waves for 40 s at 150 W before coating. The suspension was diluted from 10^{-1} to 10^{-5}, and 0.1 ml of three dilutions, 10^{-3}, 10^{-4}, and 10^{-5}, was used to coat the plates and cultivated for 10 to 25 days at 28°C. Subsequently, single actinomycete colony was picked up and inoculate to a slant with the same isolation medium.

6.7. Isolation of rare actinobacteria

The actinomycetes except streptomycetes are named rare actinomycetes. In recent years, a large number of novel bioactive substances were discovered from the rare actinomycetes. Thus, isolation methods of rare actinomycetes have been received much attention.

1. Basic media: YIM 7 HV medium, YIM 212 histidine–raffinose medium (histidine 1 g, raffinose 5 g, K_2HP_4 $3H_2O$ 1 g, $MgSO_4$ $7H_2O$ 0.5 g, agar 20 g, pH 7.2), oligotrophic medium

(peptone 1 g, ye$_a$st extracts 0.5 g, K_2HPO_4 H_2O 1 g, $MgSO_4$ $7H_2O$ 0.5 g, $CaCO_3$ 0.3 g, NaCl 5 g, vitamin mixtures, agar 15 g, pH 7.5), and minimal medium:glucose 0.5 $_g$, yeast extract 0.5 g, $MgSO_4$ $7H_2O$ 0. 5 g, NaCl 0.5 g, K_2HPO_4 1 g, agar 15 g, pH 7.5–8.0. Special carbon or nitrogen sources (e.g., chitin, lignin, xylan, methanol, propionate, keratin, coconut milk, special amino acids, etc.) can be used to replace the carbon or nitrogen of the four media.

2. Various chemicals or antibiotics (Table 4) can be used for the selective isolation of different rare actinomycetes; for example, leucomycin can be used for isolating selectively the members of *Actinomadura* and *Streptosporangium*; Tunicamycin for *Actinoplanes*, *Dactylosporangiu*, and *Micromonospora*.

Chemicals	Target genera	Chemicals	Target genera
Bruneomycin	*Actinomadura*	Benzoate	*Micromonospora*
Streptomycin	*Actinomadura*	Polymyxin	*Streptomyces*
Gentamicin	*Actinomadura*	Kanamycin	*Microtetraspora*
	Streptosporangium	Nalidixic acid	*Microtetraspora*
Leucomycin	*Streptosporangium*	Nofloxacin	*Microtetraspora*
Fradiomycin	*Actinokineospora*	Penicillin	*Saccharothrix*
Kanamycin	*Actinokineospora*	Neomycin sulfate	*Amycolatopsis*
	Thermomonospora	Lysozyme	*Streptoverticillium*
Nalidixic acid	*Actinokineospora*	Novobiocin	*Glycomyces*
Trimethoprim	*Actinokineospora*	Streptomycin	*Glycomyces*
Tellurite	*Actinoplanes*	Lincomycin	*Micromonospora*
Tunicamycin	*Actinoplanes*	Novobiocin (25°C)	*Micromonospora*
	Dactylosporangium	(50°C)	*Thermomonospora*
	Micromonospora	Oxytetracycline	*Streptoverticillium*
Rifampicin(30°C)	*Actinomadura*	Rubomycin	*Actinomadura*
(50°C)	*Saccharomonospora*	Tetracyclines	*Nocardia*

Table 4. Selective chemicals for the isolation of rare actinomycetes.

3. Combination of Chemical pretreatment and different media can isolate different rare actinomycetes. For example, HV medium with chloramine T treatment can isolate the members of *Herbidospora*, *Microbispora*, *Microtetraspora*, and *Streptosporangium* (Table 5)

Chemical treatment	Target genera
Phenol, benzethonium chloride, and chlorhexidine gluconate	*Microbispora, Streptomyces, Streptosporangium*
Quaternary ammonium compounds	*Mycobacterium, Rhodococcus*
Antibiotics	
Nalidixic acid, penicillin G	*Rhodococcus*
Kanamycin, nalidixic acid, trimethoprim	*Actinokineospora*
Gentamicin	*Streptosporangium, Actinomadura, Micromonospora*
Novobiocin	*Actinoplanes, Thermoactinomyces*
Penicillin, nalidixic acid	*Saccharothrix*
Rifampicin, Streptomycin, Kanamycin	*Actinomadura*
Tunicamycin	*Micromonospora*
Isolation media	
Humic acid vitamin agar (HV agar) with Chloramine T treatment	*Herbidospora, Microbispora, Microtetraspora, Streptosporangium*
Hair hydrolysate vitamin agar	*Actinoplanes, , Microbispora, Micromonospora, Streptosporangium*
HV agar containing nalidixic acid with SDS and yeast extract treatment	*Actinomadura, Microbispora, Micromonospora, Microtetraspora, Streptosporangium, Nocardia*

Table 5. Combinatory methods for the isolation of rare actinomycetes.

7. Cultivation of actinobacteria

7.1. Liquid fermentation

The cultivation here is limited to small liquid and solid fermentation for studying the bioactive substances produced by actinomycetes. Fermentation is extremely important procedure for the discovery of new drug leads. Different strains need different fermentation conditions, including components, concentration, and pH of broth, and time, temperature, and aeration of fermentation. In general, the goals of the fermentation are as follows:

1. All of potential bioactive substances in actinomycete strains should be produced in fermentation broth as much as possible.
2. Studying main or target compounds should be produced as much as possible.

3. Background of the fermentation broth should be as less as possible for eliminating the obstruction from broth itself.

The following fermentation broths can be used for studying the bioactive substances of actinomycetes. Each strain should be fermented with 4 to 8 broths for 4 to 7 days in choosing the optimum broth and fermentation times.

7.1.1. Seed broth (for 1000 ml water)

YIM 38 broth: yeast extracts 4 g; glucose 4 g; malt extracts 10 g; thiamine–HCl, riboflavin, niacin, pyridoxin-HCl, inositol, calcium pentothenate, *p*-aminobenzoic acid, each 0.5 mg, and biotin 0.25 mg; pH 7.2.

YIM 306 broth: glucose 10.0 g; glycerol 10.0 g; casamino acids 15.0 g; oatmeal 3.0 g; peptone 10.0 g; yeast extract 5.0 g; $CaCO_3$ 1.0 g; pH 7.0.

Fermentation time of seed broth on shaker is 36–60 h.

7.1.2. Fermentation broth

YIM 61 broth: soybean meal 20 g; peptone 2 g; glucose 20 g; soluble starch 5 g; yeast extracts 2 g; NaCl 4 g; K_2HPO_4 0.5 g; $MgSO_4$ $7H_2O$ 0.5 g; $CaCO_3$ 2 g; pH 7.8.

YIM 301 broth: soluble starch 24.0 g; meat extracts 3.0 g; yeast extracts 5.0 g; peptone 3.0 g; glucose 1.0 g; $CaCO_3$ 4.0 g; pH 7.0.

YIM 302 broth: soybean meal 20 g, mannitol 20 g; pH 7–7.5.

YIM 305 broth: mannitol 30.0 g; glucose 10.0 g; yeast extracts 5.0 g; $(NH_4)C_4H_4O_4$ (ammonium succinate) 1.0 g; K_2HPO_4 1.0 g; $MgSO_4$ $7H_2O$ 0.1 g; pH 7.0.

YIM 307 broth: mannitol 20.0 g; peptone 20.0 g; pH 7.5.

YIM 308 broth: glucose 10.0 g; meat extract 3.0 g; peptone 3.0 g; soluble starch 20.0 g; yeast extract 5.0 g; $CaCO_3$ 3.0 g; pH 7.0.

YIM 310 broth: glucose 5.0 g; peptone 3.0 g; soluble starch 10.0 g; yeast extract 3.0 g; $CaCO_3$ 2.0 g; NH_4 NO_3 3.0 g; pH 7.2.

YIM 312 broth: glucose 10.0 g; glycerol 10.0 g; cornsteep powder 2.5 g; peptone 5.0 g; soluble starch 10.0 g; yeast extract 2.0 g; $CaCO_3$ 3.0 g; NaCl 1.0 g; pH 7.3.

7.2. Solid fermentation

Solid fermentation procedures were also used for cultivation of actinomycete sometimes in research stage. The content of bioactive substances produced by actinomycetes in solid fermentation is more than in liquid fermentation. A handy method is introduced as follows: rice 100 g + YIM 61 broth 100 ml, sterilized for 1 h

Five milliliters of seed broth was inoculated into solid medium, mixed, and incubated for 5 to 7 days at 28°C. Figure 3 is the photography of two strains of streptomycetes with solid

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fermentation. The optimum component of solid medium for different actinomycetes is different from each other. It is has to emphasize that no all of actinomycetes can grow in solid fermentation.

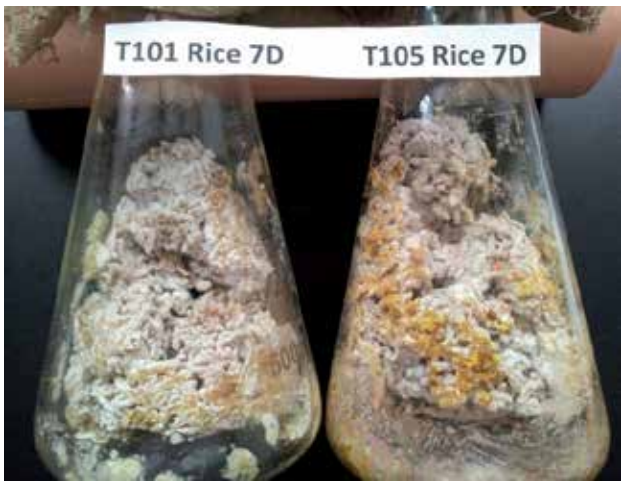

Figure 3. Solid fermentation of two streptomycete strains in rice+YIM 61 broth for 7 days at 28°C.

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Chapter 3

Morphological Identification of Actinobacteria

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Additional information is available at the end of the chapter

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Abstract

Actinobacteria is a phylum of gram-positive bacteria with high G+C content. Among gram-positive bacteria, actinobacteria exhibit the richest morphological differentiation, which is based on a filamentous degree of organization like filamentous fungi. The actinobacteria morphological characteristics are basic foundation and information of phylogenetic systematics. Classic actinomycetes have well-developed radial mycelium, which can be divided into substrate mycelium and aerial mycelium according to morphology and function. Some actinobacteria can form complicated structures, such as spore, spore chain, sporangia, and sporangiospore. The structure of hyphae and ultrastructure of spore or sporangia can be observed with microscopy. Actinobacteria have different cultural characteristics in various kinds of culture media, which are important in the classification identification, general with spores, aerial hyphae, with or without color and the soluble pigment, different growth condition on various media as the main characteristics. The morphological differentiation of actinobacteria, especially streptomycetes, is controlled by relevant genes. Both morphogenesis and antibiotic production in the streptomycetes are initiated in response to starvation, and these events are coupled.

Keywords: Actinobacteria, Morphology, Morphological characteristics, Cultural characteristics

1. Introduction

The history of the classification of prokaryote clearly demonstrates that changes were caused by the availability of new techniques [1]. The development of prokaryotic classification has experienced different stages: (i) the classical or traditional classification mainly based on

microbial morphological traits, growth requirements, physiological and biochemical features [2]; (ii) numerical taxonomy analyzing huge volumes of phenotypic data to derive meaningful relationships amongst a large number of microorganisms can be carried out using computer programs [3, 4]; (iii) chemotaxonomic methods studied the chemical variation in actinobacteria and used chemical characters in classification and identification, and it dealt with the discontinuous distribution of specific chemicals, especially amino acids, lipids, sugars, proteins, and other substances in whole cells, parts of cells or fermentation products, and with enzymes [5, 6]; (iv) genotypic classification based on genetic relatedness, inferred mainly from DNA-DNA hybridization (DDH) and comparative sequence analyses of homologous macromolecules, especially, rRNA [7, 8]. In recent years, more and more genotypic approaches were applied on the classification of actinobacteria, such as multilocus sequence analyses (MLSA) [9], average nucleotide identity (ANI) [10, 11], and whole genome analysis [10, 12-14]. Recently, the most widely accepted system is the polyphasic approach [15]. This approach combines as many different data as possible, for instance, phenotypic, chemotaxonomic, genotypic, and phylogenetic information. The modern classification method is an important means to understand the biological origin and species diversity. On one hand, the quantitative determination results are more objective; on the other hand, the research results of polyphasic taxonomy not only enrich the taxonomic content greatly, but also enrich the essence of life phenomenon. But the characterization of a strain is a key element in actinobacteria systematics in any period and prokaryotic morphologies are consistent with their phylogenetic reconstructions [16, 17].

Actinobacteria are currently characterized using the polyphasic approach that brings together a variety of phenotypic, chemotaxonomic, and genotypic data that comprise the formal description of a novel taxon. The key elements that should be acquired and analyzed in characterization studies of prokaryotes were outlined [18]: the phenotypic features are the foundation for description of taxa. Most actinobacteria are characterized and classified on the basis of their morphology in the first place. The morphological characteristics are still one of the most basic indexes which provide in-depth information on a taxon.

2. The basic morphological characteristics of actinobacteria

Actinobacteria display the greatest morphological differentiation among gram-positive bacteria; however, the cell structure of actinobacteria are typical prokaryotes and totally different with fungi. The whole structure of a hyphae cell corresponds to bacterial organization: the cytoplasm contains genomic DNA regions, ribosomes, and various inclusions, presumably reserve substances such as polyphosphates, lipids, or polysaccharides. Classic actinomycetes have well-developed radial mycelium. According to the difference of morphology and function, the mycelia can be divided into substrate mycelium and aerial mycelium (Figure 1). Some actinobacteria can form complicated structures, such as spore, spore chain, sporangia, and sporangiospore. The growth and fracture modes of substrate mycelium, the position of spore, the number of spore, the surface structures of spore, the shape of sporangia, and whether sporangiospore have flagella or not are all important morphological characteristics of actinobacteria classification.

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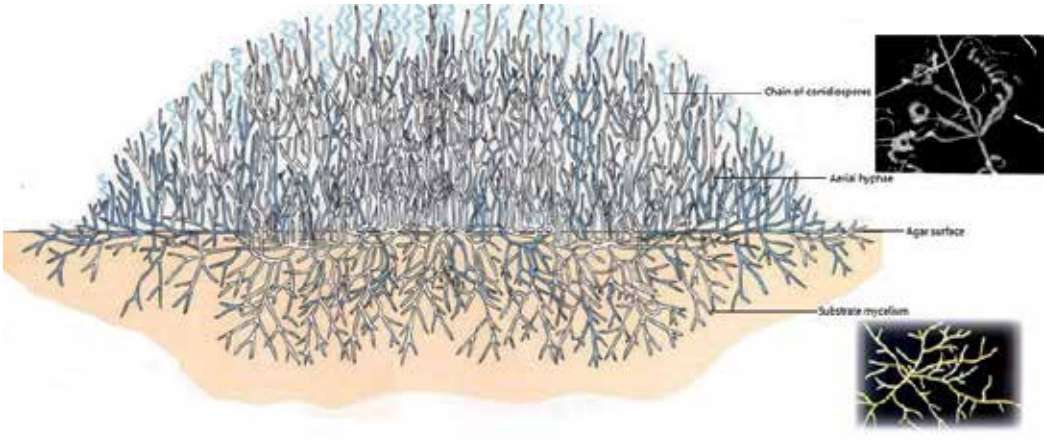

Figure 1. Actinomycetes colony growing on agar (common morphology of actinomycetes, the cross section of an actinomycete colony showing the substrate mycelium and aerial mycelium with chains of conidiospores).

2.1. Substrate mycelium

As known as vegetative mycelium or primary mycelium, the substrate mycelium grows into the medium or on the surface of the culture medium. The main function of the substrate mycelium is the absorption of nutrients for the growth of actinobacteria. Under the microscope, the substrate mycelia are slender, transparent, phase-dark, and more branched than aerial hyphae. The single hyphae is about 0.4 to 1.2 μm thick, usually do not form diaphragms and fracture, capable of developing branches. Minority groups (such as *Nocardia*), rudimentary to extensively branched like the roots, substrate hyphae often fragment in situ or on mechanical disruption into coccoid to rod-shaped, nonmotile elements when grown to a certain stage (Figure 2). The *Actinosynnema* are differentiated into substrate mycelia with long-branching hyphae that penetrate the agar and also grow into and form synnemata (Figure 3). In some genus, the hyphae form sclerotium (Figure 4).

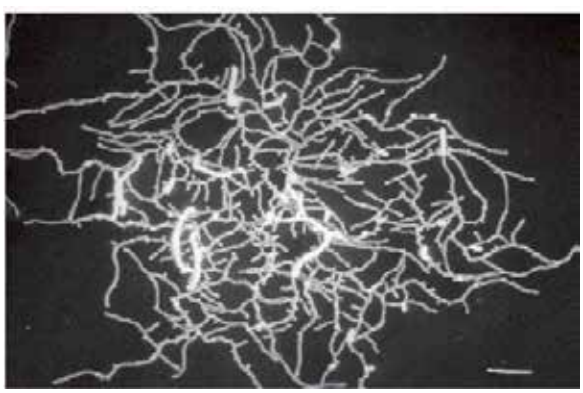

Figure 2. The fragmentation of substrate mycelium and true branching of *Nocardiaasteroides*. (Y. Mikami). [19]

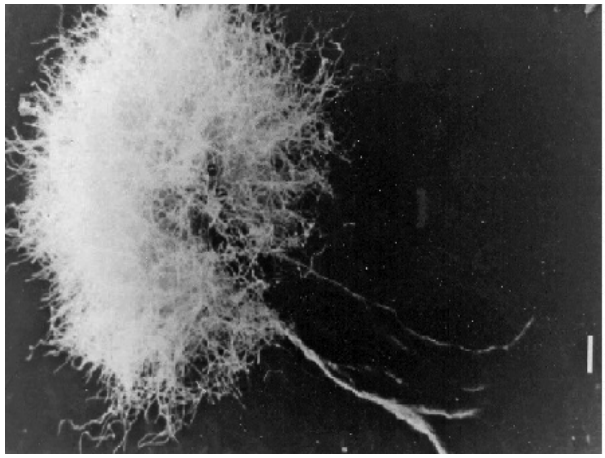

Figure 3. *Actinosynnema mirum* IFO 14064^T (by T. Hasegawa & T. Tamura). Synnemata are formed on medium. [19]

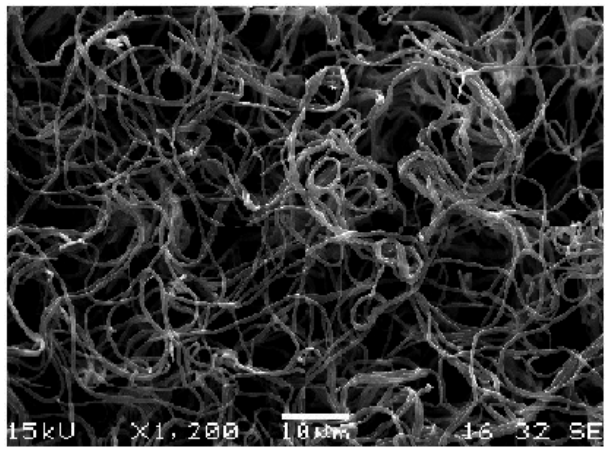

Figure 4. *Streptoalloteichus tenebrarius* NBRC 16177^T (by T. Tamura). [19]

The substrate mycelia are white, yellow, orange, red, green, blue, purple, brown, black, and other colors; some hyphae can produce water-soluble or fat-soluble pigment. The water-soluble pigment can seep into culture medium, which make the medium with the corresponding color. The non-water-soluble (or fat-soluble pigment) make the colony with the corresponding color. The color of the substrate mycelia and whether there are soluble pigments provide important references in the determination of new species.

2.2. Aerial mycelium

Aerial mycelium is the hyphae that the substrate mycelium develops to a certain stage, and grows into the air. Sometimes, aerial hyphae and substrate mycelia are difficult to distinguish.

This is easy to distinguish by an impression preparation on a cover slip, viewed in a dry system with a light microscope: substrate hyphae are slender, transparent, and phase-dark; aerial hyphae are coarse, refractive, and phase-bright. The hyphae of the aerial mycelium are characterized by a fibrous sheath, except the genera *Pseudonocardia* and *Amycolata* [20]. Ultramicroscopic, it is composed of fibrillar elements and short rodlets, forming a characteristic pattern. The fibrous sheath is also present on sporulation aerial hyphae, causing the different surface ornamentations of the spore [21, 22]. Forming all kinds of actinobacteria aerial hyphae is depending upon the species characteristics, nutritional conditions, or environmental factor. The aerial mycelium of some genus develops to a certain stage in the top form spore chain, which is a reproductive hyphae producing spore.

2.3. Spore chain

Actinobacteria grow to a certain stage, differentiated in its aerial hyphae, can form reproductive hyphae called spore-bearing mycelium. Indeed, this type of spore formation occurs in most actinobacteria genera. According to observation [23], spore chains can be divided morphologically respecting their length and number of spore: di- or bisporous with two spores, oligosporous with a few spore, and poly-sporous with many spores. Actinomycete spore chain length, shape, position, color are the important basis for classification.

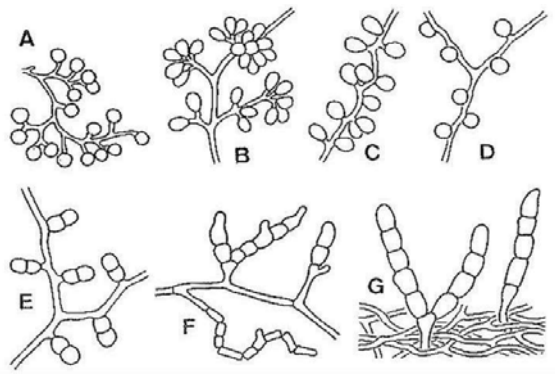

Figure 5. Single spore production and spores in short chains [24] Monosporous: (A) *Micromonospora*, (B) *Thermomonospora*, (C) *Saccharomonospora*, (D) *Thermoactinomyces*. Disporous: (E) *Microbispora.* Oligosporous: (F) *Nocardia brevicatena*, (G) *Catellatospora.*

The monosporous is the mode of single spore production. This form occurs in various suprageneric groups, represented by several well-known genera, such as *Micromonospora, Thermomonospora, Saccharomonospora*, and *Thermoactinomyces* (Figure 5, Figure 6). They all are developed from the blown-out end of a hyphal branch. The disporous chain contains a longitudinal pair of spores. The species of the genus *Microbispora* are representative of this type of sporulation (Figure 5, Figure 6). The spores are arranged either directly on the aerial hyphae or on very short side branches. The spore formation is initiated by lateral budding along an aerial hypha, producing short side branches. Oligosporous actinomy-

cetes develop short spore chains. The majority of the representatives have 7 to 20 spores per chain; at least there are 3 spores (Figure 5, Figure 6). The chains can be straight, hooked with open loops or arranged in irregular spirals having one to four turns. *Nocardia brevicatena* forms short chains of 2 to 7 spores and spore chains may be branched. The substrate mycelia tend to fragment. A reinvestigation of the spore-producing structures has revealed irregularly curled short spore chains in clusters [25].

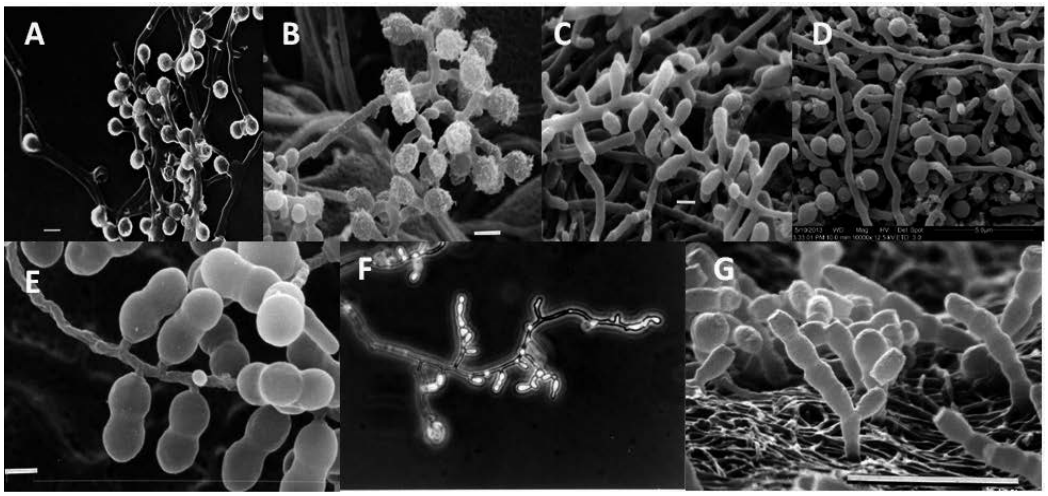

Figure 6. Microgram of single spore production and spores in short chains. [19] *(A) Micromonospora sp.* SF2259^{T} (by S. Amano, J. Yoshida & T. Shomura) *(B) Thermobifida alba* JCM 3077^{T} (by M. Hayakawa, H. Iino & H. Nonomura) *(C) Saccharomonospora viridis* IFO 12207^{T} (by M. Hayakawa, H. Iino & H. Nonomura) *(D) Thermoactinomyces daqus* H-18 (by Su Y. et al.). [26] *(E) Microbispora rosea* JCM 3006^{T} (by M. Hayakawa, H. Iino & H. Nonomura) *(F) Nocardia brevicatena* A444 (by G. Vobis) *(G) Catellatospora sp*. MB-VE 1321 (by G. Vobis)

The genus *Streptomyces* has classical polysporous, which form long chains frequently having more than 50 spores. The spores of *Streptomyces* and other polysporous actinomycetes are often called arthrospores [27]. The sporulating aerial hyphae of *Streptomyces* can be differentiated into the following main types (Figure 7, Figure 8): (A) *Rectiflexibiles* type, straight or flexuous spore chains, partly in fascicles; (B) *Retinaculiaperti* type, spore chains with hooks, open loops or short, irregular spirals having 1 to 4 turns; (C) *Spira* type, spore chains in spirals demonstrating two different subtypes: (a) Closed, compact spiral and (b) open, loose, and stretched spirals; (D) *Verticillati* type, spore chains formed in whorls and branched in umbels. Another typical genus that forms spores in long chains is *Nocardiopsis*, which has well-developed aerial hyphae, which may either be straight-flexuous or zigzag shaped, fragmenting completely into spores of various lengths [28].

The length, shape, position, and color of actinobacteria pore chain are an important basis for classification. Spore chains of the genus *Streptomyces* have various types of spore-bearing structures: straight, flexous, fascicied, monovericillate (no spirals), open loops (primitive spirals hooks), open spirals, closed spirals, monoverticillate (with spirals), biverticillate (no spirals), biverticillate (with spirals). Mature spores shows a variety of colors such as white, gray, yellow, pink, lavender, blue or green, and so on.

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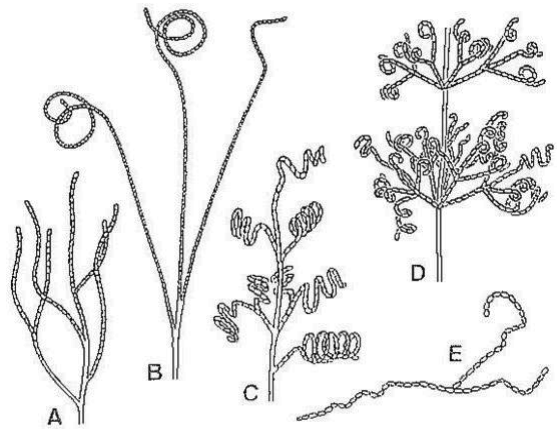

Figure 7. Spore production in long chains. [24] *Streptomyces:* (A) *Rectiflexibiles* type, (B) *Retinaculiaperti* type, (C) *Spira* type, (D) *Verticillati* type (Hütter, 1967). *Nocardiopsis*: (E) fragmenting branched aerial hyphae.

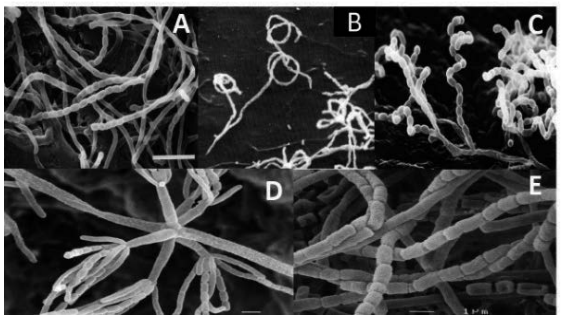

Figure 8. Microgram of spore production in long chain. [19] (A) *Rectiflexibiles* spore chains of *Streptomyces actuosus* U 227 (by T. Mikawa & R. Sashida) (B) Looped (*Retinaculiaperti*) spore chains of *Streptomyces vinaceus*. (C) *Spira* spore chains of *Streptomyces* sp. SF 2587 (by T. Shomura, J. Yoshida & S. Amano) (D) *Verticillati* spore chains of *Streptomyces verticillus* AT 291 (by T. Harada & Masa Hamada) (E) Fragmenting branched aerial hyphae of *Nocardiopsis lucentensis* IFO 15854^T (by Y. Gyobu)

2.4. Spore

The division of a hyphae and the production of a spore start with the formation of a cross-wall. In general, there are three kinds of methods of actinomycetes sporulation process (Figure 9): (i) when substrate hyphae are fragmented, the septum, which is known as a split septum, may occur and form spore, like the genus *micromonospora*. (ii) Spores are formed by septation and disarticulation of pre-existing hyphal elements with a thin fibrous sheath. The spore wall is formed, at least in part, from wall layers of the parent hypha; this is termed as *holothallic development* [29], and was found to be typical for many other spore actinomycetes, like the genus *Streptomyces*. (iii) Globose spores are formed in aerial and substrate mycelium and product spore wall, such as some strains of *Thermoactinomyces.* The spores are classical endospores with all the properties of bacterial endospores, relative to the formation process,

ultrastructure, and physiology. Aside from the mycelial growth, spore formation is the most important morphological criterion that can be used to recognize an actinomycete. Conventionally, the formation of spores is restricted to the morphological group of sporoactinomycetes, where sporulation takes place in well-defined parts of the mycelium. It is known that a number of different genes are involved in spore formation [30, 31] and that different cultivation conditions can have an influence on the spore formation.

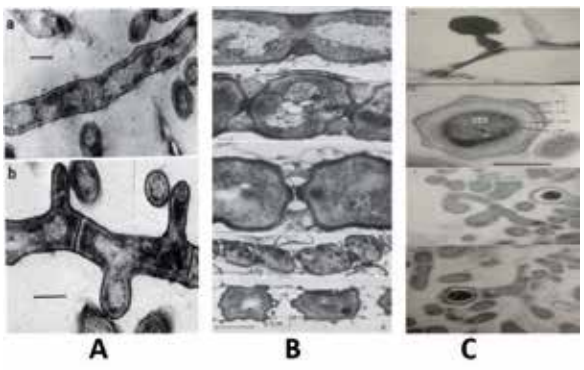

Figure 9. Models of spore formation. (A) Electron micrographs of *M. chalcea* hyphae. Bar, 250 nm. (a) Substrate nonramified hypha showing a vegetative septum. (b) Reproductive hypha showing frequent septa, two straight ramifications which will originate sporophores, a young sessile spore not yet individualized from the hypha, and a small ramification primordium. Substrate mycelium cell wall membrane, forming spores cell wall of substrate mycelia produce diaphragm, form spores [32]. (B) Electron micrographs of sporogenesis *Streptomyces melanochromogenes*. Stage 1: Initiation of septum formation, Stage 2: Septation. Stage 3: Delimination of the spore compartments. Stage 4: Separation and release of the spores. G gap; N nozzle; *rN* reduced nozzle; *Nu* nucleoid; *PL* primary spore wall layer; *SL* secondary spore wall layer; *SM* amorphous septal material; *rSM* remnant of the amorphous septal material; *SS* surface sheath. *Arrows:* initiation points of the septa [33]. (C) Electron micrographs of sporulation *Thermoactinomyces*. (A), the spores are true endospores with all the properties of bacterial endospores. (B), the mature endospore consists of an inner forespore membrane (im), cortex (co), inner spore coat (ic), and an outer spore coat (oc). (C), Spore formation starts with septation and engulfing of a portion of cytoplasm with nuclear material, terminally on short sporophores. (C, D) During the process of maturation, the spore is always surrounded by the mother cell [24].

The characteristics of spores have played a very important role in species descriptions for many years. The spores produced individually or in short chains are in general thicker than the hyphae, while those which are developed in long chains usually have the same diameter as the hyphae. Spores are about 1 to 2 μm thick and vary in term of shape and surface characteristics (Figure 10). Common spore morphology is globose, ovoid, coliform, rod-shaped, allantoid, and reniform. The motile spores are equipped with flagella which provide active movement (Figure 11). In some species, like *Kineococcus radiotolerans* SRS30216^T [34], monotrichous spores possess only one flagellum. As in *Catenuloplanes japonicas*, the spore is said to be peritrichous if numerous flagella are distributed over the whole spore. Polytrichous spores are characterized by a tuft of flagella, which can be inserted in one polar (monopolar polytrichous), as in *Actinoplanes regularis*, subpolarly (*spirillospora*), or laterally (*Pilimelia*). Non-motile spores may be smooth or present a special surface ornamentation. Spore surface ornamentation has also been adopted as a taxonomic character. The ultrastructures of the different types are very well studied in some genus. They can be grouped into several forms: smooth, rugose, warty,

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spiny, knobby, verrucose, or irregular (Figure 12). In the genus *Micromonospora*, nonmotile spores are borne singly, sessile, or terminally on short sporophores. Sporophore development is monopodial or in some cases sympodial. Spores are spherical to oval in shape (0.7–1.5 μm) and in most species have blunt spiny projections. The spores are often carried in branched clusters on short hyphae of the substrate mycelium. Additionally, the spores have blunt-spiny surfaces with variable spine sizes; this characteristic is not a diagnostic characteristic for the differentiation of *Micromonospora* species [35] (Figure 13). As the above, spore type, shape, position, spore-bearing arrangement, the number of spores, spores swim or not, spore surface textures are an important basis for classification.

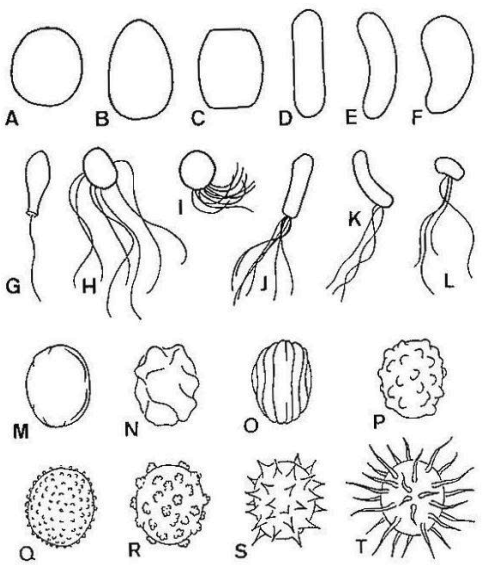

Figure 10. Morphological features of spores. [24] General shape of spores: (A) globose, (B) ovoid, (C) doliform, (D) rod-shaped, (E) allantoid, (F) reniform. Type of flagellation: (G) monopolar monotrichous, (H) peritrichous, (I) polytrichous, (J) monoploarpolytrichous (=lophotrichous), (K) subpolar polytrichous, (L) lateral polytrichous. Surface ornamentation: (M) smooth, (N) irregular rugose, (O) parallel rugose, (P) warty, (Q) verrucose, (S) spiny, (T), hairy.

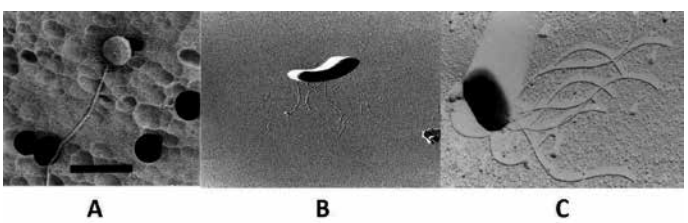

Figure 11. The type of flagellation. (A) Scanning electron micrograph of *Kineococcus radiotolerans* SRS30216^T SEM of a motile cell of strain SRS30216^T exhibiting a single flagellum. Bar, 2 lm. [34] (B) Electron micrograph of *Catenuloplanes japonicus* NBRC 14176^T. Numberous flagella are distributed over the whole spore. (T. Tamura, A. Yokota & T. Hasegawa) [19] (C) *Actinoplanes regularis* A11079. Sporangiospores are motile by a tuft of polar flagella. (N. Muto & K. Ishizawa) [19]

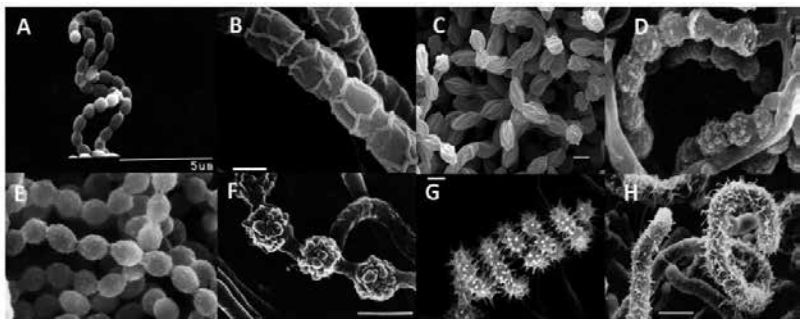

Figure 12. Surface ornamentation of spores. [19] *(A) Streptomyces otagonensis* SANK 62589 (T. Okazaki & R. Enokita). Spiral chains of spores with smooth surfaces are developed. Bar, 5 μm. *(B) Streptomyces* sp. OM-6519 (Y. Takahashi, T. Nakashima & S. Omura). The spore chain is rectiflexibiles section, and the spores have irregular rugose surface. *(C) Actinomadura rugatobispora* AS 6321 (S. Suzuki Spores are oval). Usually two but sometimes three spores per chain. Spore surface rugose with vertical ridges. Bar, 1 μm. *(D) Actinomadura* sp. ATCC 53676 (L.H. Huang, H. Maeda & J. Tone). The strain was characterized by short straight to flexuous spore chains with a warty surface. *(E) Streptomyces routienii* ATCC 39466 (L.H. Huang, H. Maeda & J. Tone). The strain has tubeculate spores that are arranged in a straight to flexuous chains. *(F) Actinomadura verrucosospora* JCM 3147^T (S. Kinoshita, K. Ochiai & K. Ando). Spore chains, in hooks, curves or spirals of one turn, are borne on the aerial hyphae, often as short lateral branches arranged in bundles. Bar, 1 μm. *(G) Streptomyces* sp. WK-1875 (Y. Takahashi, T. Nakashima & S. Omura). The spore chain is Spiral section, and the spores have spiny surface. *(H) Streptomyces finlayi* JCM 4637^T (S. Amano & S. Miyadoh). Spores of the species are oval to ellipsoidal in shape and have a hairy surface. Bar, 1 μm.

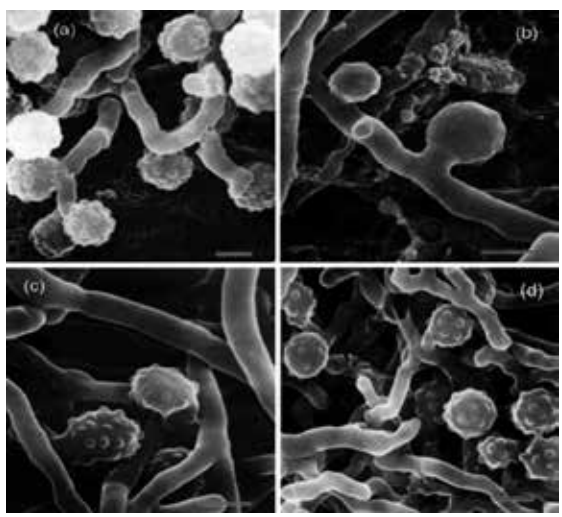

Figure 13. Scanning electron micrographs of: (a) *Micromonospora carbonacea* NRRL 2972^T; (b) *Micromonospora chalcea* ATCC 12452^T; (c) *Micromonospora purpureochromogenes* ATCC 27007^T; and (d) *Micromonospora echinospora* NRRL 2985^T. Bar = 0.5 μm [36].

2.5. Sporangia

Many genera of phylogenetically different groups form spores enclosed in sporangia. The sporangium is a sack-like structure, in which the spores are developed and held together until

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they are released, usually leaving an empty sporangial envelope. Sporangia vary considerably both in terms of size and shape. They measure between 2 to 50 μm in diameter with 10 μm being the most common size. They can be cylindrical, clavate, tubular, bottle-shaped, campanulate, digitate, irregular, lobate, umbelliform, pyriform, or globose (Figure 14, Figure 15). The sporangia arise from the substrate hyphae or aerial hyphae. Sporangia formation is largely divided into two forms: in some genera, sporangia are formed by spore filament winding; in some genera, sporangia are expanded by sporangiophores. Sporangia has sporangial envelope, which has no wall called pseudosporangial. The classical internal structure of latter type of sporangium shows coiled or parallel oriented rows of spores, held together by the sporangial envelope, which continues into the outer layer of the sporangiophore. Sporangiospore is formed by differentiation of protoplasm within sporangia. As spores, sporangial types can be classified on the basis of the number of enclosed spores. Sporangia with few spores may be called oligosporous, with the special consideration given to those with one (monosporous) or two spores (bisporous). Sporangia containing numerous spores are called polysporous. Most sporangiate genera produce motile spore, except for the *Stretosporangium* and *Kutzneria*.

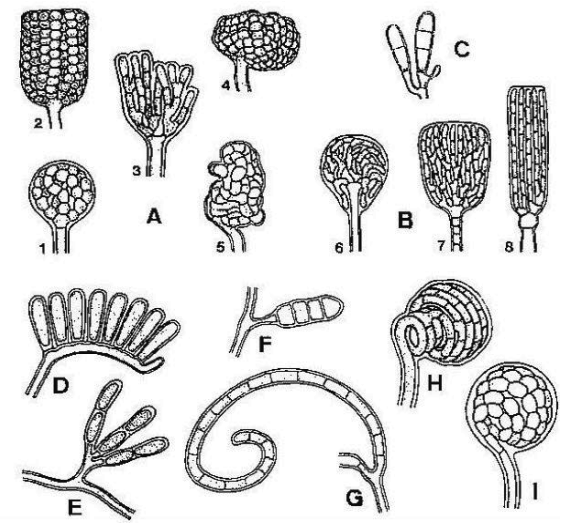

Figure 14. Spore production within sporangia. [24] Sporangia developed on substrate mycelium. (A) *Actinoplanes* (including *Ampullariella*): polysporous, (1) golobose, (2) cylindrical, (3) lobate, (4), subglobose, (5) irregular; (B) *Pilimelia*: (6) ovoid, (7) campanulate, (8) cylindrical; (C) *Dactylosporangium*: oligosporous, claviform. Sporangia developed on aerial mycelium. (D) *Planomonospora*: monosporous, clavate; (E) *Planobispora*: disporous, cylindrical; (F) *Planobetraspora*: tetrasporous, cylindrical; (G) *Planopolyspora*: polysporous, tubular; (H) *Spirillospora*: polysporous, globose; (I) *Streptosporangium*: polysporous, spherical.

In conclusion, sporangia position, sporangia shape, and sporangiospores with or without flagella, are important indications of the genus confirmation, a possible morphological evolutionary series can be observed in the genera with sporangia produced on the aerial mycelium and characterized by a single row of sporangiospores. There is gradation from

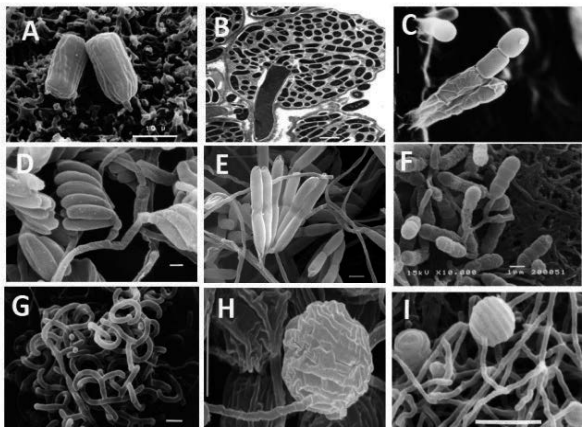

Figure 15. Basic morphological model of Sporangia. [19] *(A) Actinoplanes regularis* SANK 66080^T (Okazaki & R. Enokita). This strain forms cylindrical sporangia on substrate hyphae, and contain motile, rod-shaped sporangiospores. Candiplanecin, a new antifungal antibiotic, is produced. Bar, 10 μm. *(B) Pilimelia columellifera* MB-SK 6^T (G. Vobis). The species is characterized by a columella inside the sporangium, a continuation of the sporangiophore. Bar, 1 μm. *(C) Dactylosporangium fulvum* SF2113^T (T. Shomura). The genus Dactylosporangium is characterized morphologically by the formation of finger-like sporangia containing a single row of two to five zoospores. Bar, 1 μm. *(D) Planomonospora parontospora* ATCC 23863^T (M. Hayakawa, H. Iino & H. Nonomura). A sparsely branched aerial mycelium is formed on which the sessile sporangia, each containing single spores, occur in double parallel row. Bar, 1 μm. *(E) Planobispora rosea* KCC A-0166^T (S. Suzuki). Cluster of sporangia are developed from sporangiophore. Bar, 1 μm. *(F) Planotetraspora silvatica* NBRC 100141^T (T. Tamura). Long, cylindrical sporangia are formed at the ends of short sporangiophores on aerial hyphae, with each sporangium containing four spores in a single row. Bar, 1 μm. *(G) Catenuloplanes japonicus* NBRC 14176^T (T. Tamura, A. Yokota & T. Hasegawa). Pale yellow to tan substrate mycelium is formed. Spores are rod-shaped with smooth surfaces and are flagellated. They are formed by fragmentation of the aerial hyphae. Bar, 2 μm. *(H) Spirillospora albida* ATCC 15331^T (G. Vobis). A mature, spherical sporangium, supported by a laterally inserted sporangiophore, is shown. It has the same dimensions as a common aerial hypha. Bar, 5 μm. *(I) Streptosporangium amethystogenes* IFO 15365 (S. Iinuma, A. Yokota & T. Kanamaru). On the tips of short sporangiophores, which arise from the aerial mycelium, are borne spherical sporangia (5-8 μm) which contain nonmotile spores. Bar, 5 μm.

monosporous, bisporous, tetrasporous, to polysporous sporangia, just like *Planomonospora, Planobispora, Planotetraspora*, and *Planopolyspora* [37-41].

Some sporulation types are hard to classify according to the traditional scheme of morphological differentiation. These include the genus *Intrasporangium*, *Dactylosporangium*, *Catellatospora*, *Ampullariella*, and *Kibdelosporangium*, and so on. Reasons of forming these structures and phylogenetic relationship need to further explore in work in the future.

2.6. The stability of morphological characteristics

The morphological characteristics of actinobacteria due to gene regulation are generally quite stable, andit is an important basis for classification. The development and formation of some structures, like aerial mycelium, spore, and sporangia, are affected by culture conditions. In some media, strains produce a lot of sporangia or spore, while in other media have little or none. Figure 16 is the diagram of some genera of actinobacteria.

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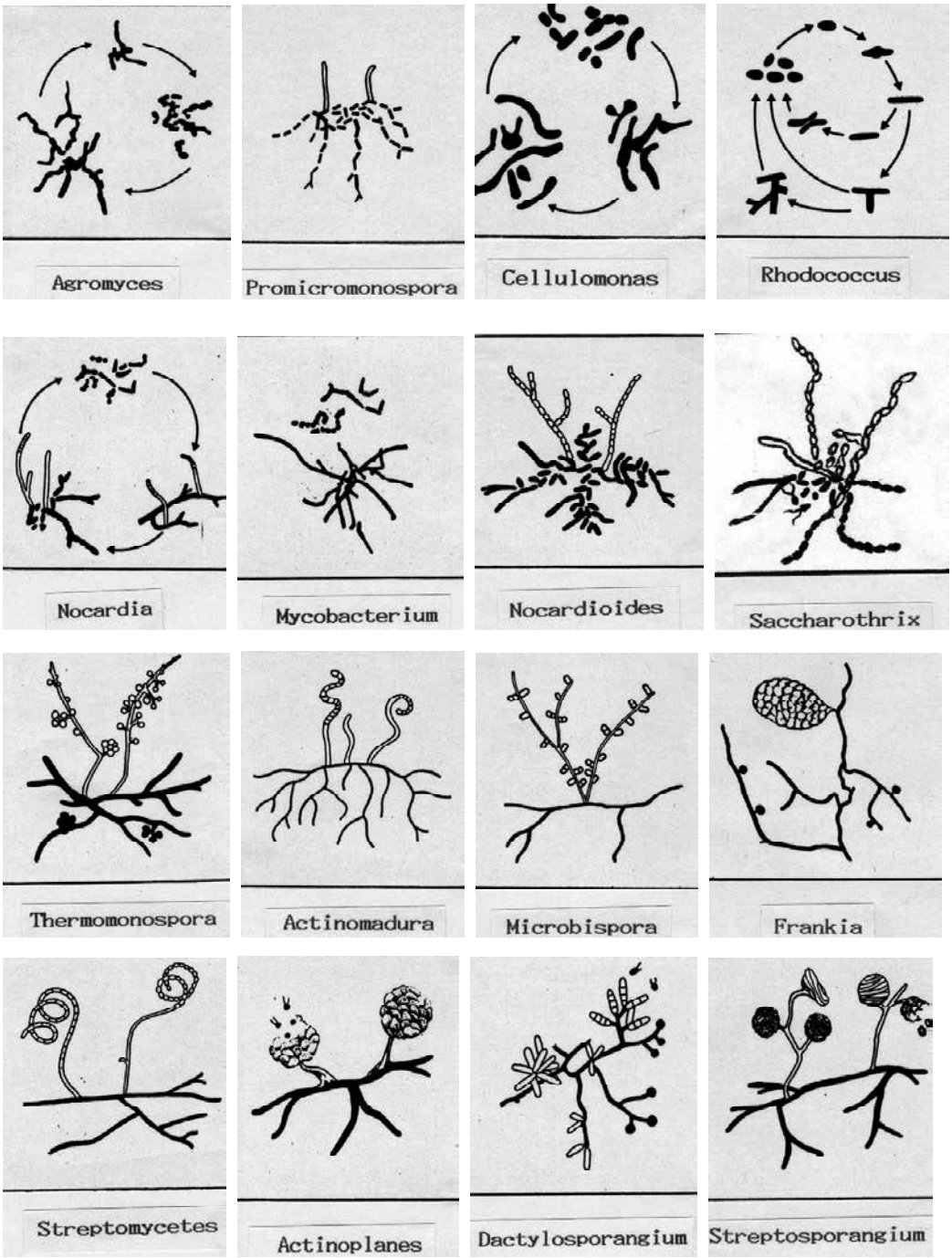

Figure 16. Diagram of some genera of Actinobacteria.

3. Experiment methods of morphological and cultural characteristics

3.1. Cultural characteristics

Cultural characteristics of actinobacteria refer to the growth characteristics and morphology in various kinds of culture media. It is usually determined after incubation for 14 days at 28°C strictly according to methods used in the *International Streptomyces Project* (ISP) [42]. The colors of substrate and aerial mycelia and any soluble pigments produced were determined by comparison with chips from the ISCC-NBS color charts [43].

Classical taxonomy attaches great importance to the role of culture characteristics in the classification identification, general with spores, aerial hyphae, with or without color and the soluble pigment, different growth condition on various media as the main characteristics (Figure 17). The colors of the mature sporulating aerial mycelium are recorded in a simple way (white, grey, red, green, blue, and violet). When the aerial mass color fell between two colors series, both the colors are recorded. If the aerial mass color of a strain to be studied showed intermediate tints, then both the color series are also noted. The media used are yeast extract-malt extract agar and inorganic-salt starch agar. The groupings are made on the production of melanoid pigments (i.e., greenish brown, brownish black, or distinct brown, pigment modified by other colors) on the medium. The strains are grouped as melanoid pigment produced (+) and not produced (–). In a few cases, the productions of melanoid pigments are delayed or weak, and therefore, it is not distinguishable. This is indicated as variable. This test was carried out on the media ISP-1 and ISP-7, as recommended by International *Streptomyces* Project (Table 1). The strains are divided into two groups, according to their ability to produce characteristic pigments on the reverse side of the colony, namely, distinctive (+) and not distinctive or none (–). In case, a color with low chroma such as pale yellow, olive, or yellowish brown occurs, it is included in the latter group (–). The strains are divided into two groups by their ability to produce soluble pigments other than melanin: namely, produced (+) and not produced (–). The color is recorded (red, orange, green, yellow, blue, and violet).

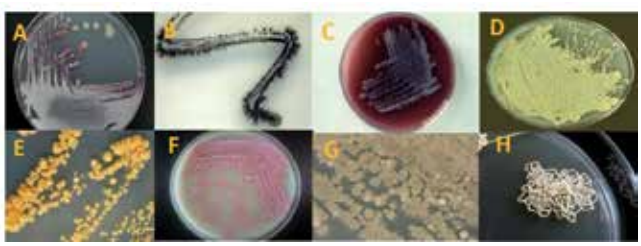

Figure 17. Cultural characteristics of some actinobacteria strains [19]. *(A) Streptomyces violaceoruber* NBRC 12826^{T} (C. Shibata & H. Komaki). This is the type strain of *Streptomyces violaceoruber* grown on an agar medium. Some morphological differentiation stages such as cream-colored colonies, white aerial mycelium, and red pigment production. (*B) Micromonospora* sp. (S. Mochales). Strains of *Micromonospora* form their spores on the substrate mycelium. These spores accumulate a slimy black mass on the surface of the colonies. *(C) Dactylosporangium vinaceum* SF2127^{T} (H. Tohyama). Note the production of a wine-red diffusible pigment. *(D) Actinomadura rugatobispora* SF2240 (S. Miyadoh). This species is characterized by its green aerial mycelium bearing longitudinally paired spores, which have rugose surfaces with

vertical ridges. *(E) Actinoplanes* sp. (S. Mochales). This strain forms bottle-shaped sporangia, which are formed directly on the substrate hyphae. *(F) Catenulispora graminis* KACC 15070^T (H.J. Lee & K.S. Whang). Colonies on an oatmeal agar are red. *(G) Kitasatospora arboriphila* NBRC 101834^T (T. Tamura & Y. Ishida). The strain produces a yellowish brown to dark brown or olive substrate mycelium and a grey to dark grey aerial spore mass on agar media. Soluble pigments are not formed. *(H) Nocardia pseudobrasiliensis* IFM 0623 (A. Takahashi-Nakaguchi & T. Gonoi). Colony (stereomicroscope). Colony color; yellow-orange. Colony; coiled pasta-like colony.

Medium	Approximate Formula Per Liter[1]	
ISP Medium 1	Yeast Extract	3.0 g
(Tryptone-yeast extract broth	Tryptone	5.0 g
agar)	pH 7.0 to 7.2	
ISP Medium 2	Yeast Extract	4.0 g
(Yeast extrac-malt extract agar)	Malt Extract	10.0 g
	Dextrose	4.0 g
	pH **7.3**	
ISP Medium 3	Oatmeal	20.0 g
(Oatmeal agar)	Agar	18.0 g
	pH **7.2**	
ISP Medium 4	Soluble Starch	10.0 g
(Inorganic salts-starch agar)	K_2HPO_4	1.0 g
	$MgSO_4 \cdot 7H_2O$	1.0 g
	NaCl	1.0 g
	$(NH_4)_2SO_4$	2.0 g
	$CaCO_3$	2.0 g
	Trace salt solution[2]pH 7.0 to 7.4	1.0 ml
ISP Medium 5	L-asparagine	1.0 g
(Glycerol-asparagine agar)	Glycerol	10.0 g
	K_2HPO_4	1.0 g
	Trace salts solution	1.0 ml
	pH 7.0 to 7.4	
ISP Medium 6	Peptic digest of animal tissue	15.0 g
(Peptone-yeast extract iron agar)	Proteose peptone	5.0 g
	Yeast extract	1.0 g
	$C_{12}H_{22}FeN_3O_{14}$	0.5 g
	K_2HPO_4	1.0 g
	$Na_2S_2O_3$	0.08 g
	pH 7.0 to 7.2	
ISP Medium 7	Glycerol	15.0 g
(Tyrosine Agar)	L-tyrosine	0.5 g
	L- aspar agine	1.0 g
	K_2HPO_4	0.5 g
	$MgSO4 \cdot 7H_2O$	0.5 g

Medium	Approximate Formula Per Liter[1]	
	NaCl	0.5 g
	$FeSO_4 \cdot 7H_20$	0.01 g
	Trace salt s solution	1.0 ml
	pH **7.2-7.4**	

[1]Agar 15-20 g

[2]Trace salt solution: $FeSO_4$ $7H_2O$ 0.1 g, $MnCl_2$ $4H_2O$ 0.1 g, $ZnSO_4$ $7H_2O$ 0.1 g, Distilled water 100. 0 ml.

Table 1. ISP Medium

As the result of cultivation characteristics that are susceptible to cultural conditions (factors such as culture medium, temperature, pH, and light), the influence of culture characteristics was declining in importance. Usually, only use it as one of many indicators of polyphasic taxonomy. And the cultivating characteristic experiment must be in strict accordance with the International *Streptomyces* Project (ISP). If the identified strains have affiliated clearly to a genus, it is necessary to culture strain spawn in similar strains of known bacteria on the culture characteristics of the medium used, observe the characteristics, and contrast.

3.2. Morphological observation

Microscopes are the traditional instruments used for assessing actinobacteria, and they remain as indispensable tools for exploring the morphological, physiological, and genetic diversity present in actinobacteria. Usually, the basic morphology of hyphae and spores is observed by light microscopy, and the microscopic structures of hyphae and spores on the surface are observed by scanning electron microscope (SEM), and the ultramicroscopic structure of the spore flagella and cell is observed by transmission electron microscopes (TEM) (Figure 18).

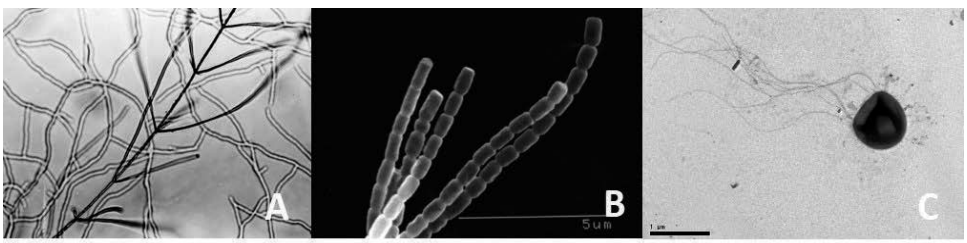

Figure 18. Morphological observation. [19] (A) Light micrograph of *Streptomyces nobilis* SANK 60192^T (Okazaki & R. Enokita). (B) SEM micrograph of *Streptomyces nobilis* SANK 60192^T (Okazaki & R. Enokita), Bar, 5 μm. (C) TEM micrograph of *Kineococcus gynurea* 103943^T (K. Duangmal & A. Matsumoto). Motile cocci with polar flagella. Bar, 1 μm.

Transplantation embedding method is usually used in morphological observation of actinobacteria [16]. The selected appropriate agar flat (2 to 4 media) were dug into 1 cm wide rectangular hole, inoculated at the edge of hole, and then covered with sterile coverslip. The flat is cultivated at proper temperature. The coverslips are taken out at different times (usually

5, 10, 14, and 20 days) and observed using light microscopy. According to the graph of light microscopy, the good area is chosen, which is cut into 1 x 1 cm pieces, sprayed directly on the cover sheet, taken pictures using scanning electron microscopy (Figure 19). In order to prevent shape deformation, fixation is usually performed by incubation in a solution of a buffered chemical fixative, such as glutaraldehyde (2.5%, 1.5 h), sometimes in combination with formaldehyde and other fixatives and optionally followed by post fixation with osmium tetroxide. The fixed tissue is then dehydrated. Because air-drying causes collapse and shrinkage, this is commonly achieved by replacement of water in the cells with organic solvents such as ethanol (respectively 30, 50, 70, 90, 100%, dehydration each 15 min) or acetone, and replacement of these solvents in turn with a transitional fluid such as liquid carbon dioxide by critical point drying. The carbon dioxide is finally removed while in a supercritical state, so that no gas–liquid interface is present within the sample during drying. The dry specimen is usually mounted on a specimen stub using an adhesive such as epoxy resin or electrically conductive double-sided adhesive tape, and sputter-coated with gold or gold/palladium alloy before examination in the microscope.

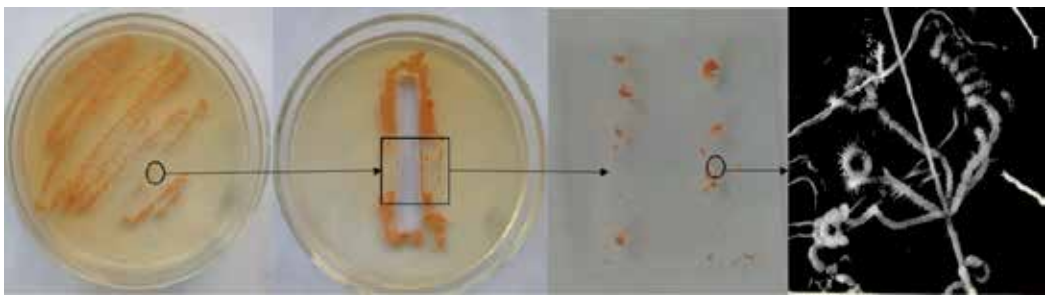

Figure 19. Observation of actinobacteria with transplantation embedding method.

4. The molecular mechanisms of morphological differentiation

Filamentous microorganisms involved two main groups, filamentous fungi and filamentous actinomycetes, particularly the *streptomycetes*. In terms of cellular growth mechanisms, these groups differ greatly. Eukaryotic fungi possess subcellular organelles and cytoskeletal structures directing growth while prokaryotic actinomycetes have no such cellular organization. Despite these fundamental differences, both groups exhibit similar morphologies, growth patterns, growth forms, hyphal and mycelial growth kinetics, spore, sporangia, and conidiospore. The study found that two groups have very similar molecular mechanisms of morphological differentiation [44].

The actinomycetes developmental life cycle is uniquely complex and involves coordinated multicellular development with both physiological and morphological differentiation of several cell types, culminating in the production of secondary metabolites and dispersal of mature spores [45, 46]. *Streptomyces* development has been the subject of intense genetic and

molecular biology research since the isolation of the first mutants specifically blocked in the process [47]. *Streptomyces coelicolor* A3 (2) is the most extensively characterized actinomycete at the genetic level. These have been used to study various aspects of its biology, notably secondary metabolism and its life cycle [48]. Genes required for aerial growth (*bld* genes) are often also needed for secondary metabolism. At least six further genes (*whiA, B, G, H, I, J*) are needed to initiate the subdivision of multigenomic aerial hyphal tips into unigenomic prespore compartments, while several more (including *sigF*, *whiD*, and the *whiE* spore pigment gene cluster) are in spore maturation. As is often the case in cascades of gene expression bacteria, at least two RNA polymerase signal factors (the *whiG* and *sigF* gene products) play specific and crucial roles in sporulation (Figure 20).

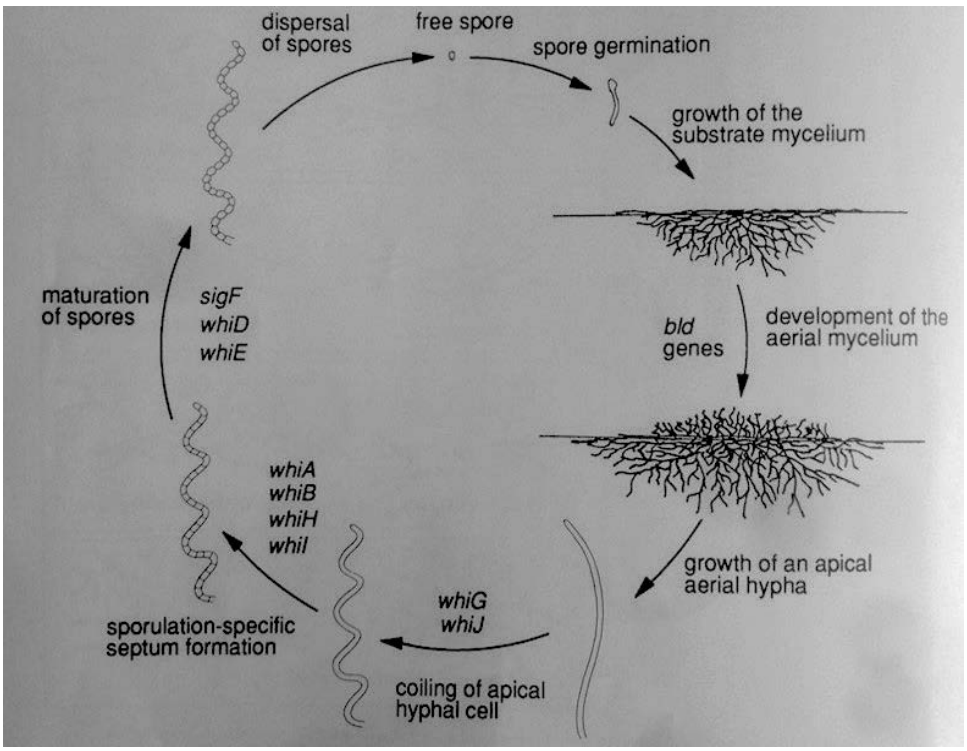

Figure 20. The life cycle of *Streptomyces coelicolor* A3 (2). [24]

Growth of actinomycetes is from the hyphal, which is similar with filamentous fungi [49]. Using the modern fluorescence microscopy, *Streptomyces* apical hyphal growth was observed (Figure 21) [50]. The apical cell is extending its cell wall only at the tip (green). Once this cell has divided by forming a new hyphal cross wall, the subapical daughter cell is unable to grow, and eventually switches its polarity to generate a lateral branch with a new extending tip. A consequence of tip growth is that DNA, which replicates along most of the hyphal length, has to move towards the tip and into new branches - a process we propose to designate nucleoid

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migration. For clarity, only a few schematic nucleoids are drawn (red), and they are not meant to reflect the actual number of chromosomes per cell. Furthermore, individual nucleoids are typically not observed in vivo as separated bodies in growing hyphae.

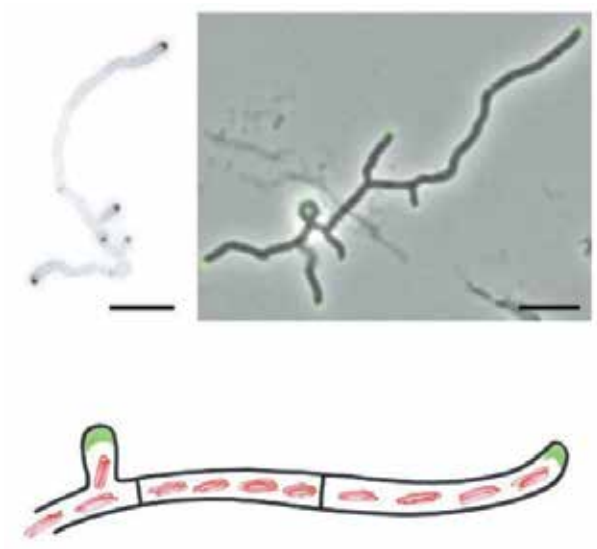

Figure 21. Apical growth in *Streptomyces*. [50]

In general, when nutrients become limiting, a developmental switch occurs during which hyphae start to escape the moist environment and grow into the air. These so-called aerial hyphae can further differentiate into long chains of spores, which can withstand the adverse conditions. Following their dispersal, these spores will reinitiate growth in suitable environments. Some of the key processes involved in the formation of aerial hyphae by streptomycetes and fungi appear to be very similar. Both groups secrete highly surface-active molecules that lower the surface tension of their aqueous environment enabling hyphae to grow into the air. In the case of filamentous actinomyces, small peptides (i.e., SapB and streptofactin) are secreted, while filamentous fungi use proteins known as hydrophobins to decrease the water surface tension. Although these fungal and bacterial molecules are not structurally related, they can, at least partially, functionally substitute for each other (Figure 22) [51]. The *bld* cascade (for bald, meaning unable to form aerial hyphae) controls the checkpoints that (eventually) lead to the onset of aerial growth, resulting in the formation of surface-active molecules that lower the water surface tension and enable hyphae to grow into the air. Moreover, the *bld* cascade seems to potentiate hyphae to undergo full development [52, 53]. Another regulatory pathway is the shy pathway [54], which controls the expression of the chaplin and rodlin genes. These genes encode proteins that assemble into a rodlet layer that provides surface hydrophobicity to aerial hyphae and spores. Both pathways control the production of structural proteins that are involved in the formation of aerial hyphae (Figure 23).

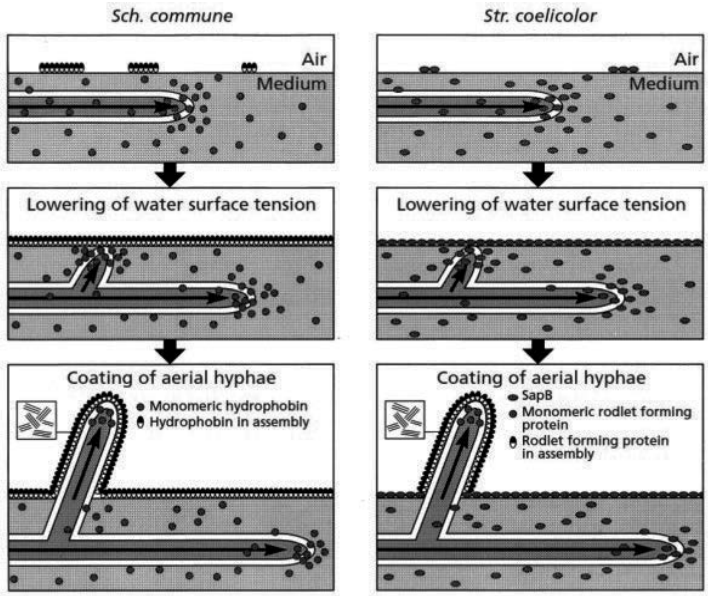

Figure 22. Model for the formation of aerial hyphae in the filamentous fungus *Sch. commune* and the filamentous bacterium *Str. Coelicolor.* [51] After a submerged feeding mycelium has been formed, *Sch. Commune* secretes SC3 into the medium, while *Str. coelicolor* produces SapB. These molecules lower the surface tension of the aqueous environment, enabling hyphae to escape the substrate and to grow into the air. SC3 lowers the surface tension by assembling into an amphipathic membrane at the water–air interface. SC3 secreted by aerial hyphae of *Sch. commune* assembles at the interface between the hydrophilic cell wall and the hydrophobic air exposing its hydrophobic side, which is characterized by a mosaic of rodlets. The hydrophobic surface of aerial hyphae of *Str. coelicolor* is also typified by a rodlet layer. Although the molecules forming this layer have not yet been identified, evidence suggests it is not SapB.

When hyphal growth is limited, much of the biomass becomes converted into spores through the extraordinary parasitic growth of a fluffy white aerial mycelium. The syncytial aerial hyphal tips (which may contain more than 50 copies of the genome) undergo multiple cell divisions to generate a string of unigenomic compartments, destined to become tough, desiccation-resistant spores [55]. Thus, substantial growth is interpolated between the first sporulation related decisions, made in the substrate mycelium, and the decisions involved in the formation and maturation of the spore compartments themselves (Figure 24) [56]. Additionally, the *Streptomyces* spore wall synthesizing complex (SSSC) does not only direct synthesis of the peptidoglycan layer but is also involved in the incorporation of anionic spore wall glycopolymers, which contribute to the resistance of spores. The SSSC also contains eukaryotic type serine/threonine kinases which might control its activity by protein phosphorylation [57]. Genetic analysis of differentiation in *Streptomyces coelicolor* has identified two classes of regulatory mutants, blocked in distinct stages of differentiation. White (*whi*) mutants form aerial hyphae in the normal way, but these hyphae are unable to complete the developmental process to form mature chains of spores [46]. They appear white when grown on solid media because they fail to produce the grey polyketide pigment associated with mature, wild-type spores. *bld* mutants are blocked at an earlier stage of development; they are unable to erect aerial hyphae and therefore appear "bald", lacking the characteristic fuzzy morphology of the wild type.

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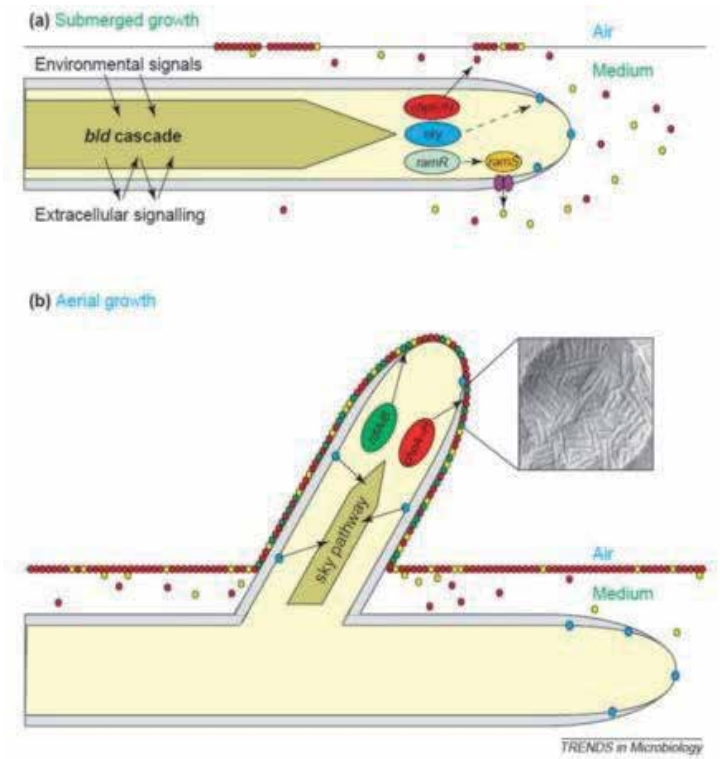

Figure 23. Integrated model for the formation of aerial hyphae in the filamentous bacterium *Streptomyces coelicolor*. (a) Extracellular signaling and environmental signals exert their influence on development through the bld cascade. This cascade induces the formation of RamR, the chaplins ChpE and ChpH (red circles), and components of the sky pathway, such as a sensor of aerial growth (blue circles). RamR activates the synthesis of RamS, which is converted to SapB (yellow circles). This morphogenetic peptide is secreted by the RamAB transporter (purple ovals) and, together with ChpE and ChpH, initiates aerial growth by lowering the water surface tension. (b) From this moment, the sky pathway takes over regulation of development. This pathway would include a sensor of aerial growth (blue circles). As a consequence, the rodlin and chaplin genes, and probably other genes, are activated. Rodlins (green circles) and chaplins (red circles) assemble into a hydrophobic rodlet coat at the outer surface of aerial hyphae. This layer provides surface hydrophobicity and prevents aggregation of aerial hyphae. The insert shows the typical appearance of the rodlet layer, as assessed by scanning electron microscopy. [54]

Morphological differentiation, which coincides with the production of various secondary metabolites, including antibiotics antitumor drugs and enzyme inhibitors, is initiated, when partial nutrient limitation is encountered. Both morphogenesis and antibiotic production in the streptomycetes are initiated in response to starvation. Upon sensing starvation, the substrate mycelia release small molecules that act as signals for the initiation of aerial hyphal growth, as well as for the production of antibiotics. Besides sensing of the nutritional situation, quorum sensing and other environmental stress signals are also involved and controlled by the hierarchical cascade of *bld* and *whi* regulatory genes [58, 59]. Mutants that fail to produce aerial hyphae are, called *bld* mutants, or those that initiate aerial hyphal growth but fail to produce mature spores, are called *whi* mutants. Some studied show that BldD is a key regulator of morphological differentiation and antibiotic production and that it connects the regulons

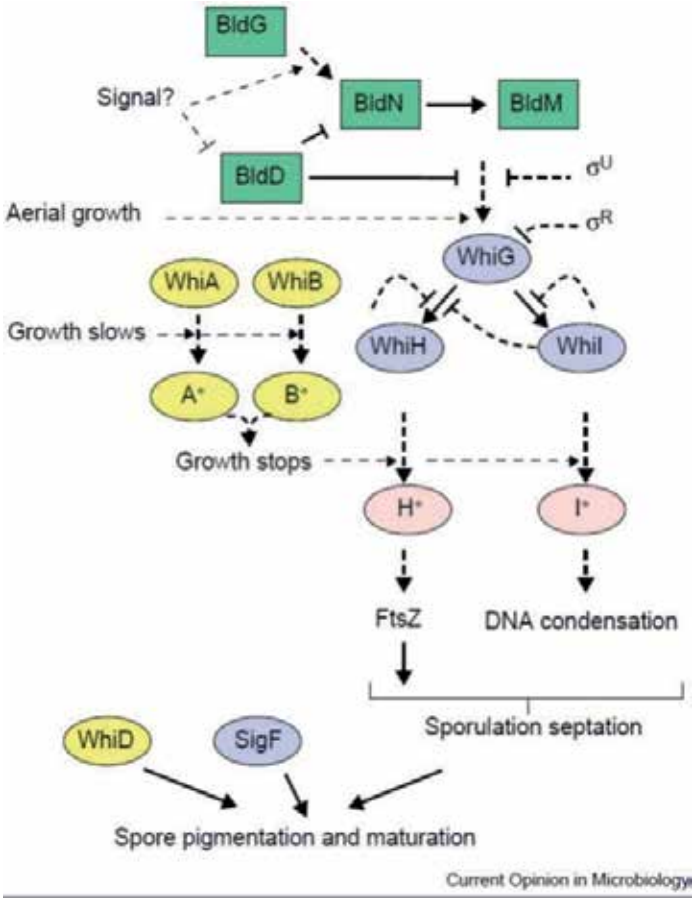

Figure 24. Regulatory and checkpoint network for *Streptomyces coelicolor* sporulation. [56] The diagram is a tentative interpretation of available information about the regulatory connections among *bld* and *whi* gene products and the likely events that influence the transitions to consecutive stages of development. In the dense substrate mycelium preparing for aerial growth, BldG protein is needed for transcription of *bldN*. BldN, a sigma factor, directs the transcription of *bldM*, which encodes a response-regulator-like protein needed for aerial growth. In the growing aerial hyphae, WhiG, a sigma factor, becomes activated to transcribe *whiH* and *whiI*. One factor in this spatially specific expression is BldD, which directly binds to, and represses, the *bldN* and *whiG* promoters in the growing substrate mycelium. Some unknown signal(s) releases this repression in aerial hyphae, and also presumably causes the BldG anti-sigma factor to remove an anti-sigma factor from an unknown sigma factor that is needed for development. The aerial hyphae also contain the WhiA and WhiB proteins, which may sense when aerial growth is slowing down and be converted to modified forms (A* and B*) that coordinate orderly growth cessation. It is postulated that growth cessation gives rise to signals that cause WhiH and WhiI to adopt altered configurations (H* and I*), in which they lose their autorepressor activities and become activators of processes involved in sporulation septation. At this time, late sporulation regulators such as WhiD and SigF activate spore maturation functions. Regulatory steps are indicated by bold lines (solid when well-established, broken when the evidence is more limited), and putative checkpoints are indicated by light dashed lines.

of several other regulators that play pivotal roles in these two central aspects of *Streptomyces* biology [60]. Furthermore, the researcher found the TeRt gene of *Streptomyces coelicolor* SC01135 controls the morphological differentiation and antibiotic synthesis [61].

Benefited from recent advances in determining prokaryotic phylogeny, our understanding of actinobacteria taxonomy is constantly improving. The early assumption that the evolution of actinobacteria went from simple to complex in morphology and that the morphological similarities reflect phylogenetic relationship must have been wrong. It is common to see convergence in morphology between totally different organisms as a result of adoption to environmental factors during evolution. The phylum actinobacteria is a large and ancient group of bacteria with many interesting features. Various members represent a gradient of morphological and developmental complexity, from simple coccoid cells like the *Micrococcus*, and rod-shaped orpleiomorphic organisms like the industrially important *Corynebacterium*, and pathogens like *Mycobacterium tuberculosis*, to the highly complex mycelium of *Streptomyces* and related genera. An informative dimension has now been added by the rapidly growing genome sequence information, which opens fantastic possibilities for comparative and evolutionary studies, both within *Streptomyces* and among the actinobacteria [58, 62].

Acknowledgements

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Chapter 4

Cultural, Physiological, and Biochemical Identification of Actinobacteria

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Additional information is available at the end of the chapter

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Abstract

The traditional phenotypic tests are commonly used in actinobacterial identification. They constitute the basis for the formal description of taxa, from species and subspecies up to genus and family. The classical phenotypic characteristics of actinobacteria comprise morphological, physiological, and biochemical features. The morphology of actinobacteria includes both cellular and colonial characters. The physiological and biochemical features include data on growth at different temperatures, pH values, salt concentrations, or atmospheric conditions, and data on growth in the presence of various substances such as antimicrobial agents, the presence or activity of various enzymes, and with respect to metabolization of compounds. The phenotype is the observable expression of the genotype. Gene expression is directly related to the environmental conditions. Actinobacterial phenotype cannot be based on the simple observation of the organism. Strains of the most closely related taxa should be compared in their phenotypic analysis using identical methods. The comparisons must include the type strain of the type species of the appropriate genera. Furthermore, with the development of technology, microbial physiological and biochemical identification technology is becoming fast, simple, and automated.

Keywords: Phenotype, Physiological and biochemical characteristics, Automatic identification system

1. Introduction

The polyphasic approach [1], the comprehensive results of various methods, such as morphological, physiological, rRNA gene sequencing, chemotaxonomic markers and pathogenicity,

are used to compile a description of a new species. Classification of actinobacteria is now based largely on analysis of the nucleotide sequences, especially 16S rRNA genes [2]. However, a comprehensive characterization of a new species should still be identified, because from even a complete genomic sequence, it would be difficult to predict many of the phenotypic features of a new species [3]. Physiological and biochemical characteristics are directly related to the activity of microbial enzymes and regulatory proteins. Enzymes and proteins are gene products; so the comparison on physiological and biochemical characteristics of actinobacteria is the indirect comparison of genome, and determination of physiological and biochemical characteristics is much easier than direct analysis of the genome. Therefore, the physiological and biochemical characteristics in actinobacterial systematics and identification are still meaningful [4]. In addition to a thorough phenotypic characterization of a new species, it is important to determine which phenotypic features are the ones most useful for identifying new species. Moreover, the particular methods used for characterizing an organism should always be stated, because the results of phenotypic tests can vary with methodology.

Phenotypic analysis is a very tedious task in the classification of actinobacteria. The classical phenotypic characteristics of actinobacteria comprise morphological, physiological, and biochemical features. Individually sufficient as parameters for genetic relatedness, yet as a whole, they provide descriptive information enabling us to recognize taxa [5]. The morphological traits include both cellular (cell shape and size, spore, sporangia, sporangiospore, the location of the spores or sporangia and their size, flagella, motility, intracellular structures, etc.) and colonial characters (shape and size, color, dimensions, form, etc.). The physiological and biochemical traits include data on growth at different temperatures, pH values, salt concentrations, atmospheric conditions (aerobic/anaerobic), growth in the presence of various substances such as antimicrobial agents, and data on the presence or activity of various enzymes, metabolization of compounds, and so on (Table 1). Physiological and biochemical tests should be carried out in test media and under conditions that are identically standard or at least comparable. There are three major inter-related areas in actinobacterial identification: taxonomic relevance; methodological reliability and cost effectiveness; and data portability [6]. It must be noted that novel taxa should be described based on the characteristics of more than one related strain and the type strain of the type species of the appropriate genera [7]. It should be emphasized that some phenotypes are encoded by extrachromosomal inheritance factors and the influencing factors that affect the expression of physiological and biochemical traits are complicated. To determine the genetic relationships based on physiological and biochemical characteristics,systematic classification, must be integrated with other characteristics, particularly genotype characteristics analysis.

Identification of a species is a constant basic work in any microbiology laboratory. Regardless of the type of microorganisms, the working steps are inseparable from the following three items: (i) to obtain the pure cultures of microorganism, (ii) to determinate the necessary appraisal indicators, (iii) to find authoritative identification manuals and publications, and related site information. Different organisms often have their own different identification priorities. For example, the identification of microorganisms with rich morphological characters, such as fungi, often bases on their morphological features as the main indicators; the

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identification of actinobacteria and yeasts, synthesizes the morphological, physiological and biochemical characteristics; the identification of bacteria lacking morphological difference, often uses more physiological, biochemical, and genetic parameters.

Characteristics	The difference between groups
Adaptability of temperature	The optimal, the lowest, and highest growth temperature and die temperature
Adaptability of pH values	The range of pH values at which it can grow, as well as the optimal pH for growth
Adaptability of osmotic pressure	The salt concentration and halophilism
Utilization of nitrogen source	Utilization of proteins, peptone, amino acids, nitrogen, inorganic salt, N_2, etc.
Utilization of carbon source and acid-producing ability	Utilization of various monosaccharides, disaccharides, polysaccharides, alcohols, and organic acids, etc. Acid production from carbohydrates
Needs of growth factors	Special vitamins, amino acids, X and V factor requirements
Atmospheric condition	Aerobic, microaerophilic, anaerobic, facultative anaerobic
Antimicrobial activity	Inhibition to Gram-positive and Gram-negative bacteria, filamentous fungi and yeast, etc.
Metabolization	Various characteristic metabolites tests, such as MR test, V-P test, iodole production, etc.
Activity of various enzymes	Oxidase, catalase, urease, etc.
Sensitivity	The sensitivity to antibiotics, potassium cyanide (potassium sodium), antimicrobial agents, dyes, etc.

Table 1. Common physiological and biochemical characteristics used for classification and identification of actinobacteria

2. Cultural characteristics of actinobacteria

Cultural characteristics of actinobacteria refer to the growth characteristics and morphology in various kinds of culture media. Pure culture should be taken before morphological observation. The pure culture of actinobacteria can be obtained through the use of spread plates, streak plates, or pour plates and are required for the careful study of an individual microbial species [Figure 1]. Cultural characteristics on 4 to 6 media are usually determined after incubation 14 to 28 days at 28°C strictly according to the methods used in the *International Streptomyces Project* (ISP) [8]. Sometimes, other media can be chosen, such as nutrient agar and czapek's agar. The colors of substrate and aerial mycelia and any soluble pigments produced were determined by comparison with chips from the ISCC-NBS color charts [9].

Nutrient agar medium	**(G/Liter)**
Peptone	10.0 g
Beef extract/yeast extract	3.0 g
NaCl	5.0 g
Agar	15.0 g
Final pH (at 25°C)	7.0±0.2
Czapek's agar medium	**(G/Liter)**
Sucrose	30.0 g
$NaNO_3$	3.0 g
$MgSO_4 \cdot 7H_2O$	0.5 g
KCl	0.5 g
$FeSO_4 \cdot 4H_2O$	0.01 g
K_2HPO_4	1.0 g
Agar	15.0 g
Final pH (at 25°C)	7.2±0.2

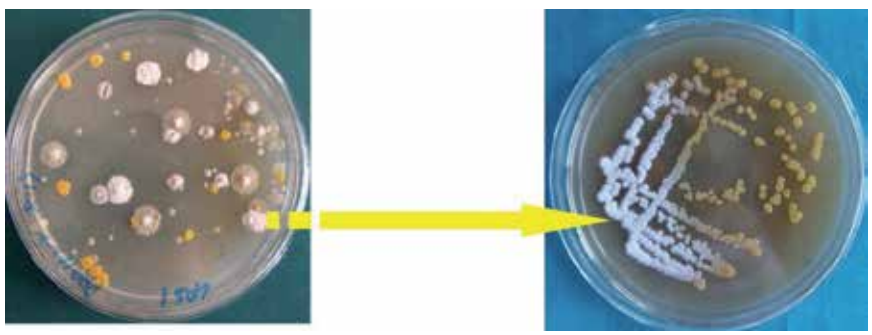

Figure 1. Acquisition of pure culture

3. Physiological and biochemical characteristics for identification of actinobacteria

Some phenotypic characteristics of actinobacteria are of such primary importance to a genus or species description. Several problems must be considered when planning the physiological and biochemical tests of actinobacteria. One of them, according to the phylogenic information based on 16S rRNA analyses, strains of the most closely related taxa and the type strain of the type species of the appropriate genera should be chosen for comparison in their phenotypic traits. Other problems are concerned with methodology. In classifying actinobacteria, it is desirable to use an established approach based on common sense, and to use tests that are pertinent. If novel methods are used, the researcher must provide evidence that the new methods produce comparable results to established methods. Furthermore, phenotypic

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characteristics of actinobacteria are influenced by cultural conditions and other factors, so tests should be performed in duplicate or triplicate. More importantly, design reasonable positive and negative controls in the experiments.

3.1. Temperature range and optima for growth

Incubate cultures at a range of temperatures; using constant temperature incubators or water baths, measure the growth response of the actinobacteria. The tested temperature range is usually from 0°C to 75°C. In general, temperature experiments employ solid medium instead of broth in order to better observe. The basal medium is Bennett's medium or YIM38 medium or nutrient medium.

Bennett's medium	**(G/Liter)**
Yeast extract	1.0 g
Beef extract	1.0 g
Casein	2.0 g
Glucose	10.0 g
Agar	15.0 g
Final pH (at 25°C)	7.3±0.2
YIM 38 medium	**(G/Liter)**
Malt extract	10.0 g
Yeast extract	4.0 g
Glucose	4.0 g

Vitamin mixture (0.5 mg each of thiamine-HCl, riboflavin, niacin, pyridoxine-HCl, inositol, calcium pantothenate, and p-aminobenzoic acid and 0.25 mg biotin)

Agar	15.0 g
Final pH (at 25°C)	7.2±0.2

Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 min. Mix well and pour into sterile Petri plates.

Note: If the temperature is above 100°C, use screw-cap culture tubes or screw-cap glass bottles and seal the screw caps to prevent the evaporation of the medium. Incubate the cultures in commercial water baths filled with dimethyl silicone oil. If the temperature is below 0°C, use an ethylene glycol water bath. For marine actinobacteria that require seawater, open ocean seawater has NaCl concentration of about 35 g/L or 3.5% (wt/vol).

3.2. Optimum pH and pH range for growth

An essential part of the description of any actinobacteria is the range of pH values at which it can grow, as well as the optimal pH for growth. Measure growth responses from a standar-

dized inoculum using basic medium (Bennett's medium or YIM38 medium or nutrient medium) at various pH values. Liquid medium is to be used for pH tests, to measure the growth responses turbidimetrically. The selection of buffer is critical. A buffer should be used in most media to maintain a stable pH for growth of the test strain. Buffers are most effective at their pK_a values and should be chosen with this in mind. Some useful biological buffers are listed in Table 2. Some buffers such as citrate, succinate, or glycine may be metabolized by the test organism. Others may be toxic. Sometimes, a combination of buffers may be helpful. Certain buffers (Good buffer) are non-metabolizable, non-toxic, have low reactivity with metal ions, and have other desirable features [10]. Phosphate salts are most commonly used because they are effective in the growth range of most bacteria, are usually non-toxic, and provide a source of phosphorus for the organism.

Buffer	**Effective pH range**	**pK_a (25°C)**
Maleate (Salt of maleic acid)	1.2–2.6	1.97 (pK_a1)
Phosphate (Salt of phosphoric acid)	1.7–2.9	2.15 (pK_a1)
Glycine	2.2–3.6	2.35 (pK_a1)
Citrate (Salt of citric acid)	2.2–3.5	3.13 (pK_a1)
Malate (Salt of malic acid)	2.7–4.2	3.40 (pK_a1)
Citrate (Salt of citric acid)	3.0–6.2	4.76 (pK_a2)
Succinate (Salt of succinic acid)	3.2–5.2	4.21 (pK_a1)
Acetate (Salt of acetic acid)	2.6–5.6	4.76
Malate (Salt of malic acid)	4.0–6.0	5.13 (pK_a2)
Succinate (Salt of succinic acid)	5.5–6.5	5.64 (pK_a2)
MES 2-(*N*-Morpholino)-ethanesulfonic acid	5.5–6.7	6.10
Maleate (Salt of maleic acid)	5.5–7.2	6.24 (pK_a2)
Citrate (Salt of citric acid)	5.5–7.2	6.40 (pK_a3)
ACES (*N*-(2-Acetamido)-aminoethanesulfonic acid)	6.1–7.5	6.78
BES (*N,N*-Bis-(2-hydroxyethyl)-2-aminoethanesulfonic acid)	6.4–7.8	7.09
MOPS (3-(*N*-Morpholino)-propanesulfonic acid)	6.5–7.9	7.14
HEPES (*N*-(2-Hydroxyethyl)-piperazine-*N'*-ethanesulfonic acid)	6.8–8.2	7.48
Phosphate (Salt of phosphoric acid)	5.8–8.0	7.20 (pK_a2)
Imidazole	6.2–7.8	6.95
TES (2-[Tris(hydroxymethyl)-methylamino]-ethanesulfonic acid)	6.8–8.2	7.40
Tricine (N-[Tris(hydroxymethyl)-methyl]-glycine)	7.4–8.8	8.05
Tris (Tris(hydroxymethyl)-aminomethane)	7.5–9.0	8.06
TABS (*N*-tris[hydroxymethyl]-4-amino-butanesulfonic acid)	8.2–9.6	8.90
CHES (Cyclohexylaminoethanesulfonic acid)	8.6–10.0	9.50
Glycine	8.8–10.6	9.78 (pK_a2)
CAPSO (3-(Cyclohexylamino)-2-hydroxy-1-propanesulfonic acid)	8.9–10.3	9.60
CAPS (3-(Cyclohexylamino)-propanesulfonic acid)	9.7–11.1	10.40

Table 2. Common biological buffers, their effective range, and their p*Ka* values at 25°C

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Usually, it is necessary to test the growth of actinobacteria from pH 4.0 to 13.0 and determine the strain growth pH range and the optimum pH value by using the following buffer system: pH 4.0–5.0: 0.1 M citric acid/0.1 M sodium citrate; pH 6.0–8.0: 0.1 M KH_2PO_4/0.1 M NaOH; pH 9.0–10.0: 0.1 M $NaHCO_3$/0.1 M Na_2CO_3; pH 11.0: 0.05 M Na_2HPO_4/0.1 M NaOH; pH 12.0–13.0: 0.2 M KCl/0.2 M NaOH [11]. Negative controls for each buffer were used and the final pH was determined by using an indicator of acidity.

3.3. NaCl ranges and optima for growth

Salt tolerance experiments mainly test the tolerance ability of the organism to NaCl and other salts, and determine the optimum concentration for growth. Inoculate liquid media containing a range of NaCl (usually 0–30%, W/V, or relative molar concentrations) concentrations and measure the growth response turbidimetrically. Bennett's medium or YIM38 medium or nutrient medium can be used as basal medium.

For some marine actinobacteria, NaCl alone may not substitute for filtered seawater, which should be sterilized by filtration and added aseptically to the sterilized medium. Even if seawater is used, it may need to be aged for a few weeks in a glass vessel in the dark to be effective. Seawater contains 3% NaCl, and testing marine organisms for growth at levels below this can be done by using various proportions of distilled water to seawater in the medium, or by using artificial seawater in which the level of NaCl can be varied.

3.4. Utilization of carbon source

Utilization of carbon source tests usually uses turbidimetric method. Use a chemically defined basal medium that lacks a carbon source, but otherwise is suitable for growth of the actinobacteria being tested. The basal medium is Pridham and Gottlieb carbon utilization medium [8]. Add carbon sources to a concentration (sugar alcohols 0.5–1%, others 0.1–0.2%). After growth has occurred, measure the growth response turbidimetrically with a spectrophotometer.

Basic medium for carbon source utilization	**(G/Liter)**
$(NH_4)_2SO_4$	2.64 g
KH_2PO_4	2.38 g
K_2HPO_4	5.65 g
$MgSO_4 \cdot 7H_2O$	1.0 g
$CuSO_4 \cdot 5H_2O$	0.0064 g
$FeSO_4 \cdot 7H_2O$	0.0011 g
$MnCl_2 \cdot 4H_2O$	0.0079 g
$ZnSO_4 \cdot 7H_2O$	0.0015 g
Final pH (at 25°C)	7.2–7.4

Note:

1. Use liquid medium to avoid the influence of agar.
2. Thermolabile carbon sources should be sterilized by filtration (filter sterilize 10% solution through bacteriological filter) or ether sterilization (weigh an appropriate amount of the dry carbon source and spread in a pre-sterilized Erlenmeyer flask fitted with a loose cotton plug. Add sufficient acetone-free ethyl ether to cover the carbohydrate. Allow ether to evaporate at room temperature under a ventilated fume hood overnight or longer. When all ether has evaporated, add sterile distilled water aseptically to make a 10% w/v solution of the carbon source).
3. Controls required for the test: No carbon source (negative control); D-glucose (positive control).
4. For marine actinobacteria, instead of distilled water, use a synthetic seawater and sterilize the media by filtration.

3.5. Utilization of nitrogen source

Use the turbidimetric method to test the utilization of nitrogen source, especially sole nitrogen sources. Basal medium that omit nitrogen source but include a suitable carbon source are used. Add nitrogen source to a concentration (usually 0.5%).

Basic medium for nitrogen source utilization	(G/Liter)
D-Glucose	1.0 g
$MgSO_4 \cdot 7H_2O$	0.05 g
NaCl	0.05 g
$FeSO_4 \cdot 7H_2O$	0.001 g
K_2HPO_4	0.01 g
Final pH (at 25°C)	7.2–7.4

Note:

1. Use liquid medium to avoid the influence of agar.
2. Thermolabile nitrogen sources should be sterilized by filtration or ether sterilization.
3. Controls required for the test: negative control (no carbon source).

3.6. Enzymological characteristics

Some enzymological characteristics are of such primary importance to a genus or species description that they must appear in every published description.

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Oxidase test

This method tests for an enzyme that transfers electrons from a donor molecule to O_2, thereby forming H_2O. Oxidase-positive organisms are usually aerobes or microaerophiles that can use O_2 as their final electron acceptor. The test reagent, N,N,N′,N′-tetramethyl-*p*-phenylenediamine (TMPD), acts as an artificial electron acceptor for the oxidase and the reduced form is the colored compound indophenol blue.

Prepare a 1% (wt/vol) solution of TMPD in certified-grade dimethylsulfoxide (DMSO). The solution is stable for at least a month under refrigeration. Test methods:

a. Method described by Kovacs [12]: Soak a small piece of filter paper in 1% Kovács oxidase reagent and let dry. Use a loop and pick a well-isolated colony from a fresh (18- to 24-h culture) bacterial plate and rub onto treated filter paper (please note on recommended media and loops). Observe for color changes. Microorganisms are oxidase positive when the color changes to dark purple within 5 to 10 sec. Microorganisms are delayed oxidase positive when the color changes to purple within 60 to 90 sec. Microorganisms are oxidase negative if the color does not change or it takes longer than 2 min.

b. Method of Tarrand and Gröschel [13]: Place a piece of Whatman no. 40 ashless filter paper, quantitative grade, in a petri dish and wet it with 0.5 ml of the TMPD-DMSO solution. Use a cotton-tipped swab to pick up one large isolated colony and allow the inoculum on the swab to dry for 5 sec. Tamp the swab lightly 10 times on the wet filter paper. Development of a blue-purple color in 15 sec is a positive test.

c. Colony methods: 1% TMPD directly drops on the colonies. The color from pink to scarlet in 15 sec is a positive test. To speed up the color change, add one drop of toluene first on colonies.

Note:

1. For this test, do not use cultures grown on selective media. Results from old cultures may be unreliable. Use the organism in exponential growth period. Do not use cultures grown on media containing fermentable carbohydrates, as acid from fermentation may inhibit oxidase enzyme activity and result in false negatives.

2. This test should avoid iron pollution, otherwise easy to produce false positive results. Reagent is fresh.

3. Reagent is highly toxic; contact with skin should be avoided.

Catalase test

Catalase catalyzes the disproportionation reaction $2H_2O_2 \rightarrow 2H_2O + O_2$, thereby helping to prevent oxidative damage to cells caused by H_2O_2. Add 0.2 ml of a 3–10% H_2O_2 solution to a screw-cap test tube. Using a platinum loop, disposable plastic loop, or glass rod, remove some growth from a colony or agar slant and rub the growth on the inner wall of the tube. Cap the tube (to prevent escape of aerosols) and slant it, so that the H_2O_2 solution covers the growth. Effervescence within 30 sec indicates a positive reaction.

Note: For this test, do not use cultures grown on blood-containing media, as blood contains catalase; however, cultures grown on a medium containing heated blood, such as chocolate agar, can be used. Some bacteria can make catalase only when provided with heme; these organisms are negative when cultured on media lacking blood, but are positive when cultured on chocolate agar. Some bacteria make a pseudocatalase (a non-heme catalase) when grown on media lacking blood, but containing little or no glucose; they are negative for catalase when cultured on media containing 1% glucose. With anaerobic organisms, expose the culture to air for 30 min before performing the test, as some anaerobes have an inducible catalase.

Urease test

Urease test check he ability of an organism to produce an exoenzyme, called urease. Urease catalyzes the reaction $(NH_2)_2CO + H_2O \rightarrow 2NH_3 + CO_2$. The ammonia that is formed causes the medium to become alkaline: $NH_3 + H_2O \rightarrow NH_4^+ + OH^-$. The alkalinity can be detected with a pH indicator.

Prepare the following medium (per liter of distilled water): peptone 1 g; NaCl 5 g; glucose 1 g; KH_2PO_4 2 g; phenol red 0.012 g; agar 15 g; pH 6.8–6.9. Autoclave and cool to 55°C. Add 30% (W/V) filter-sterilized (or ether-sterilized) solution of urea to make final concentration of 2%. Mix and dispense appropriate portions into tubes or plates. Prepare a control medium lacking urea. Inoculate the surface of the slant or plate for 4 days. Look for development of a red-violet color compared to an uninoculated control. The medium can also be prepared as a liquid medium by omitting the agar [14].

For other organisms that grow poorly or not at all on Christensen's medium, use a test medium of the composition (per liter of distilled water): BES buffer 1.065 g; urea 20.0 g; phenol red 0.01 g; pH 7.0. Also prepare a control medium lacking urea. Sterilize both media by filtration and dispense 2.0-ml portions into sterile tubes. Culture the test organism in a suitable liquid medium. Centrifuge the cells and suspend them in sterile distilled water to a dense concentration. Add 0.5 ml of the suspension to the test medium and the control medium. Incubate the tubes for 24 h at the optimal temperature for the organism. Look for the development of a red-violet color in the test medium, but not in the control medium [15].

Lipase test

Lipase activity can be shown by using Tweens, for example, Tween 80 (polyethylene sorbitan monooleate, an oleic acid ester), Tween 40 (a palmitic acid ester), and Tween 20 (a stearic acid ester). Lipolytic organisms split off the fatty acid, and the calcium salts of the fatty acids produce opaque zones around the colonies.

Prepare a basal medium containing the following (per liter of distilled water): peptone 10.0 g; NaCl 5.0 g; $CaCl_2$ $2H_2O$ 0.1 g; agar 9.0 g; pH 7.4. Sterilize by autoclaving (121°C, 20 min). Autoclave the desired Tween separately (121°C, 20 min). Cool the basal medium to 45–50°C, add the Tween to give a final concentration of 1.0%, shake until the Tween is completely dissolved and pour into plates. Inoculate the cultures as lines on the surface of the agar. Incubate for up to 7 to 14 days, inspecting daily. Look for an opaque halo around the growth [16].

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Gelatin liquefaction

The gelatin hydrolysis tests for an organism's ability to break down the protein gelatin, which is derived from collagen. Gelatin causes the media to thicken, especially at cooler (below 28°C) temperatures. If the organism can release gelatinase enzymes, the gelatin is broken down or liquefied. The media is checked over a period of about a week after inoculation and incubation at room temperature, for gelatinase activity. The tube is placed on ice for a few minutes; and if the media fails to solidify, it is considered a positive test. The gelatinase reaction may be slow or incomplete.

The conventional methods require long periods of growth, long periods for development, or are difficult to interpret. Now, commonly use the trichloroacetic acid (TCA) enhancement to be more rapid and sensitive [17]. Prepare gelatin agar plates (per liter of deionized water): tryptic soy agar powder 40.0 g, gelatin 16.0 g. Make a single streak or spot of the microorganism from a stock culture onto a gelatin agar plate and/or casein agar plate and incubate at 30–35°C. Prepare a stock solution of 35% (W/V) TCA in deionized water. After incubation for 3 h (or 24 h for the casein hydrolysis test), flood the plate with the TCA solution. Look for occurrence of a clear zone around the growth within at least 4 min. With casein hydrolysis, clear zones may be visible without adding TCA, but the TCA enhances the visibility.

Coagulation and peptonization of milk

Milk coagulation and peptonization test the ability of actinobacteria to produce protease. Coagulation is that mild protein is preliminarily degraded into big pieces by organism. Further degradation is peptonization.

Prepare milk coagulation and peptonization medium (per liter of distilled water): skim milk powder 200 g; $CaCO_3$ 0.2 g. Dispense 3–5 ml portions into narrow tubes and sterilize by autoclaving (115°C, 15 min) or fractional sterilization for 2–3 times. Inoculate the tubes and observe in 5, 10, 20, 30 days, respectively. Milk solidification occurrence is the phenomenon of coagulation. Clots further hydrolyzed into liquid, is the phenomenon of peptonization. Peptonized exudates is translucent, typically begins after coagulation.

Starch hydrolysis

Starch hydrolysis tests the ability of an organism to produce certain exoenzymes, including a-amylase and oligo-1, 6-glucosidase, that hydrolyze starch. Starch molecules are too large to enter the bacterial cell, so some bacteria secrete exoenzymes to degrade starch into subunits that can then be utilized by the organism. Make a single spot of the test organisms on a plate of the agar (within 5 mm in diameter) and incubate.

Prepare a basal medium containing the following (per liter of distilled water): soluble starch 10 g; K_2PO_4 0.3 g; $MgCO_3$ 1 g; NaCl 0.5 g; KNO_3 1 g; agar 15 g; pH 7.2–7.4. Prepare Gram's iodine solution by grinding 1.0 g of iodine crystals together with 2.0 g of KI in a mortar (slowly add 300 ml of distilled water while grinding until the iodine is dissolved). After growth occurs, flood the plate with the iodine solution. Starch stains blue with iodine, so look for colorless areas around the microbial growth.

Cellulose hydrolysis

Cellulose hydrolysis tests the ability of an organism to produce cellulase.

Conventional method: Prepare a cellulose hydrolysis medium (per liter of distilled water): $MgSO_4$ 0.5 g; NaCl 0.5 g; K_2HPO_4 0.5 g; KNO_3 1 g; pH 7.2. A filter paper (5 × 0.8 cm) submerges in liquid medium. Sterilize by autoclaving (121°C, 20 min). Inoculate the cultures on the filter paper. Incubate for 1 month to observe whether filter paper is decomposed.

Congo red-polysaccharide method [18]:The interaction of the direct dye congo red with intact β-D-glucans provides the basis for a rapid and sensitive assay system for bacterial strains possessing β-(1 → 4),(1 → 3)-D-glucanohydrolase, β-(1 → 4)-D-glucanohydrolase, and β-(1 → 3)-D-glucanohydrolase activities. Prepare basal medium contain cellulose. Inoculate for 7 to 14 days. After growth occurs, flood the plate with the congo red (1 mg/ml) for 10–15 min. Wash with NaCl (1 mol/L) 2–3 times (15 min/time). Cellulose hydrolysis can produce transparent circle. Congo red can also be added directly to the medium.

Other method [19]: Prepare mineral agar culture media in which cellulose is to be provided as a sole carbon source (per liter of distilled water): KNO_3 0.5 g; K_2HPO_4 1.0 g; KCl 0.5 g; $MgSO_4$ $7H_2O$ 0.5 g; and agar, 15.0 g. Add 0.5 ml of a suitable trace metals solution. Autoclave, cool, and dispense into plates. Prepare a series of dilutions of the organism to be tested and spread 25–50 μl portions over the surface of the plates. Place a sterile disc of lens paper on the seeded surface of the plates and incubate for 3–7 days. Look for colonies that form visible holes in the paper. To increase visibility of the holes, stain the paper on the plates or after the paper is removed with 0.2% irgalan black in 2% acetic acid.

Nitrate reduction

Many organisms can respire anaerobically by using NO_3^- as a terminal electron acceptor for an electron transport system (nitrate respiration or dissimulator nitrate reduction). Nitrate broth is used to determine the ability of an organism to reduce nitrate (NO_3) to nitrite (NO_2) using the enzyme nitrate reductase. It also tests the ability of organisms to perform nitrification on nitrate and nitrite to produce molecular nitrogen. The Griess reaction has more recently been employed to detect nitrite and nitrate as products of nitric oxide synthase in bacterial identification [20].

Preparation of recipes:

Nitrate reduction medium (per liter of distilled water): beef (meat) extract 3.0 g; KNO_3 1.0 g; peptone 5.0 g; pH 7.2–7.4.

Nitrite reduction medium (per liter of distilled water): beef (meat) extract 3.0 g; KNO_2 1.0 g; peptone 5.0 g; pH 7.2–7.4.

For either broth substrate, carefully weigh the ingredients and heat gently into solution. Dispense into test tubes and add inverted Durham tubes. Autoclave for 15 min at 121°C.

Reagent A (Griess A): Sulfanilic acid 0.8 g; acetic acid (5N) 100 ml.

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Reagent B (Griess B): N, N-Dimethyl-α-naphthylamine 0.6 ml (replace alpha-naphthylamine); acetic acid (5N) 100 ml. (Fresh reagent has a very slight yellowish color.)

5N acetic acid is prepared by adding 287 ml of glacial acetic acid (17.4 N) to 713 ml of deionized water.

Zinc dust must be nitrate- and nitrite-free.

Protocol:

For either substrate, NO_3^- or NO_2^-, inoculate the medium with a heavy inoculum from well-isolated colonies of the test organism. Incubate at 28°C for 18–24 h, some actinobacteria need 7–14 days. When sufficient growth is observed in the tube, test the broth for reduction of the substrate.

For NO_3^- substrate: Observe for gas production in the Durham tube. Mix two drops each of reagents A and B in a small test tube. Add approximately 1 ml of the broth culture to the test tube and mix well. If the test organism has reduced the NO_3^- to NO_2^-, a red color will usually appear within 2 min, indicating the presence of NO_2^- in the tube. If no color change is seen within 2 min, there are several possible reasons. Either the organism (i) was unable to reduce NO_3^- at all, (ii) was capable of reducing NO_2^-, or (iii) reduced NO_3^- directly to molecular nitrogen. Zinc is a powerful reducing agent. If there is any NO_3^- remaining in the tube (option (i) above), a small amount of zinc dust will rapidly reduce it to NO_2^-. Therefore, the appearance of a red color after the addition of zinc dust to a colorless reaction tube indicates a negative reaction, i.e., the organism has failed to reduce NO_3^-. Zinc is added to the tube by dipping a wooden applicator stick in nitrate- and nitrite-free zinc powder, just enough to get the stick dirty, and then dropping it into the tube containing the culture broth and the reagents. If too much zinc is added, the color reaction may fade rapidly. If the broth remains colorless after the addition of zinc, the organism has also reduced the NO_2^-, intermediate product to N_2 gas or some other nitrogenous product. N_2 gas is usually visible in the Durham tube. In the absence of gas, the product is assumed to be other than N_2 gas.

Occasionally, a lighter pink color will appear after the addition of zinc dust because of partial reduction, i.e., some of the primary NO_3^- substrate remains in the tube. The original tube may be reincubated and retested the following day.

For NO_{2-} substrate: Observe for gas production on the surface and in the Durham tube. Mix two drops each of reagents A and B in a small test tube. Add approximately 1 ml of the broth culture to the test tube and mix well. If the test organism has reduced the NO_2^-, there will be no color change, indicating that all of the original NO_2^- is gone, i.e., reduced. Reduction is often confirmed by the presence of N_2 gas in the Durham tube or on the surface of the broth, but other nitrogenous products may be produced. Therefore, the absence of gas does not rule out reduction of NO_2^-. If a red color appears, it indicates the presence of NO_2^-, and therefore a negative reaction. Occasionally, a lighter pink color will appear because of partial reduction, i.e., some of the primary NO_2^- substrate remains in the tube. The original tube may be reincubated and retested the following day. There is no need to add zinc dust to this reaction.

Note:

1. Be sure to run a negative control, uninoculated broth, to illustrate that the remaining NO_2 will be reduced by zinc dust, producing a red color.

2. Because reduction of NO_3^- is assumed to be anaerobic, many published procedures warn that the medium needs to be anaerobic or deep enough to support an anaerobic process. However, later experiments have shown that the metabolism on the surface of the broth for most organisms that grow well in the broth will reduce enough dissolving oxygen for the reaction to take place.

3.7. Metabolic products

Thousands of characterization tests have been described in the microbiological identification. Those that follow are designed for detection of metabolic products and they are useful for physiological characterization beyond the more general features of an actinobacterial genus or species.

a. MR test (Methyl red test)

A type of fermentation called the mixed acid fermentation results in the formation of formic acid, acetic acid, lactic acid, succinic acid, ethanol, CO_2, and H_2 in a buffered medium. The combination of acids in the mixed acid fermentation usually lowers the pH of the culture below 4.2. The test is used mainly in the differentiation of enteric bacteria. The organism being tested must be capable of catabolizing glucose [21].

Prepare MR-VP medium containing the following (per liter of distilled water): peptone 7.0 g; K_2HPO_4 5.0 g; glucose, 5.0 g; pH 7.5. Dispense 2–3 ml portions into narrow tubes and sterilize by autoclaving. Inoculate the tubes lightly and incubate for 4 days at the optimum temperature for the organism. Add one drop of methyl red reagent (0.25 g methyl red dissolved in 100 ml of ethanol). Look for a red colour (MR positive). A weakly positive test is red orange and a yellow or orange color indicates a negative test.

b. V-P test (Voges-Proskauer test)

Some fermentative organism catabolizes glucose by the butanediol pathway, in which acetoin (acetylmethylcarbinol) occurs as an intermediate in the formation of 2, 3-butanediol. In the presence of KOH and O_2, the acetoin is oxidized to diacetyl, which in turn reacts with the guanidine group associated with arginine and other molecules contributed by peptone in the medium to form a pink- to red-colored product. The α-naphthol intensifies this color [21].

Prepare MR-VP medium containing the following (per liter of distilled water): peptone 7.0 g; K_2HPO_4 5.0 g; glucose, 5.0 g; pH 7.5. Make reagent A by dissolving 5.0 g of α-naphthol in 100 ml of absolute (100%) ethanol; the reagent must not be darker than straw color.

Prepare reagent B by dissolving 40.0 g of KOH in 100 ml of distilled water. Inoculate the tubes lightly and incubate for 2 days (routine test) and for 4 days (standard test) at the optimum temperature for the organism being tested.

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Add 0.6 ml of reagent A and agitate to aerate the medium. Add 0.2 ml of reagent B and again agitate the medium. Slant the tube to increase the aeration. Allow to stand for 15–60 min. Look for development of a strong cherry red color at the surface of the medium. A negative reaction shows no color or a faint pink to copper color.

c. Tryptophan decomposition (indole production)

Organisms that possess tryptophanase can carry out the following reaction: L-tryptophan → indole + pyruvic acid + NH_3. The indole can be detected by its ability to react with p-dimethylaminobenzaldehyde to form a quinoidal red-violet condensation compound [21].

Xylene extraction test is more sensitive than the conventional test. Grow the test organism in a suitable culture medium supplemented with 0.1–1.0% tryptophan. Avoid using media containing carbohydrates, nitrate or nitrite, as these may interfere with the test. Distribute in 2–3 ml portions and sterilize by autoclaving. When cool, inoculate with the organism to be tested and incubate for up to 3 days.

Prepare Ehrlich's reagent as follows: 1.0 g of p-dimethylaminobenzaldehyde, 95 ml of 95% ethanol and 20 ml of HCl.

Add 1 ml of xylene to the broth culture, shake vigorously, and allow the mixture to stand for about 2 min. Then add 0.5 ml Ehrlich's reagent slowly down the side of the tube so as to form a layer between the medium and the xylene. Do not shake the tube after addition of the Ehrlich's reagent. Look for development of a pink or red ring below the xylene layer.

d. Hydrogen sulphide production

Some anaerobic and facultatively anaerobic actinobacteria can produce abundant H_2S by the anaerobic reduction of $S_2O_3^{2-}$. The H_2S can be detected by its reaction with iron salts contained in the medium, which form a black precipitate of FeS. A different type of H_2S production is based on the ability of some organisms to form low levels of H_2S from sulfur-containing amino acids (cysteine, cystine, and/or methionine) by means of amino acid desulfurases. The gaseous H_2S so produced is detected by its reaction with lead acetate strips suspended above the surface of the medium.

Thiosulfate iron H_2S test [22]: Prepare peptone–iron agar medium (Tresner medium) as follows (per liter of distilled water): peptone 10.0 g; ferric ammonium citrate 0.5 g; agar 15 g. To achieve more satisfactory results, inoculums from actively growing cultures were used to streak the surface of the agar slants. After a short incubation period (15 to 20 h) at 28°C, the slants were observed. A pronounced bluish-black discoloration of the medium surrounding the colonies effected no change and indicated the production of hydrogen sulfide, whereas those organisms not producing H_2S in the medium emitted only faint tints of other colors.

Paper strip method [21]: Inoculate a suitable semisolid (0.2% agar) growth medium that contains a peptone or other source of sulfur amino acids. Suspend a strip of sterile, lead acetate-impregnated paper about a centimeter above the surface of the culture, fold the upper end over the lip of the tube, and hold it in place with the screw cap or cotton plug. During growth of the organisms, H_2S gas reacts with the lead acetate to form black PbS, beginning at the lower

part of the strip. Lead acetate strips can be prepared by soaking 5-cm strips of filter paper in a 5% aqueous solution of lead acetate, sterilizing them separately in tubes by autoclaving and drying them in an oven.

3.8. Relation to oxygen

Aerobes use O_2 as a terminal electron acceptor for an electron transport system, can tolerate a level of O_2 equivalent to or higher than that present in an air atmosphere (21% O_2), and have a strictly respiratory type of metabolism. Anaerobes are incapable of O_2-dependent growth and cannot grow in the presence of 21% O_2. Facultative anaerobes can grow both in the absence of O_2 and in the presence of 21% O_2. Microaerophiles respire with O_2 but cannot grow, or grow very poorly, under 21% O_2. They grow best at low O_2 levels; some require levels as low as 1%. Some microaerophiles can also respire anaerobically with electron acceptors other than O_2.

Semisolid agar method [23]: Autoclave a narrow culture tube that has been filled to 60% of its capacity with an appropriate culture medium containing 0.2% agar. After the medium has cooled to 45°C, add the inoculum, mix to distribute the organisms uniformly and then allow the agar to solidify. Alternatively, inoculate the medium by stabbing with an inoculating needle after the agar has gelled; this avoids the mixing that otherwise might add dissolved O_2 to the medium. Growth occurring only at the surface of the medium suggests that the organism is aerobic. However, a fermentable substrate should be present in the medium, because the organism might be a facultative anaerobe that not only respires with O_2 but also grows anaerobically by fermentation. Growth occurring only in the bottom region of the tube suggests that the organism is anaerobic. However, some extremely oxygen-intolerant anaerobes may not be able to grow even in the lowest region of the medium, because of the presence of small amounts of O_2 dissolved in the medium during the addition of the inoculum. Growth occurring throughout the tube suggests that the organism is a facultative anaerobe. It is important that no potential terminal electron acceptors other than O_2 should be present, as some aerobes can respire anaerobically. Growth occurring only in a disc several millimeters below the surface of the medium suggests that the organism is a microaerophile. Motile microaerophiles usually exhibit negative or positive aerotaxis, which results in their migration to a zone where the rate at which O_2 is diffusing to them matches the rate it is used by the organisms.

3.9. Susceptibility to antibiotics

Antibiotic sensitivity is the susceptibility of actinobacteria to antibiotics. Antibiotic susceptibility testing (AST) is usually carried out to determine which antibiotic [Table 3] will be most successful in treating a bacterial infection in organism.

Testing for antibiotic sensitivity is often done by the Kirby-Bauer method [24]: wafers containing antibiotics are placed on an appropriate agar plate where actinobacteria have been placed, and the plate is left to incubate. If an antibiotic stops the actinobacteria from growing or kills the actinobacteria, there will be an area around the wafer where the bacteria have not grown enough to be visible. This is called a zone of inhibition. The size of this zone depends

on how effective the antibiotic is at stopping the growth of the bacterium. A stronger antibiotic will create a larger zone, because a lower concentration of the antibiotic is enough to stop growth.

Antibiotics	Concentration (per milliliter)
Amikacin	30 μg
Aureomycin	30 μg
Ciprofloxaci	10 μg
Chloramphenicol	30 μg
Erythromycin	15 and 30 μg
Gentamicin sulfate	10 μg
Kanamycin	15 μg
Netilmicin	10 μg
Novobiocin	5 and 30 μg
Oleandomycin	10 μg
Penicillin G	10 U
Polymyxin B	10 and 30 U
Streptomycin sulfate	10 and 25 μg
Terramycin	2.5 and 30 μg
Tetracycline	10 and30 μg
Tobramycin	10 μg
Vancomycin	10 μg

Table 3. Common antibiotics and the suitable concentration

3.10. Antibacterial activity detection

Actinobacteria are the most economically and biotechnologically valuable prokaryotes. They are responsible for the production of the discovered bioactive secondary metabolites, notably antibiotics, antitumor agents, immunosuppressive agents, and enzymes. Because of the excellent track record of actinobacteria in this regard, it is necessary to preliminarily screen antibacterial activity of isolated actinomycetes.

Common test strains: *Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Mycobacterium tuberculosis avium, Candida albicans, Aspergillus niger*. Also test strains can be selected according to necessity.

Cross streak method [25]: The actinomycetes isolates were inoculated at the centre of the sterile agar plates and the plates were incubated at 28°C for 5–7 days. After incubation, the nutrient broth (peptone 5 g; beef extract 3 g; NaCl 5 g; distilled water 1000 ml) cultures of test bacteria were streaked perpendicular to the growth of the actinomycete isolates. The plates were incubated at 37°C for 24–48 h and the extent of growth inhibition of the test bacteria was observed. The absence of growth or a less dense growth of test bacteria near the growth of actinomycete isolate was considered positive for the production and secretion of antibacterial metabolite.

Double-layer agar diffusion method [4]: Prepare double-layer agar plates; the lower is water agar (0.8–1% agar). Cool the basal medium (YIM 38 agar medium or LB agar medium) to 45–50°C, add the suspension of test strains, mix and spread on the water agar layer. Inoculate tested actinobacteria for 7 days at 28°C. Or pour fermentation broth of tested actinobacteria into sterilized steel rim or holes on medium and inoculate for 24–48 h at 37°C. Or inoculate the filter paper containing fermentation broth on the plates for 24-48 h at 37°C. Observe and record the diameter of inhibition zone. Results: no antibacterial activity (no zone of inhibition); weak antibacterial activity (diameter of zone, 6–15 mm), strong antibacterial activity (diameter of zone, >15 mm).

4. Commercial multitest system for identification of actinobacteria

In recent 40 years, with the development of microelectronics, computer, molecular biology, physics, chemistry, and subject crossing universality, a significant contribution of scientific and technological development to the clinical microbiology was the development of miniaturized identification systems based on classical method. Several systems are commercially available [Table 4], and new systems are being developed continually. These systems were mostly based on modifications of classical methods and were improved by the incorporation of highly sophisticated, computer-generated identification databases tailored for each system [26, 27]. Each manufacturer provides charts, tables, coding systems, and characterization profiles for use with the particular multi-test system being offered. These systems offer the advantages of miniaturization and are usually used in conjunction with a computerized system for identification of the organisms. As mentioned earlier, the use of these systems can increase standardization among various laboratories because of the high degree of quality control exercised over the media and reagents. Now actinobacteria is defined as a phylum of Gram-positive bacteria with high G+C content in their DNA. Although classical actinobacteria have the largest and most complex bacterial cells, some groups of actinobacteria possess the small and simple cell. For these simple (rod, cocci-shaped, without hyphae differentiated) actinobacteria, physiological and biochemical experiments are more important. Their physiological and biochemical tests can be carried by the automatic identification systems like the common bacteria.

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Manufacturer	Test system	Designed for	Number of tests
bioMérieus	An-Ident	Anaerobes	21
	API 20E; API 20NE; API Rapid 20E	Enteric Gram-negative rods	20
	API 20 strep	*Enterococcoi*, *streptococci*	20
	API 50 CH	General use; based on carbohydrate catabolism	50
	API Campy	*Campylobacters*	10
	API Coryne	Coryneform rods	20
	API NH	*Haemophilus, Neisseria, Moraxella*	10
	API STAPH	*Staphylococci*	10
	API ZONE	Non-enteric Gram-negative rods	20
	GNI+	Aerobic and facultative anaerobic Gram-negative rods	28
	Vitek GPI	Gram-positive cocci; coryneform rods	30
	Vitek NGI	*Neisseria, Moraxella*, non-enteric Gram-negative rods	15
Biolog	Gen III	General use; based on reduction of tetrazolium salts when cells are oxidizing carbon sources	98
BD Diagnostic System	Crystal E/NF	Enteric and non-enteric Gram-negative rods	30
	Crystal Gram-Positive ID	Aerobic Gram-positive bacteria	29
	ID TRI Panel	Gram-negative and Gram-positive bacteria	30
	Oxi/Ferm Tub II	Fermentative, oxidase positive and non-fermentative Gram-negative rods	14
	Enterotube II	Enterobacteriaceae and other oxidase negative Gram-negative rods	15
Dade Behring Microscan	Rapid NEG ID3	Enterobacteriacea*e* and non-enteric Gram-negative rods	36
	Pos ID Type 2	*Streptococci, enterococci, staphylococci*	27
Remel	RapidID ONE	Enteric Gram-negative rods	19
	RapidID CB Plus	Coryneform rods	20
	RapidID NF Plus	Non-enteric Gram-negative rods	17
	RapidID NF	*Haemophilus, Neisseria, Moraxella*	13

Manufacturer	Test system	Designed for	Number of tests
	RapidID POS ID	*Streptococci* and *enterococci*	34
	RapidID STR	*Enterococci* and *streptococci*	14
Trek Diagnostic Systems	Sensititre AP80	Enterobacteriaceae and non-enteric Gram-negative rods	32
	Sensititre AP90	*Enterococci* and *streptococci*	32

The products information of manufacturer come from:

bioMérieux, Marcy l'Etoile, France (http:// www.biomerieux.com/servlet/srt/bio/portail/home);

Biolog, Hayward, CA. (http://www.biolog.com);

BD Diagnostic Systems, Franklin Lakes, NJ (http://www.bd.com);

Dade Behring, Inc., MicroScan Inc., West Sacramento, CA (now owned by Siemens Medical Solutions, Henkestraße 127, Erlangen 91052, Germany) (http://www.medical.siemens.com/webapp/wcs/stores/servlet/SMBridgeBq_catalogIdBe_-999B a_catTreeBe_100001Ba_langIdBe_-999Ba_storeIdBe_10001.htm);

Remel, Lenexa, Kansas (http://www.remel.com/clinical/ microbiology.aspx);

Trek Diagnostic Systems, Ltd., East Grinstead, West Sussex, UK (http://www.trekds.com).

Table 4. Some commercial multitest systems for prokaryote identification

4.1. API Numerical identification system

API (analytical profile index) is a classification of bacteria based on experiments, allowing fast identification. This system is developed for quick identification of clinically relevant bacteria. Because of this, only known bacteria can be identified. It was invented in the 1970s in the United States by Pierre Janin of Analytab Products, Inc. Presently, the API test system is manufactured by bioMérieux [28]. The API range introduced a standardized, miniaturized version of existing techniques, which up until then were complicated to perform and difficult to read.

API systems can determinate simultaneously more than 20 items of biochemical indicators. Choose appropriate API strip according to different bacterial groups. The API strip consists of more than 20 microtubes containing dehydrated substrates. The conventional tests are inoculated with a saline bacterial suspension which reconstitutes the media. During incubation, metabolism produces color changes that are either spontaneous or revealed by the addition of reagents. The assimilation tests are inoculated with a minimal medium and the bacteria grow if they are capable of utilizing the corresponding substrate. The reactions are read according to the Reading Table and the identification is obtained by referring to the Analytical Profile Index or using the identification software. Such as API 20NE is an identification system for non-fastidious, non-enteric Gram-negative rods [Figure 2].

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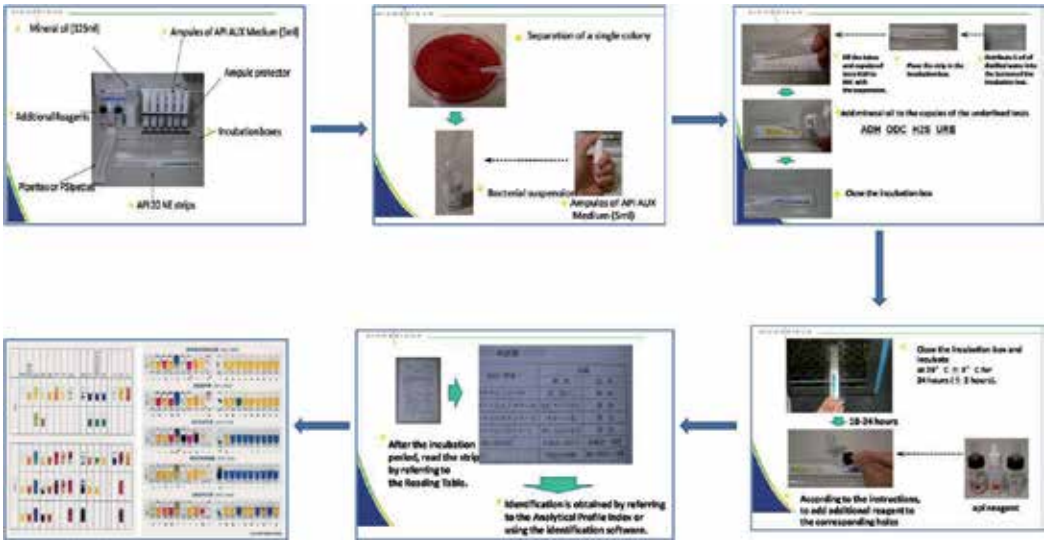

Figure 2. API 20NE operational flowchart

4.2. Biolog automatic bacterial identification system

The Biolog Microbial ID System (Biolog, Inc., Hayward, Calif.) can rapidly identify over 2,500 species of aerobic and anaerobic bacteria, yeasts, and fungi. These easy-to-use systems provide reference laboratory quality identifications. Biolog Systems do this without the labor-intensive requirements of conventional strips or panels [29, 30]. Biolog's latest generation redox chemistry enables testing and microbial identification of aerobic Gram-negative and Gram-positive bacteria in the same test panel. Gram stain and other pre-tests are no longer needed. A simple 1-min setup protocol and microbial samples are ready to be analyzed. Expanded GEN III database is designed to meet the needs of Biolog's broad customer base covering diverse disciplines of microbiology. All Biolog Microbial Identification Systems (manual, semi-automated, or fully automated) use the powerful new GENIII MicroPlate, allowing users to determine the most appropriate system to fit their current budget and level of throughput [Figure 3].

Figure 3. A common procedure of Biolog Microbial ID System [http://www.biolog.com]

It is important to realize that most such systems are designed for the identification of particular taxa and not for determining the physiological features of other taxa or new taxa. Indeed, a particular system may not even be applicable to other taxa. With these precautions in mind, multitest systems can provide useful information about the physiological characteristics of other organisms. For describing new taxa, the characterization systems that are used, as well as the inoculum age and size and the incubation temperature, must always be stated because reactions may not always agree with the results from classical characterization tests or with the results with other multitest systems.

Acknowledgements

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Chapter 5

Chemotaxonomy of Actinobacteria

Yongxia Wang and Yi Jiang

Additional information is available at the end of the chapter

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Abstract

Actinobacterial classification was originally based largely on morphological observation; it is not adequate in itself to differentiate between many genera, because some are so similar morphologically, but differ from their diagnostic chemical composition. In search of reliable classification methods reflecting phylogenetic relationships, at least to the genus level, it has been demonstrated that the analyses of chemotaxonomic markers fulfill these requirements. Chemotaxonomy of actinobacteria is concerned with the distribution of specific chemicals of the cell envelope such as amino acid, sugar, polar lipids, menaquinones, and fatty acid. For some coryneform genera of actinobacteria, analysis of mycolic acid composition is required specially. In this chapter, we will introduce the methods of chemotaxonomy including the extraction, fractionation, purification, and analysis of the target compounds.

Keywords: Chemotaxonomy, Amino acid, Sugar, Polar lipids, Menaquinones, Fatty acid, Mycolic acid

1. Introduction

Chemotaxonomy is the study of the chemical variation in microbial cell and the use of chemical characteristics in the classification and identification of bacteria including actinobacteria. In search of reliable classification methods reflecting phylogenetic relationships, it has been demonstrated that the analyses of chemotaxonomic markers fulfill these requirements [1]. Therefore, chemotaxonomy is an essential tool in the modern classification of bacteria; it has been recommended in a polyphasic approach to apply to the species, genus, and higher taxa level [2, 3]. Chemotaxonomy of actinobacteria is concerned with the distribution of specific chemicals of the actinobacteria cell envelope such as amino acid, sugar, polar lipids, menaquinones, mycolic acid, and fatty acid (Table 1) by using chemical techniques, including the extraction, fractionation, purification, and resolution of the target compounds [4].

Categories	Site in cell	Composition
Chemotaxonomic	cell	Sugars
	Cell wall	Amino acid
	Plasma membranes	Polar lipids
	Plasma membranes	Menaquinones
	Plasma membranes	Fatty acids
	Plasma membranes	Mycolic acids

Table 1. Chemotaxonomic markers applied in polyphasic approach of Actinobacteria

2. Amino acid of cell wall

Actinobacteria could be separated into broad groups at the generic level on the basis of morphology and cell wall composition [5]. For such grouping, the compositions of cell wall diaminopimeric acid isomers and whole cell sugars have become widely accepted as the taxonomic markers [6, 7]. Cell wall of actinobacteria consists of a single 20–80 nm thick homogeneous peptidoglycan and frequently represents more than 20% of the cell dry weight. Peptidoglycan constitutes between 40 % and 80 % of the wall weight, while the remainder is made up largely of other macromolecules (lipids, teichoic acids, and acidic polysaccharides and proteins) covalently linked either directly to peptidoglycan or to one another. The structure of peptidoglycan is very stable; it is an enormous polymer composed of many identical subunits; the polymer contains β1–4 linked disaccharides, N-acetylglucosamine and N-acetylmuramic acid, and several different amino acids. The backbone of this polymer is composed of alternating β1–4 linked disaccharides of N-acetylglucosamine and N-acetylmuramic acid. A peptide chain of four alternating D- and L-amino acids is connected to the carboxyl group of N-acetylmuramic acid. Chains of linked peptidoglycan subunits are joined by cross-links between the peptides. Often, the carboxyl group of the terminal D-alanine is connected directly or through a peptide inter-bridge to the amino group of diaminopimelic acid (Figure 1).

The differences in the amino acid sequence of the peptide chains, the mode of cross-links between the chains, and the diaminoacids present give important information for the classification of actinobacteria and have been used for the description of peptidoglycan type [9]. Detection of the presence of diaminoacids at position 3 of the peptide chain is useful for the classification of actinobacteria. *Meso*- and LL-diaminopimelic acid, L-ornithine, L-lysine, and L-diaminobutyric acid are found present at position 3 of the peptide chain [9–11].

A method for analysis of the diaminoacids of peptidoglycan from whole cells has been described by Staneck and Roberts, Lechevalier, Hasegawa et al., Bousfield et al., and Busse et al. [1, 12–15]; this method is rapid, simple, inexpensive equipment and requires only small amount of biomass. The procedure for rapid determination of the diaminoacids present in the

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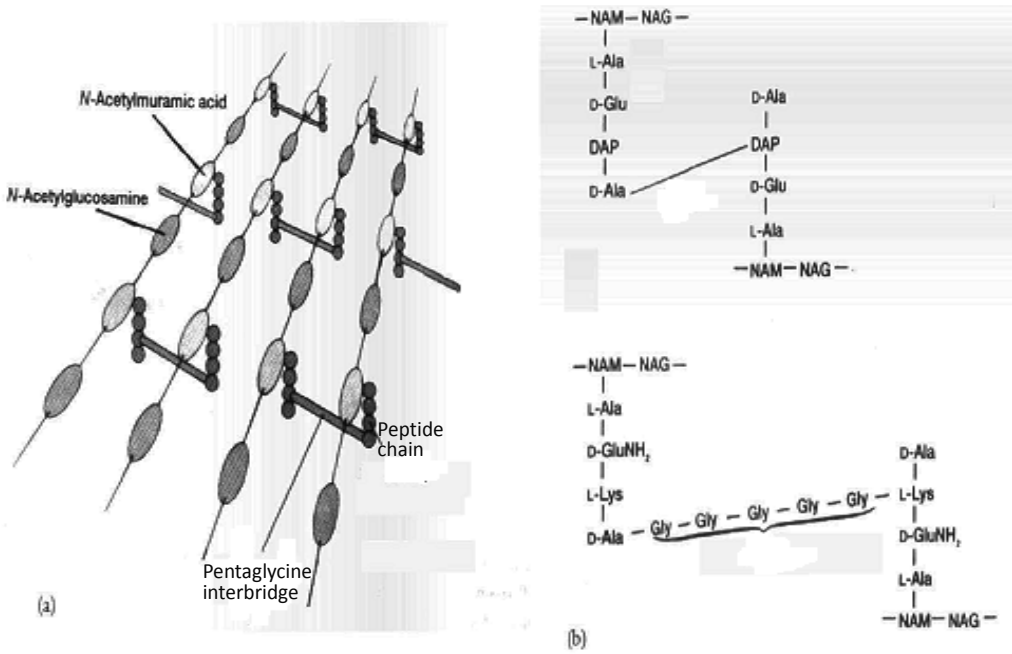

Figure 1. Peptidoglycan structure (a) and peptidoglycan cross-links type (b) (from Prescott et al. [8])

cell, described by Hasegawa et al. [14] with the solvent system of thin-layer chromatography [12], is quite suitable for separation of diaminoacids (Table 2 and Figure 2). However, for analysis of the amino acid in the peptide chains or inter-peptide bridge of the peptidoglycan, the cell wall extraction is required. Detailed cell wall extraction was described by Schleifer and Hancock [16, 17]. Here, we describe a method of cell wall extraction cited from the library of Yunnan Institution of Microbiology, Yunnan University (YIM) (Table 3).

2.1. Extraction of whole cell amino acid

1. A loop of cell mass is added into an ampule, add 0.2 ml of 6 N HCl into the ampule, seal and sand bath to hydrolyze for 16 h at 121°C.

2. Spot 1 μl to the bottom of a 10 × 20 cm of thin-layer plate coated with cellulose.

3. Spot 1 μl of 0.01 M DL-A_2pm containing both LL-and meso-A_2pm on the same plate as a standard.

4. Develop with methanol–water–6 N HCl–pyridine (80:26:4:10, v/v) for 3 h and dry the plates in a fume cupboard.

5. Repeat the fourth step once.

6. Spray the plate very lightly with 0.4% of ninhydrin and heated at 100°C for 2 min to reveal the spots; amino acids are shown as pink spots.

Table 2. Method of extraction and analysis of whole cell amino acids (modified from [12, 14])

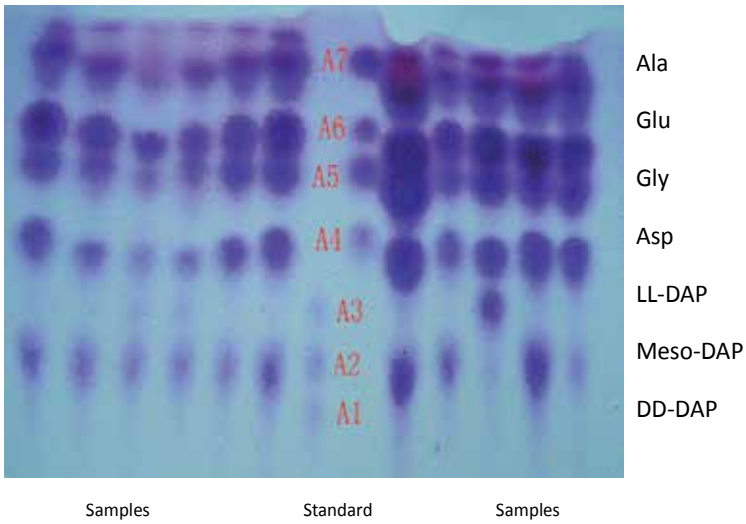

Figure 2. Separation of A_2pm isomers from the hydrolysate of whole cell by thin-layer chromatography

2.2. Preparation of cell wall amino acid

1. Add 1 g of freshly harvested or 0.3 g lyophilized cell mass into 10 ml screw cap test tube; add 1.5 ml of 1 % NaCl (w/v) into the test tube, mix , cap tightly, and stand for 10 min.
2. Add 7 ml of 0.05 mol/l PBS (pH7.6) into the test tube; sonicate for 40 min to lysis the cells (46 w, treatment 5 sec and standing 8 sec, total 40 min).
3. Centrifugate for 15 min at 4,000 rpm; remove the supernatant into a new 10 ml screw cap test tube and discard the precipitate.
4. Centrifugate for 40 min at 12,000 rpm, remove, and discard the supernatant.
5. Add 1 ml of 4 % SDS into the test tube containing precipitate, boiling water bath for 15 min or at room temperature overnight, centrifugate for 30 min at 12,000 rpm, and discard the supernatant.
6. Add 1 ml deionized distilled water, mix and centrifugate for 30 min at 12,000 rpm, and discard supernatant. The addition of deionized distilled water is repeated once.
7. The final insoluble pellet (precipitate) is dried at 65°C; add 200 μl of 6 N HCl into the test tube, mix until the dried pellet dissolved completely, and transfer the solution into the ampule, seal and sand bath overnight at 100°C.
8. Neutralize with 0.2 M NaOH to pH 7.0 and add three volumes sodium borate and mix.

Filter the mixture solution by using 0.45 μm fiber membrane, place the filtered solution into the sample bottle for detecting the amino acid composition by HPLC.

Table 3. Preparation method of cell wall amino acid

2.3. Detection of cell wall amino acid

Amino acids in cell wall hydrolysates were analyzed by precolumn derivatization with o-phthalaldehyde (OPA): ten amino acids standards (10 ml, 0.2 mM) and 10 ml hydrolyzed

purified cell wall were dissolved in 0.1 M (30 ml) borax buffer, and 10 ml OPA was added and allowed to react for 50 sec at room temperature and analyzed by high-performance liquid chromatography (HPLC). The elution time of 10 amino acids standards by HPLC is shown in Figure 3.

High-performance liquid chromatography (HPLC):

Agilent 1100, HPLC system equipped with an Agilent four-unit pump, a 7125 injector, a G1314A UV detector

Columns: ZORBAX Eclipse-AAA (4.6 × 150 mm, 3.5 μm; Agilent)

Columns temperature: 40°C

UV detect wavelength: 338 nm

Mobile phase A: 0.05 mol l^{-1} CH_3COONa and 0.3 % tetrahydrofuran

Mobile phase B: acetonitrile/methanol (1:1, v/v)

Gradient elution: 0–50–50 % buffer B by a linear increase from 0 to 25 to 30

Elution flow rate: 1.0 ml min^{-1}

Injection volume: 20 μl.

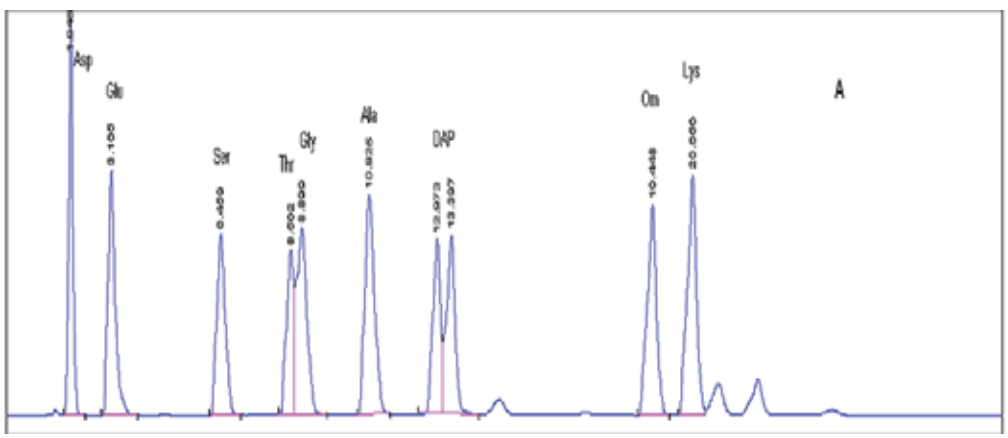

Figure 3. The elution time of 10 amino acids standards by HPLC

The presented method of cell wall amino acids analysis by HPLC will not separate well the LL-, meso-, and dd-A_2pm. So, analysis of the cell wall type of actinobacteria to the genus level requires a combination of HPLC and TLC.

3. Sugar of whole cell hydrolytes

For the classification and identification of actinobacteria, the analysis of sugars from whole cells is needed [10, 11, 19–23]. For discrimination of meso-diaminopimelic acid containing

actinomycetes, five whole cell sugar patterns have been recognized [24], based on the presence of distinct sugars (A: arabinose and galactose; B: madurose; C: no diagnostic sugars; D: arabinose and xylose; E: rhamnose). The combination of the characteristic diaminoacid and some amino acids used cell wall sugars to describe eight wall chemotypes to distinguish actinomycetes [13] (Table 4).

Cell wall chemotype	Characteristic cell wall components
I	L-Diaminopimelic acid, glycine
II	*meso*-Diaminopimelic acid, glycine
III	*meso*-Diaminopimelic acid
IV	*meso*-Diaminopimelic acid, arabinose, galactose
V	Lysine and ornithine
VI	Variable presence of aspartic acid and galactose
VII	Diaminobutyric acid, glycine
VIII	Ornithine
IX	*meso*-Diaminopimelic acid, various amino acids
X	*meso*-Diaminopimelic acid, L-Diaminopimelic acid

Table 4. Chemotypes of cell wall [13]

The analysis of diaminoacids and sugars from whole cell preparations is less time-consuming and often allows an allocation to the correct wall chemotype, but the resulting pattern may be contaminated by non-peptidoglycan-linked saccharides from the cytoplasm, capsules, or slimes. Different methods have been described for whole cell preparations [14, 19, 25], cell wall preparations [9, 26, 27], as well as analysis of sugars [12, 27, 28].

As the methods used to prepare whole cell extracts are similar, the procedure of extraction and analysis of whole cell sugar reported by Hasegawa et al. [14] are briefly described. Although the procedure from Staneck and Roberts [12] for thin-layer chromatography of diagnostic sugar on cellulose plates works reasonably well, it is not able to separate the mannose and arabinose. We described a modified method by changing the developed solvent to separate the mannose and arabinose (Table 5 and Figure 4).

1. Add a loop of cell mass into an ampule, add 0.1 ml of 0.25 N HCl into the ampule, seal and sand bath to hydrolyze for 15 min at 121°C.

2. Spot 2 μl to the bottom of a 10 × 20 cm of thin-layer plate coated with cellulose.

3. Spot 1 μl of standard solution 1 containing rhamnose, xylose, and mannose, and standard solution 2 containing ribose, madurose, arabinose, and glucose on the same plate, respectively.

4. Develop with ethyl acetate–pyridine–acetic acid–water (8:5:1:5, v/v) for 3 h and dry the plates in a fume cupboard.

5. Repeat the fourth step once.

6. Spray the plate very lightly with acid aniline phthalate and heated at 100°C for 4 min to reveal the spots.

Table 5. Extraction and analysis of whole cell sugars (modified from [12, 14])

Besides the procedure of TLC [12, 14], a better procedure to analyze whole cell sugars has been described in our laboratory [18]. It described a method to extract sugars of whole cell and a procedure for preparation of sugar sample for HPLC analysis (Table 6).

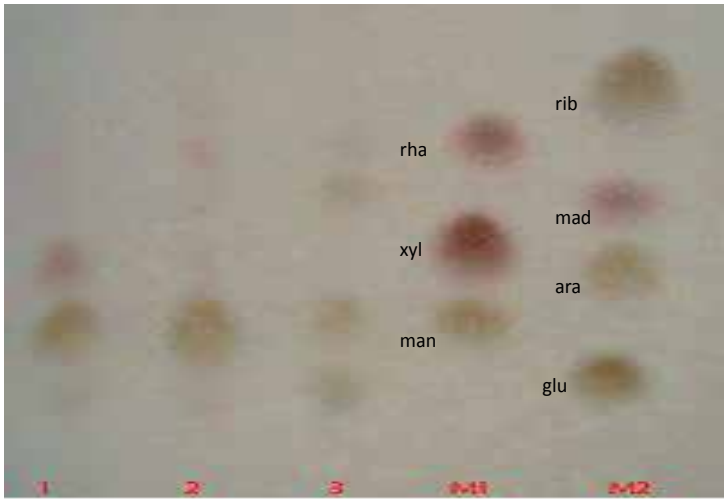

Figure 4. Separation of whole cell sugars from whole cell hydrolysate by thin-layer chromatography (rha = rhamnose, xyl = xylose, man = mannose, rib = ribose, mad = madurose, ara = arabinose, glu = glucose)

3.1. Extraction and preparation of whole cell sugar

1. Add 1 g of freshly harvested or 0.3 g lyophilized cell mass into ampule; add 0.5 ml of 0.5 N HCl into the ampule, seal and sand bath for 2 h.
2. Unseal the ampule, 80 μl hydrolysed whole cell solution and 80 μl 0.25 M methanol solution of 1-phenyl-3-methyl-5-pyrazolone (PMP) and 80 μl 0.2 M NaOH were mixed.
3. Mixture was allowed to react for 30 min at 70°C, cooled to room temperature and neutralized with 80 ml 0.2 M NaOH to pH 7.0, and extracted with isoamyl acetate.
4. After vigorous shaking and centrifugation, the organic phase was carefully discarded to remove the excess reagents.
5. The extraction process was repeated three times, using chloroform instead of isoamyl acetate for the third process; the aqueous layer was then collected and 10 ml was taken for HPLC analysis.

Table 6. Method for extraction and preparation of whole cell sugar [14, 29]

3.2. Analysis of sugar of whole cell hydrolytes

The sugar of whole cell hydrolytes was analyzed by high-performance liquid chromatography (HPLC). The elution time of nine sugar standards by HPLC is shown in Figure 5.

Agilent 1100, HPLC system equipped with an Agilent four-unit pump, a 7125 injector, a G1314A UV detector

Columns: ZORBAX Eclipse XDB-C18 (4.6 × 150 mm, 5 μm; Agilent)

Columns temperature: 40°C

UV detect wavelength: 250 nm

Mobile phase A: acetonitrile

Mobile phase B: 0.05 M sodium acetate (pH 6.9)

Elution: A : B = 17 : 83 (v/v).

Elution flow rate: 1.0 ml min^{-1}

Injection volume: 10 μl

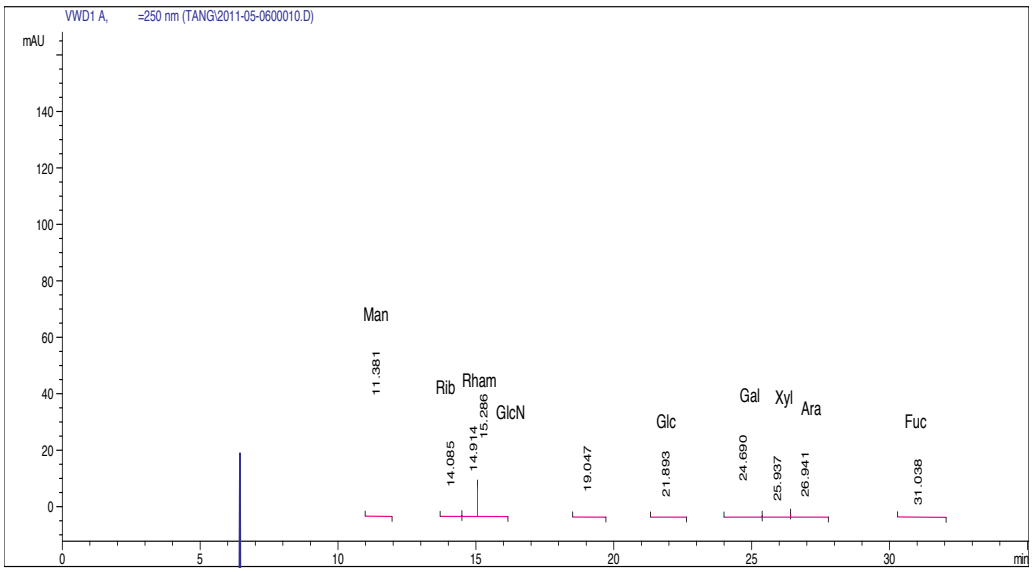

Figure 5. The elution time of nine sugar standards by HPLC (from YIM library) (Man = mannose, Rib = ribose, Rha = rhamnose, GlcN = glucosamine hydrochloride, Glc = glucuronic acid, Gal = galactose, Xyl = xylose, Ara = arabinose, Fuc = fucose)

4. Polar lipids

Polar lipids are important components of bacterial plasma membranes. Bacterial plasma membranes are composed of amphipathic polar lipids associated with specific membrane proteins. Amphipathic polar lipids consist of hydrophilic head groups usually linked to two hydrophobic fatty acid chains. Phospholipids are the most common polar lipids, including phosphatidyglycerol, diphosphatidyglycerol, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidyliositol, and other phosphatidylglycolipids. In addi-

tion, glycolipids and acylated ornithine or lysine amides also fall into this category. For the description and differentiation of actinobacteria, five phospholipid types (PI–PV) have been recognized (Table 7) [30, 31].

Phospholipid types	Characteristic phospholipids
P I	No nitrogenous phospholipids
P II	Only one nitrogenous phospholipid phosphatidyl ethanolamine
P III	Phosphatidyl choline and characteristic phospholipid
P IV	Glucosamine-containing phospholipids
P V	Phosphatidylglycerol and glucosamine-containing phospholipids

Table 7. Phospholipid types according to Lechevalier et al. [31]

In taxonomic studies, polar lipids have largely been analyzed by one- or two-dimensional thin-layer chromatography.

4.1. Extraction of polar lipids

The classic method of polar lipid extraction [13] is a time-consuming process taking at least 13 days from start to finish. Subsequently, more rapid procedures have been proposed by Minnikin et al. and Tindall [32, 33]. This utilizes a monophasic methanol for polar lipids extraction; the addition of more chloroform and water forces a phase separation. The lower, mainly chloroform, layer contains the polar lipids, whereas non-lipid components remain in the upper aqueous phase. Minnikin et al. [34] introduced a modified procedure, in which an initial extraction with hexane removes non-polar components such as isoprenoid quinones; in this way menaquinones and polar lipids can be extracted from a single sample of biomass. In this section, a modified procedure for polar lipids extraction is described (Table 8).

1. Place approximately 100~200mg of dried cell mass into a 50 ml tube with Teflon-lined screw cap.
2. Add 2 ml of 0.85% aqueous NaCl, followed by 15 ml methanol.
3. Heat for 10 min at 100°C in a boiling bath and cool to room temperature.
4. Add 10 ml chloroform and 6 ml 0.85% aqueous NaCl , then shake for 10 min.
5. Centrifuge at 8,000 rpm for 10 min, collect the lower layer.
6. The lower layer in a flask is evaporated to dryness under reduced pressure at 40°C on a rotary evaporator.

Table 8. Extraction of polar lipids (modified by Minnikin et al. and Tindall [32, 33])

4.2. Two-dimensional thin-layer chromatography

Separation of the mixture of polar lipids is performed by two-dimensional TLC (Table 9) on silica gel GF254 plate.

1. Dissolve the dried polar lipids in 100 μl of petroleum ether (boiling point: 70-90°C). Spot 10 μl to the bottom of a 10 × 10cm of thin-layer plate coated with silica gel (Merck F_{254}). Develop with chloroform–methanol–water (65:25:4, v/v) in the first dimension and dry the plates overnight in a fume cupboard.
2. Develop with chloroform–acetic acid–methanol–water (80:18:12:5, v/v) in the second dimension. Dry the plates in a fume cupboard.

Table 9. Two-dimensional thin-layer chromatography (modified by Minnikin et al. [35])

4.3. Identification of polar lipid component

Identification of the various lipids is carried out by comparison of their R_f values in the plates and staining behavior (Table 10) with references.

Molybdophosphoric acid for total lipids [36]
1. Dissolve 10% (w/v) molybdophosphoric acid in 95% (v/v) ethanol.
2. Spray the TLC plate and heat at 150°C for at least 10 min. Lipids show as dark spots on a light-green background.
Ninhydrin reagent for lipids containing free amino groups (modified by Consden and Gordon, [37])
1. Dissolve ninhydrin (0.1%, w/v) in acetone.
2. Spray plate and heat at 100°C for 5 min to reveal lipids which contain amino groups as pink spots. Mark the pink spots with a soft pencil to prevent them from fading on storage.
3. The same plate can be used for the detection of lipid phosphorus using molybdenum reagent
α-Naphthol reagent for containing sugar groups [38]
1. Dissolve 15 g α-naphthol in 100 ml 95% (v/v) ethanol.
2. Mix 10.5 ml of this solution with 6.5 ml H_2SO_4, 40.5 ml ethanol, and 4 ml water to make a working solution.
3. Spray the plate lightly and heat a 100°C for 10 min. Glycolipids appear as purple-brown or brown spot.
Dragendorff reagent for lipids containing quaternary nitrogen groups
1. Add bismuth nitrate (1.7 g) to 100 ml of 20 % acetic acid (solution A).
2. Add potassium iodide (40 g) to 100 ml water (solution B).
3. Mix solution A (3.5 ml) and solution B (5 ml) with acetic acid (20 ml) and water (50 ml) to make a working solution.
4. Spray the plate lightly at room temperature; lipids containing quaternary nitrogen shown as orange-red spots. Mark the orange-red spots with a soft pencil to prevent them from fading on storage.
5. The same plate can be used for the detection of lipid phosphorus using molybdenum reagent.
Zinzadze reagent for phosphorus-containing lipids [39]
1. Add molybdenum trioxide (40.11 g) to 1 L of 25 N H_2SO_4 and boil gently in a fume cupboard until all the residue dissolves (solution A).
2. Add powdered molybdenum (1.78 g) to 500 ml of solution A, and boil the mixture gently for 15 min and leave a cool (solution B).
3. Mix equal volumes of solutions A and B and dilute with two volumes of distilled water to make a working solution.
4. Spray the plate very lightly at room temperature, lipids containing phosphorus shown as blue spots.

Table 10. Spray reagents for identification of individual components

5. Menaquinones

Respiratory isoprenoid quinones are constituents of the bacterial cytoplasmic membrane as well as the mitochondrial membrane where they play an important role in the electron transport chain. The potential of analyzing the quinone system for the characterization of bacteria is based on the different types of quinones (e.g., ubiquinones, menaquinones and their derivatives dihydromenaquinone, demethylmenaquinone, and rhodoquinone), the length of isoprenoid side chain, and the number of saturated isoprenoid units. To date, menaquinones are the only type of respiratory isoprenoid quinones found in actinobacteria, and the variations in the number of isoprene units and hydrogenated double bonds make these membrane constituents of considerable chemotaxonomic value [40].

5.1. Extraction and purification of menaquinones

Menaquinones are free lipids that can be readily extracted from freeze-dried cells. Different methods have been described for the extraction of menaquinones [13, 33, 34, 41]. Menaquinones are normally extracted with organic solvents or with their mixture such as acetone, chloroform, and hexane (Table 11). However, they are susceptible to strong acid or alkaline, and photo-oxidation in the presence of oxygen and strong light conditions. But it is not necessary to work in a nitrogen atmosphere or dim light [42]. The menaquinones in these extracts are purified by preparative thin-layer chromatography (TLC), and analysis is then performed by HPLC.

1. Approximately 100 of lyophilized cells are extracted with a volume (40 ml) of chloroform–methanol (2:1 v/v) for approximately 1 h or overnight using a magnetic stirrer.
2. The cell/solvent mixture is passed through filter paper to remove cell debris.
3. The eluate is collected in a flask and evaporated to dryness under reduced pressure at 40°C on a rotary evaporator.

Table 11. Extraction of menaquinones (from Collins et al. [43, 44]).

The menaquinones can be readily purified from extracts by thin-layer chromatographic procedures using silica gel with hexane–diethylether as the developing solvent [43, 44]. Purified menaquinones are revealed by using UV light at 254 nm. In this section, we describe a new developing solvent to purify the menaquinones (Table 12).

1. Dissolve the dried menaquinones in 800 μl of acetone.
2. Apply extract (with 200 μl pipette) as a uniform streak (5 cm long) to a silica gel F254 sheet.
3. Develop the plate in methylbenzene, developing time ~20 min.
4. Allow plate to dry in a fume cupboard (~5 min), view menaquinones by brief irradiation with ultraviolet light at 254 nm. The menaquinones appear as dark-brown/purple bands on a green fluorescent background, R_f ~0.7.
5. Scrape gel containing menaquinones from the plate with spatula, dissolve scraped gel in 500 μl of methanol and elute through syringe and 0.45 μm filter membrane.

Table 12. Purification of menaquinones by thin-layer chromatography

5.2. High-Performance Liquid Chromatography (HPLC) analyzing the menaquinone component

TLC techniques generate only qualitative data. In contrast, high-performance liquid chromatography can be used to generate quantitative data. The resolving power and sensitivity of HPLC are also superior to that of thin-layer chromatographic techniques. The purified menaquinones are rapidly analyzed by reverse-phase high-performance liquid chromatography (rpHPLC) [41, 45].

Menaquinones series is analyzed by HPLC with a UV detector, a C18 column, and an online computer integrator. A large number of different mobile phases have been described [44, 45, 46]. Here, we prefer methanol/isopropanol mixtures (65:35, v/v) as the mobile phase to analyze the menaquinones. The column should be maintained at a constant temperature (40°C).

High-performance liquid chromatography (HPLC):

Agilent 1100, HPLC system equipped with an Agilent four-unit pump, a 7125 injector, a G1314A UV detector

Columns: Zorbax Eclipse XDB-C18 (4.6 × 250 mm, 5 μm; Agilent)

Columns temperature: 40°C

UV detect wavelength: 269 nm

Mobile phase A: methanol

Mobile phase B: isopropanol

Elution: A: B = 65:35 (v/v).

Elution flow rate: 1.0 ml min^{-1}

Injection volume: 20 μl.

6. Mycolic acid

Mycolic acids are high molecular-weight long-chain (up to 90 carbon atoms) 2-alkyl 3-hydroxy fatty acids found in representative of *Corynebacterium, Dietzia, Gordona, Myobacterium, Nocardia, Rhodococcus, Turicella*, and *Tsukumurellu* [47–53]. For the extraction and analysis of mycolic acids, different methods have been described based on TLC, GC, or HPLC [54–59].

6.1. Extraction of mycolic acids from whole cell

In this section, we describe the extraction and analysis of mycolic acids based on TLC [55].

1. Add 50–100 mg freeze-drying cells into a clean, dry test tube.
2. Add 3 ml mixture solvent of methanol, toluene, and conc. sulfuric acid (30:15:1) into the test tube and tightly seal the test tube.

3. Place the test tubes into a water bath at 75°C overnight or 16–18 h.
4. Cool the test tube down to room temperature and add of 2 ml petroleum ether (b.p. 60–80), the mixture is shaken and centrifuged for 10 min at low speed (3,000 rpm), collect the upper solvent phase.
5. Prepare a small column of ammonium hydrogen carbonate and prewash the small column with diethyl ether.
6. Pipette the upper solvent phase into a small column (ca. 1 cm) of ammonium hydrogen carbonate and collect the eluent in a small eppendorf tube (5 ml), then wash the small column again with diethyl ether.
7. Combine the washed eluent and evaporate to dryness under reduced pressure at 40°C on a rotary evaporator.

6.2. Analysis of mycolic acids from whole cell

The mycolic acids of whole cells were analyzed according to the described by Minnikin et al. [55].

1. Dissolve the dried mycolic acids in 200 μl of petroleum ether.
2. Spot 10 μl to the bottom of a 10 × 10cm of thin-layer plate coated with silica gel (Merck F_{254}).
3. Develop with petroleum ether, acetone (95:5, v/v) used for single-dimensional development and dry the plates in a fume cupboard. Develop with petroleum ether acetone (95:5, v/v), followed, in the second direction, by toluene, acetones (97:3) used for two-dimensional development system and dry the plates in a fume cupboard.
4. Staining, spray plate with molybdophosphoric acid and heat at 150°C for 5 min to reveal mycolic acids.

The evaluation of the presence of mycolic acid is only advisable, if other results (e.g., coryneform morphology) allocate the isolate to be identified to the group of coryneform bacteria, but the detection of mycolic acids strongly reduces the number of possible relatives. Further identification to the genus level is often possible by additional application of a few of the other described chemotaxonomic methods (quinones, fatty acids, polar lipids, and/or sugars).

7. Fatty acids

Fatty acid profiles analysis is well introduced for chemotaxonomy of bacteria. Fatty acids most commonly found in the cytoplasmic membrane and lipopolysaccharides of the outer membrane of Gram-negative bacteria as well as lipoteichoic acids in Gram-positive bacteria are relatively simple in structure and possess between 8 and 20 carbon atoms. The variation of carbon chain length, presence of saturated and unsaturated, occurrence of methyl groups fatty acids (iso-, anteiso-, and methylated within the molecule), occurrence of cyclopropane fatty acid (cyclo 17:0, cyclo 19:0), and occurrence of hydroxyl-fatty acid with an OH-group at position 2 or 3 of the molecule all have a taxonomic utility.

Commonly, different bacteria can have different fatty acids. Some fatty acids have a restricted distribution and may be diagnostic for particular groups. Branched fatty acids of the iso and/or anteiso type are important constituents of the *Flavobacterium/Cytophaga/Bacteroides* [60–62]. Cyclohexyl and cycloheptyl fatty acids are characteristic components of some acidothermophilic bacilli [63–65]. Cyclopropane fatty acids are often found in Campylobacter and Lactobacillus [66–68]. 10-Methyloctadecanoic acid and its homologs distribute in many actinomycetes [46, 69].

As the fatty acid composition of bacteria is dependent on the growth phase, temperature, and growth medium, preparing the biomass for analysis of the fatty acids should be taken to ensure that bacteria are grown under standardized conditions. The extraction of fatty acids can be performed with biomass (approximately 40 mg wet weight harvested from agar plates). For most actinomycetes, the reader can select the trypticase soy agar as the growth medium, but for the actinomycetes from extreme environment, the reader should select the optimum growth medium, as well as the possible media should omit material containing fatty acids, such as Tweens and serum.

7.1. Methods for analyzing fatty acids

Different methods have been described involving acid or base [55, 62, 70–72]. In taxonomic studies, it is important to use a consistent method. Here, we introduce a method for preparation of fatty acid methyl esters from whole wet cell material, which is developed by Sasser [73].

7.2. Preparation of reagents

Four reagents are required to liberate, esterify, and extract the fatty acids from living cells.

Reagent 1 Saponification Reagent

Sodium hydroxide (Certified ACS) 45 g

Methanol (reagent Grade) 150 ml

Deionized distilled water 150 ml

Add water and methanol to NaOH pellets in bottle. Stir until NaOH pellets have dissolved.

Reagent 2 Methylation Reagent

12 N hydrochloric acid 195 ml

Methanol (reagent Grade) 275 ml

Deionized distilled water 130 ml

Add acid to water, then to methanol while stirring

Reagent 3 Extraction Solvent

Hexane (HPLC Grade) 200 ml

Methyl-tert-Butyl-ether (HPLC Grade) 200 ml

Add MTBE to hexane and stir

Reagent 4 Base Wash

Sodium hydroxide (Certified ACS) 10.8 g

Deionized distilled water 900 ml

Add water to NaOH pellets in bottle. Stir until NaOH pellets have dissolved.

Warning

Reagent 1 and 2 are caustic, wear safety glasses and gloves.

Methyl-tert-Butyl-ether is extremely flammable. Extinguish all flames and heat sources before use.

Handle in a chemical fume hood.

7.3. Extraction of fatty acids.

Five steps involved in extraction of fatty acids from biomass [73] (see Figure 6):

1. Harvesting: removal of cells from the culture media. Scrap cells (~40 mg for each culture) on growth medium by using inoculation loop; add the scraped cells into a clean, dry 13mm × 100mm Teflon-lined screw cap test tube.

2. Saponification: lysis of the cells to liberate the fatty acid. Add 1 ml of reagent 1 into the test tube, tightly seal with a Teflon-lined screw cap, vortex tube for 5–10 sec, and place each samples tube into a rack. Then, place the rack of the batched samples tubes into a boiling water bath for 5 min, take out the samples tubes, vortex each tube for 5–10 sec, check the tubes for leakage, continue heating the samples tubes in a boiling water bath for 25 min. Remove the rack and cool the tubes at room temperature in a water bath.

3. Methylation: formation of methyl esters of the fatty acid. Add 2 ml of reagent 2 to each tube. Cap each tube tightly and vortex for 5–15 sec. Heat the tubes in an 80 ± 1°C water bath for 10 min. Remove and cool quickly by placing the rack of the batched samples tubes at room temperature in a water bath.

4. Extraction: transfer of the fatty acid methyl esters from the aqueous phase to an organic phase. Uncap each tube in the batch; add 1.25 ml of reagent 3 to each tube. Cap each tube tightly, place bath of tubes in rotator and mix end-over for 10 min. Uncap each tube in the batch, remove and discard the lower (aqueous) phase with a Pasteur pipette.

5. Base wash: aqueous wash of the organic extract prior to chromatographic analysis. Add 3 ml of reagent 4 to each tube, cap each tube tightly and rotate tubes end-over for 5 min. centrifugate for 3 min at 2,000 rpm. Remove the 2/3 of upper solvent phase and place into a GC sample bottle as the sample for detected of fatty acid profiles on the gas chromatograph.

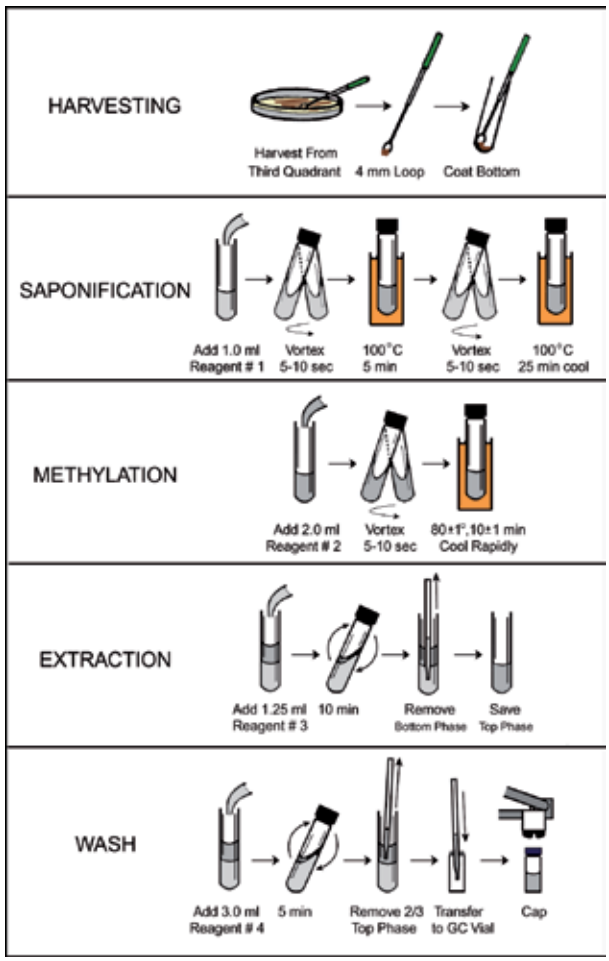

Figure 6. Five steps involved in extraction of fatty acids

Warning:

In the methylation step, excess time or excess temperature of the water bath can degrade some fatty acids.

7.4. Identification of fatty acids

For identification of the fatty acid profiles at the species level, integrated system, including a gas chromatography apparatus with identification software (the Sherlock Microbial Identification System) are required.

Gas chromatographic conditions

Gas chromatographic: Agilent 7890

Agilent 7890, column, flame ionization detector (FID)

Columns:

Agilent 19091B-102, 25 × 200 m × 0.33 μm

Oven temperature 170°C

Injector 250°C

Detector 300°C

Carries gas (hydrogen) 1 ml/min

Inlet pressure 9.000 psi

Electrometer setting 4 × 10^{12}amps

Splitting 1:20 0.4bar

Hydrogen (for flame) 30 ml/min

Synthetic air (for flame) 400 ml/min

Septum purge 10 ml/min

Auxiliary gas (N2) 30 ml/min

Sample 2 μl

8. Gas chromatography of fatty acids

In general, >5% of fatty acids as "major fatty acid" should be recorded. Fatty acid component of strain YIM 47672 analyzed with gas chromatography, as an example, is showed in Table 13 and Figure 7.

RT	Response	Ar/Ht	RFact	ECL	Peak Name	Percent	Comment1	Comment2
1.683	3.664E+8	0.026	----	7.008	SOLVENT PEAK	----	< min rt	
1.838	868	0.019	----	7.305		----	< min rt	
1.877	191	0.018	----	7.379		----	< min rt	
1.912	679	0.023	----	7.446		----	< min rt	
1.956	190	0.020	----	7.530		----	< min rt	
2.045	1201	0.046	----	7.701		----	< min rt	
2.202	1522	0.024	----	8.001		----	< min rt	
2.296	3546	0.031	----	8.181		----	< min rt	
2.628	223	0.021	----	8.817		----	< min rt	
3.028	1020	0.025	----	9.581		----		

RT	Response	Ar/Ht	RFact	ECL	Peak Name	Percent	Comment1	Comment2
3.134	837	0.044	----	9.783		----		
3.285	453	0.025	----	10.053		----		
4.676	795	0.032	----	11.754		----		
4.910	221	0.024	1.051	12.001	12:0	0.03	ECL deviates 0.001	Reference -0.002
5.135	567	0.035	----	12.192		----		
6.953	881	0.042	0.994	13.619	14:0 iso	0.13	ECL deviates 0.000	Reference -0.002
7.334	1399	0.044	0.986	13.892	14:1 w5c	0.21	ECL deviates -0.009	
7.479	6652	0.047	0.984	13.996	14:0	0.98	ECL deviates -0.004	Reference -0.005
7.555	4193	0.055	----	14.045		----		
7.674	3021	0.051	----	14.121		----		
7.768	1640	0.038	----	14.181		----		
7.868	1764	0.054	----	14.245		----		
7.976	1064	0.040	----	14.314		----		
8.049	2496	0.059	----	14.361		----		
8.156	2643	0.043	----	14.429		----		
8.231	1716	0.044	0.972	14.477	Sum In Feature 1	0.25	ECL deviates -0.001	13:0 3OH/15:1 i H
8.322	1111	0.044	0.971	14.535	15:1 anteiso A	0.16	ECL deviates 0.008	
8.459	7432	0.037	0.969	14.623	15:0 iso	1.08	ECL deviates 0.000	Reference -0.001
8.600	5278	0.037	0.967	14.713	15:0 anteiso	0.77	ECL deviates 0.000	Reference -0.001
8.821	533	0.039	0.964	14.854	15:1 w6c	0.08	ECL deviates -0.002	
9.049	39473	0.041	0.961	15.000	15:0	----	ECL deviates 0.000	
9.824	3366	0.043	0.952	15.460	16:1 iso H	0.48	ECL deviates -0.001	
10.109	**216646**	**0.041**	**0.949**	**15.629**	**16:0 iso**	**30.90**	ECL deviates 0.002	Reference 0.001

RT	Response	Ar/Ht	RFact	ECL	Peak Name	Percent	Comment1	Comment2
10.264	2016	0.042	0.948	15.721	16:0 anteiso	0.29	ECL deviates 0.003	
10.428	14559	0.042	0.946	15.819	Sum In Feature 3	2.07	ECL deviates -0.003	16:1 w7c/16:1 w6c
10.732	**96750**	**0.042**	**0.944**	**16.000**	**16:0**	**13.71**	ECL deviates 0.000	Reference -0.001
11.465	10036	0.043	0.938	16.419	Sum In Feature 9	1.41	ECL deviates 0.003	17:1 iso w9c
11.652	4552	0.045	0.937	16.527	17:1 anteiso w9c	0.64	ECL deviates 0.003	
11.832	19563	0.042	0.936	16.630	17:0 iso	2.75	ECL deviates 0.000	Reference -0.001
11.996	**77103**	**0.043**	**0.935**	**16.724**	**17:0 anteiso**	**10.82**	ECL deviates 0.001	Reference 0.000
12.122	**50353**	**0.045**	**0.934**	**16.796**	**17:1 w8c**	**7.06**	ECL deviates 0.004	
12.286	10094	0.051	0.933	16.890	17:0 cyclo	1.41	ECL deviates 0.002	
12.479	**41837**	**0.043**	**0.932**	**17.001**	**17:0**	**5.85**	ECL deviates 0.001	Reference -0.001
13.202	6556	0.046	0.928	17.408	17:0 10-methyl	0.91	ECL deviates -0.001	
13.307	1495	0.044	0.927	17.468	18:1 iso H	0.21	ECL deviates 0.004	
13.511	1684	0.052	0.927	17.583	18:3 w6c (6,9,12)	0.23	ECL deviates 0.006	
13.598	3730	0.044	0.926	17.632	18:0 iso	0.52	ECL deviates 0.000	Reference -0.002
13.763	**69473**	**0.047**	**0.925**	**17.725**	**Sum In Feature 5**	**9.66**	ECL deviates -0.002	18:0 ante/18:2 w6,9c
13.847	**35208**	**0.048**	**0.925**	**17.772**	**18:1 w9c**	**4.89**	ECL deviates 0.003	
13.937	3878	0.041	0.925	17.823	Sum In Feature 8	0.54	ECL deviates 0.000	18:1 w7c
14.034	14713	0.048	----	17.878		----		
14.141	1445	0.052	----	17.938		----		

RT	Response	Ar/Ht	RFact	ECL	Peak Name	Percent	Comment1	Comment2
14.248	8805	0.046	0.924	17.998	18:0	1.22	ECL deviates -0.002	Reference -0.004
14.601	1763	0.079	----	18.199		----		
14.742	1095	0.043	----	18.279		----		
15.507	2979	0.061	----	18.714		----		
15.583	1055	0.047	0.920	18.757	Sum In Feature 6	0.15	ECL deviates 0.001	19:1 w11c/19:1 w9c
15.784	3100	0.047	0.919	18.871	Sum In Feature 7	0.43	ECL deviates 0.004	19:0 cyclo w10c/19w6
16.010	573	0.038	0.919	19.000	19:0	0.08	ECL deviates 0.000	Reference -0.003
17.223	6351	0.051	----	19.699		----		
17.347	440	0.030	0.916	19.770	20:1 w9c	0.06	ECL deviates 0.000	
----	1716	---	----	----	Summed Feature 1	0.25	15:1 iso H/13:0 3OH	13:0 3OH/15:1 i H
----	14559	---	----	----	Summed Feature 3	2.07	16:1 w7c/16:1 w6c	16:1 w6c/16:1 w7c
----	69473	---	----	----	Summed Feature 5	9.66	18:2 w6,9c/18:0 ante	18:0 ante/18:2 w6,9c
----	1055	---	----	----	Summed Feature 6	0.15	19:1 w11c/19:1 w9c	19:1 w9c/19:1 w11c
----	3100	---	----	----	Summed Feature 7	0.43	un 18.846/19:1 w6c	19:1 w6c/.846/19cy
----	-----	---	----	----		----	19:0 cyclo w10c/ 19w6	
----	3878	---	----	----	Summed Feature 8	0.54	18:1 w7c	18:1 w6c
----	10036	---	----	----	Summed Feature 9	1.41	17:1 iso w9c	16:0 10-methyl

Volume: DATA; File: E068264.61A; Samp Ctr: 5; ID Number: 1107; Type: Samp; Bottle: 3

Method: TSBA6; Created: 5/26/2015 12:39:52 PM; Sample ID: **YIM 47672**

ECL Deviation: 0.003; Reference ECL Shift: 0.002; Number Reference Peaks: 13

Total Response: 756936; Total Named: 708097; Percent Named: 93.55%; Total Amount: 703700; *** No Matches found in TSBA6

Table 13. Fatty acid component of strain YIM 47672 with gas chromatography

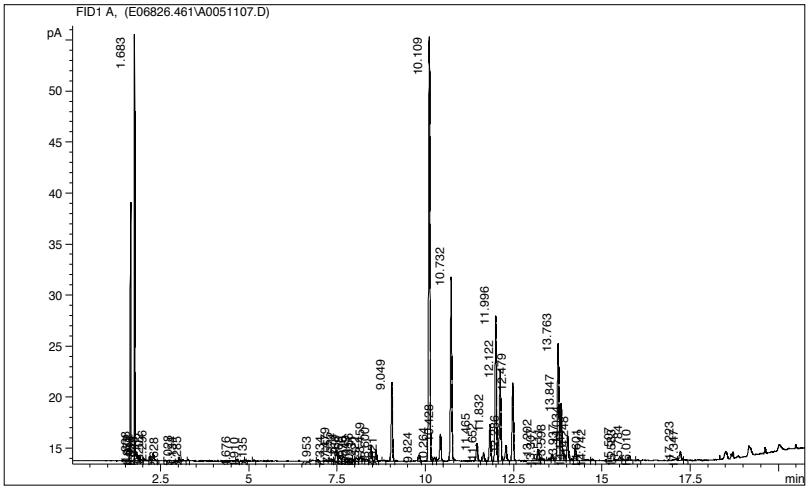

Figure 7. Gas chromatography of fatty acid of strain YIM 47672

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Chapter 6

Molecular Phylogenetic Identification of Actinobacteria

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Additional information is available at the end of the chapter

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Abstract

Molecular phylogenetics plays an important role in prokaryote taxonomy and identification. The content of this chapter is to introduce the common application of genetic criteria including 16S rRNA gene sequence nucleotide similarity and phylogeny, DNA G+C content, and DNA–DNA hybridization. However, the genomics era might put forward some new criteria. This chapter emphasizes the methods and basic principles of molecular identification and taxonomy of actinobacteria.

Keywords: 16S rDNA, molecular phylogenetic, genetic criteria

1. Introduction

Currently, the taxonomy and identification of prokaryotes rely on polyphasic combinations of phenotypic, chemotaxonomic and genotypic characteristics. Initially, taxon of actinobacteria is based on phenotypic markers such as morphology, growth requirements or pathogenic potential [1]. Later, physiological and biochemical properties of bacteria were also used for this purpose [2, 3]. Chemotaxonomy [4] and DNA–DNA hybridization techniques [5, 6] were widely used subsequently. The advent of DNA amplification and sequencing techniques, in particular of the 16S rRNA gene, constituted the crucial criteria forward for determining the taxonomic status of prokaryotes [7–9], greatly increased the rate of discovering novel species [10] and now routinely carried out as the first step in identifying novel organisms [11–13]. 16S rRNA gene was the best target molecule for studying the phylogenetic relationships because it is present in all the bacteria, functionally constant and composed of highly conserved as well as more variable regions. Some other molecular methods have been used in the classification of prokaryotes, such as multilocus sequencing typing (MLST) [14,15], SDS-PAGE analysis of whole cell soluble proteins [16], secondary structure and signature nucleotides analysis of variable areas of the 16S rRNA gene [17,18]. However, genomic age put forward that some

genomic characteristics have great potential in the taxonomy of bacteria and archaea as a substitute for the traditional method of determination of G+C content mol% and the labour-intensive DNA–DNA hybridization (DDH) technique [19-22].

2. Extraction and purification of genomic DNA

DNA is the carrier of genetic information and also the basis of gene expression. Molecular phylogenetic is the basic method for identification of actinobacteria. Before genetic-based methods, the first thing is the extraction and purification of DNA, and the quality of DNA is the premises to the success or failure of the experiment.

2.1. The principle of extraction and purification of genomic DNA

DNA contains all the genetic information which is all stored in the primary structure of DNA. Therefore, to ensure the quality of the DNA in the preparation of DNA samples is of great significance. Otherwise, it is difficult to get the right result. To ensure the quality of DNA, the following should be noticed: firstly, to avoid high temperature; secondly, to control the pH at a certain pH range (pH 5–9); Thirdly, maintain the ionic strength of buffer which is of significance to maintain the space configurations of DNA; And lastly, reduce the disruption of DNA in the course of extraction by physical factors, such as high speed oscillation, mixing and freezing–thawing. There are a lot of DNA enzymes in the environment that can digest the DNA or RNA, therefore some material used in the extraction has to be sterilized and enzyme inhibitors should be added in the extraction buffer at the same time. In addition, avoiding contamination of exogenous DNA is also important.

2.2. The main steps of extraction and purification of genomic DNA

2.2.1. Cell disruption

Genomic DNA is an intracellular constituent, so the first step of genomic DNA extraction is cell disruption. For cell disruption of microbial cells, the following several kinds of methods are commonly used: enzyme digestion, ultrasonic, grinding with liquid nitrogen, alkali treatment, microwave preparation, freeze–thawing and surfactant treatment.

2.2.2. Removal of nucleoprotein from genomic DNA

The binding force of nucleic acid and protein is mainly electrostatic forces, hydrogen bonding and Van der Waals interactions. The most difficult thing in the extraction is to separate the closely integrated protein from genomic DNA and avoid the degradation of DNA. There are some commonly used methods such as adding the concentrated solution of NaCl, which makes the nucleoprotein to depolymerize. Adding the SDS makes the protein free from genomic DNA in phenol/chloroform extraction. However, there are many kits which can remove nucleoprotein from the genomic DNA, and the result is always optimized.

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2.2.3. Precipitation of genomic DNA

Precipitation is the best way to concentrate DNA and is widely used. The advantage of precipitation is that it can remove some salt ions from the solution. It is also a step for nucleic acid purification. Ethanol, isopropanol and polyethyleneglycol (PEG) are commonly used for DNA precipitation. Ethanol is the most preferred precipitant. Two times the volume of ethanol is effective for precipitation of DNA and 2.5 times for RNA if under the appropriate salt concentration. The advantage of isopropanol is a small volume requirement and it is suitable for large DNA samples in a low concentration. The disadvantage of isopropanol is that it is easier to make salt coprecipitation with DNA and is difficult to volatilize. So washing with 70% ethanol several times to remove the isopropanol and salt is necessary. The PEG can select DNA fragments of different length. In addition, $MgCl_2$, NaAC, KAC, NH_4AC, Nil and LiCl are useful as assisted components.

2.2.4. Time and temperature of the nucleic acid precipitation

It is generally believed that the nucleic acid precipitation should be carried out at low temperature, such as –20℃ or –70℃ for a few hours or even an overnight. But this kind of treatment is easy to cause the salt coprecipitation with DNA, so at 0℃ or 4℃ for 30–60 min is recommended.

2.3. Some specific methods for extraction and purification of genomic DNA

2.3.1. Enzymatic disruption method

This method is frequently used and suitable for most actinobacteria.

i. 50 mg of the pretreated cell samples are suspended in 480 μl TE buffer and 20 μl of lysozyme solution (50 mg/ml), then put them in shaker at 37℃ for overnight.

ii. Add 50 μl of 20% SDS and 5 μl of proteinase K (20 mg/ml), vortexing for 1 min, then put incubate at 55℃ for 60 min.

iii. Supernatants are transferred to fresh microcentrifuge tubes after centrifugation at 10,000 × g for 5 min at room temperature.

iv. Add 550 μl of mixer of phenol: chloroform: isoamyl alcohol (25:24:1), vortexing for 1 min, centrifuge for 10 min (12,000 rpm). The aqueous phase containing the community DNA was transferred to another sterile microcentrifuge tube.

v. Repeat IV.

vi. Add 50 μl of 3 mol/L sodium acetate (pH 4.8–6.2), vortex gently, add 500 μl of isopropanol, or 800 μl ethylalcohol, then keep in the room temperature for more than 10 min or put it at 4℃ for 30 min to 2 h or overnight if necessary.

vii. Centrifuge for 10 min (12,000 rpm), discard the supernatant, add 200 μl of 70% ethanol, shake slightly, then centrifuge for 5 min (12,000 rpm), discard the ethanol. Repeat 1–2 times. Then dry at room temperature or at a higher temperature (≤55°C).

viii. Add 50 μl of 1×TE buffer to dilute the DNA (depend on the volume of DNA), preserve it at –20℃ for later use.

2.3.2. Extraction of genomic DNA using chelex-100

This method is fast, simple and convenient, but the extracted DNA is not suitable for long periods storing.

i. 5–10 mg of the pretreated cell samples are suspended with 50 μl chelex buffer (5% Chelex-100), incubate at 100°C for 20–40 min.

ii. Centrifuge for 10 min (12,000 rpm), then the supernatant was transferred to another sterile microcentrifuge tube, and keep it at 4℃ or –20℃ for later use.

2.3.3. Extraction of genomic DNA using microwave

i. 50 mg of the pretreated samples are suspended in 1 ml washing buffer.

ii. Centrifuge (5,700 rpm) for 1 min, discard the supernatant.

iii. Add 50 μl lysis buffer, vortex for 30 s, then treat with microwave at 600 w for 45 s.

iv. Add 500 μl preheat (65℃) extraction buffer, vortex for 5 s.

v. Follow step IV–VIII in Section 6.2.3.1

2.3.4. Extraction of genomic DNA by grinding with liquid nitrogen

This method is always used for mass extraction of DNA.

i. Put 1–2 g of wet cell mass into mortar, take a suitable amount of liquid nitrogen to cover the mass, and grind to freeze.

ii. Repeat grind for 4–5 times.

iii. Transfer the product to a sterile microcentrifuge tube (50 ml) with 7 ml TE buffer.

iv. Add 700 μl 20% SDS and 800 μl of proteinase K (20 mg/ml), vortex for 1 min, then incubate at 55℃ for 60 min (the final concentration of proteinase K is 20 μg/ml).

v. Supernatants are transferred to another tube after a centrifugation at 10,000×g for 5 min at room temperature (optionally).

vi. Add 8 ml of mixer of phenol: chloroform: isoamyl alcohol (25:24:1), vortex for 2 min, centrifuge for 10 min (12,000 rpm); the aqueous phase containing DNA is transferred to another sterile microcentrifuge tube (50 ml) (do not suck the waste in the middle).

vii. Repeat IV.

viii. Add 800 μl of 3 mol/L sodium acetate (pH 4.8–6.2) into the supernatant, vortex gently, then add 8 ml of isopropanol, or 16 ml of absolute ethyl alcohol, keep in the room temperature for more than 10 min or put it at 4℃ for 30 min to 2 h or overnight if necessary.

ix. Centrifuge for 10 min (12,000 rpm), discard the supernatant, add 4 ml of 70% ethanol, shake slightly, centrifuge it for 5 min (12,000 rpm), then discard the ethanol. Repeat 1–2 times, dry at room temperature or at a higher temperature (≤55°C).

x. Add 1×TE buffer (≥1 ml) to dilute the DNA (depend on the volume of DNA), preserve it at –20℃ for later use.

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2.4. Purification of genomic DNA

A high purity of DNA is necessary for determination of G+C content, DNA–DNA hybridization and sequencing. The following protocol could be a reference.

i. Add 480 μl 1×TE buffer to resuspend the extracted DNA from 100 mg of cell mass.

ii. Add 5 μl of proteinase K (20 mg/ml) and 15 μl RNase A (400 μg/ml), incubate at 37℃ for 30–60 min.

iii. Add 550 μl of mixer of phenol: chloroform: isoamyl alcohol (25:24:1), vortex for 2 min, centrifuge for 10 min (12,000 rpm), the aqueous phase containing DNA was transferred to another sterile microcentrifuge tube after centrifugation (do not suck the waste in the middle).

iv. Add 550 μl chloroform, vortex for 2 min, centrifuge for 10 min (12,000 rpm), and the aqueous phase containing DNA is then transferred to another sterile microtube.

v. Add 50 μl of 3 mol/L sodium acetate (pH 4.8–6.2) into the supernatant, vortex gently and then add 500 μl of isopropanol, or 800 μl absolute ethyl alcohol, vortex gently again, keep at room temperature for more than 10 min or put it at 4℃ for 30 min to 2 h or overnight if possible.

vi. Centrifuge for 10 min (12,000 rpm), discard the supernatant, add 200 μl of 70% ethanol, vortex slightly, centrifuge it for 5 min (12,000 rpm), discard the ethanol. Repeat 2–3 times. dry at room temperature or at a higher temperature (≤55°C).

vii. Add 50 μl of 0.1×SSC or deionized water to dilute the DNA (depend on the volume of DNA), preserve it at –20℃ for later use.

3. Amplification of 16S rDNA sequence

Polymerase chain reaction (PCR) is an ingenious technique used to exponentially amplify a specific target DNA sequence. PCR was developed by Kary Mullis in 1983. He won a Nobel Prize in chemistry in 1993 for his invention. PCR has been elaborated in many ways since its introduction and is now commonly used for a wide variety of applications including genotyping, cloning, mutation detection, sequencing, microarray, forensics and paternity testing.

Typical PCR is a three-step reaction (Figure 1.). The sample containing a dilute concentration of template DNA is mixed with a heat-stable DNA polymerase, primers, deoxynucleoside triphosphates (dNTPs) and buffer (including magnesium). In the first step of PCR, the sample is heated at 94–98℃ for 3–8 min, which pre-denatures the double-stranded DNA and splitting it into two single strands. In the second circulate step, the sample is heated at 94–98℃ for 30–60 s to denature the double-stranded DNA continually, then the temperature is decreased to approximately 52–65℃ (depend on the annealing temperature of primers) to allow the primers to bind or anneal with specific site in single strands which is also known as the template. Lastly, the temperature is typically increased to 72℃, allowing the DNA polymerase to react by the addition of dNTPs to create a new strand of DNA. The times of extension are varied depending upon the length of target sequence and the kind of polymerase. Generally, the extend speed

of *Taq*-polymerase is 1 kb/min. In the third step, it is a final extension, which is to repair and to fill some gaps of the products in the second step, and the reaction rate reaches a plateau in this step.

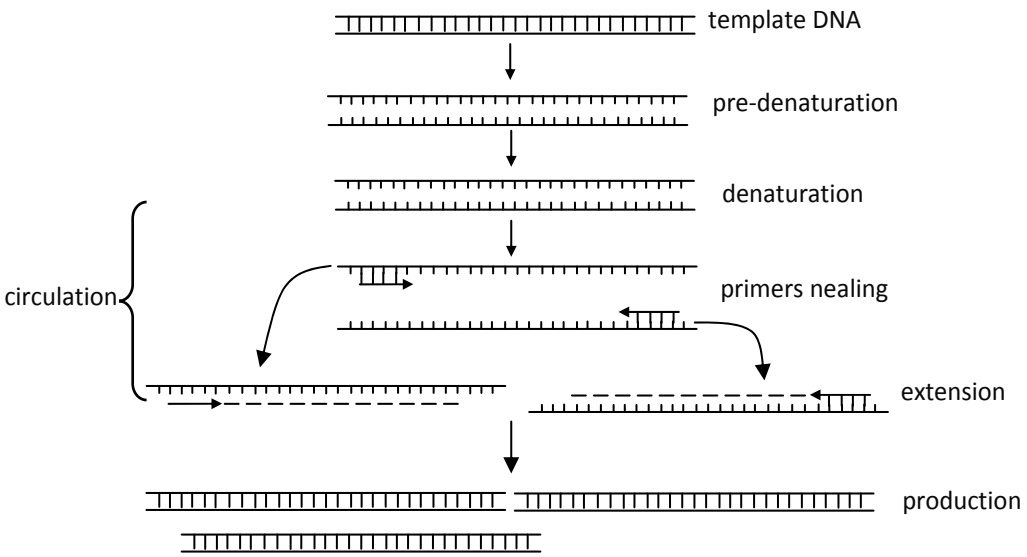

Figure 1. The principle of DNA amplification

3.1. Amplification of 16S rDNA

Universal primers of 16S rDNA for actinobacteria:

27F: AGAGTTTGATCCTGGCTCAG

1492R: GGTTACCTTGTTACGACTT

16S rRNA gene is the best target for studying the phylogenetic relationships because it is present in all bacteria, functionally constant, composed of highly conserved as well as more variable regions. As described above, determination of 16S rDNA sequence is routinely carried out as the first step in identifying novel organisms. The ingredients for amplification of 16S rDNA are listed in Table 1..

Generally, the condition for 16S rDNA amplification is:

1. 94℃ 4 min
2. 94℃ 45 s
3. 55℃ 45 s
4. 72℃ 90 s
5. Repeat steps 2–4 for 35 times

Ingredient*	Addition
10×PCR buffer	5.0 μl
dNTPs	4.0 μl
27F (25 pmol/μl)	1.0 μl
1492R (25 pmol/μl)	1.0 μl
Template DNA	1.0 μl
Taq DNA polymerase	0.3 μl
dd H_2O	37.7 μl
Total volume	50 μl

*The ingredients of the system are bought from TaKaRa.

Table 1. Composition and dosage of amplification

6. 72℃ 10 min
7. 4℃ hold (optional)

3.2. Potential problems in amplification of 16S rDNA sequence

i. Positive and negative controls must be used and run every time; if the negative controls become positive, the amplification should be carried out again.

ii. No products or all the stripes are weak. Higher temperatures and long reaction time for high GC content should be optional. Increase the dose of polymerase and template DNA and the number of cycles. Reducing the annealing temperature might be also useful. However, checking whether the system is out of date is also necessary.

iii. Nonspecific products appeared. In contrast to no products, reduce the dose of polymerase and template DNA and raise the annealing temperature.

iv. Impurities such as phenol or too much salt will result in no PCR product or nonspecific products.

v. Impure template DNA would result in sequencing failure or double peak for pure strain identification.

vi. If PCR product of wrong band, likely causes include: incorrect primer, template mutations, contamination and incorrect annealing temperature.

vii. Too many primers will lead to the annealing of themselves.

3.3. Detection of polymerase amplification products

Since the world's oldest electrophoresis experiment was carried out for nearly 200 years, electrophoresis technology has been continuously improved and developed. Now, electrophoresis is one of the most commonly used methods for biological macromolecule detection

and has played a huge boost. Electrophoresis is a technique also used to purify macromolecules, especially proteins and nucleic acids, which are different in size, charge or conformation. When charged molecules are placed in an electric field, they migrate towards either the positive or negative pole according to their charge. Nucleic acids have a consistent negative charge imparted by their phosphate backbone and migrate towards the anode. Nucleic acids are electrophoresed within a matrix or 'gel'. Commonly, the gel is cast in the shape of a thin slab with wells for loading the sample. The gel is immersed within an electrophoresis buffer (TAE or TBE) that provides ions to carry a current and to maintain the pH at a relatively constant value. The gel itself is composed of either agarose or polyacrylamide, each of which has attributes suitable to particular tasks: agarose is a polysaccharide extracted from seaweed. It is typically used at concentrations of 0.5–2%. Agarose gels are extremely easy to prepare: simply mix agarose powder with buffer solution, melt it by heating and pour the gel. It is also non-toxic. Agarose gels have a large range of separation but with relatively low resolving power. By varying the concentration of agarose, fragments of DNA from about 100 bp to 50,000 bp can be separated using standard electrophoretic techniques. Polyacrylamide is a cross-linked polymer of acrylamide. The length of the polymer chains is dictated by the concentration of acrylamide used, which is typically between 3.5% and 20%. Polyacrylamide gels are significantly more annoying to prepare than agarose gels and have a rather small range of separation, but with very high resolving power. Because oxygen inhibits the polymerization process, they must be poured between glass plates (or cylinders). Acrylamide is a potent neurotoxin and should be handled with care. Wear disposable gloves when handling solutions of acrylamide, and a mask when weighing out powder. In the case of DNA, polyacrylamide is used for separating fragments of less than 500 bp. However, under appropriate conditions, fragments of DNA differing in length by a single base pair are easily resolved. In contrast to agarose, polyacrylamide gels are used extensively for separating and characterizing mixtures of proteins. The protocol of detection of 16S rDNA sequences by agarose gel electrophoresis is:

i. To slot the organic glass mold in a horizontal position, put the comb in the right position based on your needs.

ii. Prepare 1.0% (w/v) agarose gels with TAE or TBE buffer and heated by microwave oven.

iii. Add the nucleic acid dye (GoodViewTM, EB-Ethidium bromide, GeneFinder™, or SYBER greenI) to the agarose gels after it cools down (<50℃), mix it gently.

iv. Pool the mixed agarose gels to the mold; if there are air bubbles, get rid of it.

v. Pull out the comb slightly after the agarose gels are hardened, make sure the pore is intact.

vi. Mix 5 μl amplification products with DNA loading buffer (depending on the concentration; 1–2μl for 5×loading buffer) and pipe the mix to the gel pore gently. Add the marker lastly.

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vii. Put the agarose gels which with samples into electrophoresis bath (TAE or TBE) gently, make sure the gel pore is closed to negative pole.

viii. Run the electrophoresis for about 30 min of a voltage at 4~7 V/cm.

ix. Take out of the agarose gels and the results were analyzed by gel imaging system.

x. Send the positive products for sequencing.

3.4. Analysis of 16S rDNA sequence

There are two main cases of 16S rDNA sequence analysis. One is a partial sequence of 16S rDNA sequenced from one direction and there is no need to assemble. Another kind is a contig assembled by two sequences which is always produced by clone to get an almost complete 16S rDNA sequence with high quality. Two types of files will be received from sequencing company, one is ablformat and could be opened by using Chromas, and another is Editseq format which could be opened using Editseq, Bioedit or Notepad. Quality map of sequence is shown in first one and an editable sequence is listed in the later. The qualified sequence is aligned in database (http://www.ezbiocloud.net/eztaxon and http://blast.ncbi.nlm.nih.gov are usually used). The analysis of alignment as well as construction of a phylogenetic tree will be detailed later. The SeqMan in the DNAStar package, Sequencher or vector NTI can be used for assembling. The contig can be assembled by SeqMan as in the following steps:

i. Open SeqMan, click 'sequence' and then click 'add', add the two sequences of abl format, click 'done' (Figure 2.).

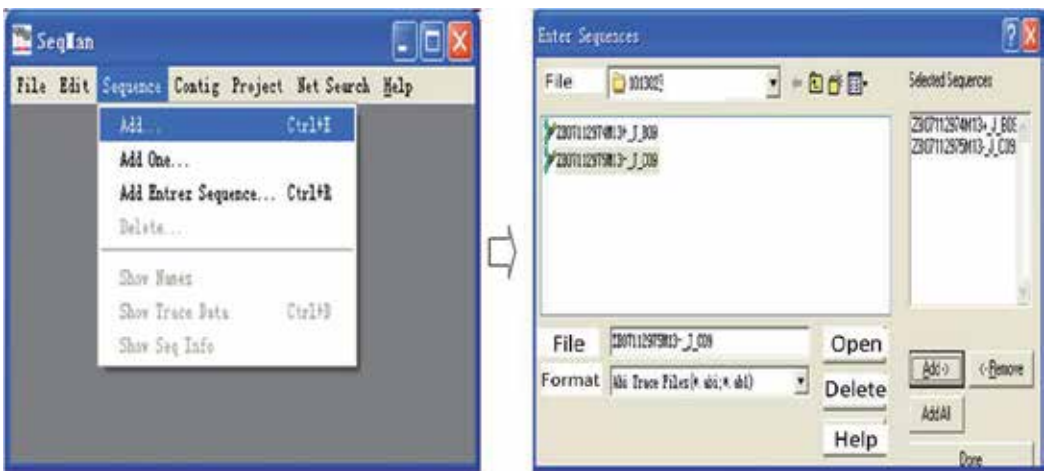

Figure 2. Add sequence to SeqMan

ii. Click 'assemble', double click the assembled file name to open the contig.

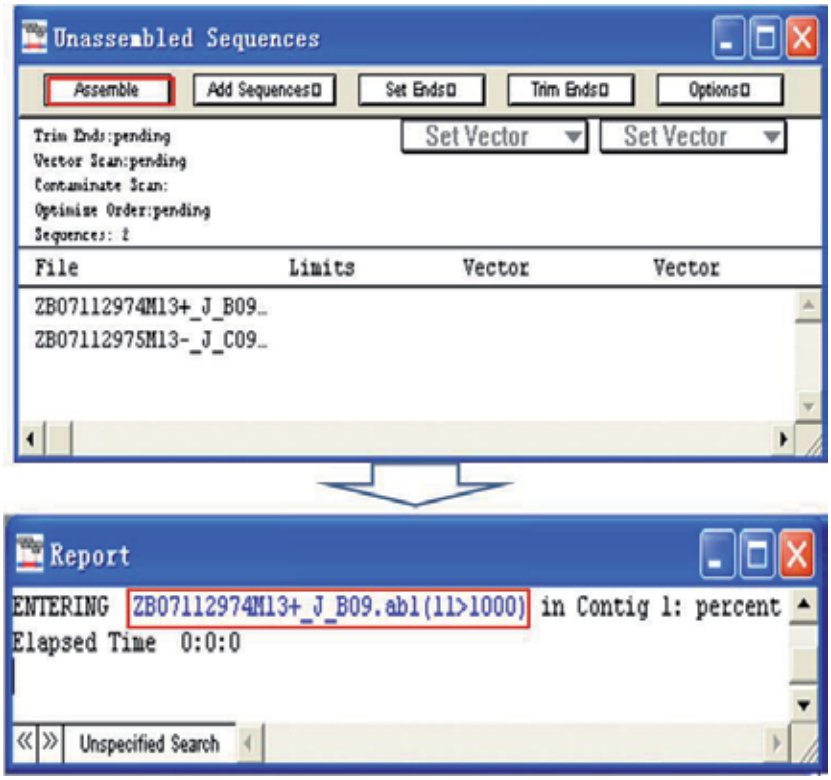

Figure 3. Assemble sequence

iii. Click the '▼' in front of file name to see the quality map; it needs to compare the quality of two maps and to decide which base could be used if the consensus does not match perfect.

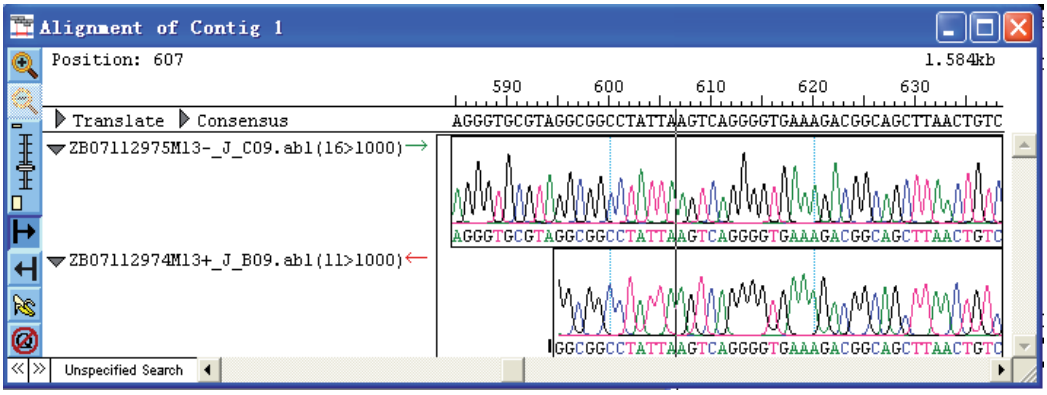

Figure 4. Check the assembled sequence

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iv. Click 'contig', then 'save consensus' and 'single file' to save the result.

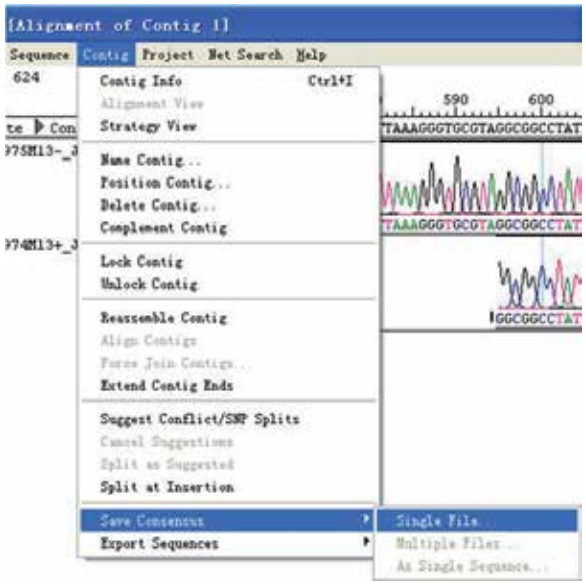

Figure 5. Save the assembled sequence

If the contig comes from clone, the vector should be cut out as follows:

i. Open the webpage (http://www.ncbi.nlm.nih.gov/tools/vecscreen/) and paste sequence to the following window, click 'Run VecScreen'.

Figure 6. Run vecscreen

ii. The following is the graphic summary in the report. Cut out the matched sequence, i.e. just the sequence from 37–1,584 could be used to construct a phylogenetic tree.

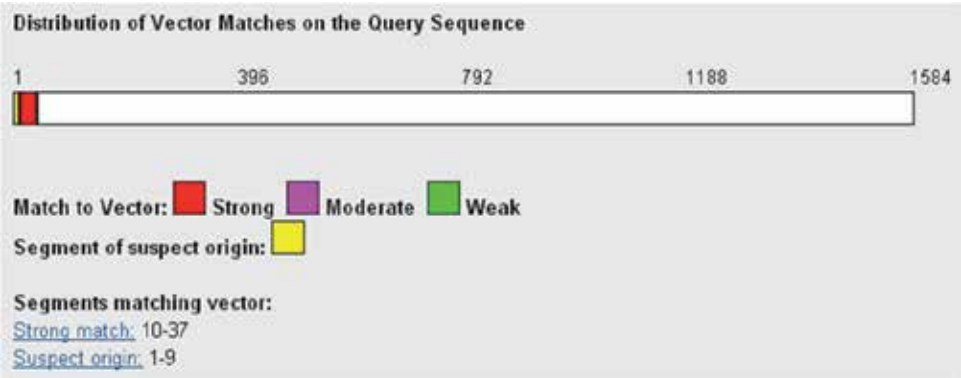

Figure 7. The report of vecscreen

4. Construction of phylogenetic tree based on 16s rDNA sequences

During the course of evolution, the genes, the numbers of genes, their functions and the sizes of the genomes are continually modified. If genes originate from a common ancestor gene and fulfill the same function in a cell, they are said to be homologous. The degree of divergence between homologous genes is considered a measure for their relatedness. In molecular phylogeny, the relationships among organisms, usually extant, are examined by comparing homologous DNA or protein sequences. The relationships are displayed as phylogenetic trees with branch (or edge) lengths reflecting the degrees of genetic divergence. Each branch tip represents an extant sequence, the internal nodes or vertices represent unknown ancestors to the terminal nodes. The branching pattern and branch lengths describe the evolutionary pathways leading to the sequences at the terminal nodes. Clusters of terminal branches connected to a common ancestor are termed clades [23].

4.1. Access to reference sequences

After alignment in the database (http://www.ezbiocloud.net/eztaxon or http://blast.ncbi.nlm.nih.gov are usually used), the closed bacteria are listed in a column and the sequences of these bacteria can be downloaded.

Access of reference sequences from Ezbiocloud is according to the following steps:

i. Upload or paste sequence in the place A or C according to the requirement respectively, or type in the accession number of Genbank in place B, click 'identify' to blast the sequence.

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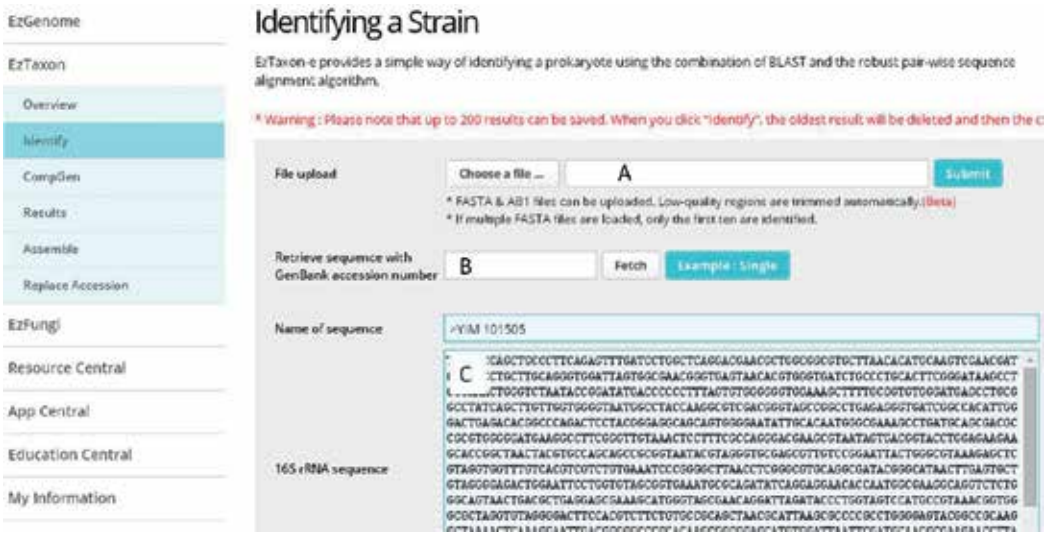

Figure 8. Add sequence to Ezbiocloud

ii. Click query number to view details.

iii. Click 'FASTA(zZ)' to download the reference sequences file in fasta format.

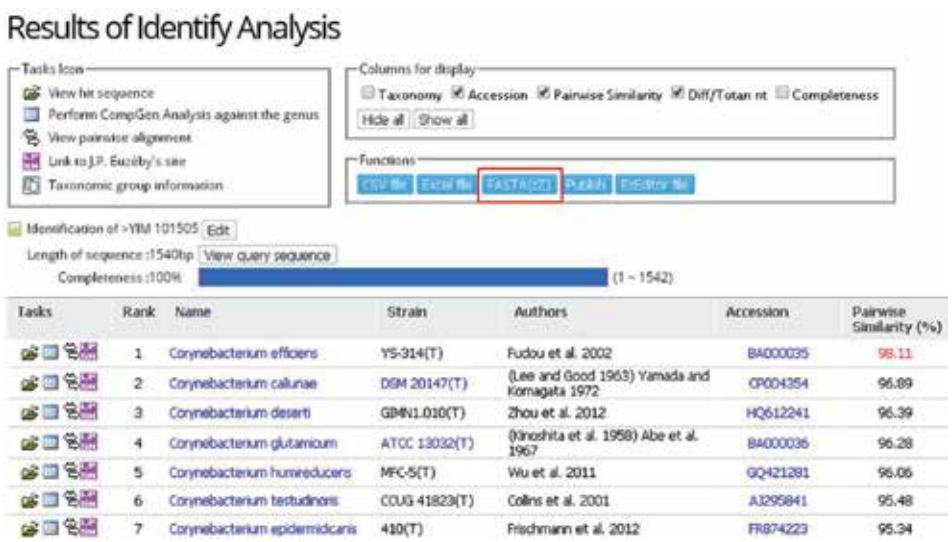

Figure 9. Download reference sequences from Ezbiocloud

Get reference sequences from NCBI according to the following steps:

i. Choose 'blastn' in the blast webpage, paste sequence in place A or upload a file in place B, choose others (nr, etc.) in the column of database, then click 'blast' in the program selection to blast.

Figure 10. Add sequence to blast of NCBI

ii. Click the accession number to see details and download sequence one by one, or click the option in the download to download selected sequences.

Figure 11. Download reference sequences from NCBI

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4.2. Sequences alignment

Prior to the phylogenetic analyses, an alignment of the sequences has to be assembled. If sequences of homologous genes show differences in lengths due to insertions or deletions, gaps have to be inserted to place functionally corresponding positions in the same vertical column of the alignment.

CLUSTAL X and CLUSTAL W are the versions of windows for multiple sequence alignment. CLUSTAL X provides a platform for multiple sequence alignment and analysis results. Users can cut and paste the sequence to change the order, can also realign selected sequences and highlighted low score snippets or abnormal residues, etc. Anyway, the interface of CLUSTAL X is more friendly, intuitive and easier to operate than CLUSTAL W. The basic approach to working in data of CLUSTAL X and CLUSTAL W will be introduced in this part.

Sequences alignment by CLUSTAL-X1.83:

i. Load sequences are in fasta, aln or clustal format, etc., make sure that the mode is for multiple alignment.

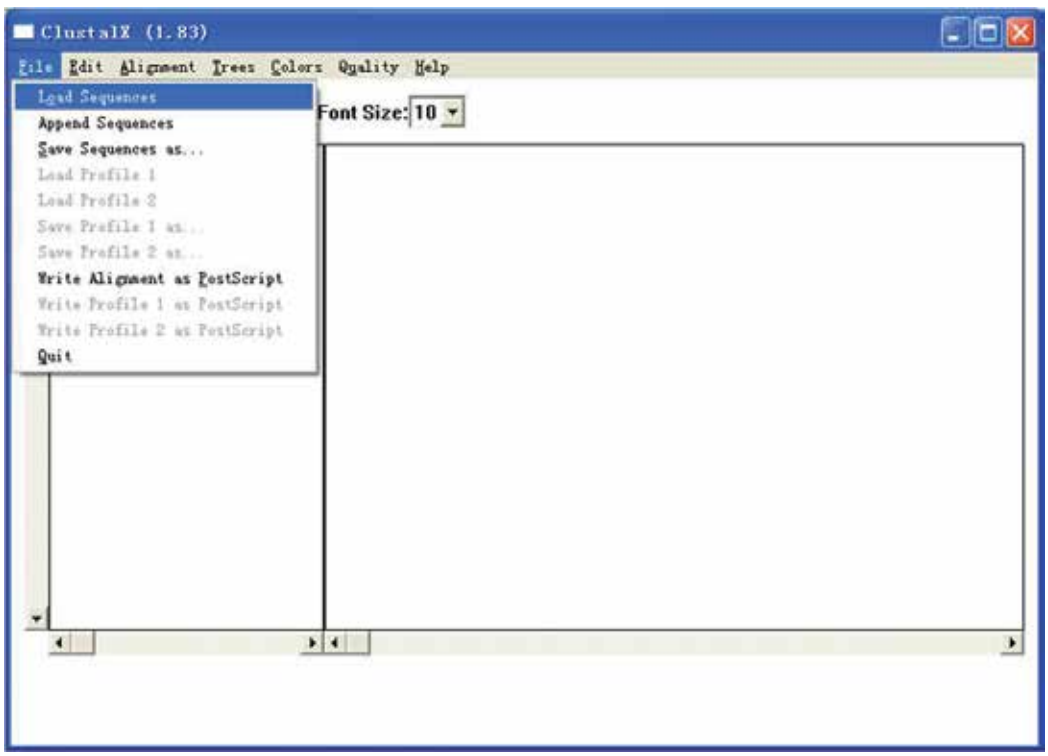

Figure 12. Add sequence to CLUSTAL-X1.83

ii. Multiple sequences alignment. Click 'do complete alignment', then there will be an interface for setting the memory way. There are two file formats, one is dnd which can be opened by treeview and another is aln in which the aligned sequences can be opened by CLUSTAL X and can be converted into MEGA file.

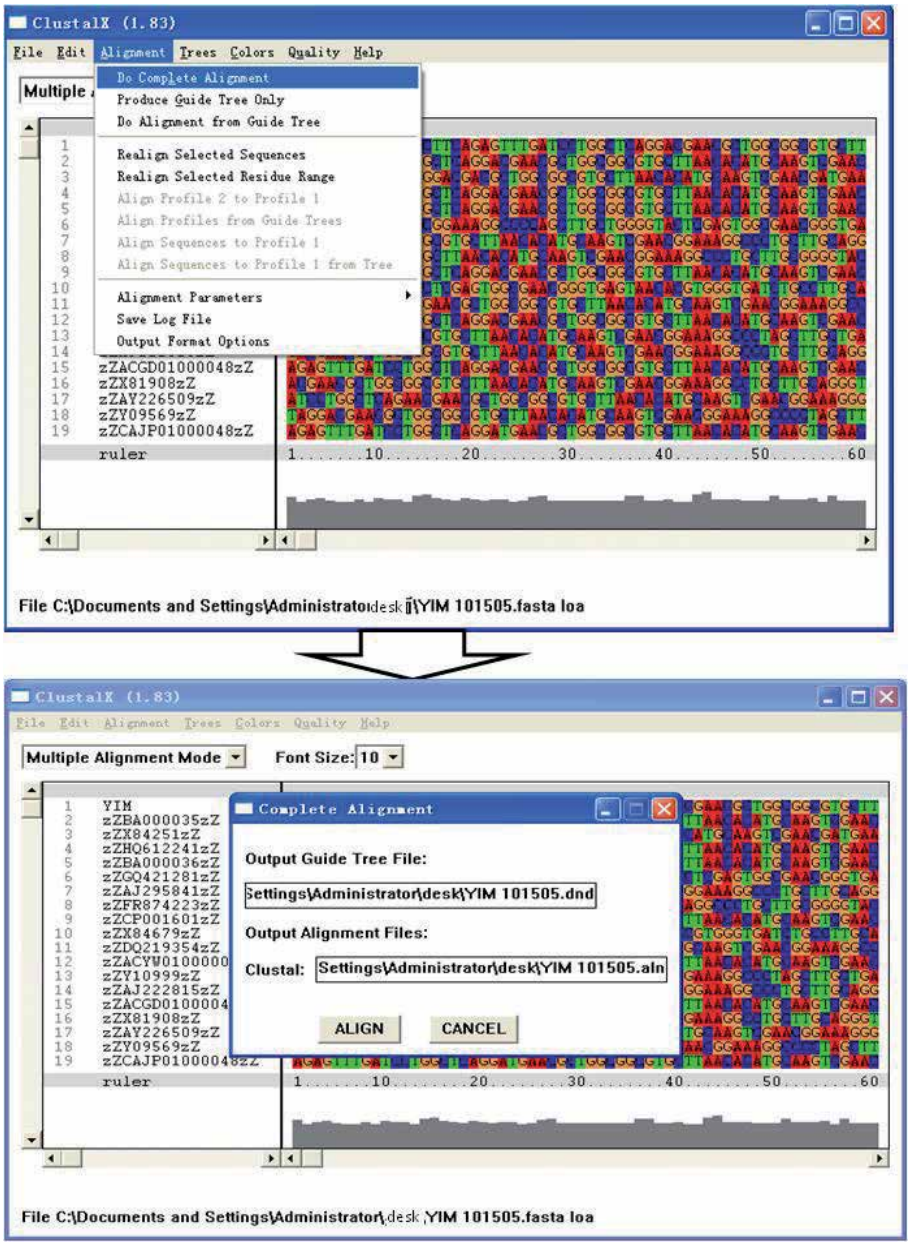

Figure 13. Align sequences by CLUSTAL_X1.83

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iii. Save sequence from the column with first '*' to the column with last '*' (see Figure 14. save range from 96–1,394) as file in clustal format.

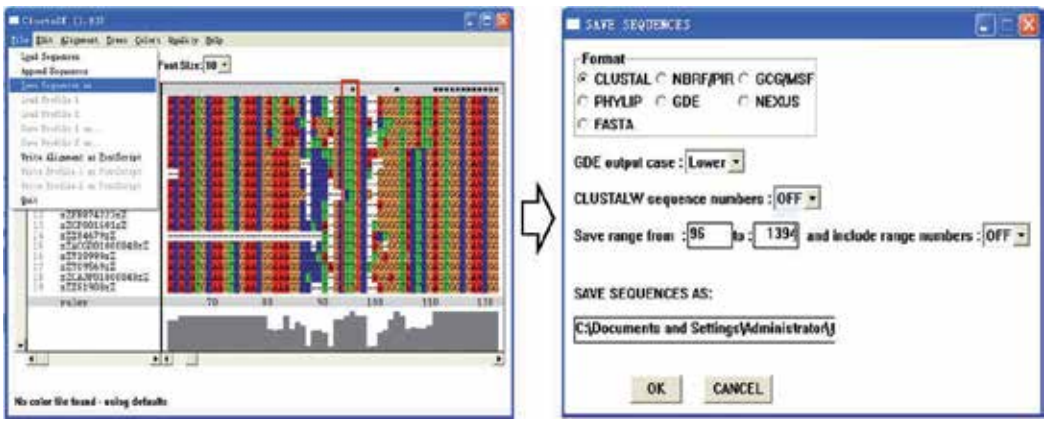

Figure 14. Save aligned sequences from CLUSTAL_X1.83

iv. Convert file in aln format into mega file format. Open MEGA6 and choose as shown in Figure 15..

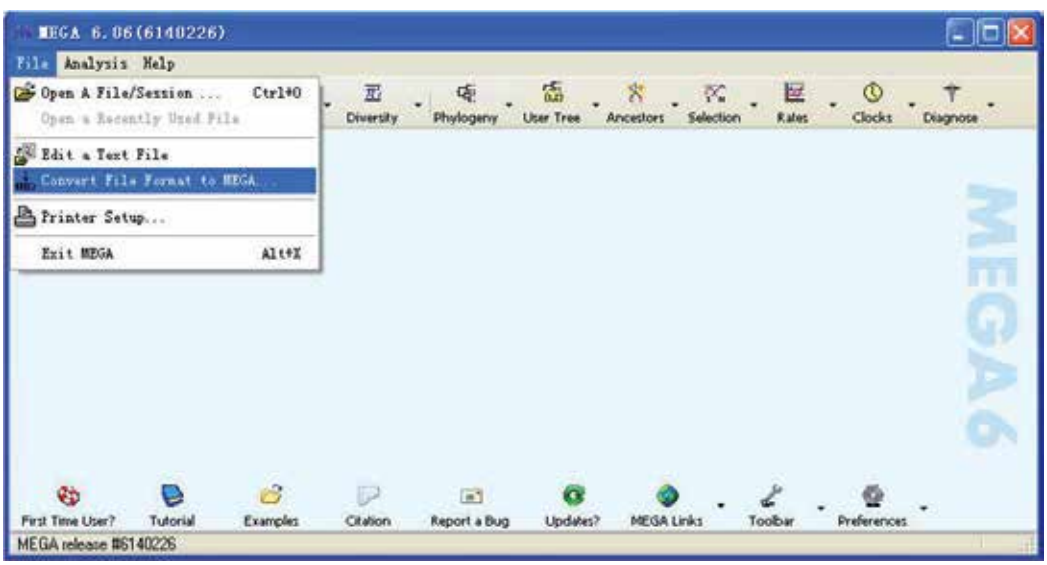

Figure 15. Convert file in aln format into mega file format

v. Save the converted file as a mega format (Figure 16).

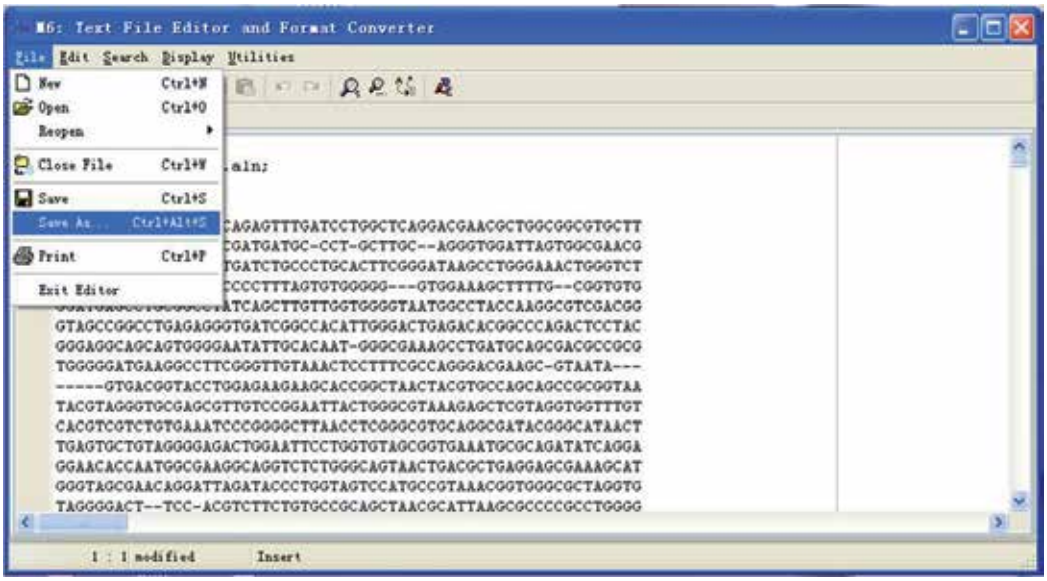

Figure 16. Save the converted file

Sequences alignment by CLUSTAL W in the platform of MEGA6:

i. Open MEGA6 to build alignment as in Figure 17.

Figure 17. Build a DNA alignment by CLUSTAL W in MEGA6

ii. Open a file from native computer as in Figure 18.

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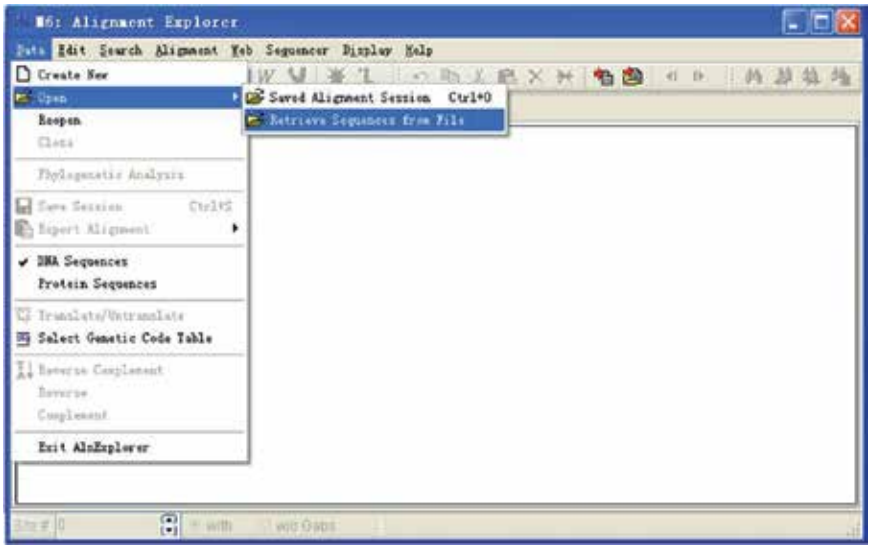

Figure 18. Add sequences to CLUSTAL W in MEGA6

iii. Multiple sequences alignment. Select all sequences and click 'align by ClustalW' to do alignment (Figure 19.). The parameters could be changed according to the illustration. Then there is an interface for setting the memory way. There are three file formats (mega\fasta\paup; Figure 20.); however, the mega format file is convenient for constructing a phylogenetic tree by MEGA6.

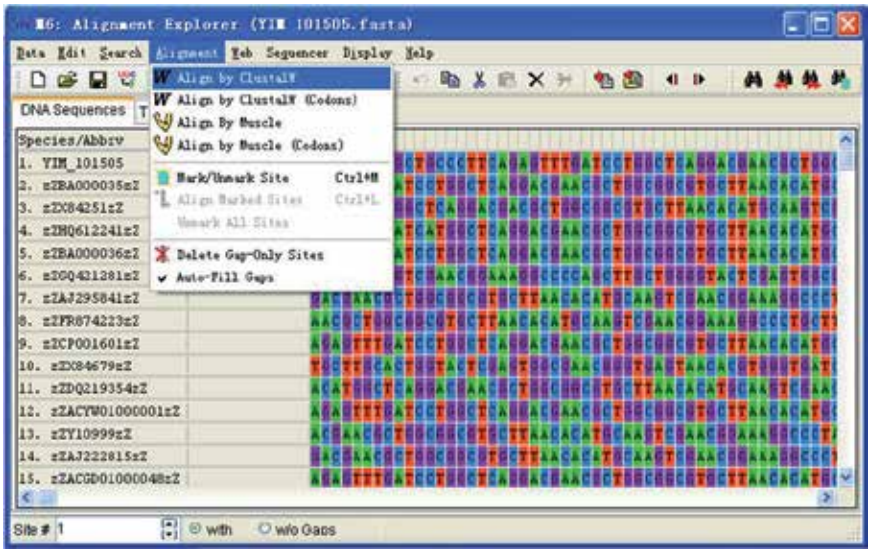

Figure 19. Multiple sequences alignment

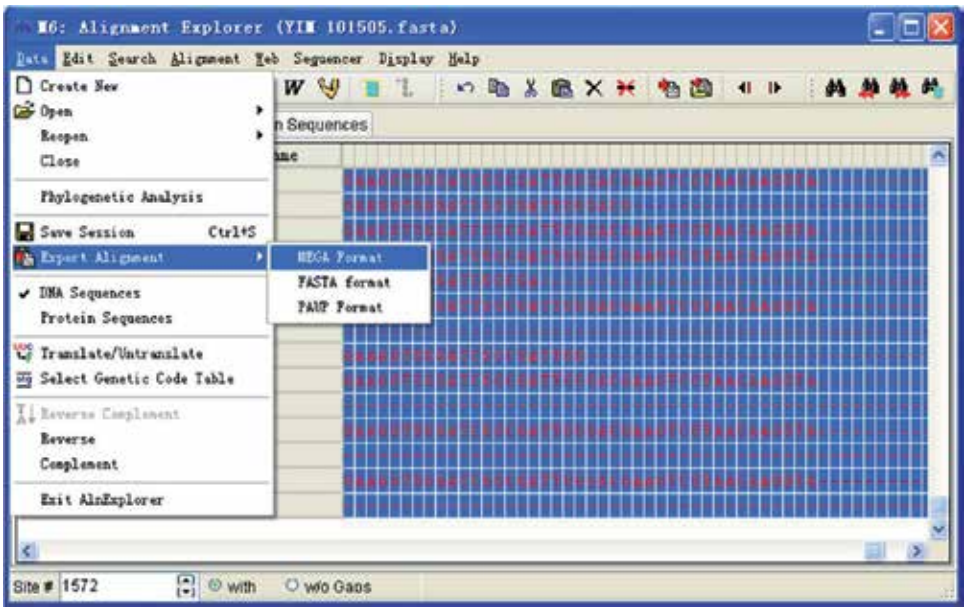

Figure 20. Save the result of alignment

4.3. Construction of phylogenetic tree by MEGA6

The Molecular Evolutionary Genetics Analysis (MEGA) software is developed for comparative analyses of DNA and protein sequences that are aimed at inferring the molecular evolutionary patterns of genes, genomes and species over time. It provides tree-making algorithms of maximum-likelihood, neighbour-joining, minimum evolution, UPGMA and maximum-parsimony. Bootstrap analysis is also included. In version 6.0, it added facilities for building molecular evolutionary trees scaled to time (timetrees), which are clearly needed by scientists as an increasing number of studies are reporting divergence times for species, strains and duplicated genes [24]. The following steps are used for construction of a neighbour-joining tree (some other tree can also be constructed following these steps when choosing different algorithms):

i. Open MEGA6, click 'open a file' to activate a mega file. It is also available to open a mega file with MEGA6 directly. Choose nucleotide sequences in the input data and choose 'NO' to confirm for protein-coding nucleotide sequences data (Figure 21.).

ii. Click 'phylogeny' and then choose algorithms of neighbour-joining (Figure 22.).

iii. Analysis preferences are set as in Figure 23., then click 'compute' to get a phylogenetic tree (Figure 24).

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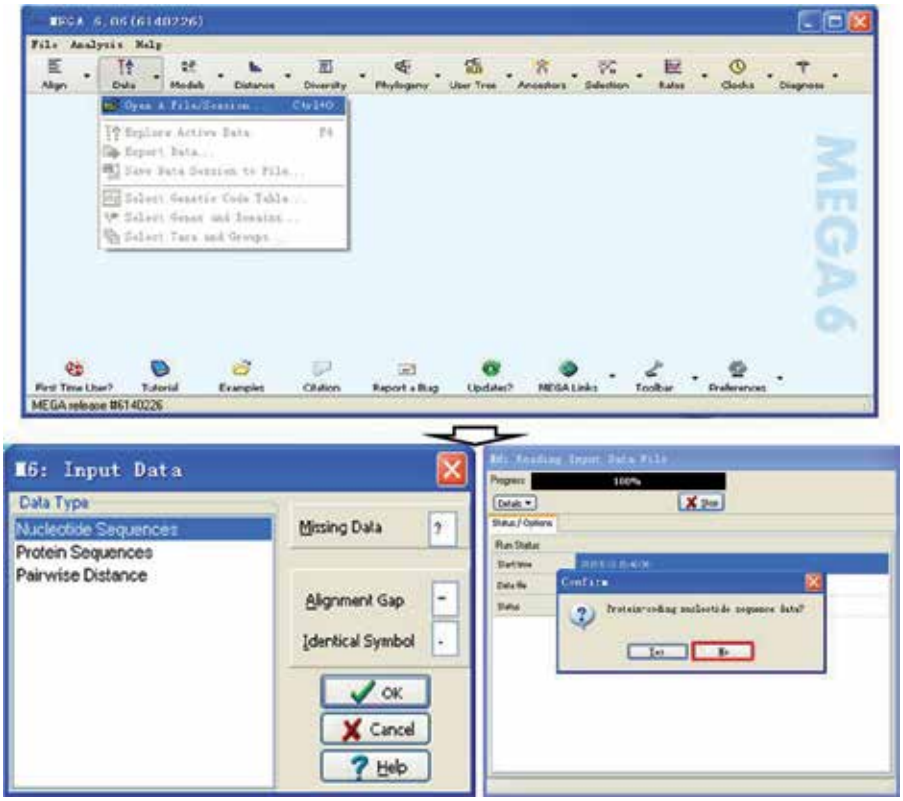

Figure 21. Choices of constructing a phylogenetic tree based on DNA sequences

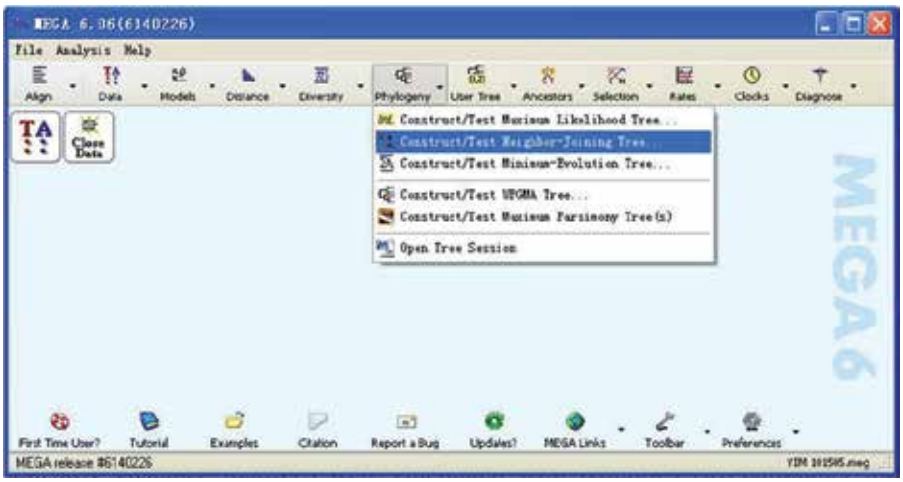

Figure 22. Algorithms in MEGA6

Figure 23. Parameters for constructing a neighbour-joining tree

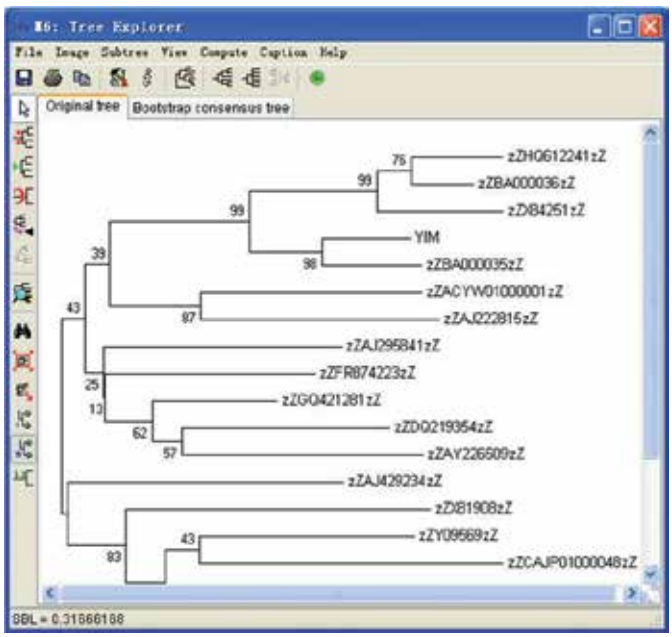

Figure 24. An example of a phylogenetic tree

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iv. Adjustment of the phylogenetic tree. The tree can be adjusted using buttons around the interface; the functions of some buttons are:

, reverse the position of nodes of two clades.

, reverse the position of two clades in one node.

, present subclade as a triangle.

, enlarge the subclade.

, definition of an outgroup for constructing a rooted tree.

, modify the shape of the tree, the width, length and bootstrap values, etc. could be set according to needs (Figure 25).

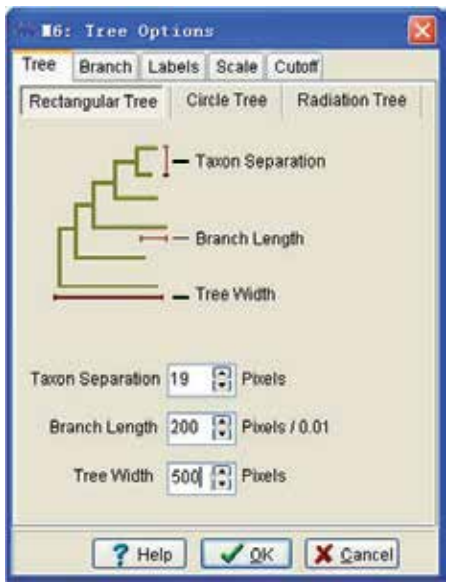

Figure 25. Interface for adjustment of tree

After the adjustment of the phylogenetic tree, save the tree in different formats or copy it into a file word format for edition.

The adjustment and editing of the phylogenetic tree in MEGA6 are limited, and there are different requirements for different journals, so the final edit of the tree is necessary. The following steps are for the edition of tree:

i. Export current tree in Newick format (Figure 26.), and upload the file to http://www.ezbiocloud.net/eztaxon/replace_accession, then there will be an output of a file in the format of tree file which can be opened by MEGA6 and the accession on the branch is replaced (Figure 27.)

ii. Copy the image to a file of.doc format, click the right click to edit the picture carefully.

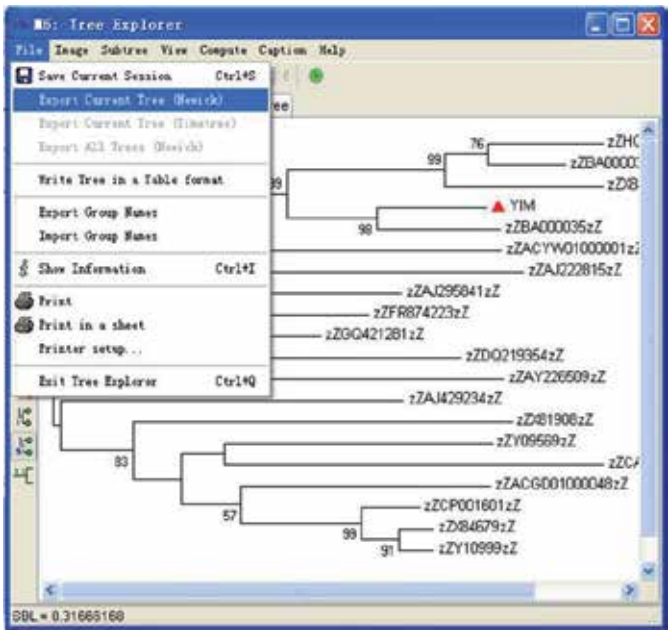

Figure 26. Save tree as Newick format

Figure 27. Upload the tree as Newick format to replace_accession in Ezbiocloud

5. Determination of G+C content

The genomic DNA of each kind of organism has a specific G+C mol%, and G+C content varies in different organisms. Among the genotypic criteria for identification of bacteria, DNA G+C content has been widely used in bacterial taxonomy [13]. It is also an important prerequisite for determining the purity of DNA. The closer the two organisms, the more similar their G+C content is. However, the reverse of this reasoning is unreliable. Because G+C content of microbes is usually constant and not affected by age, growth condition and other external factors, so the determination of G+C mol% in the taxonomy and identification of microorganisms is of importance. The G+C mol% of most actinobacteria distributes between 50 and 80. The determination methods of G+C content are usually HPLC-based, although thermal stability of the native DNA and caesium chloride density-gradient centrifugation are alternative methods, these are now largely of historical interest [13]. HPLC-based is not affected by contamination with ribonucleic acid. Because this method yields a direct measurement, it may also be more accurate than indirect methods, such as the buoyant density and thermal denaturation methods. However, use of whole genome sequences to determine the G+C content of prokaryote will be more convenient in the future.

5.1. Determination of the G+C content of genomic DNA by High-Performance Liquid Chromatography (HPLC)

Escherichia coli should be performed as a control in using this method. Following steps are mainly referred to the method described by Mesbah [25].

i. Extraction and purification of genomic DNA. The methods are detailed in section 6.2.

ii. Determination of DNA concentration.

The absorption value at 280 and 260 nm is measured by using ultraviolet spectrophotometer, from which the purity and concentration of DNA can be determined. The value of OD_{260}/OD_{280} between 1.8 and 2.0 is qualified. The concentration of double-stranded DNA (μg/μl) between 0.1 and 1.0 is suggested.

iii. Degradation of DNA.

- **a.** Pipe 10 μl of a solution of DNA into a 200 μl microfuge tube and heated in PCR amplifier at 99℃ for 15 min. Take out the tube rapidly and cooled in an ice water.
- **b.** Add 10 μl of P1 nuclease (63 U/ml in sodium acetate buffer; the buffer contains 2 mmol/l ZnSO4 and 40 mmol/l NaAc and the pH is 6.3), vortex gently. Then the sample is incubated for 2 h at 37℃ (the temperature can be improved, but the enzymic stability will decrease).
- **c.** Add 10 μl of alkaline phosphatase (70 U/ml in Tris-HCl buffer; the pH of buffer is 8.3; In addition, the pH of the sample was between 7.5 and 8.5). Then the sample is incubated for 2 h (up to 6 h) at 37℃. Store the sample at –20°C for later use.

iv. Determination of G+C content by HPLC.

The chromatography condition is listed in Table 2.

Chromatograph	Agilent 1100
Chromatographic column	(ZORBAX Eclipse XDB-C18) Analytical 4.6 × 150 mm 5-Micron
Mobile phase	0.05 mol/l $NH_4H_2PO_4$:C_2H_3N = 20:1
Detection wavelength	270 nm
Flow	1 ml/min
Column temperature	40℃
Injection volume	5–10 μl
Run time	10 min

Table 2. Chromatography condition of determination of G+C content

v. Calculation of G+C content

G+C content can be calculated from the total component of DNA or from the ratio of certain bases. G+C content is defined as 100×M, where M is the mole fraction of deoxyguanosine (dGuo) plus deoxycytidine (dCyd). Thus, M = (G + C)/(G + C + A + T), where G, C, A and T are the mole fractions of the nucleosides dGuo, dCyd, deoxyadenosine (dAdo) and thymidine (dThd) (Figure 28.), respectively. When there is deviation for *E.coli*, the results should be revised. When unmodified bases are presented, G, C, A and T are the sums of the mole fractions of the modified and unmodified nucleosides.

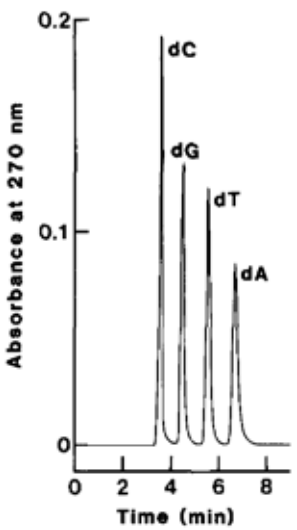

Figure 28. Four kinds of DNA nuclear nucleosides from standard HPLC chromatograms

http://dx.doi.org/10.5772/62029

6. DNA–DNA Hybridization (DDH)

DNA–DNA hybridization is one of the main procedures for identification of new species. Generally, DNA–DNA hybridization (DDH) is necessary when strains share more than 97% 16S rRNA gene sequence similarity. If a new research strain shows this high degree of similarity to more than one known species, DDH should be performed with all relevant type strains to ensure that there is sufficient dissimilarity to support the classification of the strain(s) as a new taxon. In 1987, the international system, International Committee on Systematic Bacteriology ICSB), provided that if the value of DDH is above 70% or the difference of melting temperature of hybrid molecular chain is less than 2℃, the two strains should be one species.

DDH can be performed using a number of techniques [13]. The first is liquid-phase DDH in which the hybridization reaction is in solution. The second is solid-phase DDH. The commonly used method of DDH is determined in micro-wells using covalent attachment of DNA. Total DNA for hybridization reactions is labelled with photoreactive biotin (photobiotin). The biotinylated DNA is hybridized with single-stranded unlabelled DNAs which had been immobilized on the surfaces of micro-dilution wells in this method. However, the DDH may be replaced by some genome relatedness indices, such as average nucleotide identity (ANI) [9], maximal unique matches index (MUMi) [26], genome BLAST distance phylogeny (GBDP) [27], and digital DDH (dDDH) which is computed using the recommended settings of the Genome-to-Genome Distance Calculator (GGDC) web server [28] version 2.0, etc. The protocol of DNA–DNA hybridization determined in micro-wells and DNA hybridization based on renaturation rates will be introduced in this part.

6.1. DNA–DNA hybridization determined in micro-wells

Ezaki *et al*. [5] compared the fluorometric hybridization method with a radioisotope method and firstly made it an alternative procedure to determine genetic relatedness among bacteria. Christensen *et al.* [6] made the modification by the addition of streptavidin conjugate alkaline phosphatase acting on the substrate 4-methylumbelliferyl phosphate in 2000. The protocol here mainly refers to the two articles. The salmon sperm DNA is performed as the control.

Extraction and purification of genomic DNA

i. The methods are described in section 2. **Pretreatment of DNA (steps ii, iii)**

ii. Diluted the DNA to 10 OD and 2 OD with 0.1×SSC.

iii. Heat the DNA from step II at 100℃ for 15 min in tube, then transfer the tube to ice bath for 5 min immediately.

Binding of DNA to micro-wells (steps iv - viii)

iv. Centrifuge at 1,000 rpm for 3 min, dilute the DNA (2 OD) to 0.2 OD with 1×PBS-$MgCl_2$.

v. Add 100 μl DNA (0.2 OD from step IV) to each well.

vi. Incubated micro-wells (with DNA) sealed with plastic bags at 30–50°C for a minimum of 4 h without shaking.

vii. Wash the wells two times with 300 μl 1×PBS in one well each time.

viii. Incubate the wells (with its original lid) at 40°C for about 30 min (until the well is dried).

DNA labelling with photo-activatable biotin (PAB), (under subdued light; step ix-xii)

ix. Pipe 10 μl denatured DNA (10 OD from steps II and III) to mix with 10 μl PAB (prepare two tubes; salmon sperm DNA is included).

x. Tubes are illuminated with their lids open, 10 cm below a 400 W Philips sun-lamp (SGR 140) for 90 min on crushed ice.

xi. Add 200 μl TE buffer (pH 9) into the solution, vortex gently, and the solution is extracted twice (until the water phase is no longer to red) with 200 μl 2-butanol.

xii. Shear the labelled DNA into fragments of 300–700 bp by ultrasonic wave (detect with agarose gel electrophoresis).

Pre-hybridization with unlabelled salmon sperm DNA to DNA attached to micro-wells (steps xiii– xv)

xiii. Prepare pre-hydridization solution. 200 ml pre-hydridization solution including 40 ml 10×SSC, 20 ml 50×denhardt solution, 2 ml denatured salmon sperm DNA (10 mg/ml), 38 ml MilliQ water and 100 ml formamide.

xiv. Add 200 μl DNA pre-hydridization solution (use it right after it was ready) in each well, sealed with plastic bags.

xv. Incubate at hybridization temperature until the probe can add in (at least 60 min). The formula of TOR= 0.51 × (G+C mol%)+47, however, the TOR'=TOR-36+(0-5) when formamide is used.

Hybridization with PAB-labelled DNA to DNA attached to micro-wells (steps xvi–xxi)

xvi. Mix 50 μl PAB-labelled DNA with 950 μl pre-hydridization solution to prepare hydridization solution, then incubate it for 10–15 min at hybridization temperature.

xvii. Remove the pre-hybridization solution in the micro-wells completely.

xviii. Add 100 μl DNA hydridization solution (use it right after it was ready) in each well, sealed with plastic bags and cover the micro-wells plate with silver paper, then incubate for at least 8 h at hybridization temperature.

xix. Remove the hydridization solution in the micro-wells completely.

xx. Add 300 μl 1×SSC into each micro-well, incubate for 15 min at hybridization temperature, then remove the 1×SSC solution in the micro-wells completely for washing. Repeat three times.

xxi. Add 300 μl 1×SSC into each micro-well, incubate for 5 min at room temperature and then remove the 1×SSC solution from micro-wells completely for washing. Repeat three times.

Detection of DNA hybridization (steps xxii–xxvi)

xxii. Dilute streptavidin-conjugated alkaline phosphatase (VECTOR laborators) with alkaline phosphatase reaction buffer in a ratio of 1 : 3000.

xxiii. Add 100 μl mixture from step XXII into each micro-well, sealed with plastic bags and cover with silver paper, incubate for 1 h at 37℃.

xxiv. Wash the micro-wells with 100 μl alkaline phosphatase reaction buffer for each well, incubated for 5 min at room temperature every time. Repeat three times.

xxv. Add 100 μl 4-methylumbelliferyl phosphate (4-MUP) solution (1 mM; diluted with 4-MUP buffer) into each micro-well and which then be sealed with plastic bags and covered with silver paper, incubate for 1 h at 37℃.

xxvi. Fluorescence intensities were measured using a fluostar optima microplate reader (BMG LABTECH) at a wavelength of 360 nm for excitation and 460 nm for mission.

Quantification (step xxvii)

xxvii. The percentage DNA similarity was calculated as $100\times[(I_{test}-I_{blank})]/[(I_{ref}-I_{blank})]$, where I_{test} is the intensity of hybridization between the strain to be tested and the reference strain, I_{ref}, is the intensity of hybridization of the reference strain with itself, and I_{blank} is the background hybridization (hybridization with salmon sperm DNA). Each experiment is performed with at least three replicates. The differences of mean DNA similarities between experiments are evaluated statistically by the d-test. The final similarity is the mean value of two independent experiments in which one is the DNA of tested strain as probe and another is the DNA of reference strain as probe.

6.2. DNA–DNA hybridization based on renaturation rate

This method needs a large amount of DNA and mainly according to the method described by De Ley *et al.* [29].

i. Extraction and purification of genomic DNA. The protocols are listed in section 2

ii. Dilute the DNA to 0.1 OD with 0.1×SSC.

iii. Shear the genomic DNA into fragments of 200–1,000 bp (optimal 600 bp) by ultrasonic wave.

iv. Denature NDA according to the set procedure. The procedure is set as Table.3

Procedure Number	Temperature/℃	Retention time/min	Run time/min
1-1	25	2	2
1-2	60	2	4
1-3	70	2	6
1-4	72	2	8
1-5	74	2	10
1-6	76	2	12
1-7	78	2	14
1-8	80	2	16
1-9	82	2	18
1-10	84	2	20
1-11	86	2	22
1-12	88	2	24
1-13	90	2	26
1-14	92	2	28
1-15	94	2	30
1-16	99	2	32
2-1	99	7	39
2-2	Tor*	2	41
2-3	Tor*	40	81
2-4	25	2	83

*Tor (Renaturation temperature)= 0.51 G+C mol% + 47.0

Table 3. Procedure of temperature and retention time

v. Preincubate 20×SSC in boiling water.

vi. Add 20×SSC to dilute the DNA following Procedure Number 1-16 to adjust the ion concentration, the final SSC concentration is 2×SSC.

vii. Determine the renaturation rate, obtain the data of resilience curve.

viii. Copy the data to Excel file, calculate the formula of velocity of renaturation based on the data above, and calculate renaturation velocity V.

ix. The DNA similarity is calculated as $100\times[4V_m-(V_A+V_B)]/[(2\times(V_A\times V_B)^{1/2})]$, V_A and V_B represent the renaturation velocity of sample A and B, V_m represents the renaturation velocity of mixture of sample A and B.

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Section 2

Bioprospecting

Chapter 7

Production of Antibacterial Compounds from Actinomycetes

Letizia Lo Grasso, Delia Chillura Martino and Rosa Alduina

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/61525

Abstract

Actinomycetes are soil-dwelling Gram-positive bacteria, industrially relevant as producers of a wide range of bioactive secondary metabolites, including many antibiotics of clinical and commercial importance.

The understanding of actinomycete biology has been based on extensive studies on the model organism *Streptomyces coelicolor* over many years and on the availability of its complete genome sequence. This bacterium has an unusual complex developmental cycle that includes programmed cell death phenomena that make this bacterium a multicellular prokaryotic model.

Morphological differentiation in *S. coelicolor* is strictly related to physiological differentiation: indeed the onset of morphological differentiation generally coincides with the production of secondary metabolites. During cell death, degradative proteins are synthesized and involved in an extensive degradation of some cellular constituents (proteins and lipids) used for a second growth phase, that is accompanied by antibiotic production.

If on one hand, many factors with pleiotropic activity have been identified as key players to control both morphological and physiological differentiation in *S. coelicolor*, on the other hand, for most actinomycetes, mechanisms and factors governing morphological and physiological processes have not been deeply investigated.

This chapter reviews the regulatory mechanisms known to control antibiotic production in actinomycetes and both genetic and physiological methods adopted to improve antibiotic yields.

Keywords: Antibiotic production, Actinomycetes, genetic engineering, regulation, heterologous expression

1. Introduction

The discovery by Alexander Fleming of penicillin opened up a completely new era of chemotherapy. Antibiotics have saved a large number of lives and also contributed to the increase in life expectancy. They are mainly produced by the fermentation of fungi (e.g., *Penicillium*) and bacteria (e.g., *Actinomycetes*). In particular, 80% of antibiotics are sourced from the genus *Streptomyces* and rare actinomycetes, such as *Actinomadura*, and only the 20% is produced by fungal species.

Actinomycetes are soil-dwelling Gram-positive bacteria that have extensive arsenals of secondary metabolites, metabolism products that, differently from primary metabolites such as vitamins, amino acids, nucleotides, etc., are not essential for the bacterial growth, at least in laboratory conditions; indeed, many mutants in antibiotic biosynthesis have been generated revealing that they are still vital and were used as models to understand molecular mechanisms governing antibiotic production.

Secondary metabolites include antitumorals (e.g., doxorubicin and bleomycin), antifungals (e.g., amphotericin B and nystatin), immunosuppressives (e.g., FK-506 and rapamycin), insecticides (e.g., spinosyn A and avermectin B), herbicides (e.g., phosphinotricin) and many antibiotics of clinical and commercial importance.

This chapter reviews common regulatory mechanisms that control antibiotic production in actinomycetes and both genetic and physiological methods to improve antibiotic yields.

2. Antibiotics and their targets

Antibiotics are molecules that selectively inhibit bacterial growth without damaging the eukaryotic organisms. The selectivity of action of these substances is given by the fact that they interfere with processes essential for the bacterial cell and absent or different in the eukaryotic cell.

Antibiotics essentially target bacterial structures or functions, such as cell wall biosynthesis (e.g., vancomycin), translation (e.g., streptomycin), RNA transcription (e.g., rifampicin), DNA replication and synthesis (e.g., novobiocin and metronidazole), membrane (polimyxins), and in general they inhibit bacterial growth (Figure 1).

Among the antibiotics that target the cell wall, glycopeptides are a class of drugs produced by Actinomycetes and are composed of glycosylated cyclic or polycyclic non-ribosomal peptides. Glycopeptides bind to the dipeptide D-alanyl--D-alanine (D-Ala-D-Ala) within the cell wall of Gram-positive bacteria preventing the addition of new units to the peptidoglycan and inhibiting the peptidoglycan synthesis. Significant glycopeptide antibiotics include the anti-infective antibiotics vancomycin, teicoplanin, telavancin, ramoplanin, decaplanin, and the antitumor antibiotic bleomycin. Vancomycin is used as antibiotic of last resort for infections with methicillin-resistant *Staphylococcus aureus*. Toxigenic *S. aureus* strains can be isolated from food making it an actual problem for public health [1].

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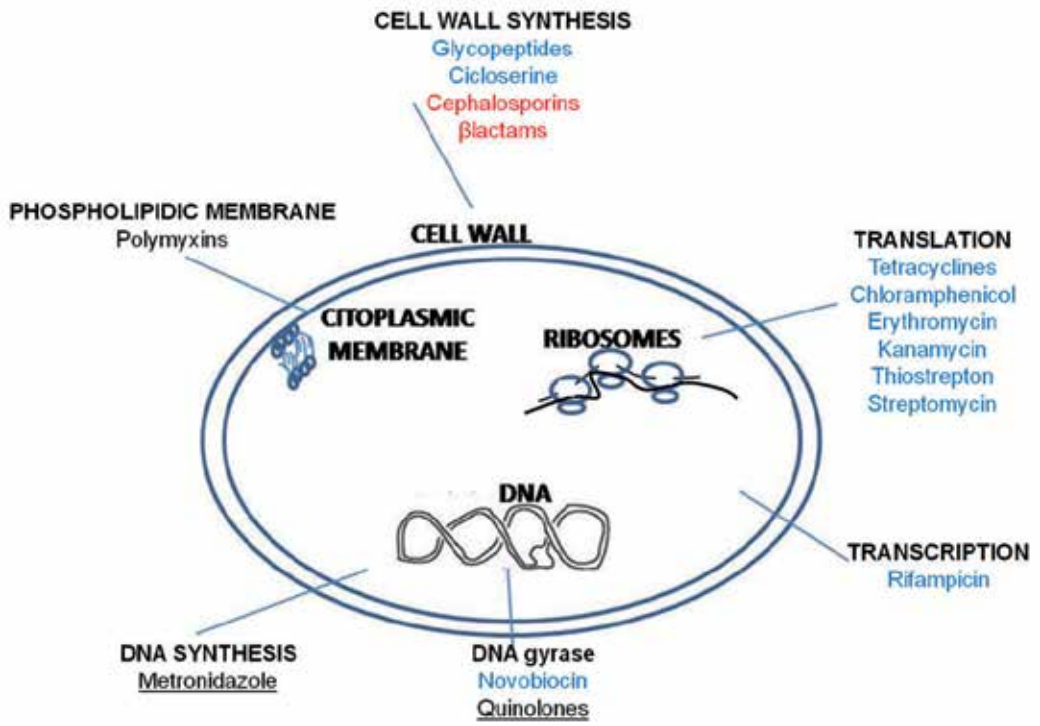

Figure 1. Principal targets of antibiotics. The antibiotics produced by Actinomycetes and by fungi are indicated in blue and red, respectively. The synthetic antibiotics are underlined.

Cycloserin, produced by *Streptomyces orchidaceus*, is a cyclic analogue of D-alanine that acts against two crucial enzymes important in the cytosolic stages of peptidoglycan synthesis: alanine racemase and D-Ala-D-Ala ligase. When both of these enzymes are inhibited, D-alanine residues cannot form and previously formed D-alanine molecules cannot be joined together.

Penicillins and cephalosporins are the most important antibiotics obtained from fungi P*enicillium notatum* and *P. chrysogenum*, respectively. Penicillins and cephalosporins mimic the D-alanyl-D-alanine groups found at the terminus of the pentapeptide in most newly synthesized peptidoglycan monomers. Binding of the drug to the transpeptidase inhibits the formation of cross-links between the rows and layers of peptidoglycan in the cell wall. Collectively, this results in degradation of the peptidoglycan and osmotic lysis of the bacterium [2].

Many different classes of antibiotics block protein synthesis. Tetracycline (produced by *Streptomyces aureofaciens*), for example, inhibits aminoacyl-tRNA binding, while chloramphenicol (*Streptomyces venezuelae*) and erythromycin (produced by *Saccaropolyspora erythraea*) bind to 50S subunit, blocking the peptidyl transferase activity; kanamycin (produced by *Streptomyces kanamyceticus*) binds to 30S subunit; thiostrepton (produced by *Streptomyces laurentii*) inhibits ribosome-dependent EF-Tu and EF-G GTPase, while streptomycin (produced by

Streptomyces griseus) prevents formation of initiation complex by insertion of improper amino acids.

Rifampicin is a semisynthetic antibiotic produced by the fermentation of a strain of *Amycolatopsis mediterranei* and it is a key component of anti-tuberculosis therapy. It inhibits the bacterial RNA polymerase by binding a pocket of the RNAP β subunit within the DNA/RNA channel and destabilizing the DNA-RNA polymerase-oligonucleotide-complexes [3].

Novobiocin, also known as albamycin or cathomycin, is an aminocoumarin antibiotic that is produced by the actinomycete *Streptomyces niveus*. Novobiocin is a very potent inhibitor of bacterial DNA gyrase and functions by targeting the GyrB subunit of the enzyme involved in energy transduction [4–5].

Polymyxins are antibiotics produced by nonribosomal peptide synthetase systems in Gram-positive bacteria, such as *Paenibacillus polymyxa*. Their structure consists of a cyclic peptide with a long hydrophobic tail. They act by disrupting the structure of the bacterial cell membrane and interacting with its phospholipids [6].

A few antibiotics are produced by chemical synthesis (e.g., quinolone and metronidazole). Quinolones are synthetic, bactericidal agents that inhibit the enzyme topoisomerase II, a DNA gyrase necessary for the replication of the microorganism. Topoisomerase II enzyme produces a negative supercoil on DNA, permitting transcription or replication; thus, by inhibiting this enzyme, DNA replication and transcription are blocked.

Metronidazole is a synthetic compound used in the treatment of infections caused by Gram-negative anaerobic bacteria and protozoa. It was shown to induce base-pair substitutions [7] and to be a potent mutagen in bacteria and low eukaryotic systems [8].

3. Genetic organization of antibiotic biosynthesis

Genes involved in the biosynthesis of antibiotics and other secondary metabolites are typically clustered within the respective genome or, rarely, in circular plasmid. A biosynthetic gene cluster contains many genes, often located within a few thousand base pairs of each other that encode for proteins participating in a metabolic pathway that leads to the production of a particular secondary metabolite (Figure 2).

Figure 2. Schematic example of a gene cluster for antibiotic production. Different colors indicate different functions.

The size of gene clusters can vary significantly, from a few to several hundred genes. Commonly, 10–50 genes are required for the synthesis of an antibiotic. A gene cluster usually contains genes involved in the precursor biosynthesis, tailoring steps, export, resistance, and regulation.

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Some peptide antibiotics are formed by amino acidic precursors that are then assembled by non-ribosomal machinery. In the case of non-ribosomal peptide synthesis, non-proteinogenic amino acids, such as 3,5-dihydroxyphenylglycine (DPG) and 4-hydroxyphenylglycine (HPG), can be found. In many cases, the backbone of the antibiotic is modified by the so-called tailoring steps, i.e., chlorination, methylation, glycosylation, N-acylation, and so on.

The polyketides are another class of natural antibiotics synthesized through the decarboxylative condensation of malonyl-CoA-derived extender units in a process similar to the fatty acid synthesis. The polyketide chains produced by a minimal polyketide synthase are often further modified (e.g., glycosylated) into bioactive natural products.

Actinorhodin and undecylprodiginines are two of the antibiotics produced by *S. coelicolor*. Actinorhodin is a red/blue pH-indicating benzoisochromanequinone made by a type II polyketide synthase-based pathway, while undecylprodiginines are red hydrophobic tripyrroles made by a fatty acid synthase-like.

Usually, a gene cluster for antibiotic production encodes for regulatory genes, named pathway specific, with positive or negative control on the cluster. Moreover, there could be some pleiotropic regulators that affect antibiotic production, morphological development, and primary metabolism of the bacteria. As examples, actinorhodin biosynthesis is regulated by the transcriptional activator ActII-ORF4 [9–10], while the undecylprodigiosin pathway is regulated via a minicascade of two cluster specific regulators, with RedZ activating the expression of *redD*, an aberrant orphan response regulator, direct activator gene for the biosynthetic genes [11].

In the bacteria producers of antibiotics, resistance genes are necessary to avoid the suicide, while transport genes are used to export the antibiotic outside the cell. Resistance to antibiotics can be caused by several general mechanisms (Figure 3): increased efflux or decreased influx of the antibiotic, target site alteration, target amplification, or antibiotic inactivation/modification [12].

The production of β-lactamase is a common mechanism found in many pathogens. This enzyme is capable of hydrolyzing and destroying the β-lactam ring of the antibiotic avoiding its antibacterial activity.

As example of alteration of the target site, the methylation of an adenine of the ribosomal RNA prevents the interaction between macrolides and ribosome.

Resistance to glycopeptides is frequently due to the presence of genes encoding for enzymes involved in the synthesis of alternative forms of peptidoglycan, with low affinity for glycopeptides. For example, the C terminal D-Ala-D-Ala is replaced by D-Ala-D-Lac or D-Ala-D-Ser [13]. Glycopeptide resistance has been explored in three different actinomycetes: *Streptomyces coelicolor*, which does not produce glycopeptide, *Streptomyces toyocaensis* and *Actinoplanes teichomyceticus*, which produce the glycopeptides A47934 and teicoplanin, respectively. For the producers of glycopeptides, activation of the resistance genes by the endogenously produced antibiotic prevents suicide, while in non-producing bacteria resistance may be due to genetic changes, such as mutation or acquisition of resistance genes through

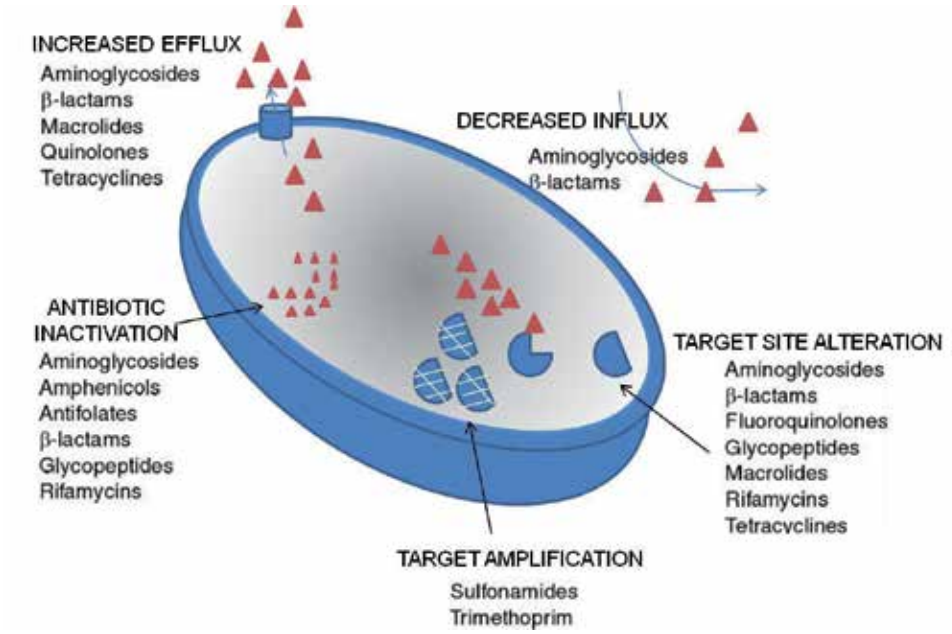

Figure 3. Mechanisms of antibiotic resistance.

horizontal transfer. In other systems, the antibiotic is acetylated by specific acetyl transferase inactivating its antibacterial property [14–15].

The best known efflux system regards the tetracycline, the gene *tetL*, carried by a transposon, codifies for a protein that transports the antibiotic outside the cell.

4. Morphological and physiological differentiation

Actinomycetes represent an important model of bacterial development; they display an unusual complex life cycle with different cell types (spores, vegetative and reproductive mycelium) and with the morphological changes strictly connected to the physiological differentiation. The understanding of Actinomycetes biology has been based on extensive studies on the model organism *Streptomyces coelicolor* over many years and on the availability of its complete genome sequence [16]. *S. coelicolor* is considered a ''multicellular'' prokaryotic model that includes programmed cell death and sporulation. After spore germination, vegetative growth leads to formation of a mycelium consisting of a ramifying network of syncytial hyphae that penetrate a moist substrate by extension of hyphal tips and subapical branching (vegetative mycelium). Subsequent reproductive growth often proceeds with the formation of filamentous aerial hyphae that eventually undergo differentiation into chains of unigenomic spores (Figure 4). This complex developmental cycle includes programmed cell death phenomena that make this bacterium a multicellular prokaryotic model [17–19].

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Genetic studies of *Streptomyces* development regulation focused largely on analyses of *S. coelicolor* mutant strains defective in different stages of development. The main studied strains are the so-called "*bald*" mutants that are defective in aerial growth and the "*white*" mutants that are defective in the formation of mature grey spores on the tips of the white aerial mycelium. The *bald* mutants are affected in genes that regulate the ''sky-pathway'' that activates the expression of genes encoding proteins forming hydrophobic cover, such as eight chaplin proteins, RdlA, and Rdl rodlin proteins; the *white* genes are responsible of the formation of grey spores [20–21].

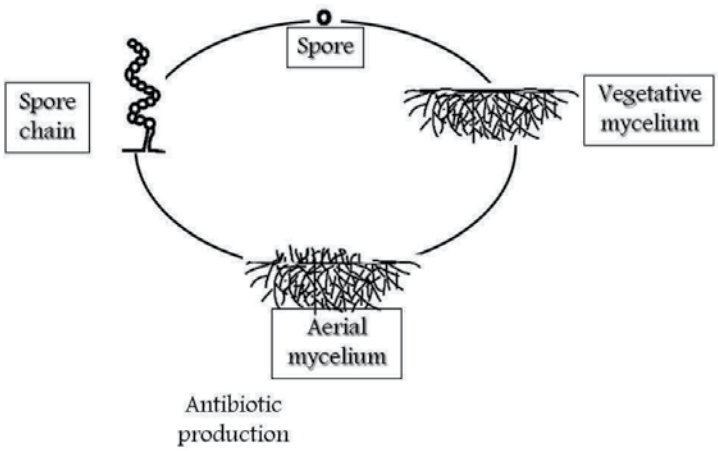

Figure 4. The *Streptomyces coelicolor* life cycle.

Morphological differentiation in Actinomycetes is strictly related to physiological differentiation: indeed the onset of morphological differentiation generally coincides with the production of secondary metabolites.

If on one hand, many factors with pleiotropic activity were identified as key players to control both morphological and physiological differentiation in *S. coelicolor*, on the other hand, for most actinomycetes, mechanisms and factors governing morphological and physiological processes were not deeply investigated yet.

5. General approaches to overproduce natural antibiotic

Natural bacterial strains often produce only small amounts of antibiotic (μg/l), while production rates in the range of g/l are needed to set up a cost-effective production process. In order to increase the industrial yield of products, different strategies can be adopted.

Random mutagenesis for the selection of overproducing mutants remains the preferred method when molecular genetic tools have not been developed for the producer microorganism. Although random mutagenesis and screening procedures have been widely used for

genetic improvement of antibiotic production, there are certain disadvantages, such as the time necessary to obtain a favorable mutation. The knowledge-driven genetic manipulation can make the optimization of strains and conditions more efficient.

In general, many approaches have been used to improve antibiotic production as schematically represented in Figure 5.

The tuning of media composition and fermentation conditions (carbon source, phosphate and nitrogen concentrations, pH, temperature) and the supply of specific precursors are the first approaches used in order to increase the yield in fermentation. Moreover, genetic manipulation of primary or secondary metabolism can be applied. Regarding primary metabolism, mutations in pathways for amino acids or other molecules that are used as precursors in antibiotic biosynthesis or mutations in the ribosome can improve indirectly the yield of secondary metabolites.

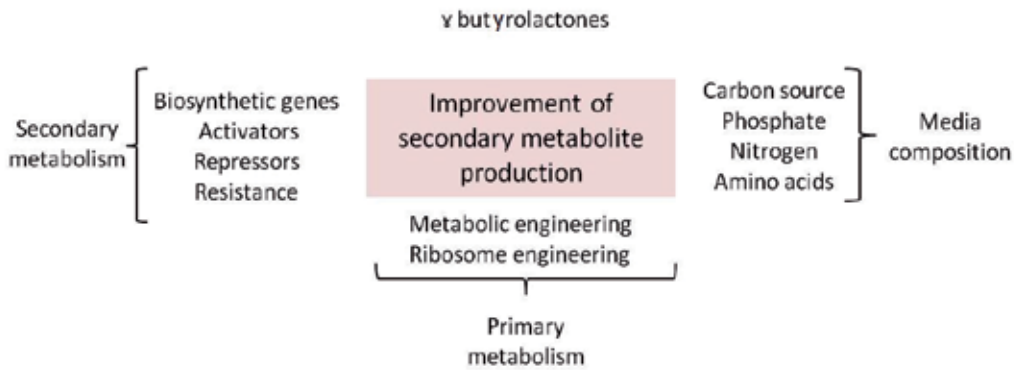

Figure 5. Approaches used to improve the antibiotic production.

Regarding secondary metabolism, the over-expression of biosynthetic genes, such as the genes that codify for antibiotic specific precursors, the over-expression of pathway-specific positive regulators or the inactivation of pathway-specific negative regulators can result in an increase of antibiotic yield. Increasing self-resistance levels in producing organisms has been also used for improving production yields. Manipulation of pleiotropic regulators, involved in both primary and secondary metabolisms, was also successfully used to improve antibiotic yields.

The production of antibiotics in some *Streptomyces spp*. depends upon diffusible butyrolactones structurally similar to homoserine lactones. γ butyrolactones have been applied for improvement of secondary metabolite production.

6. Media composition effect on antibiotic production

In bacteria, several sugars can be used as carbon sources. Although glucose is often an excellent carbon and energy source for microbial growth, it is infrequently utilized as the major carbon

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and energy source in secondary metabolite fermentation. When incubated in media containing glucose and another carbon source, bacteria metabolize first glucose that represses the transcription of genes required for the utilization of the secondary carbon sources. When glucose is exhausted, the metabolism of the second carbon source is activated, and generally this correlates with the onset of antibiotic production. This phenomenon is referred to as carbon catabolite repression and is mediated via components of the phosphoenolpyruvate:carbohydrate phosphotransferase system, which transports and phosphorylates carbohydrates.

Glucose repression in *S. coelicolor* was demonstrated to be dependent upon the formation of intermediates of carbohydrate catabolism, for example, fructose 1,6-diphosphate and glucose 6-phosphate [22–23] or enzymes of the glucose catabolic pathway, such as glucose kinase, by exerting transcriptional repression of enzymes involved in the use of glycerol, arabinose, fructose, and galactose [24].

Glucose and other carbon sources have been found to suppress production of many secondary metabolites, e.g., actinorhodin in *S. lividans* [25]. In fact, it was reported that glucose inhibits actinorhodin production by repressing the transcription of *afsR2* that encodes a global regulatory protein involved in the stimulation of secondary metabolite biosynthesis [25] (Figure 6).

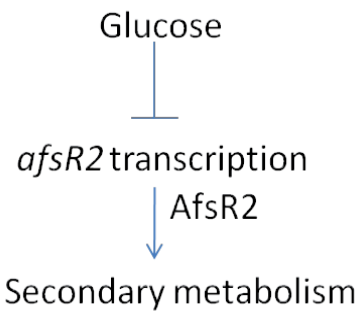

Figure 6. Effect of glucose on the secondary metabolism in *S. lividans.*

cAMP, ATP, and adenosine were reported to regulate antibiotic production [26]. When glucose is the carbon source, inhibition of the cAMP-producing enzyme, adenylate cyclase, occurs and cAMP levels are low (Figure 7). cAMP is important to activate the transcription factor cAMP receptor protein (CRP). In the absence of cAMP, CRP does not activate the transcription of target genes. When glucose is absent, cAMP is accumulated and it forms a complex with CRP, thereby activating the expression of a large number of genes, including some encoding enzymes that can supply energy independently from glucose and trigger spore germination, aerial mycelium formation, and actinorhodin production [27–30]. Extracellular ATP (exATP) was reported to massively increase actinorhodin and lightly increase undecylprodigiosin yields in *S. coelicolor* [31]. The nucleoside adenosine was reported to enhance production of undecylprodigiosin and conversely to suppress the actinorhodin production [32].

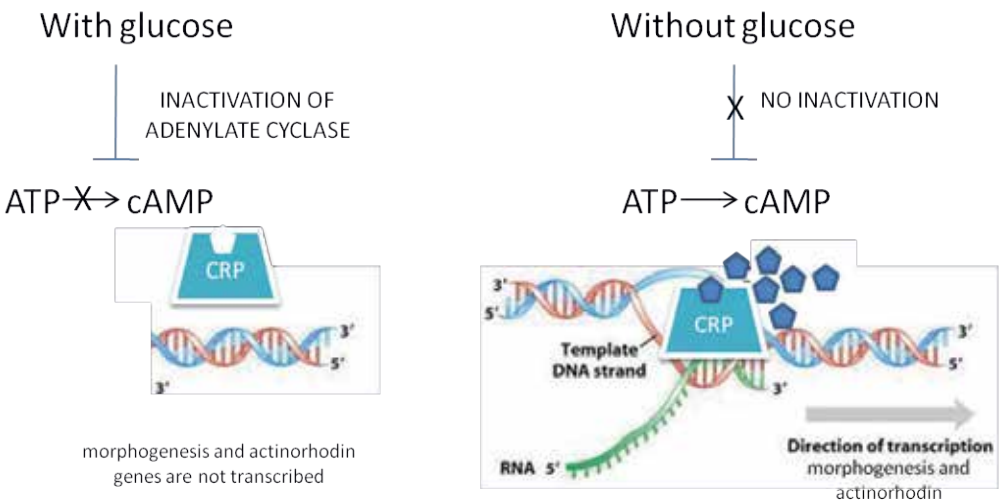

Figure 7. Effect of glucose on cAMP (blue pentagon) accumulation and antibiotic production in *S. coelicolor*.

Several microorganisms' nutrients, such as phosphate and nitrogen compounds, affect the production of antibiotics and other secondary metabolites. The lack of specific nutrients is perceived by microorganisms through complex signaling mechanisms. The study of these pathways is often the key in the understanding of regulatory processes underlying the synthesis of secondary metabolites.

Streptomycetes sense and respond to the stress of phosphate starvation via the two-component PhoR-PhoP signal transduction system (Figure 8). In *Streptomyces coelicolor*, phosphate negatively controls antibiotic biosynthesis by the two-component PhoR-PhoP system. The PhoR protein is a membrane sensor kinase, whereas PhoP is a DNA-binding response regulator (OmpR family). Primary and secondary metabolisms are interconnected. In fact, when *S. coelicolor* encounters phosphate limitation in its environment, PhoP activates the transcription of *afsS* that positively regulates secondary metabolism biosynthesis through the transcription of pathway-specific activators, such as *actII-ORF4* and *redD* for actinorhodin and undecylprodigiosin biosynthesis, respectively [33].

The biosynthesis of many antibiotics is very sensitive to phosphate. In *Nonomuraea*, A40926 production is negatively influenced by phosphate. In particular, phosphate depletion induced *dbv4* transcription that encodes an StrR-like protein, positive regulator of A40926 cluster genes [34]. In a few cases, phosphate has been reported to have a positive control, i.e., on lantibiotic production in Firmicutes strains and in *Microbispora sp*. ATCC-PTA-5024. It was surmised that phosphate has a different effect on ribosomal and non-ribosomal peptide biosynthesis [35].

A simple strategy to improve antibiotic production is to alter the PhoP concentration, by disrupting the *phoP* gene or the *phoP-R* cluster. In *S nataliensis*, the mutants obtained showed an increase of 80% in antibiotic production [36]. However, for some *Streptomyces* strains, the

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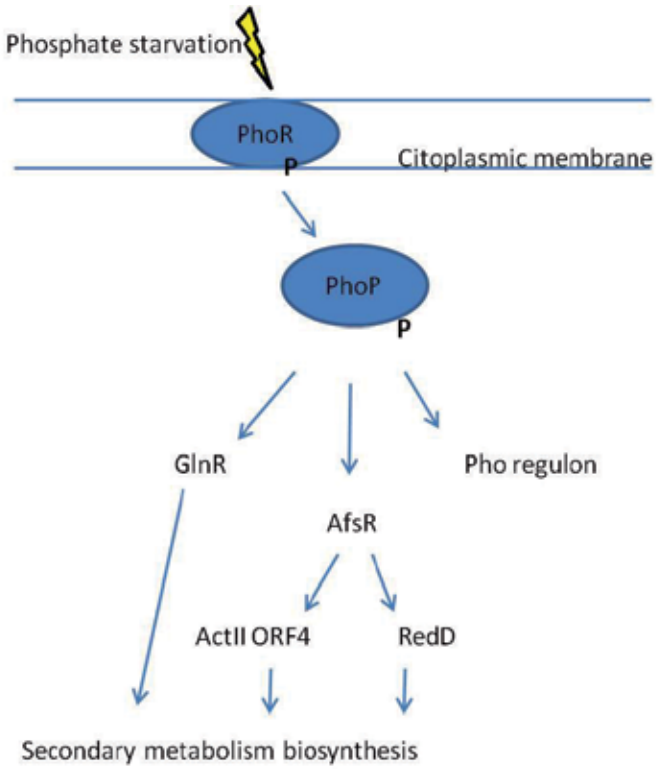

Figure 8. Cascade mechanism involved in phosphate control of actinorhodin and undecylprodigiosin biosynthesis in *S. coelicolor*.

phoP null mutant did not show an antibiotic overproduction, probably because of the complexity of the network [37].

High concentration of nitrogen sources (such as ammonium or amino acids) also suppresses the secondary metabolism. Complex fermentation media include proteins as nitrogen sources. For example, production of streptomycin antibiotic in *S. griseus* occurs in soybean meal with L-proline and low concentration of ammonium salt. Aminoglycoside antibiotic production is repressed by ammonium salt, while nitrate and certain amino acids stimulate their production [38].

In *S. coelicolor*, it was demonstrated that nitrogen assimilation is transcriptionally regulated by GlnR (Figure 8), which is an orphan response regulator with no coupled sensor kinase [39]. The Δ*glnR* mutant strain did not grow on nitrate as the sole nitrogen source and showed reduced growth on ammonium. Furthermore, no production of the pigmented antibiotics actinorhodin and undecylprodigiosin was observed [40].

Nitrogen metabolism under phosphate control exerted by the binding of PhoP to the promoter region of *glnR* reveals that crosstalk between global regulators, such as PhoP and GlnR, controls the expression of secondary metabolites [41].

In some bacteria of the phylum Actinobacteria, such as *S. lividans* and *S. coelicolor*, a shortage of nitrogen compounds is reflected in the increased concentration of tRNA discharges, a consequence of the limited availability of amino acids; this phenomenon leads to the activation of the RelA protein that binds ribosome stalling and allows the synthesis of the nucleotide pppGpp (guanine 5'- triphosphate 3'-diphosphate). In conditions of nutrient deficiency, there is a decrease of the GTP pool and the accumulation of pppGpp. pppGpp binds to the RNA polymerase subunit encoded by the *rpoB* (RNA polymerase subunit) gene, directing the transcription of genes important for the production of secondary metabolites. This mechanism of adaptation to changes in environmental conditions is called "stringent response" [42–43].

7. Genetic engineering

Antibiotic production can be improved by metabolic engineering in several ways. A flux increase in the biosynthetic pathway can be improved by directed mutagenesis or by elevated precursor availability. As an example, acetyl-CoA carboxylase was cloned into an expression vector and introduced into *S. coelicolor*; the conversion of acetyl-CoA into malonyl-CoA was enhanced and channeled to actinorhodin production [44]. Similarly, deletion of the *pfkA2* gene that encodes one of the three reported homologs of phosphofructokinase in *S. coelicolor* and led to an increased carbon flux through the pentose phosphate pathway increased the actinorhodin and undecylprodigiosin production four times [24].

In *Streptomyces clavuligerus*, deletion of the glyceraldehyde 3-phosphate dehydrogenase encoding gene *gap1* improved clavulanic acid production because of increased supply of precursor glyceraldehyde 3-phosphate [45]. Furthermore, *S. coelicolor* over-expression of acetyl-CoA carboxylase improved the yield of actinorhodin, which uses malonyl-CoA as a precursor for actinorhodin biosynthesis [44]. In *S. coelicolor*, deletion of citrate synthase or aconitase led to overproduction of organic acids, as well as changes in secondary metabolite production and morphological differentiation [46–47]. In *Streptomyces lividans*, polyphosphate kinase gene inactivation resulted in the accumulation of polyphosphates and activation of actinorhodin production, which is normally silenced in this species [48–49].

A promising method to increase antibiotic production is the ribosome engineering developed by Ochi and colleagues [50]. This method consists of the isolation of spontaneous mutants that are resistant to sub-lethal levels of antibiotic that targets the ribosomal proteins (such as streptomycin, gentamicin, kanamicin, cloramphenicol) or the RNA polymerase. Rifampicin resistance mutation in *rpoB* and streptomycin resistance mutation in *rpsL* for the ribosomal protein S12 led to an increased antibiotic production in *S. coelicolor* and *S. lividans* [51].

Some actinomycetes possess two *rpoB* genes (e.g., *Nocardia species*), in contrast to the widely accepted consensus of the existence of a single RNA polymerase in bacteria. In the *Nonomuraea sp.* strain ATCC 39727, two alleles of RNA polymerase B subunit gene *rpoB* (*rpoB*$^{(S)}$ and *rpoB*$^{(R)}$) provide the microorganism with two functionally distinct and developmentally regulated RNA polymerases [52]. *rpoB*$^{(R)}$ is characterized by an 18-bp in-frame deletion and mutations causing five amino acid substitutions located within or close to the so-called *rif*

cluster that play a key role in fundamental activities of RNA polymerase. *rpoB*$^{(R)}$ transcription is tightly regulated during *Nonomuraea* growth. Indeed, the expression of the *rpoB*$^{(R)}$ allele is growth phase-dependent in the wild type strain: the allele is silent during the pseudo-exponential phase of growth and begins to be expressed during the transition to the stationary phase. The merodiploidy might contribute to the developmental strategy of this actinomycete and the two isoforms may contribute to assemble growth phase-specific variants of RNA polymerase with different functional properties.

The constitutive expression of *rpoB*$^{(R)}$ gene in *Nonomuraea* increased the production of the glycopeptide antibiotic A40926 in this organism, while the heterologous expression in both the wild-type *Streptomyces lividans* strain 1326 and in strain K_O-421 (a relaxed mutant unable to produce ppGpp) markedly activated antibiotic biosynthesis of actinorhodin and undecylprodigiosin [43].

The basic knowledge of phosphate and nitrogen metabolic pathway can be used for rational manipulation. For example, amplification of *asfR*/*asfS* on a high copy number plasmid led to overproduction of actinorhodin and undecylprodigiosin in both *S. coelicolor* and *S lividans* [53–54]. In the same way, RelA overexpression leads to an enhanced antibiotic production in *S. coelicolor* [55].

8. Secondary metabolism to control antibiotic production

Antibiotic production is controlled at two main levels: pleiotropic regulators controlling the production of more than one antibiotic and cluster-situated regulator modulating the antibiotic biosynthetic genes of the cluster in which they are included.

The complex gene cluster for the biosynthesis of each antibiotic usually contains regulatory and resistance genes. Typically, there may be more than one such pathway-specific regulatory gene per cluster. Overexpression of positive regulators or deletion of genes that codify for repressors can be a strategy to improve antibiotic production.

Among the regulatory genes, two-component systems are the most important transduction signal mechanism in bacteria. Typically, the two-component system comprises a membrane-bound histidine kinase and a cognate response regulator. The receptor senses specific environmental stimuli, it auto-phosphorylates and activates by phosphorylation the response regulator that mediates the cellular response, mainly through the transcriptional regulation of target genes in the cluster for antibiotic [56].

The AbrC1 protein is a histidine kinase part of a two-component system in *Streptomyces coelicolor* M145. It is a negative regulator of antibiotic production and morphological differentiation. Indeed, the deletion of this repressor led to a clear increase in actinorhodin, undecylprodigiosin, and calcium-dependent antibiotic yields [57].

Other examples of regulators are those of the StrR and LuxR families. StrR was initially identified in *Streptomyces griseus* as a pathway specific positive regulator of the expression

streptomycin biosynthetic gene [58]. LuxR was initially identified in the marine Gram-negative bacteria *Vibrio fisheri,* where it regulates the quorum sensing phenomena of bioluminescence in a population density dependent manner [59]. Proteins of the LuxR family have been identified among Actinomycetes and two LAL proteins were identified as pleiotropic regulators affecting various cellular processes in S*treptomyces coelicolor* [60].

The filamentous actinomycete *Nonomuraea sp.* strain ATCC39727 codifies for *dbv4* and *dbv3*, two pathway-specific regulatory genes that code for StrR- and LuxR-type transcriptional positive regulators, respectively [61]. The over-expression of *dbv*3 led to a two-fold increase in the glycopeptide antibiotic A40926 productivity.

The increase of self-resistance levels in producing organisms was used for improving production yields. This strategy was used for *S. kanamyceticus* and *S. fradiae*, kanamycin and neomycin producers, respectively. The 6'-N-acetyltransferase derived from *Streptomyces kanamyceticus* strain M1164 was cloned into the high copy plasmid vector pIJ702 and transferred in both strains. In both cases, transformants containing the recombinant plasmid showed increased resistance to a number of aminoglycoside antibiotics and substantially increased production of kanamycin and neomycin [62]. Similar results were described in several antibiotic overproducing organisms, such as *S. aureofaciens*, producer of the chlortetracycline 6-demethylchlortetracycline, through the overexpression of a self-defense gene involved in drug efflux [63].

To increase the balhimycin production by *Amycolatopsis balhimycina*, the *dahp* and *pdh* genes, from the biosynthetic cluster that codify for key steps of the shikimate pathway, were overexpressed both individually and together. The constructed strains expressing an additional copy of the *dahp* gene and the strain carrying an extra copy of both *dahp* and *pdh* showed improved specific glycopeptide productivities by approximately a factor of three [64].

9. γ butyrolactones to control the onset of antibiotic biosynthesis

The production of antibiotics in some *Streptomyces spp*. depends upon diffusible butyrolactones structurally similar to homoserine lactones. The most intensively studied is the A factor that positively controls the pleiotropic regulator AdpA (Figure 9). A factor is gradually produced by AfsA and it is accumulated in a growth dependent manner [65]. When the concentration of A-factor reaches a critical level, it binds ArpA, which is released from the promoter of *adpA*, thus leading to *adpA* transcription. The transcriptional activator AdpA then activates a variety of genes that are required for the biosynthesis of secondary metabolites, including streptomycin, and morphological differentiation in *Streptomyces griseus*. Overexpression of *adpA* caused *S. griseus* to produce streptomycin earlier and with a ten-fold higher yield than the wild-type strain [66].

So far, various γ-butyrolactone molecules, synthases, and receptors have been identified [67–68].

γ butyrolactones have been applied for improvement of secondary metabolite production. A factor from *S. griseus* stimulated antibiotic production in *S. natalensis* showing an efficient cross-

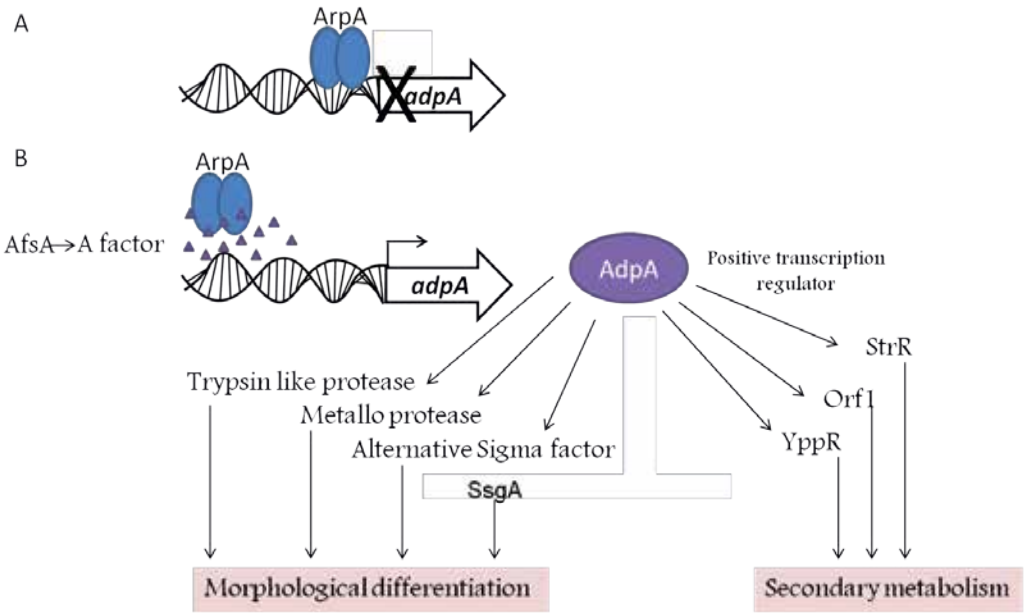

Figure 9. The A-factor regulatory cascade in *S. griseus*. (A) In the absence of A factor, ArpA binds to the *adpA* promoter and represses its transcription. (B) When A factor accumulates and reaches a threshold level, it binds to ArpA that is detached from the *adpA* promoter, leading to its expression.

talk among different species [69]. Little is known about the use of these small molecules as global elicitors of antibiotic production, but considering the effect on antibiotic production even at low concentration, this could be an interesting approach to follow.

10. Heterologous expression of actinomycetes biosynthetic gene clusters

Many antibiotic producing actinomycetes are recalcitrant to manipulation and suitable protocols for their genetic manipulation are not always available. The transfer of the genetic information for secondary metabolite production from the original producer to a model host represents a successful strategy to manipulate biosynthetic gene clusters. Heterologous expression of large biosynthetic pathways could be also useful in all those cases in which bacteria are not cultivable or to produce cryptic metabolites, revealed by genome sequencing and mining. Actinomycetes are characterized by large genomes that are GC-rich [70] and their genes are not easily expressed in *Escherichia coli*. To date, several shuttle vectors that can be maintained in model streptomycetes (*Streptomyces coelicolor, S. avermitilis, S. lividans*) have been described, and several libraries have been constructed in bacterial and P1-derived artificial chromosomes [71–74].

In some cases, a successful heterologous expression of actinomycetes biosynthetic gene clusters was obtained after changing fermentation conditions, that is, feeding with a biosynthetic

precursor, minimizing background endogenous activities, or after cloning strong promoters upstream of production genes weakly transcribed (for a review see [75]). Heterologous expression in amenable hosts can be useful to exploit and to explore the genetic potential of actinomycetes.

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Chapter 8

Biotechnological Potential of Oxidative Enzymes from Actinobacteria

Marilize Le Roes-Hill and Alaric Prins

Additional information is available at the end of the chapter

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Abstract

Oxidative enzymes are often considered for use in industrial processes because of the variety of reactions they are able to catalyse. In the past, most of these oxidative enzymes were obtained from fungi. However, in recent years, it has become evident that these enzymes are also produced by bacteria, including actinobacterial strains, which can therefore be considered as an underexploited resource of oxidative enzymes with potential for application in various industries. This chapter will focus on selected oxidative enzymes found in actinobacteria, their potential for application in industrial processes and how we can access and improve these enzymes to suit the required bioprocess conditions.

Keywords: Actinobacteria, oxidative enzymes

1. Introduction

Dating back over 100 years, organisms belonging to the class *Actinobacteria* have been the focus of different studies [1]. This should come as no surprise: the class contains five subclasses and nine orders of which the order *Actinomycetales* have received the most amount of attention, not only because it is the largest order (Figure 1), but also because of the importance of the genera and species represented in this order. For example, *Mycobacterium tuberculosis*, the causative agent of tuberculosis, marine *Micromonospora* and *Salinispora* strains with the ability to produce unique anti-cancer agents, and the genus *Streptomyces*, whose members are known to produce a variety of bio-active compounds and enzymes with potential for industrial application are all members of the order *Actinomycetales* (to name but a few).

Over the past 20 years, there has been an increased interest in the production of certain oxidative enzymes by actinobacteria, especially in the field of lignin degradation, detoxifica-

tion of organic pollutants such as polynuclear aromatic hydrocarbons (PAHs), organophosphate pesticides and azo dyes [2]. Oxidative enzymes or oxidoreductases (EC 1.x.x.x) catalyse oxidation-reduction reactions. These enzymes are further subdivided depending on the target donor molecule and the final electron acceptor, for example, the EC 1.1 grouping refers to enzymes acting on the CH-OH group of donors, while EC 1.1.3 further defines the type of electron acceptor, which in this case is oxygen (http://www.chem.qmul.ac.uk/iubmb/enzyme/). Based on the information obtained from BRENDA, the comprehensive enzyme information system (http://www.brenda-enzymes.org/), there have been more than 1,500 reports of oxidoreductase production by actinobacteria. This represents a vast number of oxidoreductases, and therefore this chapter will focus only on enzymes grouped within the EC 1.x.3.x, EC 1.11.x.x, EC 1.13.x.x and EC 1.14.x.x classes (where oxygen acts as an electron acceptor or is incorporated during the reaction). Based on the literature reported in BRENDA, the following actinobacterial genera are represented in the EC 1.x.3.x, EC 1.11.x.x, EC 1.13.x.x and EC 1.14.x.x classes: *Acidothermus*, *Actinomyces*, *Amycolatopsis*, *Arthrobacter*, *Brevibacterium*, *Cellulomonas*, *Corynebacterium*, *Dietzia*, *Gordonia*, *Kocuria*, *Leifsonia*, *Lechevalieria*, *Microbacterium*, *Micrococcus*, *Micromonospora*, *Mycobacterium*, *Nocardia*, *Nocardioides*, *Pimelobacter*, *Prauserella*, *Pseudonocardia*, *Rhodococcus*, *Saccharopolyspora*, *Streptoalloteichus*, *Streptomyces*, *Thermobifida* and *Williamsia*.

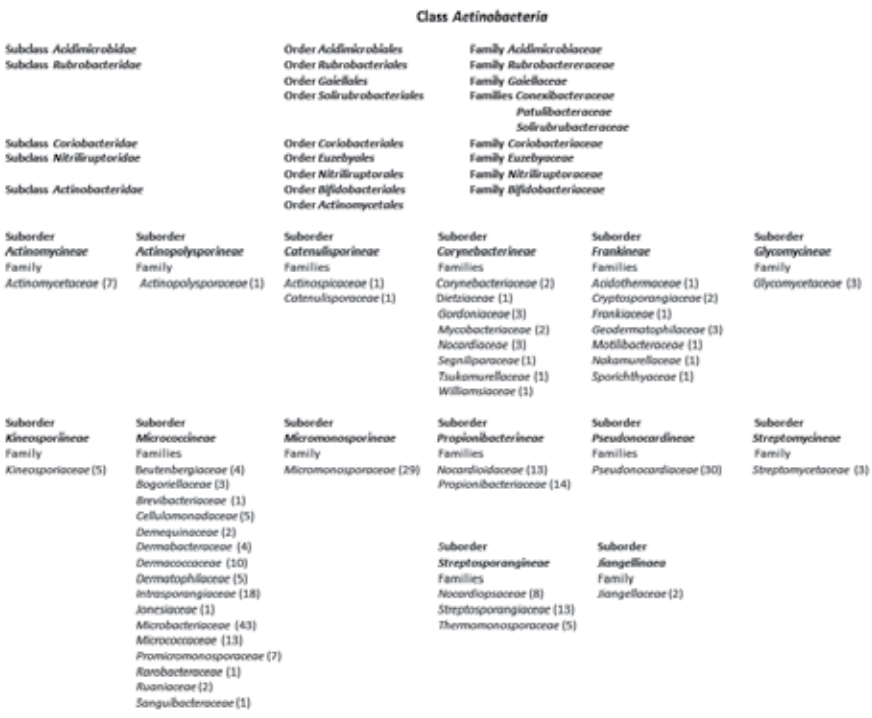

Figure 1. The class *Actinobacteria*, subclasses, orders, suborders and families. Values in brackets represent the number of genera described for each family (adapted from [3]; updated June 2015).

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2. Oxidative enzymes produced by actinobacterial strains

The development of practical biocatalytic oxidation/reduction (redox) processes is very important because many chemical and biochemical transformations involve redox processes [4]. It is therefore not surprising that oxidative enzymes have been applied to a wide range of industrial processes: in the beverage industry to remove phenolics (which causes turbidity) in drinks and to remove/diminish the cork smell in wines [5]; in the pulp paper industry to delignify wood for the bleaching process [6]; in the textile industry to decolourise the effluents from dyes [7]; to bleach textiles and to synthesise dyes [8]; in the nanotechnology industry as biosensors [9-11]; for clinical and environmental analysis and for cosmetics, for use in hair dyes and for skin lightening creams [12]. Peroxidases, laccases and tyrosinases are among the oxidative enzymes that have been widely researched and much information on their applications and mechanistic roles has been published, with a particular interest directed towards the fungal producers. In this chapter, we wish to draw attention to selected oxidative enzymes that are produced by the actinobacteria, including the well-known peroxidases, laccases and tyrosinases.

2.1. Cholesterol oxidase (EC 1.1.3.6)

Many actinobacteria carry out useful biotransformations, which allow for the production of a wide range of substances of clinical and commercial interest [13]. Of these compounds, steroids are among the most important pharmaceutical products used for the treatment of various diseases [14]. In eukaryotic organisms, cholesterol is an essential component for the maintenance of cell membrane structure and the synthesis of a number of compounds. Cholesterol oxidase (CO) is a prokaryotic enzyme that has been very useful for biotechnological applications, where it has been applied in the detection and conversion of cholesterol [15-17].

CO is a flavoenzyme that catalyses the oxidation and isomerisation of cholesterol to cholest-4-en-3-one, with the reduction of oxygen at C_3 to hydrogen peroxide (Figure 2) [18-19]. Despite having a broad substrate range, the presence of a 3β-hydroxyl group is essential for the CO activity. Whilst most microorganisms produce cell-bound CO, the actinobacteria are prolific producers of high levels of extracellular CO [18].

Actinobacterial COs have been isolated from *Corynebacterium* spp., *Rhodococcus erythropolis, Rhodococcus rhodochrous, Mycobacterium* spp., *Brevibacterium* spp. and *Streptomyces* spp. [20]. Ivshina et al. [21] demonstrated the use of CO isolated from *Rhodococcus* strains for the bioconversion of β-sitosterol to 17β-hydroandrost-4-ene-3-one (testosterone), with the addition of co-oxidant glucose and in the presence of the inhibitor, 2,2'-dipyridyl. CO from actinobacteria is currently in use in analytical practices, such as the measure of cholesterol in biological fluids and the quantitation of dehydroepiandrosterone sulphate (DHEAS) in liquids from cysts of ducts of the human mammary gland [14]. CO from *Streptomyces* spp. may also be used as a source of insecticidal proteins. CO, however, is not only beneficial but also been implicated in the causation of disease in humans. For example, *Rhodococcus equi*, a primary pathogen for horses, requires CO for opportunistic infection in immunosuppressed humans by causing membrane damage [22]. Brzostek et al. [23] demonstrated that CO also plays a key

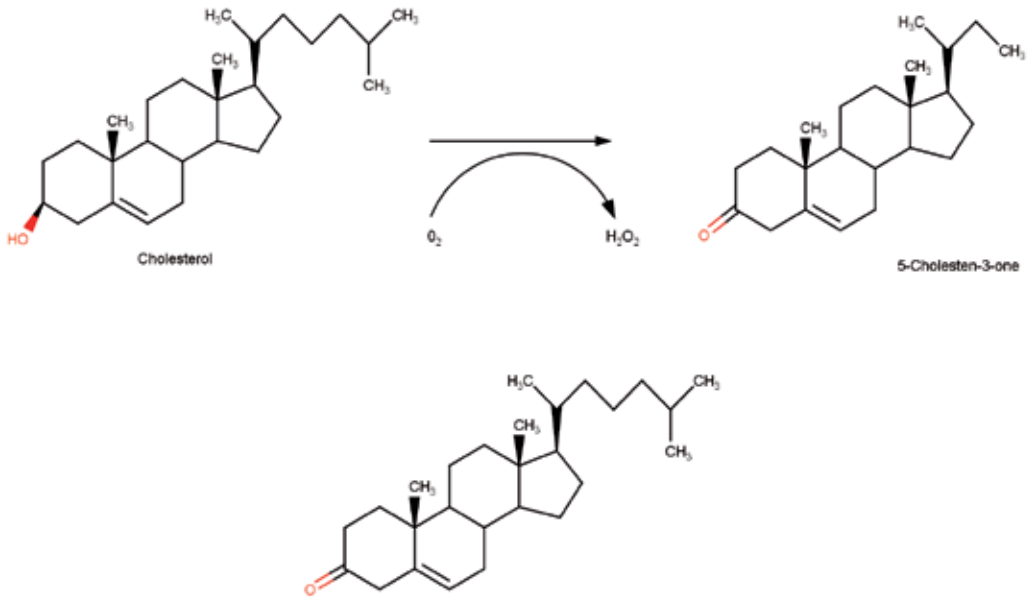

Figure 2. Mechanism of action of cholesterol oxidase on cholesterol (adapted from [20]).

role in the pathogenesis of *M. tuberculosis*. Therefore, by understanding how these COs act as virulence factors, it is possible to develop alternate means of treatment of these opportunistic infections.

2.2. Laccases (EC 1.10.3.2)

Laccases are multicopper oxidases that catalyse the one electron oxidation of four reducing substrate molecules, whilst simultaneously reducing molecular oxygen to water [24] (Figure 3). These enzymes have been extensively studied and are ubiquitous in nature, found in higher plants, fungi, insects and prokaryotes, including the actinobacteria [24, 25]. For decades, laccases of fungal origin have been at the centre of research efforts. Bacterial laccases, or laccase-like enzymes, are becoming increasingly prominent. As of 2014, less than 10 bacterial laccases from actinobacteria have been fully characterised, all of which belong to the genus *Streptomyces* [26]. As with other bacterial laccases, however, they possess characteristics that make them suitable for industrial applications, including increased thermostability, a broad pH range, stability under denaturing conditions, and for some actinobacterial laccases an atypical structure [27-29].

Whilst typical laccases consist of three cupredoxin domains, several laccases of actinobacterial origin have only two domains, which were first elucidated for the structure of the small laccase (SLAC) from *Streptomyces coelicolor* [31]. Several other two domain laccases have since been isolated, including the SilA from *Streptomyces ipomoea*, Ssl1 from *Streptomyces sviceus* and SCLAC from *Streptomyces* sp. C1 [32-34].

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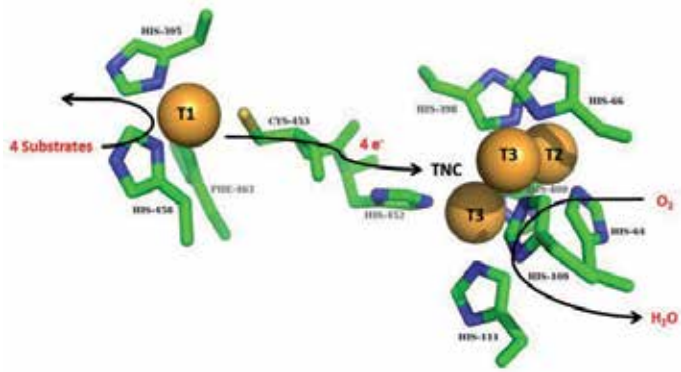

Figure 3. The typical laccase reaction mechanism [30].

Given the broad substrate range that laccases exhibit they can be applied in a wide variety of industries. Laccases have been employed in the following industrial applications: degradation of dyes in the textile industries, in the food industry where the consumption of oxygen in the packaged food is removed to avoid spoilage, in the paper and pulp industries for decolourisation of ink and the breakdown of lignocellulosic compounds, bioremediation for the treatment of toxic environmental pollutants including PAHs, pesticides, dyes from the textile industries and endocrine-disrupting chemicals [12, 35-40].

2.3. Peroxidases (EC 1.11.1.X)

Peroxidases are a large group of oxidoreductases that catalyse the oxidation of substrate molecules using hydrogen peroxide (H_2O_2) as the electron acceptor, with the majority of peroxidases using haem as a cofactor [41]. Haem peroxidases are typically grouped into two superfamilies: the first consists of bacterial, fungal and plant peroxidases and the second contains peroxidases from animals, fungi and bacteria [42]. The production of peroxidases from actinobacteria have been well described and are a good potential source of novel industrially relevant peroxidases, especially in a market that is largely dominated by the plant horseradish peroxidase (HRP) [42-44].

Antonopoulos et al. [45] investigated the biotechnological potential of the extracellular peroxidase from *Streptomyces albus* in the biobleaching of kraft pulps. It was found that the enzyme exhibited sufficiently high peroxidase activity so that it could be applied directly to the alkaline kraft pulp (alkalotolerant). The peroxidase was stable at high concentrations of H_2O_2 and no expensive co-mediators were necessary, when compared to fungal manganese-dependent and lignin peroxidases. Van Bloois et al. [41] isolated a DyP-type peroxidase from the thermophilic actinomycete, *Thermobifida fusca*, which showed high reactivity towards anthraquinone dyes, but moderate activity towards standard peroxidase substrates, aromatic sulphides and azo dyes. In 2014, Jaouadi et al. isolated a highly thermostable humic acid-biodegrading peroxidase from *Streptomyces albidoflavus*. This peroxidase exhibited a catalytic efficiency that is higher even than that of HRP [46].

2.4. Catechol 1,2-dioxygenase (EC 1.13.11.1) / Catechol 2,3-dioxygenase (EC 1.13.11.2)

Aromatic compounds are typically broken down by bacteria. However, recalcitrant varieties, such as polycyclic aromatic compounds do exist [47]. Bacterial populations often possess genes which code for enzymes that are able to degrade such toxins into protocatechuate and catechol [48]. The catechol in question is further broken down by one of two processes: (1) the action of catechol 1,2-dioxygenase (C12O) via an *ortho*-pathway or (2) via a *meta*-pathway which occurs when catechol 2,3-dioxygenase (C23O) cleaves catechol adjacent to the hydroxyl groups (Figure 4) [49-50].

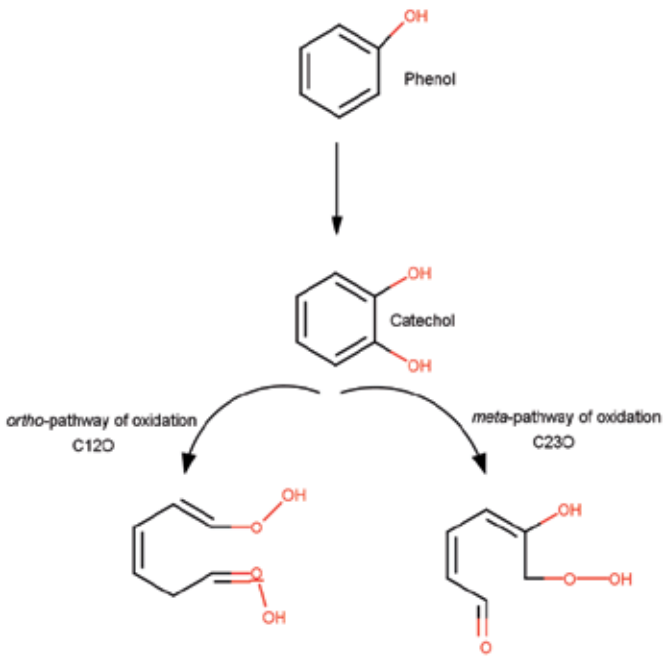

Figure 4. The breakdown of catechol through the action of (1) catechol 1,2-dioxygenase and (2) catechol 2,3-dioxygenase.

These dioxygenases are widely distributed among actinobacteria. Molecular analysis of catechol-degrading bacteria has shown that the *catA* gene which encodes for C12O is detected among several actinobacterial genera, including *Rhodococcus, Gordonia, Streptomyces, Corynebacterium* and *Mycobacterium* [48-51].

Many studies have demonstrated the use of C12O and C23O in the degradation of environmental contaminants using actinobacterial strains. Sutherland et al. [52] demonstrated C12O activity in four thermophilic *Streptomyces* strains when inducing the cultures with substituted benzoic acids. In addition, C12O activity was also induced during the culture of *Rhodococcus* sp. NCIM 2891 when using a medium supplemented with phenol [53]. An et al. [54] cloned a thermophilic C12O from the total DNA of *Streptomyces setonii* and heterologously expressed

it in *Escherichia coli*. This unique C12O exhibited remarkable thermostability, up to 65°C. Whilst C12O and C23O play a major role in (1) the breakdown of environmental contaminants, including aniline and its derivatives in agricultural soils [55] and (2) the degradation of biodiesel, diesel, chlorinated benzenes and some PAHs such as dibenzothiopene [56-57], the use of free enzymes are currently not a viable option since the enzymes are typically unstable under certain environmental conditions. Silva et al. [57] isolated C12O and C23O from *Gordonia polyisoprenivorans* and tested both the cell-free extracts and immobilised extracts. Activity was observed over a range of environmental conditions. Higher activity was observed for the immobilised C12O and C23O, thereby stabilising the enzymes and increasing the potential for greater industrial application of these enzymes.

2.5. Baeyer-Villiger monooxygenases (EC 1.14.13.X)

The interest in using Baeyer-Villiger monooxygenases (BVMOs) as biocatalysts has increased over recent years. BVMOs are flavin-dependent enzymes that are used to efficiently perform not only regio-, chemo- and/or enantioselective Baeyer-Villiger oxygenation reactions (Figure 5) using stoichiometric quantities of O_2 as an oxidant and NADPH as an electron donor, but also selected sulphoxidations and epoxidations [58-62].

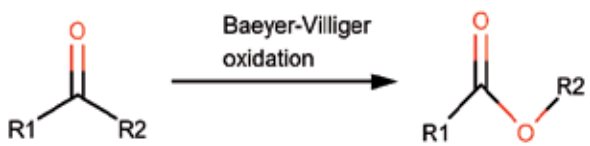

Figure 5. The Baeyer-Villiger oxidation reaction in which BVMOs convert ketones to their corresponding esters [63].

BMVOs are widely distributed among bacteria, being especially prevalent among the actinobacteria, with an average of one BVMO per genome [64]. In contrast, no Type 1 BVMOs (based on sequence similarity) have been found in plant, human or animal genomes [63]. As with many oxidases, the presence of a highly conserved protein sequence motif can be used to identify BVMOs [65]. Using this motif, Fraaije et al. [66] identified a putative BVMO from the genome of the thermophilic actinomycete, *T. fusca*. Jiang et al. [67] mined the genome of *Streptomyces avermitilis* and recombinantly expressed *PtIE*, which in the presence of NADPH and catalytic FAD exhibited Baeyer-Villiger activity. When the isolated enzyme was incubated with 1-deoxy-11-oxopentalenic acid, it gave rise to an unknown derivative of pentalenolactone, a sesquiterpenoid antibiotic, giving further insight into the pentalenolactone metabolic pathway [67].

In *Gordonia* sp. strain TY-5, BVMOs were shown to be implicated in the metabolism of acetone that is derived from propane oxidation and provides further knowledge on the poor understanding of acetone oxidation in microbes [68]. BVMOs can also be used for the synthesis of β-amino acids, compounds of considerable industrial importance due to their function as essential components in the preparation of β-peptides, terpenoids and β-lactam antibiotics [69].

2.6. Cytochrome P450 monooxygenase (EC 1.14.14.1)

Cytochrome P450 monooxygenases (CYPs) are perhaps one of the most widely studied enzymes. CYPs are haem *b* containing monooxygenases. Haem is a prosthetic group which contains an iron ion that is coordinated to four nitrogen atoms of porphyrin [70]. Similar to the BVMOs, the CYPs are remarkable in the amount of reactions they are able to catalyse, including but not limited to hydroxylation, epoxidation, peroxidation, deamination, dehalogenation, alcohol and aldehyde oxidation and C-C bond cleavage. As such, they are exploited for their potential applications in the production of drugs, vitamins, fragrances and pesticides [71-72]. CYPs play significant roles in various organisms: from carbon-source degradation and the production of metabolites in prokaryotic cells to the breakdown of toxic environmental xenobiotics found in mammals and insects [73].

CYPs, with their ubiquitous nature, have been observed in a number of actinobacteria. An environmental *Mycobacterium* strain (RP1) was isolated from contaminated activated sludge. This strain was able to use morpholine and other heterocyclic compounds as the sole carbon source for growth. Poupin et al. [74] deduced that a soluble CYP was involved in the break-down of morpholine through the cleavage of the C-N in morpholine. Lamb et al. [75] studied the entire complement of CYP (the CYPome) in *S. coelicolor* and found that many of the CYPs are involved in the biosynthesis of antibiotics. Shresta et al. [76] subsequently cloned a CYP from *Streptomyces peuceticus* and showed the hydroxylation of the macrolide, oleandomycin. This showed that CYP have flexibitiliy towards unnatural substrates and can be used in the generation of a variety of biological synthetic compounds of clinical value.

2.7. Tyrosinases (EC 1.14.18.1)

Tyrosinases (polyphenol oxidases) are copper-dependent oxidases that catalyse the *ortho*-hydroxylation of monophenols to diphenols (cresolase activity), and subsequently oxidising the resultant catechols to *o*-quinones (Figure 6) [77, 78]. To date, only three tyrosinase crystal structures have been elucidated, of which one was isolated from the actinomycete, *Streptomyces castaneoglobisporus* [79].

These enzymes are ubiquitous in nature, and serve a multitude of biological functions [80]. Most notably, tyrosinases play a key role in the production of melanin. For example, in plants tyrosinases are responsible for the browning of open surfaces in fruits [77], while in microbes melanin plays a key role in the defence of DNA against radiation and reactive oxygen species (ROS) and binds to toxic heavy metals [81-82]. Biologically active melanin has been shown to have many advantages, including anti-tumour activity and providing protection against UV radiation [83-86].

The most commonly used tyrosinase for commercial purposes is that of the fungus, *Agaricus bisporus* [87]. However, actinobacteria are well-known to produce tyrosinases, especially since many *Streptomyces* species produce a melanin-like pigment [88], and as such, actinobacterial tyrosinases have become increasingly prevalent [79, 89].

The cresolase and catecholase activities of tyrosinase are advantageous for many industrial processes, including the production of pharmaceutically important compounds such as the *o*-

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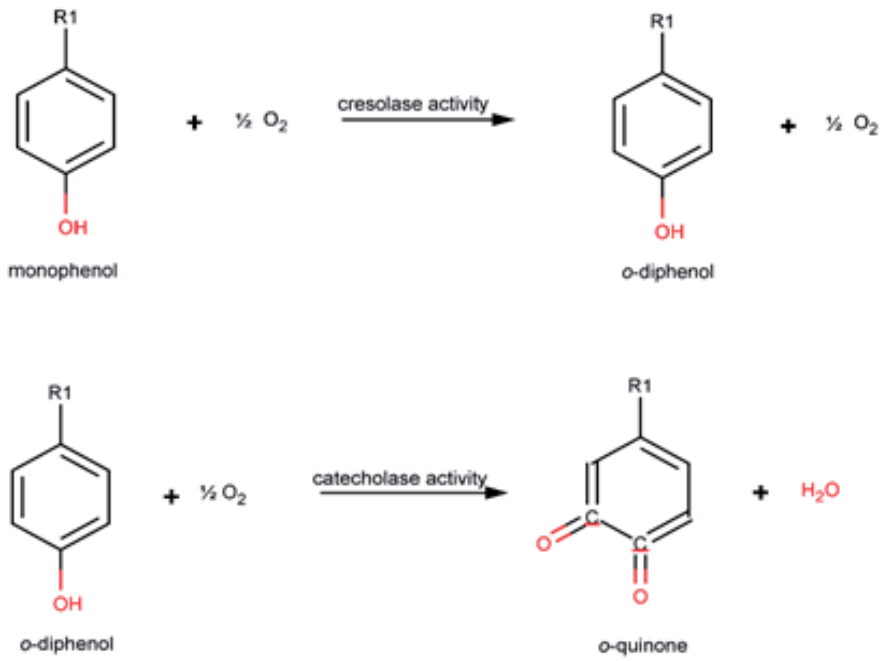

Figure 6. Enzymatic activity of tyrosinase on a monophenol (adapted from [78]).

diphenols, L-3,4-dihydroxyphenylalanine (L-DOPA) and dopamine [78]. For example, in humans, melanin plays an important role and melanin deficiencies can cause severe abnormalities and diseases. Parkinson's disease is one of these adverse conditions and is caused by a reduction of melanin in neurons [90-91]. The use of water-soluble melanin, such as that produced by several *Streptomyces* species, could therefore be useful in the treatment of Parkinson's disease. Madhusudhan et al. [92] investigated the production and cytotoxicity of extracellular insoluble and soluble melanin that were produced by *Streptomyces lusitanus* DMZ-3. Whilst their study showed that both the soluble and insoluble melanin were highly cytotoxic, it was observed that the soluble melanin was more biologically active than the insoluble melanin [92]. There has also been an increase in using safe, biologically produced compounds in the food industry, for example, the use of silver nanoparticles to control food pathogens [93]. Kiran et al. [94] further demonstrated the efficacy of silver nanoparticles as biocontrol agents by using response surface methodology to optimise the production of melanin from *Nocardia alba* MSA10. The melanin produced showed the rapid reduction and stabilisation of nanostructures and the produced structures had a broad spectrum of antimicrobial activity against common pathogens, including *Bacillus cereus, E. coli, Vibrio parahaemolyticus* and *Salmonella typhi*. Tyrosinases are also useful for the remediation of phenol-contaminated waters. Roy et al. [95] isolated and immobilised a tyrosinase from a marine actinomycete, *Streptomyces espinosus* strain LK4, which was able to effectively remove phenol from aqueous solutions.

2.8. Other oxidases

2.8.1. L-amino acid oxidases (EC 1.4.3.2)

L-amino acid oxidases (L-AAO) are oxidoreductases that catalyse the oxidative deamination of L-amino acids to yield keto-acids, ammonia and hydrogen peroxide [96]. These enzymes exhibit broad substrate ranges, and as such are commonly applied for the resolution of racemic mixtures. For example, an L-AAO isolated from *Rhodococcus opacus* DSM 43250 exhibited a broad substrate range, which includes the amino acids L-phenylalanine, L-leucine, L-alanine and L-lysine. It was able to resolve a racemic mixture of D,L-leucine and D,L-phenylalanine [96].

2.8.2. Putrescine oxidase (EC 1.4.3.10)

Putrescine is a low molecular weight diamine that belongs to a group of compounds that are termed biogenic amines. The accumulation of biogenic amines in foods, such as putrescine, can be used as a marker for food spoilage caused by microbes such as *Enterobacteriaceae* and *Clostridium* spp. [97-98]. Putrescine oxidases (PuOs) catalyse the oxidative deamination of putrescine to 4-aminobutaral, ammonia and hydrogen peroxide [99]. PuOs have been isolated from a number of actinobacteria, most notably from *R. erythropolis* and *Kocuria rosea* (*Micrococcus rubens*) [100-101]. Standard analytical methods for the detection of biogenic amines are thin-layer chromatography, gas chromatography and ultra-performance liquid chromatography [102-103]. Newer, more rapid methods of detection, such as biosensors, have been developed. PuOs from *K. rosea* was immobilized onto multi-walled carbon nanotubes for application as a biosensor, which allowed for the rapid detection of putrescine in mammalian plasma, with little interference from other biological species such as cadaverine or histamine, and without the need for prior purification of sample plasma [104]. Additionally, Bóka et al. [103] also immobilized PuO onto the surface of a spectroscopic graphite electrode and employed it for the detection of putrescine in beer samples. The biosensor measurements were compared to measurements performed through the use of high-performance liquid chromatography (HPLC), and higher sensitivity was exhibited when using the biosensor, demonstrating a rapid, efficient method for the detection of putrescine.

2.8.3. L-glutamate oxidases (EC 1.4.3.11)

In contrast to the broad substrate range of L-AAOs, there are strict substrate-specific amino acid oxidases, such as the L-glutamate oxidases [105], the first of which was isolated from the actinobacterium, *Streptomyces violascens* [106]. Glutamate oxidases have been shown to play a key role in the synthesis of pharmaceutically relevant chiral intermediates, specifically, the conversion of glutamate to α-ketoglutarate [107-108].

2.8.4. Sarcosine oxidases (EC 1.5.3.1)

Sarcosine oxidases (SOs) catalyse the hydrolysis of sarcosine and formaldehyde, while simultaneously yielding hydrogen peroxide [109]. It is predominantly being exploited in

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clinical assays for the determination of creatinine in serum. The SO from *Corynebacterium* sp. U-96 is perhaps the most extensively studied SO to date. Whilst the *Corynebacterium* SO remains the most industrially relevant, SOs have been cloned and characterized from various *Athrobacter* spp. [110-112]. In addition, a *Streptomyces* sp. SO has also been cloned and expressed in a *Streptomyces* expression system [109-113]. Furthermore, recent genome studies have identified the presence of SO genes in the genomes of many actinobacterial species, which could potentially serve as a source of novel SOs [114-116].

3. Industrial relevance of actinobacterial oxidases: how to access them and improve their functionality

With oxidising enzymes, as with most other enzyme groups, the emphasis in the discovery and development of new enzymes for industrial processes is increasingly focused on the properties of the new enzymes that need to match the stringent conditions imposed by the industrial setting [117]. Thus, while the existence of large numbers of novel enzymes is demonstrated constantly, via a host of modern gene discovery technologies, this evidence of their existence is not sufficient to guarantee our capacity to provide the enzymes that industry demands. A further consideration in the development and application of new oxidizing biocatalysts is the requirement to demonstrate the novelty which will provide market advantage [118]. Thus, as new oxidizing enzymes are discovered, they need to be characterised in terms of substrate selectivity, product scope and stability in the presence of process constraints (e.g. the presence of organic solvents, temperature conditions, pH conditions).

The majority of oxidising enzymes are co-factor-dependent, which leads to requirements for co-factor recycle or replacement in the industrial processes utilising these enzymes. While this can be overcome by application of whole-cell biocatalysts, the search for non-co-factor-dependent oxidases which can catalyse equivalent reactions is a useful goal [117-118]. The three most commonly used screening methods to screen for novel enzyme activity include: (1) screening environmental samples for organisms whose enzymes have the ability to catalyse certain reactions; (2) the use of protein engineering to manipulate an existing biocatalyst and (3) looking for novel functionality/substrate specificity in existing biocatalysts [119]. These processes along with others can further be grouped into molecular-based (*in silico* screening of genome sequences; metagenomics and PCR-based screening; reverse genetics) and non-molecular-based screening techniques (dye decolourisation; high-throughput screening with liquid-based enzyme assays; selective isolation directly from an environmental sample) (Figure 7).

3.1. Isolated strains

Based on information obtained from BRENDA, the majority of the reports on oxidative enzymes from actinobacterial strains have originated from studies based on isolated strains and yet only a fraction of the genera within the order *Actinomycetales* is represented. This could be due to various reasons, including the fact that many of the strains that have been the focus

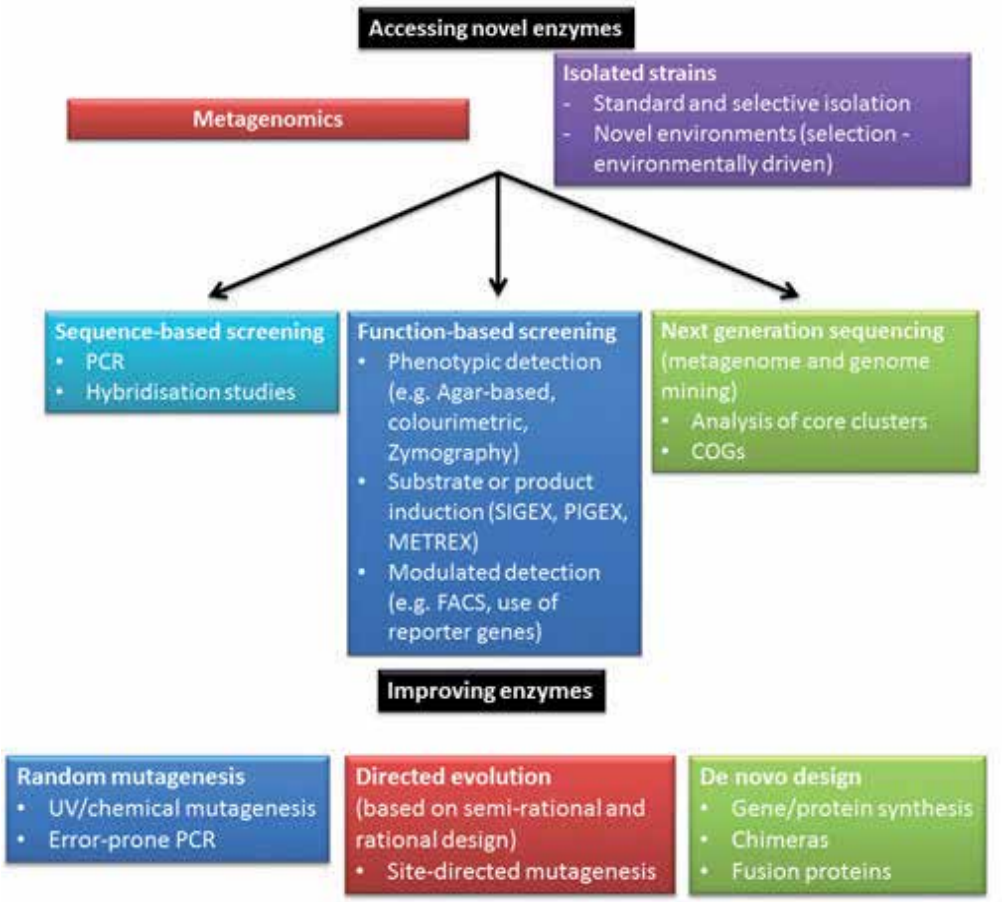

Figure 7. Summary of standard methods employed to access novel enzymes and to improve enzymes.

of studies are either pathogens (human and/or animal, e.g. *Mycobacterium* and *Rhodococcus* spp.) or are known producers of bio-active compounds (e.g. *Streptomyces* spp.). In addition, standard isolation techniques only allow for the detection of a small fraction of actinobacterial populations in environmental samples, often missing out on the isolation of the 'rare' actinobacteria (those not readily isolated) [120]. To access these strains and their genetic diversity, researchers have designed selective isolation techniques, many of which are based on the properties of the targeted organisms (e.g. motility or heat resistance) and/or the properties of the environment the sample was collected from [121]. Kurtböke [120] reiterated that the successful isolation of 'rare' actinobacterial strains or strains producing bio-active compounds/enzymes from any given environment would be dependent on our understanding of the function of the strains within the environment of interest. For example, Le Roes-Hill et al. [122] identified various oxidase-producing actinomycetes that were isolated from the hindguts of a higher termite where it is hypothesised that the oxidase-producing actinobacteria may be involved in lignin degradation. An alternative to the isolation of actinobacterial strains from

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environmental samples, the genetic diversity of 'rare' actinobacteria can also be accessed through metagenomics-based studies.

3.2. Metagenomics - Accessing the genetic information of the 'unculturables'

Over the past two decades, there has been a dramatic increase in the number of metagenomics-based studies. The belief that the majority of bacteria in the environment (>99%) are unculturable or that culture techniques for their isolation have not yet been developed have necessitated the analysis of the metagenome [123]. With the advent of metagenomics in the 1990s, numerous novel genes have been discovered [124-126]. Metagenomics allows for the cloning and expression or screening of multiple genomic DNA extracts from any given sample [127]. Accessing the gene or enzyme of interest is, however, dependent on various factors, but typically involve sequence-based screening and/or function-based screening, some of which are also applicable to isolated strains.

3.2.1. Sequence-based screening

Sequence-based screening relies heavily on prior knowledge of the enzyme of interest, e.g. the polymerase chain reaction (PCR) primers and hybridisation probes need to be designed based on conserved regions in sequences that are currently available in databases. This approach is therefore not suited for the discovery of novel protein classes [128]. In two metagenomic studies, a sequence-based screening approach allowed researchers to identify the presence of actinobacterial two-domain laccases in lignin-rich environments. Ausec et al. [129] made use of previously published primer sets, while Lu et al. [130] designed their own primers that were designed based on sequence alignments of known two-domain laccases. Both of these studies showed the vast biodiversity of these laccases in the respective environments analysed: drained peat soils [129] and compost prepared from agricultural waste [130].

Similarly, sequence-based screening has been shown to be a valuable tool for determining the diversity of *Streptomyces* genes in isolated strains. Due to the biased codon usage of actinobacterial strains [131], it is possible to design primers based on short consensus sequences. Decker et al. [132] showed with a comparison of the sequences of known dNDP-glucose 4,6-dehydratases from *Streptomyces griseus* N2-3-l, *Streptomyces violaceoruber* Tii22 and *Saccharopolyspora erythraea* DSM 5908 that several conserved regions are present. Consequently, PCR primers were designed and used to amplify genes encoding for dNDP-glucose 4,6-dehydratase from eight different actinomycetes [132].

3.2.1.1. In silico screening of genome sequences

Of the nearly 6,400 bacterial genomes available on the NCBI 'Microbial genomes' page, (http://www.ncbi.nlm.nih.gov/genomes/MICROBES/microbial_taxtree.html; accessed 24 June 2015), 871 represent the class *Actinobacteria*. Even though this represents a vast resource for the discovery of novel oxidative enzymes [133], *in silico* screening is often hampered by the misannotation of sequences [123]. Various researchers have, however, successfully identified, amplified, cloned and expressed actinobacterial oxidases discovered by genome mining, e.g.

the phenylacetone monooxygenase (EC 1.14.13.92; BVMO) from *T. fusca* [134]; laccase (EC 1.10.3.4) from *S. sviceus* [33]; cuprous oxidase from *Corynebacterium glutamicum* [135]; laccase (EC 1.10.3.4) from *S. coelicolor* [31] and cholesterol oxidase (EC 1.1.3.8) from *Mycobacterium neoaurum* [136], to name a few.

In addition, access to various genomes allows for comparative genomic analysis of the diversity of specific enzyme groups/classes among the genomes analysed. For example, Li et al. [137] performed comparative genomic analyses on 18 *Nocardiopsis* genomes. Functional analysis of homologous gene clusters allowed for the identification of genes encoding for cytochrome P450 monooxygenases, thereby broadening our knowledge-base on the distribution of these enzymes in actinobacteria.

3.2.1.2. Reverse genetics

Recently, a strategy known as 'reverse genetics' has been developed for the identification of numerous gene clusters [138]. The first step in this strategy entails the deduction of a biosynthesis hypothesis for the corresponding substance. On the basis of the chemical structures, radiolabelling studies and other available data about the substances, the enzyme systems required for backbone-synthesis and tailoring enzymes can be predicted. In the second step, characteristic target genes and enzymes are selected and compared in multiple sequence alignments with homologous genes and gene products retrieved from public and in-house databases. Subsequently, conserved protein motifs can be determined and used for primer design. In the last step, internal fragments of the selected biosynthesis genes can be amplified by PCR and used as probes to identify these genes in genome libraries [138]. This method has been successfully used in the identification of genes from *Streptomyces lavendulae* coding cytochrome P450 monooxygenases involved in the biosynthesis of the antibiotic, complestatin [139]. Piraee and Vining [140] also used the same method to identify genes encoding for a halogenase, which is involved in the biosynthesis of chloramphenicol in *Streptomyces venezuelae.*

3.2.2. Function-based screening

Function-based screening is often preferred to sequence-based screening because it allows for access to a wider range of enzymes or biological activities [128, 141]. In metagenomics, the success of function-based screening is dependent on various factors: (1) abundance of the target gene; (2) the size of the gene; (3) the presence of a full-length sequence; (4) the expression host; (5) the expression system and (6) the assay or means of detection of the enzyme activity [123]. In the majority of metagenomic studies, *E. coli* is used as the expression host. *E. coli*, however, can only express 40% of environmental genes, but for the high G+C% actinobacteria, it is predicted to drop as low as 7% [141]. Functional metagenomics may therefore underrepresent the potential of actinobacterial strains. A solution to this is the use of more than one expression host: various *Streptomyces* spp. and *Rhodococcus* spp. have been used for the expression of actinobacterial genes. McMahon et al. [141] demonstrated the importance of using more than one host. In their study, the vectors from 12 functionally active clones were transformed into *Streptomyces lividans* and *E. coli*, and activity was only observed in the *S. lividans* host.

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There are three main approaches in function-based screening: the detection of a phenotypic trait (most commonly used), production of the enzyme or compound due to substrate/product/metabolite-induced gene expression (SIGEX/PIGEX/METREX) and modulated detection where the expressed product is linked to a reporter gene allowing for detection by fluorescence or luminescence [142]. In the next few paragraphs, methods used for the detection of oxidases in actinobacteria are described. For more detail on the different function-based screening approaches mentioned above, see Ekkers et al. [142] for a complete review.

3.2.2.1. Selective isolation of oxidase-producing strains directly from an environmental sample

Kiiskinen *et al.* [143], described a method where the substrate for the oxidase of interest have been incorporated into the isolation media for new isolates, e.g. for the detection of laccase activity, guaiacol was incorporated - a laccase-producing strain would cause a change of colour in the agar from a dark red-brown to red-orange colour. Similarly, Bordeleau and Bartha [144] described a method whereby newly isolated strains on agar plates were sprayed with liquid *p*-anisidine-H_2O_2. Strains positive for the production of peroxidase developed a dark halo around the colony.

Isolated strains can also be screened for enzyme activity on solid media containing dyes. McMullan et al. [145] gives a short review on published work regarding the degradation of dyes by filamentous actinomycetes. The extracellular enzymes that are involved in the degradation of dyes are typically the enzymes involved in lignin degradation: lignin peroxidase, laccase and manganese-dependent peroxidase [146-147]. In filamentous actinomycetes, however, it was found that there is no correlation between the degradation of the polymeric dye, Poly R478, and that the enzymatic process involved is still unexplained [145]. The degradation of the azo dye, Remazol Brilliant Blue R (RBBR), was, however, found to be linked to the action of peroxidases which are similar in structure and function to the fungal manganese-dependent peroxidases [145, 147].

3.2.2.2. High-throughput screening with liquid-based enzyme assays

Oxidoreductases produced by actinobacteria can also be accessed by using various high-throughput assays. With the increased interest in finding novel enzymes for use in biocatalysis, the number of assays currently available is quite vast and most are based on the use of chromogenic or fluorogenic substrates [148]. Most of these assays employ the use of a standard UV/visible spectrophotometer or microtiter plate readers, but for certain assays, the screening process involves the use of mass spectrometry, nuclear magnetic resonance (NMR), Fourier-transform infrared (FT-IR), thin layer chromatography (TLC), capillary array electrophoresis and enzyme-linked immunosorbent assays (ELISAs) to detect the changes in the catalysed reaction [148-149]. Enzyme fingerprinting and the use of microarrays are increasingly becoming powerful tools in the high-throughput screening of enzymes [148]. In addition, fluorescence-activated cell sorting (FACS) is a powerful high-throughput screening method that allows for the screening of large (10^9) clone libraries in a relatively short time period [123]. Zhu and Fang [150] recently also reviewed the potential of droplet microfluidics as a high-throughput technique for the screening of enzyme activities.

3.2.2.3. Protein engineering

Protein engineering is an alternative approach to obtain an enzyme with novel activities and biochemical properties. Random mutagenesis through the use of UV radiation or chemical mutagenesis is often favoured when the enzyme structure or sequence is not known. Even though this approach is typically used to generate an enzyme with improved properties, it is often limited to a change in a single property [123]. Random mutagenesis, however, was successfully used by Fujii et al. [151] to improve the vitamin D_3 hydroxylase activity of *Pseudonocardia autotrophica*. The mutated enzyme (four mutations) was expressed in *R. erythropolis* and exhibited 21.6 times higher activity, while the isolated mutated enzyme showed a six times higher activity than the wild-type enzyme [151]. Dudek et al. [152] generated multiple mutations in a single step through the use of the OmniChange method (allowed for random mutation of up to five sites), resulting in a quadruple mutated BMVO with an expanded substrate specificity. In addition, Yao et al. [136] successfully applied two mutated cholesterol oxidases (mutated by UV mutagenesis) in the production of steroids to determine whether cholesterol oxidase plays a role in the transformation of sterols. These examples emphasise the fact that random mutagenesis is a powerful tool for the development of enzymes with enhanced biochemical properties and still has its place in protein engineering.

For more complicated changes, such as changes to specific amino acids, knowledge is required on sequence-structure information, allowing for a more rational or semi-rational design. Liu et al. [153] made use of a combination of site-directed mutagenesis and error-prone PCR to generate 7,800 variants of a cytochrome P450 monooxygenase from *Rhodococcus ruber* DSM44319. The best variant showed 240 times increased de-ethylation activity towards 7-ethoxycoumarin and 10 times increased demethylation activity towards 7-methoxycoumarin. Site-directed mutagenesis have also been applied to the phenylacetone monooxygenase from *T. fusca* [134], the cyclohexylamine oxidase from *Brevibacterium oxydans* [154], the small laccase of *S. sviceus* [155], the small laccase of *S. coelicolor* [156-158] and the tyrosinase of *Streptomyces kathirae* [159].

The de novo synthesis of enzymes allows for the design of enzymes with specific properties. This, however, requires a deep understanding of sequence-structure-function [160]. A similar approach is the production of chimeric proteins, where different enzymes are combined so that the properties from the different enzymes can be harnessed in one reaction setup. These fusion enzymes are designed to enhance the biocatalytic activity/function of the enzymes. They can consist of enzymes that act synergistically (e.g. enzymes involved in lignin degradation) [161] or as in the case of the BVMO from *T. fusca*, the enzyme was fused with a phosphite dehydrogenase (an NADPH regeneration enzyme), thereby supplying the BVMO with the co-factor required for function [162].

4. In conclusion

Members of the order *Actinomycetales* clearly represent a vast untapped resource for oxidative enzymes with potential for biotechnological application. Only selected genera from selected

families are currently represented in literature and databases, leaving a great scope for further exploration. Specialised research in the area of actinobacterial genetics has allowed for the development of expression systems that allow access to environmental actinobacterial genetic material. In addition, the 871 genome sequences currently available on the NCBI database (a number that would surely be doubling over the next year), as well as the multitude of type strains in culture collections, provide numerous opportunities for the discovery of new and interesting oxidative enzymes. The recent move towards the sequencing of metagenomes will also present a vast resource from which sequence information of oxidative enzymes can be accessed. Even though we would be limited to accessing known enzyme classes, the potential for the discovery of novel enzymes would be great. Researchers in the field of actinobacterial oxidative enzymes are therefore encouraged to (1) develop effective screening programs; (2) make use of a full suite of biochemical properties to determine the potential of the enzyme for industrial application; (3) demonstrate the biotechnological potential of the enzyme; (4) determine the protein sequence of isolated enzymes so that the information on characterised enzymes can be expanded and used for directed protein engineering approaches; (5) look towards novel environments for new and interesting actinobacterial strains or genetic information and (6) make use of sequence resources currently available (e.g. genome sequences) in order to expand our knowledge base on oxidative enzymes from actinobacteria.

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Chapter 9

Hypersaline Actinomycetes and Their Biological Applications

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Additional information is available at the end of the chapter

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Abstract

Actinomycetes are the potential sources of novel metabolites, therapeutic compounds, enzymes, and other chemicals. Among them, the applications of halophilic actinomycetes toward the medically and industrially important metabolites and enzymes are gaining increasing attention by the scientific community. A large number of novel compounds and enzymes from halophilic actinomycetes have been isolated and characterized from various geographic regions around the world. In this chapter, occurrence, characterization, halotolerant mechanisms, medical importance, metabolites, enzymes, and industrial applications of halophilic actinomycetes are discussed. Halophilic actinomycetes may also serve as good models for the production of important metabolites and enzymes with respect to stress response.

Keywords: Halophilic actinomycetes, occurrence, metabolites, enzymes, applications

1. Introduction

Actinomycetes are the most valuable microorganisms for the production and synthesis of economically important therapeutic compounds and antibiotics. They are the source for the production of about more than 50% of discovered bioactive compounds, including antitumor agents, antibiotics, enzymes, and immunosuppressive agents [1, 2]. Most of these bioactive secondary metabolites were isolated from terrestrial actinomycetes; however, in recent

years, the rate of discovery of new bioactive compounds has decreased. Hence, it is crucial that new groups of actinomycetes from unexplored or underexploited environments should be exploited optimally to obtain novel bioactive secondary metabolites [3]. Unexplored regions of high salt deposited areas, conditions closely related to the marine water regions, regions with increased levels of pH, and low oxygen were the suitable places to isolate halophilic actinomycetes. Actinomycetes recovered from halophilic and unexplored regions attracted the interest of researchers because of their applications in the medical and biotechnological fields [4]. This chapter deals with the characterization methods for the identification of halophilic actinomycetes and their applications

2. Occurrence of halophilic actinomycetes

Marine sediment, soil, water, contaminated regions on the seashore, salt lakes, saline soils, alkaline–saline habitats, brines, and other regions are good sources for the selection of novel halophilic actinomycetes. Recent literature claimed that the saline regions contain many significant uncultured actinomycetes [5]. In particular, actinomycetes such as *Streptomyces pharmamarensis*, *Prauserella halophila*, *Pseudonocardia* sp., *Salinispora arenicola*, *Aeromicrobium* sp., *Micromonospora* sp., *Marinactinospora thermotolerans*, *Microbacterium* sp., *Nocardiopsis xinjiangensis*, *Salinibacterium* sp., *Salinactinospora qingdaonensis*, *Rhodococcus* sp., *Actinomadura* sp., *Saccharopolyspora* sp., *Streptosporangium* sp., *Actinopolyspora algeriensis*, *Marinophilus* sp., *Streptomonospora halophila*, *Verrucosispora* sp., *Gordonia* sp., and *Nonomuraea* species were recovered and characterized from hypersaline regions [6–10]. Also, it was quoted that the characterization of novel halotolerant actinomycetes was specific with respect to their morphological, biochemical, physiological, and molecular level identification.

3. Identification and characterization of halophilic actinomycetes

In general, different pretreatment methods such as incorporation of specific antibiotics were used to prevent the growth of bacteria and fungi, and alteration in the composition of the cultivation medium was specific for the isolation of rare actinomycetes. However, humic acid and different concentrations of vitamin agar medium were the best base for the selection of rare halophilic actinomycetes. Humic acid strengthens the morphological identification and characterizes the spore chain of the actinomycetes by preventing the production of diffusible pigments in the cultivation medium. A substantial part of the microbes in environmental samples are not cultivable and therefore cannot be identified by methods based on culturing of the strains. However, protocols based on molecular techniques are not dependent on the viability of the microbes, and molecular methods for the amplification of specific genes such as 16S rRNA and *rec* A can be designed to work at different levels of specificity for the detection of whole groups of actinomycetes. Polymerase chain reaction (PCR)-based methods for the detection and identification of microbes are widely used in the cultivation of industrially important microbes [11]. Applications in environmental microbi-

ology, especially in the soil environment, are also increasing [12]. With currently used cultivation methods, only a small part of the halophilic actinomycetes diversity is detected. The cultivability values ranged from 0.001% to 15%, depending on the cultivation area and the method of cultivation medium [13]. On the other hand, PCR amplification of 16S rRNA genes from environmental samples has revealed that 7–64% of the amplified sequences originated from uncultured actinomycetes [14]. Comparisons of the amounts of total and cultivable microbes in the indoor environment have shown that the cultivable part of the microbial community ranges from 1% to 10% [15]. Therefore, careful attention must be paid in the identification of the rare halophilic actinomycetes.

4. Halotolerance mechanism

Halophilic actinomycetes require higher concentrations of salt for their growth and are classified into moderate (15% NaCl) and extreme (30% NaCl) halophiles. They survive through two mechanisms – "high-salt-in" and "low-salt, organic-solutes-in" – for the protection of intercellular proteins in the presence of salts similar to potassium chloride and production of organic acids, which will directly alter the intracellular enzyme levels. To adjust to the hyper-salt conditions, halophilic cell membranes are composed of main adaptive systems that block NaCl from penetrating into the cells, such as the accumulation of inorganic salts and the water-soluble low-molecular-weight organic compounds. Inorganic salts in the form of (Na^+, K^+, Cl^-) are mainly involved in adjusting the osmotic potentials, and the organic salts in the form of small solutes or electrolytes are concerned with the balance of the intracellular salt levels [16]. These two mechanisms are mainly involved in the maintenance of the cell structure; therefore, the cells thrive in stress conditions such as high salt [17].

5. Importance of halophilic actinomycetes

Actinomycetes identified in the halophilic regions such as salt lakes, salterns, solar salts, and subsurface salt formation have to cope up with the osmotic stress were an infinite pool of novel chemical molecules with increasing importance for several biotechnological applications in different fields. Traditionally, halophiles have been used in the food and nutraceutical industries for the fermentation of soy and fish sauces and β-carotene production; also they have been recently used in many novel and unique molecules such as compatible solutes, biopolymers or carotenoids, enzymes, biodegradable plastics, biosurfactants, bioemulsifiers, and bacteriorhodopsins for molecular biotechnology applications [18, 19]. Among the valuable products, enzymes obtained from these organisms, mainly involved in the processing of food, act as a catalyst in the bioremediation process and other biosynthetic processes. In addition to the enzymes, highly sensitive chromoproteins derived from halophiles act as a biocomputing and light-sensitive neurological probe for the treatment of blindness [20]. Ectoine and hydroxyectoine obtained from the halophilic actinomycetes are commercially used as protective and stabilizing agents for mammalian cells [21]. Ectoine derived from

Halomonas boliviensis fermentation reduces the DNA lesions induced by visible and UVA-visible lights. Besides, halophiles attracted many researchers for the development of sustainable energy production to minimize the effects of global warming. Because halophiles have superior qualities such as high tolerance levels of the enzymes toward salts and temperatures, stability in the presence of organic solvents, secretion of therapeutic compounds, and antimicrobial properties, in many applications, studies of halophilic bacteria have developed rapidly and significant advances have been made recently [22].

6. Metabolites produced by halophilic actinomycetes

In the last two decades, halophilic actinomycetes have gained significant importance as a new, promising source for novel bioactive compounds that can be used for drug development. Halophilic actinomycetes may produce a variety of bioactive compounds, which have a wide range of biological activities, including antibacterial, antifungal, antiviral, and other bioactive compounds [23]. The vast majority of bioactive compounds that isolated from halophilic actinomycetes are derived from the genus *Streptomyces*, whose species are very widely distributed in nature and cover around 80% of the total produced antibiotics [24].

7. Antibacterial

An antibacterial is an agent that may either inhibit the bacterial growth or kill the bacteria. The urgent need to find novel antibacterial agents has increased during the last decade owing to the increase of emerging multidrug-resistant bacteria. The discovery of novel antibiotics that have a potent effect against resistant pathogenic bacteria is an important aspect of antibiotics research today. The diversity of natural products makes it one of the most important sources for novel structures, which have been noticed to possess useful biological activities. Several studies are oriented toward the isolation of new actinomycetes from different habitats in the context of the search for novel antibiotics from new sources representing one of these habitats [25].

Generally, the antibacterial activity of halophilic actinomycetes from marine environments is extensively studied. Nevertheless, the exploitation of halophilic actinomycetes as a source for the discovery of novel antibiotics is still at an early stage, despite that numerous novel antibacterial compounds were isolated during the last few years (Table 1). Arenimycin (Fig. 1a) is a novel antibacterial compound isolated from the extreme halophilic actinomycete *Salinispora arenicola.* This compound was classified as a new antibiotic based on the novel structure, which belongs to the benzo [α] naphthacene quinone class of antibiotics. Arenimycin has proven to show a potent antibacterial activity against a panel of drug-resistant human pathogens such as rifampin- and methicillin-resistant *Staphylococcus aureus*. Arenimycin is a representative of the first report of this class of antibiotics from marine actinomycetes [26]. Abyssomicin C (Fig. 1b) is another example for novel antibacterial compounds extracted

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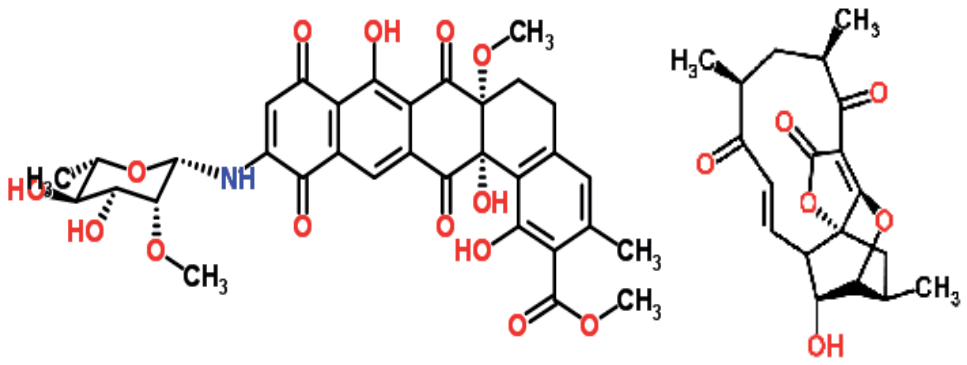
from the halophilic actinomycete *Verrucosispora* sp. [27]. Abyssomicin C was derived with other two novel structures that belong to the polycyclic polyketides abyssomicin B and abyssomicin D. Among these three compounds, abyssomicin C strongly exhibits antibacterial activity against Gram-positive bacteria, including multiresistant clinical isolates of *Staphylococcus aureus*. The mode of action of this new antibiotic is based on the inhibition of *para*-aminobenzoic acid biosynthesis resulting in the inhibition of the folic acid biosynthesis pathway.



a. Arenimycin b. Abyssomicin C

Figure 1. Chemical structure of some novel antibacterial compounds produced by halophilic actinomycetes [26, 27].

Compound	Source
1,4-Dihydroxy-2-(3-hydroxybutyl)-9,10-anthraquinone 9,10-anthrac	*Streptomyces* sp.
1-Hydroxy-1-norresistomycin	*Streptomyces chinaensis*
Abyssomicins	*Verrucosispora* sp.
Arenimycin	*Salinispora arenicola*
Bisanthraquinone	*Streptomyces* sp.
Bonactin	*Streptomyces* sp.
Caboxamycin	*Streptomyces* sp.
Chloro-dihydroquinones	*Streptomyces* sp.
Diazepinomicin	*Micromonospora* sp.
Essramycin	*Streptomyces* sp.
Frigocyclinone	*Streptomyces griseus*
Glaciapyrroles	*Streptomyces* sp.
Gutingimycin	*Streptomyces* sp.

Compound	Source
Helquinoline	*Janibacter limosus*
Himalomycins	*Streptomyces* sp.
Lajollamycin	*Streptomyces nodosus*
Lincomycin	*Streptomyces lincolnensis*
Lynamicins	*Marinispora* sp.
Marinomycins	*Marinispora* sp.
Marinopyrroles	*Streptomyces* sp.
Proximicins	*Verrucosispora* sp.
Resistoflavin methyl ether)	*Streptomyces* sp.
Tirandamycins	*Streptomyces* sp.
TP-1161	*Nocardiopsis* sp.

The listed compounds and the strains were obtained from the report of Subramani and Aalbersberg, (2012) [36].

Table 1. Novel antibacterial compounds produced by halophilic actinomycetes

8. Antifungal

The number of antifungal agents available for controlling fungal infections is still limited in comparison to antibacterial agents, and their use is still risky owing to toxicity and side effects [28]. Therefore, studies are still being conducted to isolate and identify novel antifungals that are potentially effective against pathogenic fungi [23]. Halophilic actinomycetes from marine environments are useful biological tools for the discovery of novel antifungal substances against fungi. Few studies reported the isolation of antifungal agents from halophilic actinomycetes. *Streptomyces* is the main source for antifungal agents (Table 2) which have been isolated from marine habitats.

Compound	Source
Azalomycin F4a 2-ethylpentyl ester	*Streptomyces* sp.
Bonactin	*Streptomyces* sp.
Chandrananimycin	*Actinomadura* sp.
Daryamides	*Streptomyces* sp.
N-(2-hydroxyphenyl)-2-phenazinamine (NHP)	*Nocardia dassonvillei*

The listed compounds and the strains were obtained from the report of Subramani and Aalbersberg, (2012) [36].

Table 2. Novel antifungal agents produced by halophilic actinomycetes

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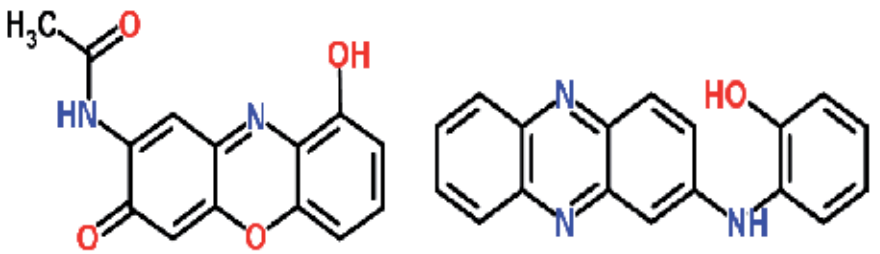
Chandrananimycin A (Fig. 2a) is a novel antifungal substance produced by marine *Actinomadura* sp. strain M048. Chandrananimycin A exhibited strong, effective antifungal activity against *Mucor miehei*. It exhibits other biological activities as an antibacterial, anticancer, and antialgal agent against the microalgae [29]. *N*-(2-hydroxyphenyl)-2-phenazinamine (NHP) (Fig. 2b) is a new antibiotic produced by halophilic actinomycetes isolated from the sediment sample *Nocardia dassonvillei* collected from the Arctic Ocean [30]. The new antibiotics possess potent antifungal activity against *Candida albicans*, with an MIC of 64 μg /ml.



a. Chandrananimycin A b. N-(2-hydroxyphenyl)-2-phenazinamine (NHP)

Figure 2. Chemical structure of novel antifungal isolated from halophilic actinomycetes [30].

9. Antiviral

Natural products represent the main source for discovering new/novel chemical structures that are used for the treatment of infections. The vast majority of secondary metabolites produced by microbes that have been developed for controlling microbial infections are directed against bacterial and fungal infections but not against viral infections. To date, antiviral agents have been isolated from natural products that are limited, and the studies in this field are few [31]. The antiviral agents available commercially in pharmacological markets are over 40 compounds, including those being tested as promising antiviral agents or alternative antiviral medicines [32]. Potential substances that possess antiviral activity have been isolated from halophilic microorganisms from marine environments. EPS-1 and EPS-2 are antiviral agents produced by *Bacillus licheniformis* and *Geobacillus thermodenitrificans*, respectively, targeting the replication of herpes simplex virus type 2 (HSV-2) [33]. Cyanovirin-N was obtained from the halophilic cyanobacteria *Nostoc ellipsosporum.* Cyanovirin-N has antiviral activity against HIV-1 and HIV-2 by targeting the replication [34]. However, halophilic actinomycetes produce several bioactive compounds, the compounds derived from halophilic actinomycetes as antiviral agents are limited. Antimycin A is an antiviral substance produced by *Streptomyces kaviengensis* isolated from marine sediments collected from the coast of New Ireland, Papua New Guinea. Antimycin A has potent antiviral activity against western equine encephalitis virus and a wide range of RNA viruses in cultured cells, includ-

ing members of the *Togaviridae*, *Flaviviridae*, *Bunyaviridae*, *Picornaviridae*, and *Paramyxoviridae* families. Benzastatin C (Fig. 3) is a 3-chloro-tetrahydroquinolone alkaloid extracted from the halophilic actinomycete *Streptomyces nitrosporeus* [35]. This compound exhibited antiviral activity against herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2), and vesicular stomatitis virus (VSV), respectively, in a dose-dependent manner with EC50 values of 1.92, 0.53, and 1.99 μg/mL.

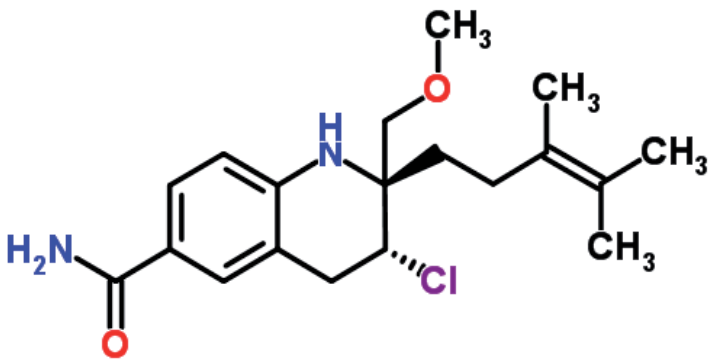


Figure 3. Chemical structure of benzastatin C produced by *Streptomyces nitrosporeus* [35].

10. Therapeutic compounds

Diseases constitute a significant threat in the life of humans; over 30,000 diseases have been described clinically. Nevertheless, only less than one third of these can be treated [36]. There is an urgent need to obtain new therapeutic agents to cover this medical requirement. Natural products are able to fulfill medical needs through the discovery of novel therapeutic compounds [37]. In this regard, investigations on halophilic actinomycetes during recent years have led to the development of numerous isolated therapeutic substances (Table 3), including anticancer, antitumor, anti-inflammatory, antioxidant, and antimalarial substances [38]. Salinosporamide A (Fig. 4a) is a novel anticancer substance isolated from the marine actinomycete *Salinispora tropica*. Its chemical structure is bicyclic beta-lactone gamma-lactam. The mode of action of salinosporamide A is the inhibition of proteasome, which leads to induced apoptosis in multiple myeloma cells [39]. Nereus Pharmaceuticals, Inc., has developed salinosporamide A under the name NPI-0052 for the treatment of cancer in humans, which represents the first clinical anticancer agent candidate for the treatment of cancer produced by halophilic actinomycetes [36]. Lodopyridone (Fig. 4b) is an anticancer agent obtained from the marine actinomycete *Saccharomonospora* sp. [40]. Lodopyridone exhibited anticancer activity against the human colon adenocarcinoma cell line HCT-116 with an IC_{50} of 3.6 μM. Cyclomarin A is a novel anti-inflammatory agent isolated from halophilic *Streptomyces* sp. It is a cyclic heptapeptide and possesses potent anti-inflammatory activity in both

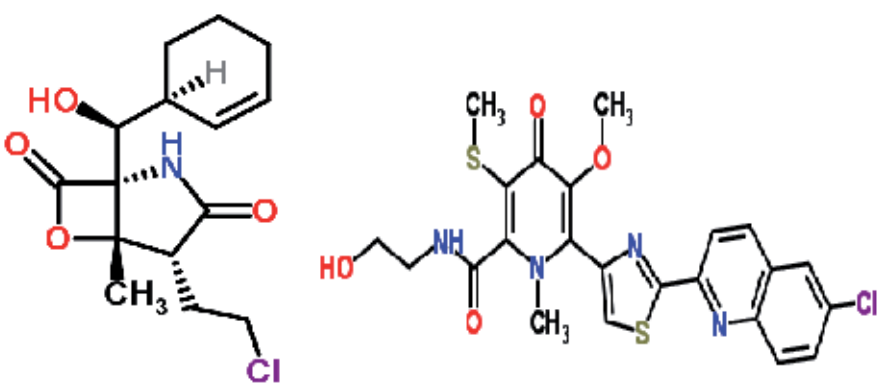
in vivo and in vitro assays. Trioxacarcin is a new antimalarial substance isolated from marine *Streptomyces* sp. [41]. Trioxacarcin has been tested for antimalarial activity. Results showed extremely high antiplasmodial activity against the pathogen of malaria in comparison to the artemisinin drug, the most effective drug against malaria. Actinosporins C and D are novel antioxidants produced by the sponge-associated actinomycete *Actinokineospora* sp. strain EG49 isolated from the marine sponge *Spheciospongia vagabunda* [42]. Actinosporins C and D showed (at 1.25 μM) a significant antioxidant and protective capacity from the genomic damage induced by hydrogen peroxide in the human promyelocytic (HL-60) cell line.

Compound	**Source**
Biological activity: anticancer	
1-Hydroxy-1-norresistomycin	*Streptomyces chinaensis*
3,6-Disubstituted indoles	*Streptomyces* sp.
Caprolactones	*Streptomyces* sp.
Chinikomycins	*Streptomyces* sp.
IB-00208	*Actinomadura* sp.
Lodopyridone	*Saccharomonospora* sp.
Marinomycins A-D	*Marinispora*
Mechercharmycins	*Thermoactinomyces* sp.
Salinosporamide A	*Salinispora tropica*
ZHD-0501	*Actinomadura* sp.
Biological activity: antitumor	
1,8-Dihydroxy-2-ethyl-3-methylanthraquinone)	*Streptomyces* sp.
Arenicolides	*Salinispora arenicola*
Aureolic acid	*Streptomyces* sp.
Aureoverticillactam	*Streptomyces aureoverticillatus*
Butenolides	*Streptoverticillium luteoverticillatum*
Chalcomycin	*Streptomyces* sp.
Daryamides	*Streptomyces* sp.
Elaiomycins B and C	*Streptomyces* sp.
Glyciapyrroles	*Streptomyces* sp.

Compound	Source
Mitomycin C	*Streptomyces lavendulae*
Piericidins	*Streptomyces* sp.
Staurosporinone	*Streptomyces* sp.
Streptokordin	*Streptomyces* sp.
Biological activity: anti-inflammatory	
Cyclomarins	*Streptomyces* sp. *Salinispora* *Arenicola*
Salinamides A and B	*Streptomyces* sp.
Biological activity: antioxidant	
Dermacozines A-G	*Dermacoccus*
Actinosporins C-D	*Actinokineospora* sp.
Biological activity: antimalarial	
Trioxacarcin A, B, and C	*Streptomyces ochraceus* *Streptomyces bottropensis*

The listed compounds and the strains were obtained from the report of Subramani and Aalbersberg, (2012) [36].

Table 3. Novel therapeutic substances isolated form halophilic actinomycetes



a. Salinosporamide A b. Lodopyridone

Figure 4. Chemical structure of some therapeutic compounds produced by halophilic actinomycetes [5].

11. Biodegradable chemicals

Saline environments, particularly waters and soils, are always exposed to contamination by heavy metals or other toxic chemical substances due to anthropogenic activities [43]. The degradation of different substances in the environment is a continuous process due to the continued activities of microorganisms. Microorganisms degrade toxic compounds to non-toxic materials such as H_2O, CO_2, or other inorganic compounds [44]. Halophiles play a significant role in the biodegradation of the materials that contaminate marine environments. Actinomycetes have been reported to be one of those microorganisms that contribute in the degradation of organic compounds in the nature and play a role in the mineralization of organic matter [36]. Petroleum substances constitute one of the materials that contaminate marine habitats [45]. In a study carried out on oil-utilizing halophilic bacteria, results of phylogenetic studies showed that 30% of all isolates belonged to actinomycetes [46]. Extremely halophilic actinomycetes, *Streptomyces albiaxialis*, were reported to be able to grow in extreme environment by using crude oil as the sole carbon source in a salinity level up to 10% NaCl. Halophilic actinomycetes isolated from the oil-polluted soil in Russia were identified as *Rhodococcus erythropolis*. It was grown in a medium containing crude oil as a unique source of carbon. Results showed degradation of n-alkanes and iso-alkanes with chain lengths of C_{11}–C_{30} and C_{14}–C_{18} [47]. A new extremely halophilic actinomycete strain that belongs to *Actinopolyspora* sp. was isolated from saline and arid surroundings of an oil field in the Sultanate of Oman. This strain exhibited the capability of degrading alkanes up to C_{15} and, at a slower rate, up to C_{25} [48].

12. Industrially important enzymes

The extracellular enzymes produced by halophilic actinomycetes have significant advantages in biotechnological applications, such as biosynthetic processes, environmental bioremediation, and food processing. Most of the enzymes are active and stable at high temperatures and pH values and have ionic strength in the presence of organic solvents [49]. They have the capability of secreting extracellular enzymes such as lipase, DNase, protease, esterase, pullulanase, galactosidase, nuclease, xylanase, inulinase, cellulose, pectinase, gelatinase, alpha-glucosidase, beta-glucosidase, alpha-mannosidase, beta-mannosidase, chitinases, xylose isomerases, fructokinases, ribokinases, etc., which exhibit higher activity in alkaline pH and stability in high concentrations of organic solvents in their environment which have been reported in the last few years [50–52]. Among the industrially important enzymes, alkaline proteases and cellulase are produced by a number of microorganisms, but limited research was done on bulk production of alkaline proteases and cellulases by alkaliphilic actinomycetes. Cellulases and proteases are the most wanted extracellular enzymes and constitute 60% of global enzymes sales [53]. Reports claimed that a certain number of alkaliphilic and halophilic actinomycetes produce extracellular protease enzymes [54]. The halophilic protease secreted by the *Nocardiopsis prasina* HA-4 withstands a wide range of pH (7–10) and temperature (20–42°C). *Nocardiopsis halotolerans* and *Saccharomonospora halophila*

recovered from Kuwait showed better keratinolytic activity under high salt concentration [55]. *Streptomyces psammoticus* that secreted lignin degrading enzymes under alkaline conditions confirmed that the enzymes have applications in delignification of pulp, textile dye decolorization, and effluent treatment [53]. Cellulases are mainly involved in the production of second-generation bioethanol and textile processing industry [56]. Halophilic cellulases obtained from actinomycetes and from metagenomic library of some marine bacteria were reported to be halostable, thermostable, and alkalostable, all favorable for textile and laundry industries [57].

13. Advantage of using halophilic actinomycetes in the industry

Halophilic actinomycetes were suitable candidates for the expression of soluble recombinant proteins [58]. A broad range of plasmid vectors obtained from halophilic actinomycetes are used for cloning native promoters and heterologous promoters for the stable expression of gene for the production of the industrially important, compatible solute ectoine [59]. Since halophilic actinomycetes have antimicrobial activity and other extracellular enzymes production capability, there is less chance of contamination during fermentation. Therefore, the major cost for the sterilization of the cultivation media is reduced.

14. Conclusions and future prospects

Several halophilic actinomycetes have special biological and chemical defense systems such as pH tolerance, and stress-tolerant metals present one of the main sources for the discovery of novel metabolites, biosurfactants, several other chemicals, and commercially important enzymes with various applications. Despite the huge demand of synthetic molecules with effective antimicrobial properties, novel methods and technologies for discovering novel natural products from microbial sources from halophilic regions should be studied. The antimicrobial metabolites producing novel actinomycetes are good bugs for unsterile cultivation and continuous bioprocessing using seawater and low-cost-contributing media components. It will be interesting to identify the mechanism of the stable properties of halophilic enzymes, which may lead to significant novel biotechnological applications. Additionally, the stability of recombinant vectors in harsh conditions such as high salt, multiple metal ions, or organic solvents should be investigated. In conclusion, halophilic actinomycetes will be a useful host for the production of commodity chemicals, antibiotics, enzymes, and biofuels in bulk with low cost.

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Section 3

Agricultural and Industrial Utility

Chapter 10

Actinobacteria as Plant Growth-Promoting Rhizobacteria

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Additional information is available at the end of the chapter

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Abstract

Actinobacteria commonly inhabit the rhizosphere, being an essential part of this environment due to their interactions with plants. Such interactions have made possible to characterize them as plant growth-promoting rhizobacteria (PGPR). As PGPR, they possess direct or indirect mechanisms that favor plant growth. Actinobacteria improve the availability of nutrients and minerals, synthesized plant growth regulators, and specially, they are capable of inhibiting phytopathogens. Different activities that are performed by actinobacteria have been studied, such as phosphate solubilization, siderophores production, and nitrogen fixation. Furthermore, actinobacteria do not contaminate the environment; instead, they help to maintain the biotic equilibrium of soil by cooperating with nutrients cycling. The aforementioned is directly related to the quality and productivity of crops. Moreover, different aspects of these microorganisms have been studied, such as production of metabolites that improve plant growth, resilience against unfavorable environmental conditions, and beneficial and synergic interactions with arbuscular mycorrhizal fungi. Taking into account the above-mentioned activities, actinobacteria can be considered as possible plant fertilizers.

Keywords: Actinobacteria, PGPR, nutrients, solubilization, growth

1. Introduction

Actinobacteria are one of the major components of microbial populations present in soil. They belong to an extensive and diverse group of Gram-positive, aerobic, mycelial bacteria that play an important ecological role in soil nutrient cycling [1-4]. These bacteria are known for their economic importance as producers of biologically active substances, such as antibiotics,

vitamins, and enzymes [5]. Actinobacteria are also an important source of diverse antimicrobial metabolites [6-8].

Historically, the most commonly described actinobacterial genus has been *Streptomyces* and *Micromonospora*. In fact, the genus *Streptomyces* is known as one of the largest sources of bioactive natural products [7-9]. Particularly, it has been estimated that approximately two-thirds of natural antibiotics have been isolated from actinobacteria, and about 75% of them are produced by members of the *Streptomyces* genus [10, 11].

In the past decade, research has focused on minor groups of actinobacteria, including species that are difficult to isolate and cultivate, and those that grow under extreme conditions, i.e., alkaline and acidic conditions [6, 12]. However, the vast majority of soil actinobacteria show their optimum growth in neutral and slightly alkaline conditions; thus, the methods of isolation have been traditionally based on this neutrophilic character. Actinobacteria have attracted special interest because these filamentous sporulating bacteria are able to thrive in extremely different soil conditions and they play an ecological role of importance in nutrient cycling. Moreover, they were recently considered as plant growth-promoting rhizobacteria [13-15].

Plant growth-promoting rhizobacteria (PGPR) are free-living beneficial bacteria of agricultural importance. The presence of PGPR produces beneficial effects on plant health and growth by suppressing disease-causing microbes and accelerating nutrient availability and assimilation. Hence, in the quest to improve soil fertility and crop yield and reducing the negative impact of chemical fertilizers on the environment, there is a need to exploit PGPR for beneficial agricultural uses. In that regard, we propose to characterize actinobacteria as PGPR.

2. Mechanisms involved in the PGPR activity

2.1. Production of plant growth regulators

Plant organ formation and their subsequent development are mediated by internal factors of vital importance. Growth regulators in plants (PGR “Plant Growth Regulators”) are known as plants hormones. PGR are small molecules that affect plant growth and development at very low concentrations [16].

One of the parameters used to determine the effectiveness of certain rhizosphere bacteria is the ability to promote the development of characteristic root system of this type of plant growth regulators [17, 18]. The rapid development of roots, either by primary root elongation or secondary lateral root emergence, allows young seeds to have easy access to nutrients and water from their environment [19].

Different mechanisms are involved such as production of siderophores and indole acetic acid, nutrient solubilization, antagonistic or beneficial synergistic effects. These mechanisms have been studied in our collection of actinobacteria and they will be further explained as follows.

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2.2. Siderophores production

Microorganisms have been forced by environmental restrictions and biologic imperatives to produce specific molecules that can compete efficiently with hydroxyl ions for ferric state of iron. Siderophores are compounds produced by various microorganisms in soil. These organisms rely on chelation phenomena to support their biological activity. Siderophores are extracellular fluorescent pigments that possess affinity to iron (III), they are water-soluble and have low molecular weight (500-1,000 Da) [20]. Furthermore, siderophores are produced by a great variety of microorganisms that grow in scarce iron conditions [21, 22]. These compounds act as specific chelate agents of ferric ion, leaving available the ionic form (Fe+2), which is easily absorbed by microorganisms [23].

Chelation is a usual phenomenon of the biologic systems. This refers to formation of chelates that can be described as a polydentate ligand in coordination with a central ion by two or more atoms [24]. When siderophores form a complex with Fe+3, these are recognized by cell membrane receptors [25, 26]. This facilitates the inclusion of the formed complex to cell. Once in the cell, Fe^{+3} ions are reduced to a Fe^{+2} becoming available to be used in different biological processes. Finally, the siderophore [27] is released again. Siderophores are classified into three groups based on their chemical nature of the bounds created with metals. They are known as catecholates, hydroxamates, and hydroxide-carboxylates [24]. Actinobacteria and Enterobacteria are among the microorganisms able to produce siderophores. Actinobacteria is one the most important groups in terms of siderophores production.

The vast majority of nitrogen-fixing microorganisms produce siderophores so as to obtain iron. This is necessary to perform the enzyme nitrogenase. The enzyme is composed of several protein units; a total of 36 iron atoms are required for operating properly [28]. According to determinations made by our research group, the highest levels of siderophores production were obtained by *Streptomyces* MCR24 that was maintained over the time; conversely, the lowest recorded levels were observed in *Streptomyces* MCR30. Despite the fact that in its great majority, the analyzed siderophores produced by actinobacteria contained high concentrations of hydroxamates, it can be assumed that some strains show a possible advantage as PGPR mechanism. The strains tested for production of siderophores hydroxamate were capable of growing in culture media without addition of any source of nitrogen. These results are similar to those found by Carson *et al.* [29] and indicate that the selected strains had such capacity and in one-way or another (high or low) produced siderophores.

Studies performed by Díaz [30] evidenced a higher performance of siderophores production when a monosaccharide like glucose is used as a source of carbon. Studies with actinobacteria have shown that the metabolic rate of this group of bacteria is higher when there is an excess of carbon. This favored the production of some organic compounds related to the physiology of the species [31-34]. In the presence of an easily assimilable source of carbon, some species of *Streptomyces* are capable of synthesizing organic acids. Those acids return to metabolic routes to produce energy and result in various secondary metabolites that in such case can be considered as siderophores [34-36].

Authors like Diaz [30] proved that strains of actinobacteria such as *Streptomyces* MCR3 and *Thermobifida* showed a great synthesis of siderophores of the hydroxamate-type using glucose as the only source of carbon, in contrast to what occurred when sodium succinate was utilized to the same purposed. Concerning *Streptomyces* sp., the production of hydroxamate-Desferrioxamine [37, 38] B by this microorganism is well documented. On the other hand, *Thermobifida* has been recently reported as the producer of a siderophore known as fuscachelin A [39].

2.3. Indole acetic acid production

Indole acetic acid (IAA) is a plant growth regulator and active form of auxins. It plays an important role in plant development through its life cycle [40, 41]. IAA stimulates the growth of the radicular system [28, 42-44], thanks to the development of lateral roots and divisions of the apical meristem that derives in roots elongation [43, 44]. This increases the access of soil nutrients to the plant [40, 46]. IAA has proved to be the main one responsible for plant growth promotion over the nitrogen fixation related to diazotrophic bacteria activity [45].

The production of IAA has been widely studied in actinobacteria [47-53]. Authors like El-Shanshoury [47] suggest that IAA can act as endogen regulator of spore germination of *Streptomyces atroolivacezlz* and can be involved in the differentiation of actinobacteria [46].

Streptomyces genus [47-53], *Frankia* sp. [47, 51, 54], *Nocardia* sp. [47, 54], *Kitasatospora* sp. [52] have been widely studied as IAA producers. Studies performed by our research group confirmed the ability of genus *Streptomyces* sp. and *Thermobifida* sp. to produce IAA. Duque and Quintana [50] affirmed that MCR14 (*Streptomyces sannanensis*) was the most important microorganism for IAA production.

2.4. Non-symbiotic nitrogen fixation

The actinobacteria are heterotrophic organisms that require carbon sources to obtain the energy necessary for nitrogen fixation. Therefore, each of the different bacteria differs in the carbon metabolism and the intrinsic ability of nitrogen fixation, showing different rates of acetylene reduction assay (ARA). This test is based on detecting indirectly the presence nitrogenase enzyme, which is in charge of reducing nitrogen (N_2) to ammonium. This essay evaluates the enzymatic reduction of acetylene to ethylene (NH_4^+) [55]. Likewise, ARA indirectly measures the microorganism capacity to fixate nitrogen, given that nitrogenase is an unspecific enzyme that catalyzes the reduction of steric analogs of N_2 [56].

Actinobacteria log phase was evaluated in free-nitrogen media. From those experiments, it was established that the best period for ARA measurement was found after 10 days of culturing. Strains MCR14, MCR27, and MCR31 were selected from the 10 evaluated strains as they turn the pH indicator in Nfb culture media. This fact did not occur for the rest of the evaluated strains. High rates of nitrogen fixation were expected in the vials where color change was observed (Figure 1) and then positively confirmed by ARA. De los Santos et al. [57] described a similar behavior when a semi-solid media inoculated with the bacteria *Burkholderia* did not display changes of pH in all vials; however, some of them showed a slight increase of pH due to microbial growth. In those vials, it was possible to observe a blue-green color.

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This confirms the need of carrying out ARA test in order to guarantee diazotrophic characteristics of these microorganisms. In Nfb semi-solid media, actinobacteria grew as a thin white film placed few millimeters below the agar surface. Bacteria were able to move through the media, thanks to Nfb consistency and found the optimum place to balance the respiration rate with diffusion oxygen rate [58].

Certain evidence indicates that diazotrophs organisms are capable of nitrogen biologic fixation in considerable wide ranges of pH. Despite the fact that Nfb has a neutral pH, hydrolysis of carbon derived from the metabolism will cause products that may change the pH. These pH values interfere with nitrogen fixation, making it difficult to alkalinize the culture media, and therefore, changing the color to blue. This behavior was evidenced in most of the isolated actinobacteria (Figure 1).

Figure 1. Actinobacteria evaluated in NFB modified media

After ARA evaluation, it was observed that isolated MCR24, MCR26, and MCR31 recorded the highest rates of ethylene production at the three incubation times. However, no statistically significant differences were noted. The highest nitrogenase activities were observed in MCR31 cultures. It is highly important to detect nitrogenase activity among our strains of free-living diazotroph actinobacteria by using ARA. The high microbial diversity present in soils of high Andean forest of Colombia (Cundinamarca and Boyacá states) derives in the ability of nitrogen fixation obtained by the isolates.

Frankia sp. have an outstanding feature related to vesicles specialized in nitrogen fixation. These vesicles are in charge of protecting the nitrogenase complex. These actinobacteria have been extensively reported by several authors [59-61], due to its fixation characteristics that have been confirmed by acetylene reduction method [62-64]. The morphology of the bacteria cultured in our laboratory facilities using Nfb media did not display any similarity with *Frankia* sp, since different microscopic descriptions such as long and short filaments, tortuous or spiral-

shaped filaments were found. The above mentioned can be an evidence of the presence of new species that have the ability to grow in nitrogen-free conditions.

Gen *nifH* detection was performed. This gene codifies for nitrogenase enzyme and is the molecular marker most widely used for detection of nitrogen-fixing bacteria and phylogenetic studies [65]. We decided to use the primers purposed by Diallo et al. [66]. These primers are very similar to those used by Valdés et al. [64] in non-Frankia actinobacteria for amplifying the *nifH* gen. Based on this protocol was possible to amplify the gen in all the isolated bacteria. Figure 2 displays a band that corresponds to a gen fragment with an expected size of 320 bp. These amplified genes were purified by means of QiaGEN Minelute PCR Purification Kit and then sequenced. The size of the observed bands is similar to that reported by Valdés et al. [64] for *nifH* gene. Furthermore, background can be observed. Studies performed by Soares et al. [67] affirmed that the "background" or "smearing" could be related with the use of degenerate primers such as PolR and PolF during nested PCR.

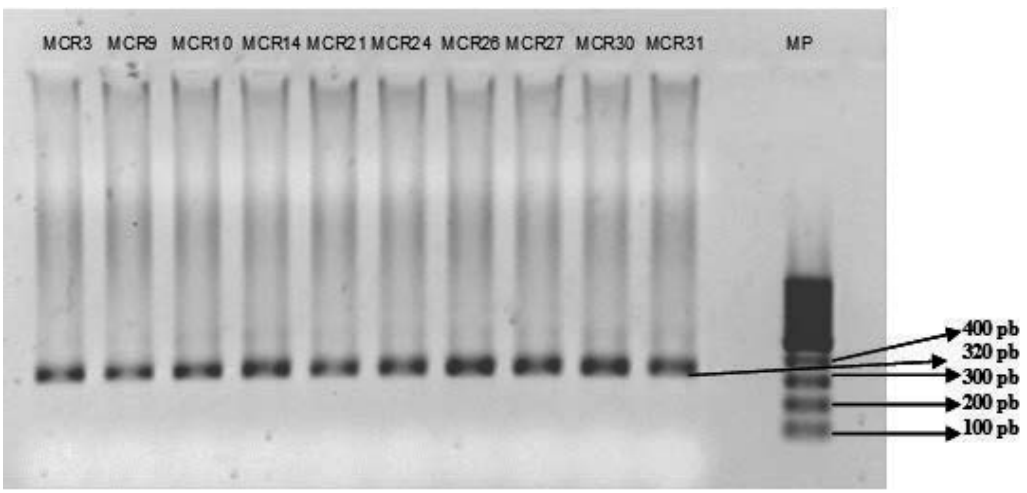

Figure 2. PCR amplification of an intern fragment of gen *nifH* of 320 bp, in 10 of the isolated diazotrophic actinobacteria MP (100 bp Invitrogen).

It is important to emphasize that the research performed by Valdés et al. [64] corresponds to one of the first studies that have recorded the use of degenerate primers to detect the presence of *nifH* gene in free-living actinobacteria different from *Frankia* and isolated from *Casuarina equisetifolia*. As *nifH* gene can act as a molecular marker, there are other genes that belong to nitrogenase complex that are capable of indicating such activity in nitrogen-fixing microorganisms. Studies performed by Fedorov et al. [68] on a new primer system for detecting and amplifying gene *nifDK* portion have pointed out the possible use of such gene as a molecular marker. The progress in the development of new primer systems that target different sites in *nif* operon can be efficiently used for searching nitrogen-fixing actinobacteria in which no nitrogenase activity has been detected before. However, the presence of *nifH* gene by itself does not indicate active nitrogen fixation since nitrogenase activity is regulated at pre- and

post-transcriptional level [69] and its activity depends on environmental conditions. Probably, the most suitable indicator of nitrogenase activity complex is ARNm of *nifH* [70].

BLAST search was carried out using GenBank in order to find the gene *nifH* sequence closely related to that found in our study. It was noted that they were similar to the sequence assigned to *Frankia* sp. According to the access produced by GenBank, some of the strains (MCR 3 and MCR24) showed similarity score between 96 and 98%. The other strains recorded *nifH* gene alignments similar to genes found in nitrogen-fixing bacteria such as *Rhizobium* and *Bradyrhizobium*, and others. This suggests that PCR fragments probably belong to the *nifH* gene. Among the noted sequences, the majority of the strains did not record any similarities with diazotrophic bacteria; this was expected to take into account that *Frankia* sp. represent the only nitrogen-fixing bacteria by means of actinorrhizal symbiosis. *Streptomyces thermoautotrophicus* UBT1 is capable of fixing atmospheric nitrogen and grows in media free of nitrogen; however, it is not capable of acetylene reduction. This type of nitrogen reduction is unusual and it is believed to be coupled to carbon monoxide and dependent of oxygen [71]. These types of microorganisms are not associated to plants and they do not have other characteristics of agronomic interest. In contrast, the atmospheric nitrogen fixed by the actinobacteria studied in our laboratory can influence plant growth. Nitrogenase activity was confirmed after analyzing the ability to reduce acetylene to ethylene and the presence of *nifH* genes by PCR amplification [64, 72].

2.4.1. Phosphorus solubilization

Phosphorus (P) is one of the major essential macronutrients for plants, which is applied to the soil in the form of phosphatic manure. However, a large portion of the applied phosphorus is rapidly immobilized, becoming unavailable for plants [73]. The free phosphatic ion in soil plays a crucial role. The orthophosphatic ion form is the only ion that can be assimilated by plants in considerable amounts [74]. Soil microorganisms are involved in a wide range of biological processes, including phosphorus transformation of soil. They solubilize soil phosphorus for plants growth [75].

Although the mechanisms used by actinobacteria to solubilize phosphorus are not fully understood, it is known that in the solubilization process, inorganic phosphorus and some organic acids (oxalic and citric, principally) are involved [76-78]; our group performed solubilization quantifications and determined organic acids associated with solubilizing of P [79]. Similarly, Hamdali et al. in 2010 and 2012 [80, 81] have reported that compounds different from organic acids, more specifically metabolites of the viridomicenas and siderophores families, are capable of solubilizing P from various sources, which lead us to explore more about the process of solubilization of inorganic phosphorus generated by this group of organisms.

2.4.2. Organic phosphate solubilization

The organic P is composed of various fractions, compromising the most labile and most resistant to mineralization. However, the main organic component of phosphorus cycle is the

microbial biomass [82]. Organic phosphate is mineralized by the phosphatase enzyme, which is excreted by some microorganisms, and is released [83, 84].

Studies performed by our research group evidenced that *Streptomyces* MCR26 has the capacity of secreting acid phosphatases, and therefore, mineralizing sources of organic phosphorous [15]. Additionally, actinobacteria not previously reported as phosphate-mineralizing microorganisms were identified and were related to genus *Saccharopolyspora, Thermobifida* and *Thermonospora*. Actinobacteria from the genus *Micromonospora* sp., *Nocardia* sp., *Actinomadura* sp., *Rhodococcus* sp., *Actinoplanes* sp., *Microbispora* sp. and *Streptosporangium* sp. produce phosphatase enzymes which have been classified according to their alkaline or acid activity, depending on reaction conditions [85].

2.4.3. Inorganic phosphate solubilization

The growth of phosphate-solubilizing bacteria (PSB) growth often causes soil acidification, playing a key role in phosphorus solubilization [86]. Therefore, PSB are considered important solubilizers of insoluble inorganic phosphate. In turn, plants reimburse PSB with carbohydrates [87].

Prada et al. [79] isolated 57 strains of actinobacteria from different sampling areas. Soil characterization showed the following: pH ranges from 4.0 to 5.9, total P from 360 to 2830 mg/kg, available P from 8.7 to 118.4 mg/kg, and organic matter from 2.95 to 13.52%. The results of two qualitative assessments are not totally consistent. Seven of the tested strains F1A, F1B, F1C, F4C, T1A, T1D, and T3A were the best solubilizing strains, in both solid and liquid evaluation media. We performed a quantitative assessment in order to find the strain that has the highest solubilizing capacity and to evaluate which of the two methods is more reliable. The strains T1C, T1H, T3A, T3C, P3E, F1A, F2A, and V2B are as good as *Streptomyces* sp. MCR24 for Ca3 (PO4)2 and these strains solubilized significantly more phosphorus than the other strains. Strains T1H, T1C, T3A, T3C, and F1A are only present in the selection obtained with the methodology reported by Mehta and Nautiyal [88], suggesting that this test can select more strains with true solubilizing ability, and thus it is more reliable.

Perez et al. [89] claimed that isolates that cause a shift of > 1.5 units could be selected for further studies. In order to confirm the usefulness of this cut-off point proposed by Perez et al. [89] and therefore, to select the best strains, we implemented a quantitative assay by measuring the release of soluble phosphorus using the NBRIP broth [90-91]. Figure 3 shows strains T1C, T1H, T3C, P3E, and V2B. They have significantly higher activity than other isolates. However, this result was not observed in the plate assay, probably because one or more acids involved in the process did not diffuse into the agar and, therefore, there was no presence of a solubilization halo. On the other hand, the evaluation in NBRIP-BPB broth revealed that isolates capable of decolorizing the broth more than 1.5 units were also more efficient in the quantitative assay. Additionally, Mehta and Nautiyal [88] assay contribute to reduce costs and efforts in microorganisms with bio-fertilizing potential screening. Studies focusing on actinobacteria physiology in Colombia are scarce, especially those focused on agriculture [15, 90-92].

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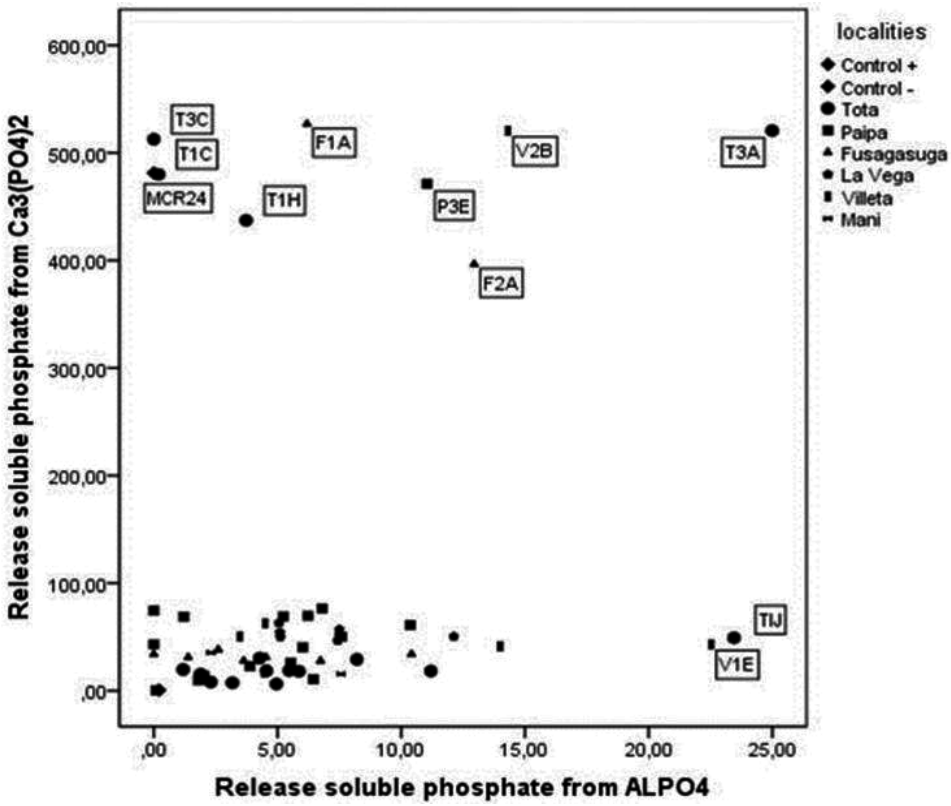

Figure 3. Released soluble phosphate activity with two sources of P. $Ca_3(PO_4)^2$ 5g L^{-1} is displayed in Y axis and activity with $AlPO_4$ 1g L^{-1} source is displayed in X axis.

2.5. Chitinases production

The chitin is a homopolymer comprised of N-acetyl-D-Glucosamine residues with α-1, 4 bonds. It is widely spread in nature as a structural component of fungi (22-44%), insects and crustaceans (25-58%), and protozoa [93-96]. The chitin is hydrolyzed by a complex chitinase that comprises three enzymes such as exochitinase, endochitinase and N-acetyl-D-Glucosamine.

Actinobacteria are considered as the dominant organisms involved in the decomposition of chitin in soil [1] and also promising antagonist agents for biocontrol due to the hydrolysis reaction over the fungi mycelium [97]. The species that belong to *Streptomyces* genus are considered as the principal chitinolytic microbial group in soil, due to its capacity to degrade this polymer [98].

Within a group of 30-isolated actinobacteria, 20 were able to hydrolyze chitin after 3 days of growth on mineral agar supplemented with chitin, as the sole source of carbon. The clearing

zones were observed around bacteria following 9 days of growth, suggesting the presence of chitinolytic activity with secreted proteins into the culture medium [99].

2.6. Antagonistic activity against phytopathogenic fungi

Antagonism is defined as a mechanism of action based primarily on the direct inhibitory activity between two microorganisms [100] that have opposite actions within the same system. In order to evaluate the chitinolytic activity of seven strains of actinobacteria against *Fusarium oxysporum*, *Phytophthora infestans*, *Rhizoctonia solani* and *Verticillium dahlie*, a set of experiments were performed. Additionally, its ability as plant growth regulator was also considered.

It was observed that the strains had a high, medium, or low inhibition on tests of antagonism against phytopathogenic fungi, but *F. oxysporum* was the most resistant fungal strain. Diverse actinobacteria may act as antagonistic microorganisms of *F. oxysporum* by producing antibiotics (antibiosis). These compounds diffuse through the medium inhibiting the growth of phytopathogenic fungus. Molano et al. [101] determined *in vitro* inhibition of *Fusarium oxysporum* growth by actinomycin, an antibiotic produced by *Nocardia* sp., strain isolated from rhizosphere soil sample lichen (Mosquera, Colombia). Production of such secondary metabolites was toxic to the phytopathogenic fungus.

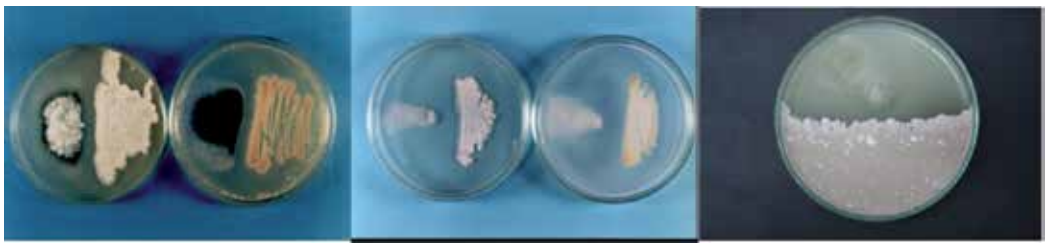

Figure 4. Dual culture confrontation method. (a) MCR26 vs *Verticillium dahliae*. (b) MCR10 vs *Phytophthora infestans*. (c) MCR14 vs *Rhizoctonia solani*

Strains MCR26, MCR10, and MCR24 proved to be the best as fungal antagonists (Figure 4). Based on these results, it can be inferred that mycelial growth inhibition is not caused by chitinase production, but rather by antifungal products. No inhibition of mycelial growth was observed by strains with chitinolytic activity. Using these results, we moved to the interaction phase with the mycorrhizal fungi, considering that chitinolytic enzymes did not directly affect fungi.

Actinobacteria that belong to *Streptomyces* genus have been commercially used to control plant damages. This genus have demonstrated antagonistic activity against *Alternaria* sp., *Pythium aphanidermatum, Colletotrichum higginsianum, Acremonium lactucum*, and *Fusarium oxysporum* [102,103]

Experiments performed at Unidad de Investigaciones Agropecuarias (UNIDIA) have proved the ability of *Streptomyces cuspidosporus* to inhibit the phytopathogen fungus *Fusarium oxysporum* following 8 days of incubation [50]. Additionally, we evidenced antibacterial activity

present in actinobacteria. Complete inhibition was observed when *Streptomyces* MCR26 was tested against *Bacillus cereus* and *Escherichia coli*, conversely, *Thermobifida* MCR24 strain which was completely inhibited by *Bacillus cereus* [104].

The antagonistic potential of the compounds produced by these strains was previously reported by our research group (UNIDIA). We evaluated the antagonistic activity in vitro of no mycorrhizal fungi generally found in soil. It was found that *Streptomyces* MCR26 and *Thermobifida* MCR24 partially inhibited *Rhizoctonia solani* and *Phytophthora infestans* growth [15].

2.7. Mycorrhiza (MA) helper bacteria

In general, the ability of certain microorganisms to influence the formation and functioning of the symbiosis MA through various kinds of activities, such as activation in fungal propagules infective pre-symbiotic stages [93,105], facilitate formation of inputs point into the root [106-108] and they increase the growth rate [109-111].

In our studies, it can be seen that the two strains of *Streptomyces* (MCR9 and MCR26) cause a stimulation of spores germination of the fungus MA, while *Thermobifida* MCR24 reduces significantly the germination of spores. It was also observed that isolated from *Streptomyces* and *Thermobifida* improved the growth of the mycelium of *Glomus* FC8 sp. Actinobacteria behavior evaluated in this study confirmed the results obtained by *Streptomyces globisporus* 1-K-4 [112], which showed that the concentration of the bacteria increases in rhizoplane seedling rice almost immediately after the inoculation.

Following the methodology described by Azcón-Aguilar et al. [113] and Barea et al. [114] we determine in this study, with, whether or not germination of *Glomus* sp spores were inhibited by the three isolated actinobacteria. Each petri dish was individually inoculated with actinobacteria (MCR9, MCR24, or MCR26) and the spores. Spores and the correspondent actinobacteria were placed on the apex of a hypothetical hexagon keeping a distance of approximately 3.5 cm between each other. After being inoculated, the germination of the spores was observed after 32 days of incubation at 25°C in dark conditions. Percentage of germination was calculated in each treatment. It was recorded that the two strains of *Streptomyces* (MCR9 and MCR26) improved germination of the spores of fungus MA. In contrast, *Thermobifida* MCR24 notably decreases spores germination. Furthermore, *Streptomyces* and *Thermobifida* improved mycelium development of *Glomus* FC8 sp.

Carpenter-Boggs et al. [111] found that actinobacteria such as *Streptomyces orientalis* have a beneficial effect on spores of *Gigaspora margarita.* They also observed that the amount of volatile compounds produced by the isolated ones have a good correlation with the germination of MA spores. Such research can explain why the actinobacteria that belong to our collection improve spore germination of *Glomus* FC8 sp. Moreover, Mousse [109] and Azcón-Aguilar & Barea [115] described that some mycorrhizosphere bacteria were capable of promoting the MA settle. This improves germination of spores.

Through confocal microscopy was observed that chitinolytic strains and strains that showed antagonistic capability against non-mycorrhiza fungi with chitin wall did not cause degradation of the mycelium wall of *Glomus* FC8 sp. or to the commercial witness. These results are

consistent with other studies that have observed bacteria inside of MA and colonizing fungal hyphae [116-118]. Different studies have proved that microbial antagonists of fungal pathogens do not cause any inhibitory effect against MA [114,119-121].

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Chapter 11

Frankia as a Biodegrading Agent

Medhat Rehan, Erik Swanson and Louis S. Tisa

Additional information is available at the end of the chapter

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Abstract

The *Frankia* actinorhizal plant symbiosis plays an important role in colonization of soils contaminated with toxic aromatic hydrocarbons. Our understanding of the bacterial partner, *Frankia*, in the actinorhizal symbiosis has been greatly facilitated by the availability of sequenced genomes. The analysis of these *Frankia* genomes has suggested that these bacteria are metabolically diverse and have potential for toxic aromatic hydrocarbon degradation. In this chapter, we explore what is known about that metabolic potential.

Keywords: *Frankias*-triazines, aromatic hydrocarbon degradation, PAH, bioremediation, bioinformatics, actinobacteria

1. Introduction

Frankia are filamentous nitrogen-fixing Gram-positive actinobacteria that are found as free-living microbes in the soil and in symbiotic associations with actinorhizal plants [1-5]. These bacteria fix nitrogen by converting atmospheric N_2 into biologically useful ammonia and supply the host plants with a source of reduced nitrogen. *Frankia* are developmentally complex and form three cell types: vegetative hyphae, spores located in sporangia, and vesicles. Hyphae are septate structures and form the growing state of this microbe. Under appropriate conditions, either terminal or intercalary multilocular sporangia are produced and contain many spores. When mature, the spores are released from the sporangia. The spores are presumed to aid in the survival and dispersal of *Frankia* in the environment. Vesicles are produced under nitrogen-limited conditions and consist of unique lipid-enveloped cellular structures that contain the enzymes responsible for nitrogen fixation. Thus, vesicles act as specialized structures for the nitrogen fixation process. *Frankia* are able to establish symbiotic nitrogen-

fixing associations with over 220 species of woody dicotyledonous plants, termed actinorhizal plants, that are found in eight families of angiosperms [1, 3-6]. The symbiosis with *Frankia* allows these actinorhizal host plants to colonize nutrient-poor soil and harsh environments. Actinorhizal plants have been used to recolonize and reclaim industrial wastelands and environments contaminated with heavy metals and toxic aromatic hydrocarbon [7-15]. The metabolic potential of these bacteria has only recently been investigated in the context of bioremediation [16-18].

1.1. *Frankia* genomics and identification of metabolic potential

Based on phylogenetic analysis, *Frankia* strains have been classified into four main lineages [19-23]. Members of lineage 1 are found infective on host plants of the Betulaceae *(Alnus*), Myricaceae, and Casuariaraceae families, while lineage 2 represents strains that are infective on Rosaceae (*Dryas*, etc.), Coriariaceae *(Coriaria)*, Datiscaceae (*Datisca),* and the genus *Ceanothus* (Rhamnaceae). Members of lineage 3 are the most promiscuous and are infective on Eleagnaceae, Rhamnaceae, Myricaceae, *Gynmmostoma,* and occasionally *Alnus.* The fourth *Frankia* lineage consists of the "atypical" strains which are unable to reinfect actinorhizal host plants or form ineffective root nodule structures that are unable to fix nitrogen. Our understanding of this genus has been greatly enhanced by the sequencing of several *Frankia* genomes from the different *Frankia* lineages [24-33]. Analysis of *Frankia* genomes has revealed new potential with respect to metabolic diversity, natural product biosynthesis, and stress tolerance, which may help aid the cosmopolitan nature of the actinorhizal symbiosis [31, 34].

In this chapter, we will describe what is known about the degradation properties of these bacteria.

2. Rhizodegradation

Among bacteria with bioremediation potential, *Frankia* are unique in that these bacteria form a symbiosis with actinorhizal plants. The implications of this trait for bioremediation efforts have only recently been explored. In the context of bioremediation, the most extensively studied system is the *Frankia–Alnus* association. Diverse assemblages of free-living *Frankia* strains are present in soils with polyaromatic hydrocarbon (PAH) contamination [8-10, 15, 35-38]. These *Frankia* strains readily form symbioses with alders, resulting in greatly increased alder fitness in harsh environments. The *Frankia*–alder symbiosis also increases the mineralization of representative organic pollutants in oil-sands reclamation sites. The *Frankia*–alder symbiosis has been used in reclamation projects because of these traits [5, 8, 36-38]. Free-living *Frankia* also appears to be part of natural degradation communities. Specifically, *Frankia* has been found to be one of the most abundant genera in wastewater treatment communities [35]. Based on these findings, *Frankia* appears to be an underutilized tool in holistic remediation approaches.

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3. *S*- triazines degradation

3.1. Overview

Triazines are a class of herbicides composed of a heterocyclic six-membered ring with alternating carbon and nitrogen atoms joined by double bonds. These herbicides have been used extensively for control of broadleaf and grassy weeds in corn, sorghum, and sugarcane cultivation. Atrazine and simazine are the most ubiquitous members of the s-triazine family. Biodegradation of atrazine is a complex process and depends on the nature and amount of atrazine in soil or water [39-41]. There are four major steps in atrazine degradation: hydrolysis, dealkylation, deamination, and ring cleavage. For the hydrolysis step, an amidohydrolase enzyme (AtzA) cleaves the carbon-chlorine (C-Cl) bond and thus dechlorinates atrazine to hydroxylatrazine. This intermediate is dealkylated and deaminated at the ethyl and isopropyl groups by the amidohydrolase enzymes, AtzB and AtzC, to produce cyanuric acid. This product is converted to ammonia and carbon dioxide by the AtzD, AtzE, and AtzF enzymes [42-44].

3.2. *S*-triazine degradation pathway in *Frankia*

In *Frankia,* the first two steps in atrazine degradation have been identified as well as the regulation of their gene expression [17]. The mineralization of atrazine to ammonia and carbon dioxide is generally initiated by hydrolytic dechlorination, catalyzed by the enzyme atrazine chlorohydrolase (AtzA). Alternatively, this reaction is catalyzed by another atrazine chlorohydrolase (TrzN), which is also able to use atrazine derivatives including desethyl-desisopropylatrazine as substrates. Analysis of the *Frankia* genomes identified candidate genes for the atrazine degradation pathway (Figure 1). The *trzN* gene was identified in *Frankia alni* ACN14a (FRAAL1474) and *Frankia* sp EuI1c (FraEuI1c_5874) genomes and its amidohydrolase gene product is predicted to remove chlorine from s-triazine compounds to produce hydroxyatrazine or ammeline from atrazine and desethyl desisopropyl atrazine, respectively. Furthermore, a putative *atzB* gene was also identified in both *Frankia* genomes (FRAAL1473 and FraEuI1c_5875) whose predicted gene product, adenosine aminohydrolase 3, is involved in the dealkylation reaction of the *N*-ethyl group from hydroxyatrazine transforming it into *N*-isopropylammelide. Physiological studies showed that *Frankia* ACN14a and EuI1c cultures are able to break down atrazine and desethyl-desisopropylatrazine producing the end products hydroxyatrazine and *N*-isopropylammelide. Although the enzymes were not purified, these data clearly showed metabolism of atrazine. Analysis of gene expression in *Frankia* ACN14a found that the two genes, *trzN* (FRAAL1474) and *atzB* (FRAAL1473) are under control of the *atzR* (FRAAL1471) gene, which encodes a predicted LysR-type transcriptional regulator.

Bioinformatics analysis of the *Frankia* genomes revealed a potential full pathway for atrazine degradation in the *Frankia* sp EuI1c genome (Figure 2). The *atzC* (FraEuI1c_4724) gene, which encodes a putative amidhydrolase enzyme, was identified and is predicted to be involved in the dealkylation of the *N*-isopropyl group from atrazine to produce cyanuric acid. With other bacterial systems, cyanuric acid is hydrolyzed to ammonium and carbon dioxide via the *atzDEF* operon [43, 45]. In *Frankia* EuI1c, the *atzD* (FraEuI1c_3137) gene product is predicted

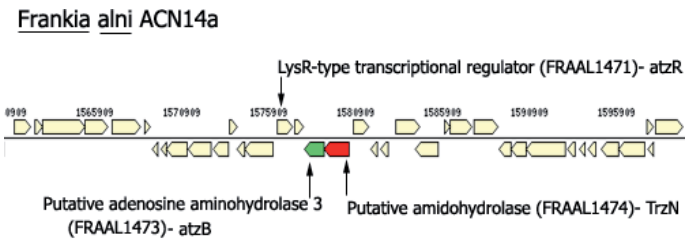

Figure 1. Gene cluster organization in *Frankia alni* ACN14a for atrazine degradation. The cluster contains a putative *trzN* (FRAAL1474), putative *atzB* (FRAAL1473), and putative LysR-family transcriptional (*atzR*).

to transform cyanuric acid into carboxybiuret, which spontaneously decarboxylates to biuret. Putative *atzE* (FraEuI1c_1007 and 1008), and *atzF* (FraEuI1c_3831) genes were also identified in the *Frankia* EuI1c genome and their gene products expected to complete *s*-triazine mineralization by converting biuret to allophanate and ammonia plus carbon dioxide. A *trzR* (FraEuI1c_3136) gene, which encodes a GntR family transcriptional regulator, is found before the *atzD* gene and is involved in the expression of that gene (Rehan unpublished).

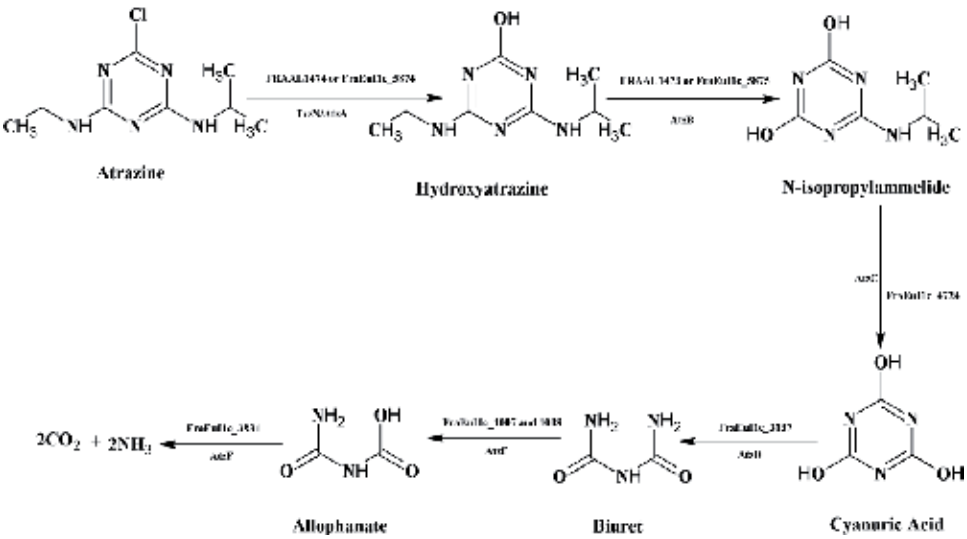

Figure 2. The atrazine degradation steps in *Frankia* strains EuI1c and ACN14a include atrazine dechlorination and dealkylation and ring cleavage by TrzN, atzB, and atzD enzymes.

4. Aromatic compounds degradation

4.1. Biphenyl and polychlorinated biphenyl

Biphenyls and polychlorinated biphenyls (PCBs) are some of the most recalcitrant xenobiotics found in the environment. The degree of chlorination differs greatly among the PCBs, ranging

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from 1 to 10, as does their position on the carbon atoms. Since the mid-1980s, the use of PCBs has been phased out in many countries. However, due to their toxicity, persistence in the environment, and potential carcinogenicity, they are still a major global environmental problem [46-48].

Bacteria degrade biphenyl and PCBs via the *meta*-cleavage pathway, which is encoded by the *bph* operon, and produces tricarboxylic acid and chlorobenzoate (CBA) as intermediates [47-50]. The first enzyme in this pathway is biphenyl dioxygenase, which is a multimeric complex consisting of the large α and small β subunits, and the ferredoxine and ferredoxine reductase subunits. The degradation process is initiated by biphenyl dioxygenase which incorporates two oxygen atoms at the 2 and 3 carbon positions of the aromatic ring (called 2,3-dioxygenation) to generate hydroxyl groups. For PCBs degradation, biphenyl dioxygenase catalyzes the initial 2,3-dioxygenation, and dihydrodiol dehydrogenase converts the product into 2,3-dihydroxybiphenyl. The enzyme, 2,3 dihydroxybiphenyl dioxygenase, cleaves the dihydroxylated ring to produce (chlorinated) 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (HOPDA). A hydrolase enzyme then hydrolyzes HOPDA to (chlorinated) benzoic acid and 2-hydroxypent a-2,4-dienoate.

4.1.1. Biphenyl degradation pathway in Frankia

At least four *Frankia* strains (ACN14a, CcI3, EUN1f, and EuI1c) are resistant to biphenyl and polychlorinated biphenyl (PCB) at concentrations up to 5mM [51, Swanson unpublished results]. Data mining for known organisms capable of biphenyl degradation [46, 52] and the availability of a *Frankia* genome database enabled the identification of genes potentially involved in biphenyl degradation in several of the *Frankia* strains listed above. Five genes were identified that encode enzymes involved in biphenyl degradation: the alpha and beta subunits of the aromatic-ring-hydroxylating dioxygenase, a Rieske (2Fe-2S) iron–sulfur domain protein, an alpha/beta hydrolase fold protein, and a short-chain dehydrogenase/reductase (SDR). These enzymes are putatively capable of oxidizing and hydroxylating benzene rings, and are also known as the upper meta-cleavage pathway. A lower pathway of aromatic ring degradation consisting of three genes (encoding the 2-hydroxypenta-2,4-dienoate hydratase; acylating acetaldehyde dehydrogenase; and 4-hydroxy-2-oxovalerate aldolase) is located downstream of this operon [53, Swanson and Tisa unpublished data]. Figure [3] shows the gene neighborhood of the Biphenyl degradation genes. These genes were also found in *Frankia* strain EUN1f and Dg1 genomes (Swanson and Tisa unpublished). Both the meta-cleavage upper and the lower pathways are commonly referred to as the *bph* operon in several other PCB-degrading bacteria. *Rhodococcus* RAH1, a species closely related to *Frankia*, utilizes *bph* genes homologous to those found in *Frankia* to metabolize PCBs as a sole carbon and energy source [54]. Since at least two genes (Aromatic-ring-hydroxylating dioxygenase, subunit alpha-like protein (FraEuI1c_4097) and short-chain dehydrogenase/reductase (FraEuI1c_4101) in the *bph* operon in *Frankia* are upregulated in the presence of biphenyl, it is likely that *Frankia* also uses the *bph* operon to metabolize biphenyl and PCBs (Rehan and Tisa unpublished)

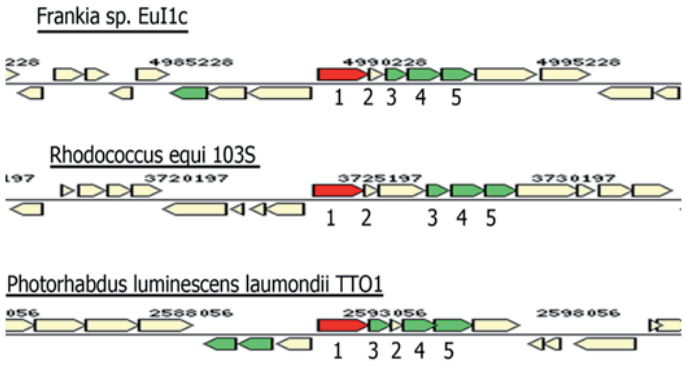

Figure 3. The gene neighborhood of *bph* operon in *Frankia* EuI1c in comparison to *Rhodococcus equi 103S* and *Photorhabdus luminescens laumondii* TTO1 operon. (1) Aromatic-ring-hydroxylating dioxygenase, subunit alpha. (2) Rieske (2Fe-2S) iron–sulfur domain protein. (3) Aromatic-ring-hydroxylating dioxygenase, subunit beta. (4) Alpha/beta hydrolase fold protein. (5) Short-chain dehydrogenase/reductase SDR.

4.2. Phenol degradation

4.2.1. Overview

Phenol (or hydroxybenzene) consists of a benzene ring substituted with a hydroxyl group. Derivatives of this molecule are colloquially known as phenolic compounds. Phenolic compounds are ubiquitous chemicals with diverse properties and uses. The simplest phenolic compound, phenol, is widely used in oil and coal processing, tinctorial and metallurgic industries, and many other industrial applications. Phenol also enters the environment via vehicle exhaust and as the product of natural metabolic processes, and chlorophenols are widely used as biocides in agricultural applications [for a review see 55]. While anthropogenic phenolics are often hazardous, natural phenolic compounds are mostly harmless in the concentrations that are found in foods such as coffee and tea, and some are used as antibiotics [56, 57]. However, the toxicity of some phenolics, particularly phenol and chlorinated phenols, has prompted considerable research activity devoted to phenol remediation. Acute and chronic exposure to phenol and chlorophenol has serious health effects. Phenol and chlorophenol cause lipid peroxidation which ultimately leads to tissue necrosis, and liver and kidney damage [58]. Additionally, chlorophenol exposure is associated with elevated risks of cancer, immune deficiencies, and teratogenic effects [59-61].

4.2.2. General phenol degradation pathway

One of the most promising techniques for removing anthropogenic phenolics from the environment is bioremediation. As was the case for many compounds, the degradation pathway for phenol was first elucidated in a *Pseudomonas* strain [62]. Most bacteria degrade phenolics using catechol catabolic enzymes, most importantly catechol-2,3-dioxygenase. Phenols are first hydroxylated to form catechol, and then catechol-2,3-dioxygenase cleaves the

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benzene ring at the meta position [62]. Therefore, the degradation pathway that begins with catechol-2,3-dioxygenase is called the meta pathway (Figure 4). While the meta pathway is most prevalent, degradation can also begin with cleavage at the para or ortho position using catechol-1,2-oxygenase [63-65]. After ring cleavage, 2-hydroxymuconic semialdehyde hydrolase catalyzes a decarboxylation reaction yielding 4-oxalocrotonate. 4-oxalocrotonate is hydrated by 2-oxopent-4-enoate hydratase to form 4-hydroxy-2-oxovalerate. 4-hydroxy-2-oxovalerate aldolase then splits 4-hydroxy-2-oxovalerate into pyruvate and acetaldehyde, which can then be incorporated into the central metabolic pathways [62].

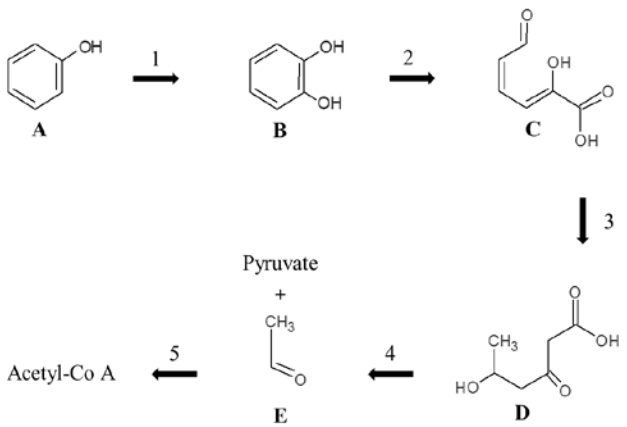

Figure 4. General phenol degradation pathway.

4.2.3. Phenolic compounds and Frankia

Frankia spp. both produce and are affected by phenolic compounds. However, it is unclear whether *Frankia* may degrade phenol and other phenolic compounds. The response of *Frankia* to phenolics was first studied in the context of plant–microbe interactions. Despite apparent functional and morphological similarities between *Frankia* nodules and leguminous nodules, the molecular and physiological mechanisms that control nodulation are distinct. Therefore, the unique process of nodulation by *Frankia* is still an area of intense research. *Alnus* spp. (Alders) plants are a major host plant for *Frankia,* and also have unusually high levels of phenolics in their root exudates, which affect the growth of *Frankia*. Most *Alnus* phenolics tested inhibit *Frankia* growth to varying degrees [66, 67]. Specifically, benzoic acids are less inhibitory than cinnamic acids such as caffeinic acid. However, one plant phenolic, o-hydroxyphenylacetic acid, promoted *Frankia* growth, and both benzoic and cinnamic acids caused increased branching of *Frankia* hyphae. Low concentration plant phenolics also mediate a global shift in *Frankia* gene expression, while higher concentrations (above 30 mg L^{-1}) simply inhibit biosynthesis [33]. Interestingly, *Frankia* also increases phenolic expression of their host plant, causing them to produce more phenol, flavonoids, and hydroxycinnamic acid [68].

Frankia may promote excretion of phenolics as a way to increase available nutrients. However, this explanation depends on *Frankia* having the ability to degrade phenolic compounds. While

no study has demonstrated that *Frankia* degrades phenolic compounds, there is genetic evidence that this bacterium may have the ability to degrade phenolics. First, some *Frankia* strains have genes coding for the production of catechol and other phenolic compounds [34]. Because bacteria often salvage the biomolecules they produce, the presence of an anabolic pathway suggests that a catabolic pathway is also present [69]. Furthermore, multiple *Frankia* strains contain catechol-2, 3-dioxygenase, the most important enzyme in the phenol degradation pathway (Swanson and Tisa unpublished data) [64]. A closely related bacterium, *Rhodococcus* spp., uses the catechol-2,3-dioxygenase pathway to grow with phenol as its sole carbon source [70]. The same species is also able to break down the more recalcitrant pentachlorophenol via the para pathway [71]. This suggests that *Frankia* may break down phenol, a trait that could be applied in bioremediation efforts. Several *Frankia* strains are able to grow on phenol, quercetin, catechol, and other phenolic compounds (Furnholm, Greenleaf, and Tisa unpublished data), but the metabolism of their breakdown has not been studied.

4.3. Naphthalene degradation

4.3.1. Overview

Naphthalene is a ubiquitous polyaromatic hydrocarbon composed of two benzene rings joined at the 9 and 10 carbons (Figure 5). Naphthalene is produced by distilling and crystallizing coal tar, and also as by-product of fossil fuel combustion and cigarette smoke [72]. Naphthalene is used in a number of industrial applications including as feed stock for the production of plastics and resins, and as a component of creosote-based wood preservatives. Naphthalene is also used in tincture and leather tanning industries [72]. Unlike many organic pollutants, naphthalene does not bioaccumulate. Instead, naphthalene is metabolized and excreted in the urine of rats and humans [72, 73]. Nonetheless, naphthalene is a problematic pollutant with numerous toxic effects. Acute exposure to naphthalene causes hemolytic anemia, and liver and neurological damage [74]. Chronic naphthalene exposure is associated with elevated cancer risk [75, 76]. The toxicity of naphthalene and its prevalence as a pollutant has spurred research on remediation techniques, including bioremediation and biodegradation.

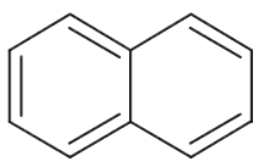

Figure 5. Structure of naphthalene.

4.3.2. Degradation pathway

The naphthalene biodegradation pathway was first studied in a strain of *Pseudomonas* which has two related naphthalene degradation pathways. The upper pathway catabolizes naph-

thalene to produce salicylate and a molecule of pyruvate [77]. The lower pathway breaks salicylate down into acetyl Co-A and pyruvate [78]. The first step of the upper pathway is catalyzed by four proteins: naphthalene dioxygenase reductase, naphthalene dioxygenase ferredoxin, and naphthalene dioxygenase Fe-S protein small and large subunits. This collection of enzymes oxidizes naphthalene to produce *cis*-naphthalene dihydrodiol, which is subsequently dehydrogenated by naphthalene *cis*-dihyrdodiol dehydrogenase to form 1,2-dihydroxynaphthalene. 1,2-dihydronaphthalene dioxygenase then produces 2-hydroxychromene-2-carboxylate which is then cleaved by 2-hydroxychromene-2-carboxylate dehydrogenase to form *cis-o*-hydroxybenzylpyruvate. 1,2-dihydroxybenzylpyruvate aldolase then splits *cis-o*-hydroxybenzylpyruvate producing pyruvate and salicylaldehyde. Finally, salicylaldehyde dehydrogenase carboxylates salicylaldehyde to form salicylate [77, 78].

In the lower pathway, salicylate hydroxylase hydroxylates salicylate to produce catechol. The remaining benzene ring is then cleaved by catechol-2,3-dioxygenase to produce 2-hydroxymuconic semialdehyde [78]. Hydroxymuconic semialdehyde dehydrogenase then produces 2-hydroxyhexa-2,4-diene-1,6-dioate which is subsequently isomerized by 4-oxalocrotmate isomerase to produce 2-oxohexa-3-ene-1,6-dioate. This is then transformed into 2-oxopent-4-enoate by 4-oxalocrotomate decarboxylase. 2-oxopent-4-enole hydratase produces 4-hydroxy-2-oxovalerate, which is subsequently split into acetaldehyde and pyruvate by 2-oxo-4-hydroxypentanoate aldolase. Finally, acetaldehyde dehydrogenase converts acetaldehyde into acetyl Co-A [78]. Both of these pathways are also found in *Rhodococcus* spp, a close relative of *Frankia* [79].

4.3.3. Naphthalene degradation in Frankia

Not surprisingly, *Frankia* also metabolizes naphthalene as a sole carbon and energy source via a related pathway [18]. Specifically, *Frankia* uses the protocatechuate pathway to convert naphthalene or a naphthalene derivative into acetyl Co-A and succinyl Co-A (Figure 6) [18]. This finding confirms the role of *Frankia* in naphthalene degradation, which was suggested by earlier field studies [8-10, 37, 38]. In symbiosis with alders, *Frankia* increases polyaromatic hydrocarbon degradation in oil-sand tailings for the first 1.5 years [8, 10, 37]. However, after 2.5 years, alders without *Frankia* symbionts demonstrated naphthalene degradation equal to the degradation or *Frankia*-inoculated alders [8]. The *Frankia*-alder symbiosis thrives in PAH-contaminated areas [15]. Interestingly, alder plants found in these PAH-contaminated areas maintained a symbiosis with *Frankia* lineage III as opposed to the normal lineage I, suggesting that this pollutant affected nodulation and/or survival of the actinorhizal plants. Taken together, these findings indicate that *Frankia* could be a useful tool in naphthalene remediation.

4.4. Protocatechuate

4.4.1. Overview

Under oxic conditions, microbial degradation of many aromatic compounds occurs through the catechol or protocatechuate branch of the ß-ketoadipate pathway via either *ortho* cleavage

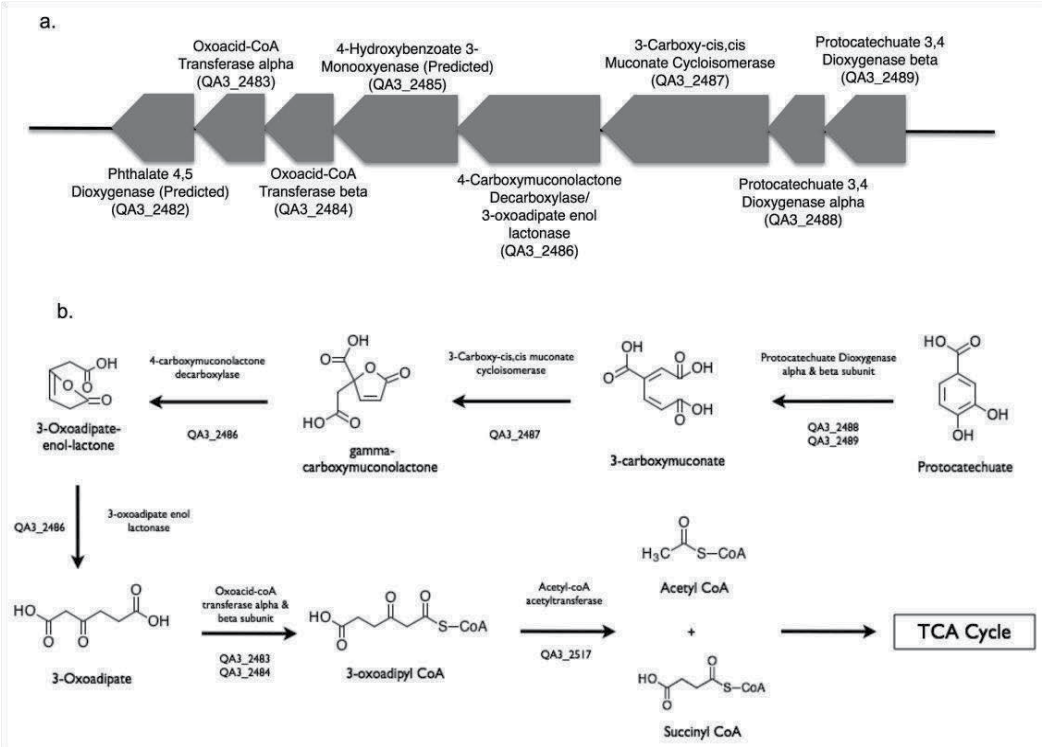

Figure 6. Putative naphthalene degradation pathway in *Frankia* [18]. (Figure is recopied with permission from *Canadian Journal of Microbiology*.)

by catechol 1,2-dioxygenase and protocatechuate 3,4-dioxygenase or *meta*-cleavage by catechol-2,3-dioxygenase and protocatechuate-4,5-dioxygenase.

4.4.2. Potential protocatechuate degradation pathway in Frankia

Besides the protochatechuate pathway found in *Frankia* QA3 [18], several other potential protocatechuate pathways have been identified from bioinformatics analysis of the available *Frankia* genomes. In *Frankia* EuI1c, a potential operon (*FraEuI1c_2560* -to- *FraEuI1c_2564*) for a putative protocatechuate pathway was identified (Figure 7). This operon encodes the predicted gene products involved in the putative pathway including protocatechuate 3,4-dioxygenase alpha and beta subunits, fumarate lyase, 3-oxoadipate enol-lactonase, and 4-hydroxybenzoate 3-monooxygenase. These gene products are similar to the protocatechuate degradation pathway found in *Rhodococcus opacus* 1CP [80, 81]. These results suggest that *Frankia* may use the protocatechuate degradation pathway to degrade many aromatic ring compounds after their conversion to protocatechuate.

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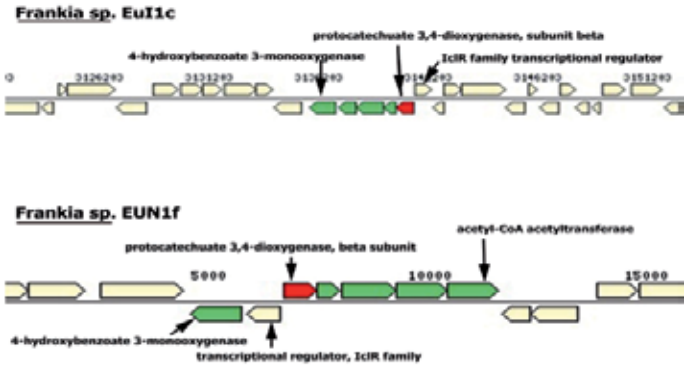

Figure 7. The proposed protocatechuate degradation pathway in *Frankia* strains EuI1c and EUN1f.

5. Hydrocarbons

5.1. Overview

Petroleum-based energy and products are used extensively around the world. The pervasiveness of petroleum inevitably leads to serious environmental pollution. Petroleum is a complex mixture of hydrocarbons, cycloalkanes, aromatic hydrocarbons, and more complex chemicals like asphaltenes. These chemicals and their derivatives, which are termed petrogenic compounds, are released into the environment as a result of oil spills and combustion of petroleum-based products [82]. Oil spills are one of the most serious sources of petroleum pollution and devastate aquatic and marine environments. Ongoing research to identify new methods for petroleum remediation is important because oil spills and other types of petroleum-derived pollution continue to pose environmental health risks.

Hydrocarbon-degrading bacteria and fungi are widely distributed in marine and freshwater environments, as well as soil habitats [83, 84]. In *Pseudomonas*, the alkane hydroxylase (monooxygenase) system consists of three components: alkane hydroxylase (AlkB), rubredoxin, and rubredoxin reductase. This system is responsible for the first oxidation step in the utilization of n-alkanes [85]. Similar alkane hydroxylase systems have been found in a variety of alkane-degrading bacteria [86, 87]. *Alcanivorax* sp. strain 2B5 will degrade C13–C30 n-alkanes and branched alkanes (pristine and phytane) from crude oil as the sole carbon source via a novel alkane hydroxylase gene (alkB). Other *Acinetobacter* are able to use n-alkanes with chain length C10–C40 as a sole source of carbon. In addition, the presence of multiple alkane hydroxylases in two *Rhodococcus* strains were characterized and both organisms contained at least four alkane monooxygenase gene homologs (*alkB1*, *alkB2*, *alkB3*, and *alkB4*) [76, 88].

A bioinformatics approach was used to identify these potential hydrocarbon degradation pathways among the sequenced *Frankia* strains. Functionally analyzed genes for the known hydrocarbon degradation pathways [84, 88] were used to probe the *Frankia* genome database

and identify potential pathways. Our preliminary results (Rehan unpublished data) revealed that the *F. alni* ACN14a genome possesses a putative alkane-1 monooxygenase (Alkane omega-hydroxylase) gene (*FRAAL1986*), which is one of the known enzymes involved in the break-down of n-alkanes (Figure 8). Furthermore, a similar gene (*Franean1_2192*) was also found in the *Frankia* sp. EAN1pec genome. These bioinformatics results support the hypothesis that *Frankia* may be able to degrade oil-spill-derived hydrocarbons. However, these preliminary results need further study.

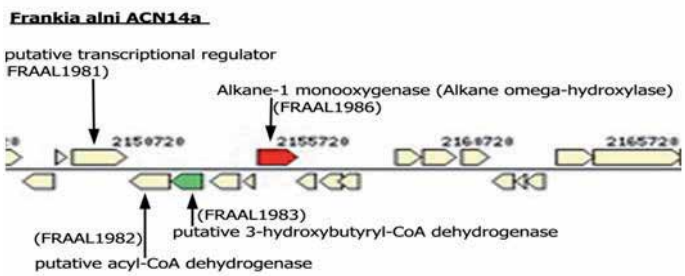

Figure 8. Potential alkane-1 monooxygenase identified in *F. alni* ACN14a.

6. Future aspects

Clearly, we have only begun to scratch the surface of the metabolism of *Frankia* and its biodegradative potential. These initial studies correlating metabolic capacity to gene function are the first step in exploiting the bacteria for their bioremediation ability. Further bioinformatics data mining are necessary to elucidate the unique metabolic potential of *Frankia*. However, these *in silico* studies require "wet lab" experiments to confirm these capabilities.

From limited field studies, actinorhizal nodule occupancy seems to be under control by environmental conditions. The presence of *Frankia* lineage III strains inside alder nodules found under PAH-stressed soils suggests that this lineage may have a greater metabolic potential. The larger genome size of this lineage compared to the other infective strains also supports this hypothesis. However, further experiments are required to confirm this postulate.

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Chapter 12

Enzymes and Phytohormones from Micromonospora

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Additional information is available at the end of the chapter

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Abstract

Actinobacteria produce diverse and huge amounts of enzymes that are widely used in different industrial purposes. Specific properties of enzymes allow to run the reactions under milder conditions with improved yield and reduced wastes. Further redesign for natural enzymes is very essential because they are often not suitable for biocatalytic processes. Recently, new microbial natural and creating enzymes are discovered synchronous with the late advanced technologies of genomics, metagenomics, proteomics, efficient expression systems and emerging recombinant DNA. The ongoing development of enzyme biotechnology aids in the improvement of the industrial biocatalysis field. Thermophilic actinobacteria produce thermostable enzymes that are widely used in industrial processes. In contrast, psychrophilic actinobacteria grow well at low temperatures and subsequently their enzymes are more effective at low temperatures. The advanced DNA sequencing technique allows determining and identifying the sequences and functions of all the genes that synthesize proteins that are widely use in the industry. Recombinant strains can be obtained by using certain biotechnological tools to potentially increase enzymes production on a large scale. The ongoing development in this field will lead to the improvement of different industrial purposes such as food, chemicals, textiles, leather, pharmaceuticals, and so on.

Keywords: Actinomycetes, biocatalysts, enzyme biotechnology, industrial microbiology

1. Introduction

Actinobacteria are recognized by their morphology thin, elongated cells with branching filaments, as its name denotes. Nevertheless, morphology alone is not enough to add an organism to the actinobacterial group, so the advanced identification depends on other techniques such as cultural, physiological, biochemical and molecular characteristics. More-

over, phylogenetic analysis is necessary to distinguish between true actinobacteria and those that have closely phylogenetic affinities, and at the same time to precisely determine the similarity among different species of the same genus. A lot of enzymes produced from actinobacteria are used in industries thus they are called "industrial enzymes". These enzymes employ in the industry of paper, leather, detergents, textiles, pharmaceuticals, chemicals, foods, beverages, biofuels, animal feed, personal care, and others [1]. The sustainable industry based on enzymes technology depends on the biotechnological development, which is represented in the modern molecular techniques such as metagenomics and genomics. The latter allows the discovery of new potential recombinant-biocatalytic enzymes used in different industries needed by society. These industrial enzymes are employed to yield over 500 products [2, 3]. Although chemical and organic methods are used in industries, they have several disadvantages non-economic, unfriendly to the environment, lacking of enantiomeric specificity for chiral synthesis, need for high temperature, low pH and high pressure and leads to organic waste and pollutants. On the other hand, enzymes are more useful in industrial applications because they act at mild reaction conditions, have a long half-life, and are friendly to the environment [4]. Moreover, gene-encoded enzymes can be modified to improve their properties stability, substrate specificity and specific activity. There are many competitive companies that participate in the enzyme industry Novozymes being the largest one followed by DSM and DuPont among others. The companies' competition relies on certain criteria such as product quality, performance, use of intellectual property rights, and the ability to innovate.

2. Enzymes detection

Recently, new enzymes can be detected by using certain biotechnological tools such as metagenome screening [5, 6], microbial genome mining [7, 8], and exploring the diversity of extremophiles [9, 10].

2.1. Metagenome screening

Metagenome is a new manner for microbial screening based on the function and/or sequence of the gene [6, 11, 12]. Function-based screening is a method by which gene isolation is carried out depending on the desired gene function through phenotype detection, heterologous complementation, and induced gene expression [13]. Whilst sequence-based screening is a method performed by either polymerase chain reaction (PCR) or hybridization procedures. Strains producing industrial enzymes are isolated from different environments such as volcanic vents, arctic tundra, cow rumen [14], marine environments [15], and termite guts [16] such as lipase [17, 18], oxidoreductase [19, 20], amidase [21], amylase and nitrilase [22], beta-glucosidase [23, 24], decarboxylase, and epoxide hydrolase [25]. Function-based screening method has main disadvantages due to its inefficiency and biased expression of foreign genes in *Escherichia coli* as an alternative host [12]. To overcome these problems, alternative bacterial host and expression systems are currently being examined including *Streptomyces lividans*, *Pseudomonas putida* and *Rhizobium leguminosarum*, among others [26, 27]. Target rate depends on other factors such as gene size and the assay method. Enzyme activity can be assayed by

agar plate method, which has a sensitivity developed by using cell lysates [28], toxics resistant genes [29, 30], correlation of enzyme activity with a phenotypic characteristic such as green fluorescent protein (GFP) [31], or β-galactosidase [32]. Also, development of flow cytometry-based screens as SIGEX are leading the way as they enable more rapid screening of large metagenomic libraries [33].

2.2. Microbial genome mining

New enzymes particularly used in industries are explored from huge genome sequence databases [34]. The advanced genome sequencing programs, such as 454 from Roche, Solexa from Illumina, or SoLiD from ABI, reduce time and cost. Using these programs support the complete and accurate reading of multiple whole genomes in less than two weeks [35]. Two approaches are being followed to discover new enzymes [36].

2.3. Exploring the diversity of extremophiles

More than 30 papers were published on extremophiles in a special issue [37]. Extremophiles are promising microorganisms for enzyme production because they can grow under extreme physical conditions such as temperature (2-12°C, 60-110°C), pressure or radiation, and/or geochemical conditions such as salinity (2-5% NaCl) and pH (< 2, > 9). High biodiversity for extremophiles is present under all extreme conditions mentioned [10, 38]. For example, there are some thermostable enzymes such as proteases, lipases, cellulases and amylases that are successfully used in different industrial applications [10, 39]. Thermostable enzymes are isolated from different thermophilic bacteria such as *Clostridium*, *Thermus*, *Thermotoga*, and *Bacillus*, as well as Archaeabacteria such as *Pyrococcus*, *Thermococcus* or *Methanopyrus*. The most common thermostable enzyme is Taq DNA polymerase which isolated from *Thermus aquaticus* [40]. Moreover, thermostable enzymes are greatly used for detergent manufacturing. Polymer-degrading enzymes such as cellulases and xylanases are used for pulp and paper industries, and are also used with other applications such as extraction and clarification of fruit juices, improvement of bakery products, polishing and stone-washing of textiles, and bioremediation of waters contaminated with oils or hydrocarbons [10, 41 - 43]. Halophites are produced for specific enzymes that tolerate salt stress by containing a considerable number of negatively-charged amino acids that prevent precipitation [44]. The most common halophites that used in industrial applications are *Halobacterium*, *Halobacillus* and *Halothermothrix* [45, 46]. Thermoalkaliphilic enzymes, such as proteases and lipases, are produced by microorganisms that grow under extreme pH values and used as additives in laundry and dishwashing detergents [10, 47].

3. Improvement of microbial enzymes

Improvement of enzyme properties can be carried out by creating a growing demand [40]. Industrial enzymes usually need further fine tuning to achieve industrial scale production [48]. Using recombinant DNA technology in large industrial scales is very urgent due to the increase

in production by 100-fold [49]. There are two main manners for enzymes modification to adapt their functions rational redesign and combinatorial methods.

3.1. Rational redesign

This strategy is based on using direct mutation to hit amino acid substitutions, which require complete information about the 3D structure and chemical mechanism of the enzymatic reaction. Databases include protein structures and sequences, so the sequence comparison of a new enzyme with the references can identify related enzymes whose functions or/and structures are already known [50 - 54].

3.2. Combinatorial methods

Combinatorial methods rely on certain factors relevant to an enzyme molecule such as chirality, biocatalytic effect, catalytic rate, solubility, specificity and stability. Combinatorial methods are faster and cheaper than methods for finding new enzymes it acts better than natural methods under specific conditions [3, 55 - 57]. Combinatorial methods, also called "directed evolution" have random mutagenesis in the protein-encoding gene using different techniques such as PCR [58], repeated oligonucleotide directed mutagenesis [59], and chemical agents [60]. The PCR technique introduces both random and point mutations in a large number of enzymes. These techniques perform random recombinant *in vitro*, typically between parent genes with homology higher than 70% [61]. The point mutation is carried out by the target active site residues (about 10–15 amino acids) and those closest to it (another 20–30 amino acids) [62]. There is another way, called CAS Ting, which is based on a combinatorial active site testing in which libraries are generated from groups of two or three residues made from the active site residues [63]. While running PCR, the amino acid alphabet is reduced and new proteins composed of only 12 amino acids are synthesized [64 - 66]. Further techniques can create shuffle exons or domains [67], loop regions [68], random truncations [69], or insertions and deletions of codons [70]. Moreover, random redesign technology is used to create new and improved enzymes that have the highest activity, are more stable at different pH values and temperatures [71], have increased chirality [72], altered substrate specificity [73], stability in organic solvents [74], novel substrate specificity and activity [75] and increased biological activity of protein pharmaceuticals and biological molecules [76, 77]. Directed evolution method could improve the activity of glyphosate-N-acetyltransferase by 10,000-fold and thermostability by 5-fold [78]. Directed evolution work has resulted in the presence of new and improved enzymatic proteins in the market since 2000 [79, 80].

4. Production of recombinant microbial proteins

Molecular biology techniques, particularly recombinant DNA, have a great effect in enzyme production from different microorganisms such as *Bacillus* spp, *Ralstonia eutropha*, *Pseudomonas fluorescens*, *Saccharomyce cerevisiae*, *Pichia pastoris*, *Hansenula polymorpha*, *Aspergillus* spp, *Trichoderma* and *E. coli* [81]. The latter is most common bacteria used in this purpose due to its

accurate genome modification, rapid growth, and good growth on different media [82, 83]. Moreover, the *Pseudomonas fluorescens* bacteria of the Pfenex Company can produce 20 g/L of protein [84]. On the other hand, *Saccharomyces cerevisiae* is more useful than bacteria because it usually used as a cloning host, it has high cell density, produces heterologous proteins, and its genetics are more advanced than any other eukaryote. Despite all the benefits mentioned above, this yeast is not convenient for use in the mammalian proteins industry at the large-scale because drawbacks may occur such as hyperglycosylation, antigenic response in patients due to accumulation of α-1,3- mannose residues, and absence of strong and tightly-regulated promoters [85]. *P. pastoris* is regarded as one of the most common industrial microorganism and could produce over 700 proteins [81, 86, 87]. Recombinant protein production had 22 g/L for intracellular proteins [88] and 14.8 g/L for secreted proteins [89]. *P. pastoris* can produce up to 30 g/L of recombinant proteins [90 - 94]. Although DNA recombination technology is more easy and available, gene insertion and deletion remain difficult [95, 96]. The problem at industrial level is that non-fungal proteins production is low compared to homologous proteins. There are many strategies used to overcome this problem such as the establishment of weak protease strains [97], insertion of a considerable number of gene copies [98], use of strong fungal promoters, efficient secretion signals [98, 99], and gene fusions with a gene that encodes part of or an entire well-secreted protein [99]. *Chrysosporium lucknowense* was found to have a great ability for protein production (50–80 g/L), and from which low-viscosity and low-protease mutants have been obtained [100, 101].

5. Biocatalysts

Biocatalysts are widely used in different economic industries such as food industry [102]. Biocatalysis can be carried out by using intact cells, immobilized cells, cell free extracts, purified enzymes, or immobilized enzymes [103,104]. Microbial industry has been developed rapidly due to due to using the recent techniques such as genome sequencing, directed evolution, protein expression, metabolic engineering, and structural biology [105,106].

5.1. Enzymes applications

Enzymes have great importance in industrial processes [107, 108]. These enzymes are used in the detergent, textiles, pulp, paper, leather, and biofuels industries. Biofuels have the highest sales [109, 110]. In the textiles industry, enzymes are used as cleaners, reducing the use of raw materials and waste production [111]. Pectate lyase is used in the cotton industry [112]. Lipases, xylanases, and laccases are used in removing pitch in the pulp industry [113 - 115]. Cellulases are widely used in the textiles industry and are also used in the degradation of lignocellulosic feed stocks. There are several cellulases such as endoglucanases that degrade cellulose randomly, cellobiohydrolases that release glucose dimers from both ends of cellulose chains, and beta-glucosidases that hydrolyze oligomer chains to liberate glucose molecules [116 - 118]. The main microbial source of cellulases is *Trichoderma reesei* which depolymerizes plant blocks to free sugar molecules [119 - 124].

5.2. Enzymatic food industry

A certain group of enzymes plays an important role in the food industry and subsequently achieves great revenue [125]. The mechanism of feed enzyme action supports nutrient digestion and therefore leads to a much easier feed utilization. Moreover, they hydrolyze complex components that can be deleterious or have no value [126]. The most common commercial feed enzymes are phytases, proteases, α-galactosidases, glucanases, xylanases, α-amylases, and polygalacturonases, which are used in swine and poultry products industries [127, 128]. Lipase is a distinguishable group of enzymes that is used in the food industry. The maximum yield obtained from lipases requires optimization of enzyme concentration, pH, temperature, and emulsion content. The most common recombinant fungal lipases are produced from *Rhizomucor miehi*, *Thermomyces lanuginosus* and *Fusarium oxysporum* [129, 130]. Protease is also an important group of enzymes that is mainly used in the dairy industry, such as cheese manufacturing where it hydrolyzes a specific peptide bond that generates para-k-casein and macropeptides [131]. Recombinant protease of *A. niger var awamori* can produce 1 g/L of chymosin after nitrosoguanidine mutagenesis and the selection for 2-deoxyglucose resistance [132, 133]. There are four recombinant proteases that have been registered by the FDA for cheese production [128, 134]. Although all enzymes mentioned above are the most common in the food industry, there are others used in this application such as invertase used for candy and jam manufacture, β-galactosidase (lactase) used for hydrolysis of lactose from milk or whey, and galactosidase used for crystallization of beet sugar [135 - 137].

5.3. Enzymatic processing of chemicals and pharmaceuticals

The chemicals industry depends on enzyme technology that is low cost, has easy methods, and high quality [138]. Enzymatic processing requires lower energy, which has many benefits such as high yield, high catalytic activity, low releasing of wastes and byproducts, and lower volumes of wastewater streams [139]. Genomic and proteomic technology development improved enzyme properties which in turn improved the chemicals industry [140, 141]. L-tyrosine is a main compound for protease conversion [142, 143]. Beta-lactam antibiotics manufacturing is one of the common pharmaceuticals industries that depend on enzymes technology [144]. Esterases, lipases, proteases and ketoreductases are used in the industry of chiral alcohols, carboxylic acids, amines or epoxides [110, 145, 146]. Sometimes, recombinant microbial enzymes raise the yield percentage up to 100% [147]. A combination of random gene mutagenesis and ProSAR analysis increase the chirality of ketoreductase enzyme toward tetrahydrothiophene-3-one from 63 to 99% [148 - 152]. The improved enzymatic biotechnology supports the production of 2-methyl pentanol as an important intermediate for pharmaceuticals manufacture [153 - 155]. Also, an improved acyltransferase aids the conversion of cholesterol-lowering agent, lovastatin, to simvastatin [156, 157]. Moreover, lipase is used in stereoselectivity for acetylation of asymmetrical diol during an antifungal agent production [158]. Enzymatic biotechnology has many advantages such as higher substrate solubility, reversal of hydrolytic reactions, and modified enzyme specificity, which result in new enzyme activities [159].

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6. Industrial enzymes

6.1. Pectinases

Actinobacterial pectinases are widely used in various industrial applications such as food and beverages industries, as well as fruit treatment including fruit maturation, viscosity rising, decreasing of must, preliminary treatment of must for wine industries, extraction of tomato pulp, and tea and chocolate fermentation [160, 161]. Pectinases are also used in the textile and paper industries in plant fiber degumming [162 - 165]. Moreover, the combination of pectinase and β-glucosidase supports the scent and volatile substances of fruits and vegetables, and raise the content of antioxidants [166, 167]. Pectinases are produced from several genera of microorganisms such as *Bacillus, Aspergillus, Rhizopus, Trichoderma, Pseudomonas, Penicillium* and *Fusarium* [168, 169].

6.2. Lipases

Lipases are used in various commercial applications, such as in the detergents industry. The most common lipase-producing microorganisms are *Penicillium restrictum*, *Candida rugosa, Candida antarctica, Pseudomonas alcaligenes, Pseudomonas mendocina*, *Burkholderia cepacia* [72], *Geotrichum candidum* DBM 4013, *Pseudomonas cepacia, Bacillus stearothermophilus, Burkholderia cepacia, Candida lipolytica, Bacillus coagulans, Bacillus coagulans* BTS-3, *Pseudomonas aeruginosa* PseA, *Clostridium thermocellum* 27405, *Yarrowia lipolytica* and *Yarrowia lipolytica* CL180 [170 - 179].

6.3. Lactases

Lactases or β-galactosidases are hydrolyzing enzymes that catalyze the hydrolysis of lactose at terminal residues and produce glucose and galactose. The microbial source of lactases are yeasts such as *Kluyveromyces lactis*, *K. fragilis* and *Candida pseudotropicalis*; bacteria such as *Escherichia coli*, *Lactobacillus bulgaricus*, *Streptococcus lactis* and *Bacillus* sp; and fungi, such as *Aspergillus foetidus*, *A. niger*, *A. oryzae* and *A. Phoenecia* [180,181]. Beta-galactosidase is widely used in the dairy industry, and also used in dairy products crystallization such as milk candy, condensed milk, frozen concentrated milk, yoghurt and ice cream mixtures [182 - 185].

6.4. Cellulases

Cellulases are hydrolyzing enzymes for cellulose substances to produce cellobiose and then glucose [186]. The combination of cellulases and pectinases provides well in applications in juice and wine industries [187]. There are a wide array of microorganisms that produce cellulases, such as *Trichoderma*, *Penicillium*, *Aspergillus*, *Fusarium, Phoma*, *Acidothermus*, *Bacillus*, *Pseudomonas*, *Staphylococcus*, *Streptomyces*, *Xanthomonas*, *Acetovibrio*, *Clostridium*, *Pseudonocardia*, and *Thermoanaerobacter* [188, 189].

6.5. Amylases

Amylases are used in the textiles, beer, liquor, bakery, infant feeding cereals, and animal feed industries. *Aspergillus* and *Rhizopus* are the most common amylases producers [190,191].

Amylases are used in the food industry in the conversion of starch into dextrin which in turn is converted to maltose, which used in the manufacture of soft drinks, beer, jellies, and ice cream. Maltose can be converted into glucose, which used in the soft-drinks and bakery industries [192,193]. Amylases are the most used enzymes in bread baking [194]. Amylases also play an important role in the pharmaceuticals industry [195 - 199].

6.6. Proteases

Proteases are used in the baking, feed and brewing industries. Proteases catalyze the splitting of peptide linkages in proteins. Proteases are classified into exopeptidases and endopeptidases according to the site of the concerned peptide bond to be cleaved. Recently proteases represent 60% of industrial enzymes on the market because they are easy to obtain and to recover. Proteases have high coagulation activity, with fewer risks [200]. Proteases are mainly used in the industry of soluble proteins, chemicals and pharmaceuticals [201]. Proteases are produced by *Bacillus*, *Thermus caldophilus*, *Desulfurococcus mucosus*, *Streptomyces* and *Escherichia coli* [202].

6.7. Glucose oxidase

Glucose oxidase can oxidize β-D-glucose with the formation of D-gluconolactone. The enzyme is used to remove harmful oxygen in the food industry to avoid toxicity [203].

6.8. Glucose isomerase

Glucose isomerase catalyzes the reversible isomerase from D-glucose and D-xylose into D-fructose and D-xylulose respectively. Glucose isomerase plays an important role in the food industry such as in the production of fructose-rich corn syrup [197]. The cloned gene xylA of *Thermus themophilus* is introduced to *Saccharomyces cerevisiae* to be expressed under the control of the yeast PGK1 promoter [205,206].

6.9. Invertase

Invertase is produced by *Saccharomyces cerevisiae* and other microorganisms. The enzyme catalyzes the hydrolysis from sucrose to fructose and glucose. The supplementation of an invertase to banana juice is to increase its sweetness and viscosity [207 - 210].

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Chapter 13

Industrial Enzymes and Metabolites from Actinobacteria in Food and Medicine Industry

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Additional information is available at the end of the chapter

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Abstract

Microbial enzymes are known to play a crucial role as metabolic catalysts, leading to their use in various industries and several applications. These enzymes are very useful for industrial applications as they increase the reaction rates by several times than normal chemical reactions.

The biochemical heterogeneity, ecological diversity and capacity of the actinobacteria to produce secondary metabolites make them an ideal objective for the production of enzymes.

Members of the actinobacteria *Micromonospora* contains 32 species, are distributed in nature and have been isolated from different environments of different geographical zones, and also form associations with plants on its leaves, roots, rhizospheres and from nitrogen-fixing root nodules of actinorhizal and leguminous plants.

The present review mainly contemplates on enzymes and metabolites of actinobacterial genus *Micromonospora*.

Micromonospora L5, isolated from *C. equisetifolia* nitrogen-fixing nodules, produces hydrolytic enzymes, cellulose, xylanase, pectinase, and also secretes chitinase. The production of these enzymes allows *Micromonospora* L5 to play a potential role to succeed for second generation biofuel production and on the composting process to meet the need in the energy crisis and solve the problem of the increasing amount of organic domestic wastes.

Keywords: *Micromonospora*, hydrolytic enzymes, biofuel, composting, industry

1. Introduction

Microbial enzymes are known to play a crucial role as metabolic catalysts, leading to their use in several industries. The demand for industrial enzymes and for novel natural products is on a continuous rise due to the growing need for sustainable, ecological and economic solutions. Microbes have been serving as one of the largest and useful sources of many enzymes.

Microbial enzymes are very practical and friendly with the environment for industrial applications as they work under mild reaction conditions (e.g., temperature, pH, atmospheric conditions). Additionally microbial enzymes are highly specific and generally increase the reaction by several times than normal chemical reactions. On the other hand, many industrial processes require high temperature, low pH and high pressure, and have low catalytic efficiency. Furthermore, the use of organic solvents leads to organic wastes and pollutants.

Actinobacteria have been known for a longtime as powerful degraders of the dominant portion of plant biomass, lignin, cellulose, xylene, pectin and other complex polysaccharides. The availability of the whole genome sequence data has opened new insights in comparing genomes; current advances in genome sequencing indicate that the potential of bacteria (including the actinobacteria) to degrade certain components of lignocellulose is widespread. From 5,123 analyzed sequenced bacterial genomes for cellulose utilization or degradation 24% synthetized cellulases and β glucosidases [1]. Later results confirmed the potential importance of actinobacteria in lignocellulose degradation [2].

The biochemical heterogeneity, ecological diversity, and ability of the actinobacteria to produce secondary metabolites make them an ideal source for the production of enzymes [3], a source of antibiotic discovery [4], and a source of novel natural products [5]. As a source of novel natural products, 18 from 20 actinobacteria isolated from the soil of the Biosphere Los Petenes in the Mexican Caribbean, have shown activity against human pathogenic bacteria and fungi including *Escherichia coli, Salmonella entiriditis, Salmonella typhymurium, Enterococcus faecalis, Staphylococcus aureus, Pseudomonas aeruginosa, Helminthosporium* sp. and *Candida albicans* [6]. This fact suggests a sustainable solution for the growing need to solve the medical problem of the pathogenic bacteria becoming resistant to the available industrial antibiotics.

Additionally actinobacteria represent the most suitable biotechnologically procaryotes for the production of a wide range of bioactive metabolites. These filamentous bacteria and their enzymes have an array of biological industrial and environmental applications e.g. soil decontamination [7], biological control of plant diseases [8], and decomposition of organic matter and domestic wastes [9,10].

Actinobacteria are key components of the soil environment and are important contributors to the sustainability of agricultural systems. The increasing energy demands have focused worldwide attention on the utilization of renewable resources, particularly agricultural and forest residues. Lignocellulose, xylan and pectin represent the dominant portion of plant biomass in terrestrial ecosystems and are considered to have great potential as a cheap and renewable feedstock for biofuel production. Alternative and renewable fuels derived from lignocellulosic biomass offer the potential to mitigate global climate change and reduce the

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dependence on fossil fuels. In addition, the decomposition of these compounds in soil environments is an essential process of the carbon cycle.

The relevant aspects of actinobacteria and their ecological, economic, and industrial importance are described in the review [11].

In this chapter, we will highlight the importance of different enzymes with a special focus on the soil actinobacteria *Micromonospora.*

2. Micromonospora

The actinobacteria *Micromonospora* is a genus that contains 32 species [12,13], are gram positive filamentous bacteria, chemo-organotrophic and aerobic characterized by their high guanine-cytosine content in its genome, do not form aerial mycelium in agar plates and produce mycelial carotenoid pigments, white, orange, brown, and when colonies sporulate they appear black in color in certain strains. This bacterium forms branched and septate hyphae of about 0.25 to 0.6 μm in diameter.

These filamentous bacteria are distributed in nature, and have been isolated from different environments of different geographical zones e.g. coastal sediments in Wales [14], marine sediments in Mexico [15] and peat swamp forests in Thailand [16]. The genus have been found forming intimate associations with plants on their leaves [17], roots [18, 19] and various plant rhizospheres [20, 21] including rhizospheres of biofuel crops growing on marginal lands [22], from nitrogen-fixing root nodules of the actinorhizal plant *Casuarina equisetifolia* [23, 24] and *Coriaria myrtifolia* [25], and also from root nodules of the leguminous plants *Lupinus angustifolius* [26] and *Pisum sativum* [27]. Furthermore, *Micomonospora* inhabits nitrogen-fixing nodules in a systematic way [28]. This fact has opened up the question as to what is the ecological role of this bacterium in the plant. The genome of *M. lupini* Lupac 08 and *Micromonospora* L5 contains different genes for hydrolytic enzymes including chitinases [29] which are directly involved in the defense against fungal pathogens by hydrolyzing the cell walls indicating that these bacteria may confer protection to the plant.

Micromonospora also acts as a plant growth promoting rhizobacteria (PGPR) [30] through its ability to promote the growth of nitrogen-fixing symbioses such as *Discaria trinervis-Frankia* [31], *Lupinus albus-Bradyrhizobium canariense* [32] and *Medicago sativa-Sinorhizobium meliloti* [33]. It is supposed, that the actinobacteria produce bioactive metabolites, which are released into the culture medium confirming its role as a PGPR. *Micromonospora* in dual inoculation with other actinobateria in the *Lotus tenuis-Mesorhizobium loti* symbiosis showed to promote root nodulation in plants fertilized with high N levels [34], indicating a high potential of agronomic application since the N fertilization has a powerful inhibition of nodulation of the nitrogen-fixing plants.

The genus shows high biochemical versatility capable of utilizing many different carbon sources given its ability to produce a very rich array of secondary metabolites: antitumor

anthraquinones (lupinadicins A and B), antibacterial polyketides, and inhibitors of tumor cell invasion (lupinacidin C) [35, 36, 37].

2.1. Micromonospora L5

Micromonospora L5 (Figure 1) was isolated from *C. equisetifolia* nitrogen-fixing nodules. In the course of isolating the diazotroph Actinobacteria *Frankia* from surface-sterilized root nodules, we obtained the filamentous bacterium *Micromonospora* strain L5. *Frankia* is hard to isolate due to its very slow growth (generation time is 24–48 h) and a very frequent contaminant is *Micromonospora*.

Indirect evidence of nitrogen fixing genes was obtained by acetylene reduction activity and partial amplification of nifH-like gene fragments in the strain *Micromonospora* sp. L5. However, its genome was screened for the presence of nitrogen-fixing genes and the result was negative.

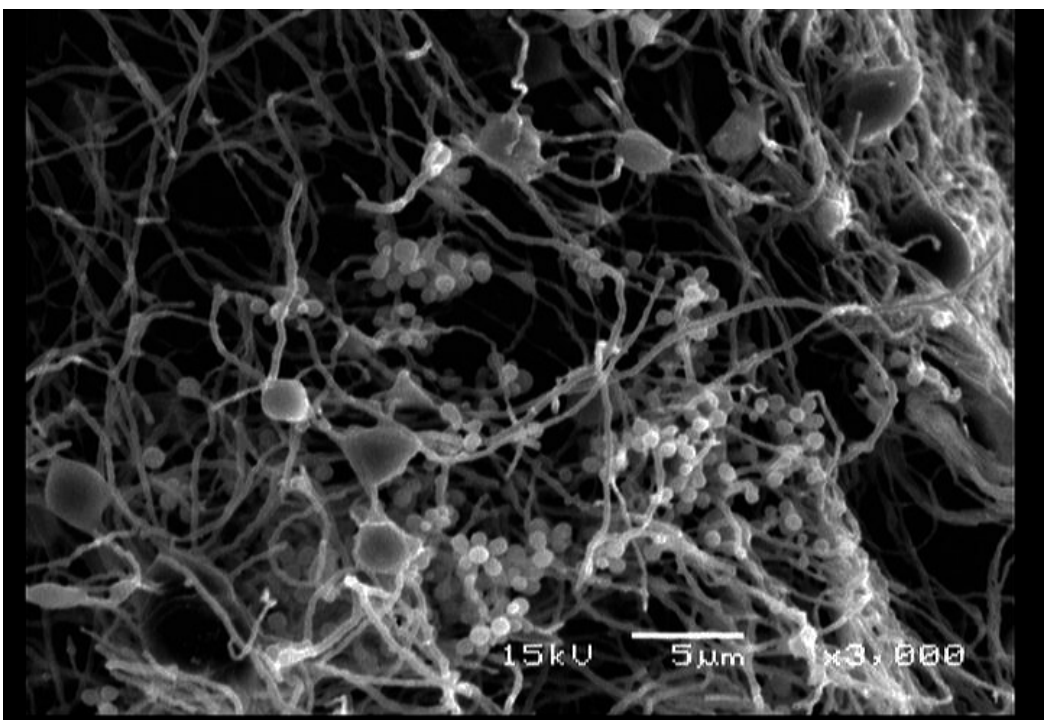

Figure 1. Scanning Electron Microscope view of *Micromonospora* L5. Branched hyphae are observed as well as microspores and large single spores.

The complete genome of *Micromonospora* L5 [38] (NCBI Reference Sequence NC_014815.1) allowed us to find the sequences of different hydrolytic enzymes, cellulases, xylanases, pectinases, and through the BIOCYC Database Collection Enzymes we found the different pathways of the biodegradation of the enzymes (Figures 2, 3, 4).

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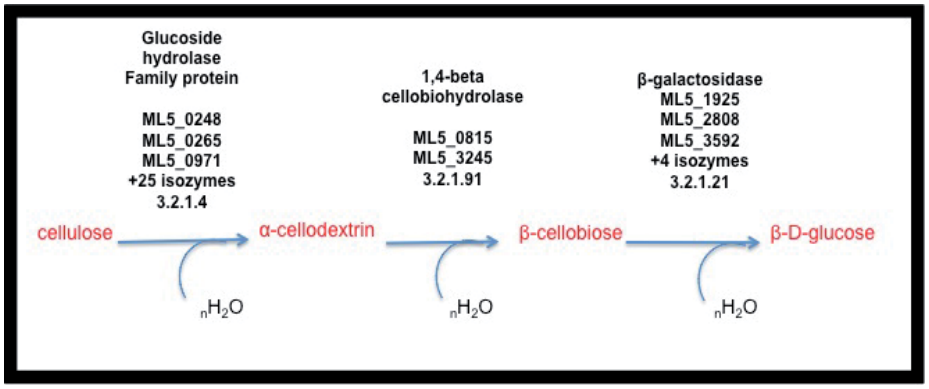

Figure 2. The different pathways of the hidrolysis of cellulose of *Micromonospora* L5 according to BIOCYC Database Collection. Letters in black color indicate the enzymes and its access number in the genome.

Figure 3. The different pathways of the hidrolysis of xylene of *Micromonospora* L5 according to BIOCYC Database Collection. Letters in black color indicate the enzymes and its access number in the genome.

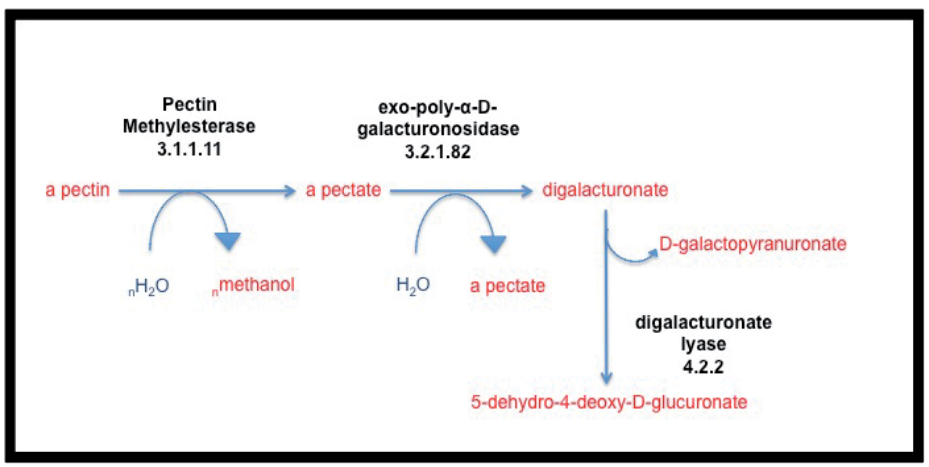

Figure 4. The different pathways of the hidrolysis of pectin of *Micromonospora* L5 according to BIOCYC Database Collection. Letters in black color indicate the enzymes and its access number in the genome.

In addition, the genome of *Micromonopora* L5 contains genes for chitinases. The pathway of the degradation of pectin is shown in Figure 5. The production of chitinases indicates that this strain may confer protection to the plant by hydrolyzing the cell walls of fungal pathogens.

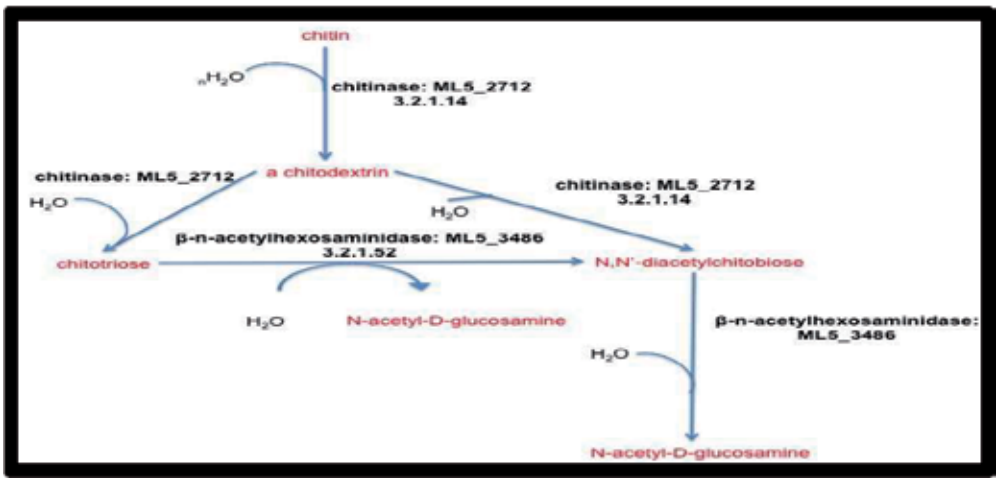

Figure 5. The different pathways of the hidrolysis of chitin of *Micromonospora* L5 according to BIOCYC Database Collection. Letters in black color indicate the enzymes and the access number of enzymes in the genome.

Production of all these enzymes was observed under laboratory conditions and activity was visualized after 8 days of incubation at 28oC and 37oC as shown in Figure 6.

The enzymes endo-β-1,4-glucanase, Exo-β-1,4-glucanase and β-glucosidase of *Micromonospora* showed to be very active at 28oC as well at 37oC (Table 1). The production of 1,4 celobiohydrolase by *Micromonospora* L5 supports its ability as a powerful degrader of cellulose since this enzyme is the most important in the hydrolysis of cellulose.

Enzyme	Enzymatic activity in IU/ml			
	pH 7.0		pH 8.0	
	Temperature		Temperature	
	28°C	37°C	28°C	37°C
Endo-beta-1,4-glucanase	0.800	1.946	0.675	1.688
Exo-beta-1,4-glucanase o celobiohydrolase	0.425	1.114	0.345	0.834
Beta-glucosidase	0.655	1.611	0.415	1.245

Table 1. Quantitative cellulolytic, xylanolytic, and pectinolytic activity of *Micromonospora* L5 after 7 days of culture.

The production of these enzymes also allows *Micromonospora* L5 to play an active role in the degradation of organic matter on its natural habitat, in the carbon cycle and during the composting process of organic domestic wastes. High amounts of solid organic waste are produced all over the world and require safe treatment. The increase of organic waste that

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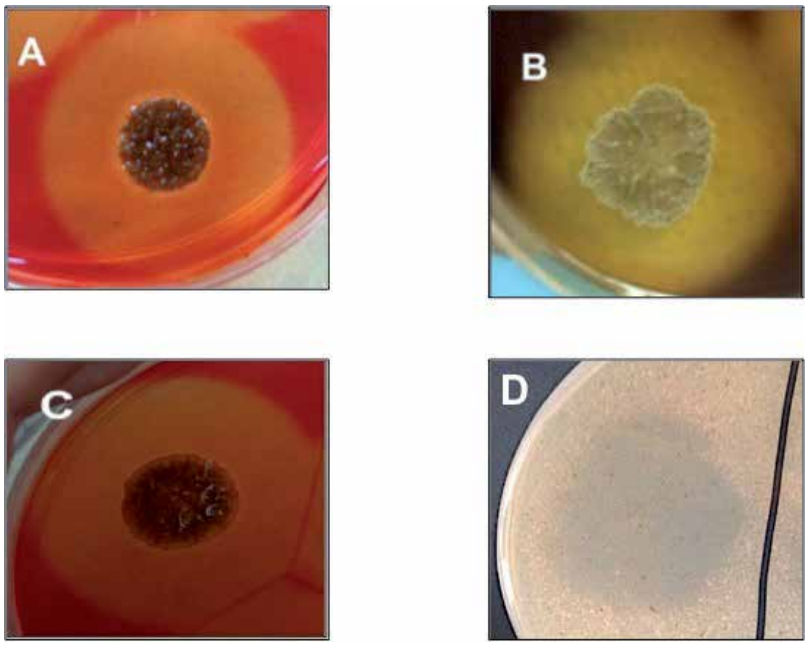

Figure 6. Expression of (A) cellulolytic, (B) pectinolytic, (C) xylanolytic and (D) Chitinase genes of *Micromonospora* L5 at 7 days after inoculation and at a temperature of 37oC.

contains polymerized hydrocarbons requires an efficient composting process. An alternative for improving this process is the search for microorganisms to accelerate the degradation of the organic residues.

3. Enzymatic hydrolysis and applications

3.1. Cellulose

Bioconversion of cellulose, nature's most abundant polysaccharide is accomplished by the enzyme cellulase. Sources of bioconversion of cellulose are wastes of the wood industry, agroindustry, and domestic and garden wastes [39].

Complete enzymatic hydrolysis of cellulose requires synergistic action of three cellulase enzymes: endoglucanase, exoglucanase and beta-glucosidases. Cellulase enzyme systems have a higher activity than the sum of the individual activities of the enzymes, a phenomenon known as synergy collective activity. Cellulase systems are not only an accumulation of enzymes representing all three types, but act in coordination to efficiently hydrolyze cellulose [40].

The cellulose enzymes Endoglucanases III and Cellobiohydrolases I are used in detergents for cleaning textiles. A recent innovation in this industry is the use of cellulases along with protease

and lipase in the detergents [41], although certain enzymes (protease, amylase, lipase, cellulase, mannanase, and pectinase) have been used as catalysts in detergents since the 1960s.

On the other hand the importance of cellulases in the industry of the production of biofuels is the bioconversion of cellulose to molecules of glucose for the fermentation process. A critical step in the development of cellulosic fuels is determining the most favorable conditions for enzymatic saccharification to hydrolyze the cellulose in biomass to fermentable sugars. For a review of cellulases for biofuels see [42].

3.2. Xylan

Xylan is the second most abundant polysaccharide in nature. Xylanases have been reported from actinomycetes [43, 44].

The xylanolytic enzyme system is composed of an array of hydrolytic enzymes, endo-1,4-β-xylanase, xylan-1,4-β-xylosidase, α-glucosiduronase,α-larabinofuranosidase, and acetylxylan esterase.

The most successful application of xylanase is in the paper industry for prebleaching of kraft pulp (process of conversion of wood into wood pulp) to minimize the use of corrosive chemicals in the subsequent treatment stages of pulp [45]. Apart from its use in the paper industry, xylanases are also used as food additives to poultry [46] for the hydrolysis of arabinoxylanes contained in the forage crops conducting to a good nutrimental efficiency of the prime materials [47]. The use of xylanase in combination with pectinase and cellulase are utilized for clarification of fruit juices and degumming of plant fiber sources such as flax [48]. For a review of xylanases and their applications see review [49].

3.3. Pectin

Pectic substances are present in the primary cell wall and are the major component of the middle lamellae, they are responsible for the structural integrity and cohesion of plant tissues. Microbial pectinases are important virulence mechanisms in the phytopathologic process and in plant-microbe symbiosis. The endophytes from soil enter the host plant by colonizing the cracks formed by the emergence of lateral roots from where they spread to the intercellular spaces in the root.

Soil microbial pectinases also participate in the decomposition of dead plant material, contributing to the natural carbon cycle.

Considering the industrial pectinase production alone occupies about 25% of the overall manufacturing of enzyme preparations for food, the use of pectinolytic enzymes in the industry for juice improves the fruit juice yield. The crushing of pectin-rich fruits results in high viscosity juice, and pectinase addition in the extraction process decreases the juice viscosity and degrades the gel structure. In several processes, pectinolytic enzymes are applied together with other cell wall degrading enzymes such as cellulases and xylanases. The mixture

of pectinases and cellulases has been reported to improve more than 100% the juice extraction yields [50]. For a review of the industrial application of microbial pectinolytic enzymes see [51].

Apart from its use in the food industry for juice production, pectinolytic enzymes are widely used in wine production. The use of pectolytic enzymes, as both clarifying and color extractors, to improve the chromaticity and stability of red wines, gives wines better chromatic characteristics that are more stable over time than the control wines. They show lower loss of red, lower increase in tonality, reach greater levels of brightness much earlier and remain less turbid. Also their chromatic intensity is maintained throughout the two years of storage at fairly acceptable levels [52].

3.4. Chitin

Chitin is the second most abundant natural polymer and distributed as a structural component of crustaceans, insects, other arthropods, and as a component of the cell walls of most fungi.

Chitinase has received attention due to its use as a biocontrol agent. Plant pathogenic fungi is the major problem for agricultural food production. Control of plant pests by the application of biological agents holds great promise as an alternative to the use of chemicals. Chitinases are directly involved in defense against fungal pathogens by hydrolyzing the cell walls. The chitinase genes can also be useful

in developing transgenic plants leading to the plant to develop resistance to various fungal and insect pests [53]. This enzyme may also be useful in the management of sea food waste industries.

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Chapter 14

Actinobacteria — A Biofactory of Novel Enzymes

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Additional information is available at the end of the chapter

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Abstract

Biocatalysis offers green and clean solutions to chemical processes and is emerging as an effective alternative to chemical technology. The chemical processes are now carried out by biocatalysts (enzymes) which are essential components of all biological systems. However, the utility of enzymes is not naive to us, as they have been a vital part of our lives from immemorial times. Their use in fermentation processes like wine and beer manufacture, vinegar production, and bread making has been practised for several decades. However, a commercial breakthrough happened during the middle of the 20th century with the first commercial protease production. Since then, due to the development of newer industries, the enzyme industry has not only seen a remarkable growth but has also matured with a technology-oriented perspective. Commercially available enzymes are derived from plants, animals, and microorganisms. However, a major fraction of enzymes are chiefly derived from microbes due to their ease of growth, nutritional requirements, and low-cost downstream processing. In addition, enzymes with new physical and physiological characteristics like high productivity, specificity, stability at extreme conditions, low cost of production, and tolerance to inhibitors are always the most sought after properties from an industrial standpoint. To meet the increasing demand of robust, high-turnover, economical, and easily available biocatalysts, research is always channelized for novelty in enzyme or its source or for improvement of existing enzymes by engineering at gene and protein levels. The novel actinobacteria and their industrially important enzymes will assist effective productivity and fulfill the requirements of industries.

Keywords: actinobacteria, extra-cellular enzymes, applications

1. Introduction

Among the microorganisms, actinobacteria are of special interest since they are known to produce chemically diverse compounds with a wide range of biological activities. Actinobac-

teria, the filamentous Gram positive bacteria are primarily saprophytes of the soils, where they contribute notably to the turnover of complex biopolymers such as lignocellulose, hemicellulose, pectin, keratin, and chitin. Undoubtedly, they are also well known as a rich source of antibiotics, enzymes, and other bioactive molecules, and are of considerable importance in pharmaceutical and other industries [1].

The ever-increasing requirement for enzymatic preparations is being met by such classical sources as animal and higher plant tissues, and that has stimulated the search for similar enzymes from the microbial world. The value of microorganisms, including actinobacteria, in the production of enzymes is enhanced by their relatively high yields, cost-efficiency, and susceptibility to genetic manipulation [2]. At present, enzymes of microbial origin are widely used in food processing, detergent manufacturing, the textile and pharmaceutical industries, medical therapy, bioorganic chemistry, and molecular biology. The wide use of enzymes reflects their characteristic specificity of action as biocatalysts. However, enzymes of identical substrate profile produced by different microorganisms may significantly vary in the optimal conditions of their productivity. For this reason, it is necessary to obtain microorganisms which produce enzymes with required substrate specificity, at particular temperature and pH ranges demanded by the production process. The biochemical heterogeneity, ecological diversity, and exceptional capacity of actinobacteria for secondary metabolites production make them an obvious target for enzymes displaying new activities and/or specificities. For many years, actinobacteria are best known as the source of majority of antibiotics. More recently, they have been found to be a promising source of a wide range of industrially important enzymes. Keeping this in mind and recognizing the significance of actinobacteria, especially *Streptomyces* species, as a source of novel enzymes, many studies have focused to understand the diversity of marine actinobacteria and to screen the enzyme-producing ability of actinobacteria occurring in the less explored hypersaline saltpan, mangrove, and marine sediments.

2. Significance of actinobacterial enzymes

Actinobacteria are one of the ubiquitous dominant groups of Gram positive bacteria. Actinobacteria have been commercially exploited for the production of pharmaceuticals, neutraceuticals, enzymes, antitumor agents, enzyme inhibitors, and so forth [3]. These bioactive compounds are of high commercial value, and hence actinobacteria are regularly screened for the production of novel bioactive compounds. A wide array of enzymes applied in biotechnological industries and biomedical fields have been reported from various genera of actinobacteria. Since there is vital information available due to the advent of genome and protein sequencing data, actinobacteria has been continuously screened for the production of proteases, cellulases, chitinases, amylases, xylanases, and other enzymes. The industrial applications of several actinobacterial enzymes are given in Table 1.

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Use	Enzyme	Applications
Detergent (laundry and dish wash)	Protease	Protein stain removal
	Amylase	Starch stain removal
	Lipase	Lipid stain removal
	Cellulase	Cleaning, color clarification, anti-redeposition (cotton)
	Mannanase	Mannanan stain removal (reappearing stains)
Starch and fuel	Amylase	Starch liquefaction and saccharification
	Amyloglucosidase	Saccharification
	Pullulanase	Saccharification
	Glucose isomerase	Glucose to fructose conversion
	Cyclodextrin-glycosyltransferase	Cyclodextrin production
Xylanase	Viscosity reduction (fuel and starch)	
Food (including dairy)	Protease	Milk clotting, infant formulas (low allergenic), flavor
	Lipase	Cheese flavor
	Lactase	Lactose removal (milk)
	Pectin methyl esterase	Firming fruit-based products
	Pectinase	Fruit-based products
	Transglutaminase	Modify visco-elastic properties
Baking	Amylase	Bread softness and volume, flour adjustment dough conditioning
	Xylanase	Dough stability and conditioning (*in situ* emulsifier)
	Lipase	Dough stability and conditioning (*in situ* emulsifier)
	Phospholipase	Dough strengthening
	Glucose oxidase	Dough strengthening
	Lipoxygenase	Bread whitening
	Protease	Biscuits, cookies
	Transglutaminase	Laminated dough strengths
Animal feed	Phytase	Phytate digestibility – phosphorus release
	Xylanase	Digestibility
	β-Glucanase	Digestibility
Beverage	Pectinase	De-pectinization, mashing
	Amylase	Juice treatment, low calorie beer
	β-Glucanase	Mashing
	Acetolactate decarboxylase	Maturation (beer)
	Laccase	Clarification (juice), flavor (beer), cork stopper treatment
Textile	Cellulase	Denim finishing, cotton softening

Use	Enzyme	Applications
	Amylase	De-sizing
	Pectatelyase	Scouring
	Catalase	Bleach termination
	Laccase	Bleaching
	Peroxidase	Excess dye removal
Pulp and paper	Lipase	Pitch control, contaminant control
	Protease	Biofilm removal
	Amylase	Starch-coating, de-inking, drainage improvement
	Xylanase	Bleach boosting
	Cellulase	De-inking, drainage improvement, fiber modification
Fats and oils	Lipase	Transesterification
	Phospholipase	De-gumming, lyso-lecithin production
Organic synthesis	Lipase	Resolution of chiral alcohols and amides
	Acylase	Synthesis of semisynthetic penicillin
	Nitrilase	Synthesis of enantiopure carboxylic acids
Leather	Protease	Unhearing, bating
	Lipase	De-pickling
Personal care	Amyloglucosidase	Antimicrobial (combined with glucose oxidase)
	Glucose oxidase	Bleaching, antimicrobial
	Peroxidase	Antimicrobial
	L-Asparagine	Antitumor
	Neuraminidase	Antiviral agents
	Aminoacylase	Regulation of urea cycle

Source: Goodfellow (1988).

Table 1. Industrial applications of actinobacterial enzymes

3. Types of actinobacterial enzymes

3.1. Aminoacylase

Aminoacylase (N-acylamino-acid amidohydrolase) catalyzes the hydrolysis of acylated D- or L-amino acids to D- or L-amino acids and an appropriate carboxylic acid: N-acetyl-(D) or (L)-amino acid> carboxylic acid+(D)-or (L)-amino acid (Figure 1). Aminoacylases are interesting and ever-increasing enzymes due to the growing demand in the pharmaceutical industry for optically active amino acids. In enzymology, an aminoacylase is an enzyme that catalyzes the following chemical reaction:

http://dx.doi.org/10.5772/61436

N-acyl-L-amino acid + H_2 *f* carboxylate + L-amino acid

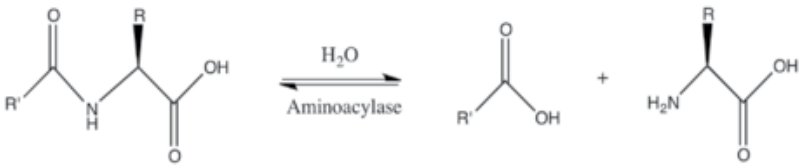

Figure 1. Catalytic reaction of aminoacylase

This enzyme belonged to the family of hydrolases, those acting on carbon-nitrogen bonds other than peptide bonds, specifically in linear amides. This enzyme mainly concerns D-amino acids, both natural and synthetic, such as D-phenylglycine and its derivatives which are used for the production of semisynthetic penicillins and cephalosporins. Phenylglycine obtained synthetically as a racemic mixture can be split into enantiomers by chemical or enzymatic reactions. The latter are usually applied because they are simpler and more efficient. Two methods have been proposed for the isolation of pure enantiomers of D-amino acids using enzymatic hydrolysis of racemic mixtures of their N-acetylated derivatives. In the first method, a stereospecific enzymatic hydrolysis of N-acetyl-DL-amino acids has been used to obtain a mixture of D-amino acid and unaffected N-acetyl-L-amino acid which has to be racemized before its reuse in the process, while in the second method enzymatic cleavage of N-acetyl-L-amino acid, a component of the racemic mixture, results in a mixture of L-amino acid and non-hydrolyzed N-acetyl-D-amino acid. D-amino acid is obtained from the latter as a result of chemical deacylation of the N-acetylated derivative. The second method of obtaining D-amino acids is the one applied in practice. D-aminoacylases are uncommon in microorganisms, though Szwajcer *et al*. [4] reported the occurrence of one such enzyme from *Micrococcus agilis*. Many examples of D-aminoacylases have been found in some species of actinobacteria; 427 strains of *Streptomyces* and 16 strains of *Streptoverticillium* were screened for D-aminoacylases and found only in four species of streptomycetes, namely *S. olivaceus, S. roseiscleroticus, S. sparsogenes,* and *S. tuirus* [5]. All the species produced D-aminoacylase intracellularly when inducers such as D-leucine, D-phenylglycine, D-valine, and their N-acetylated derivatives were supplemented with the production medium [6]. The D-aminoacylases obtained from *S. olivaceus* and *S. tuirus* were purified and characterized according to their substrate specificity. Both enzymes were active at pH 7.0 and both were hydrolyzed hydrophobic N-acetyl-D-amino acids rather than hydrophilic amino acids. Extracellular production of both L-aminoacylase and penicillin V amidase has been demonstrated in *Mycobacterium smegmatis* [7] and several strains belonging to the genus *Streptoverticillium* [8]. L-aminoacylase isolated from a *Streptoverticillium* sp. [9] displayed a high hydrolytic activity toward N-acetylated aliphatic and aromatic L-amino acids [10]. Additionally, N-acyl-L-amino acids cannot be used directly as building blocks for proteins and must first be converted to L-amino acids by aminoacylase. Again, the L-amino acid products can be used for biosynthesis or catabolized energy.

3.2. Amylase

Amylase comprises a group of industrial enzymes having approximately 25% of the global enzyme market. Specifically, an extracellular amylase with the ability to digest raw starch has found important applications in bioconversion of starches and starch-based substrates. The level of alpha amylase activity in various fluids of human body is of clinical importance, e.g., in diabetes, pancreatitis, and cancer research, while plant and microbial alpha amylases are used as industrial enzymes. Starch-degrading amylolytic enzymes are of great significance in biotechnological applications ranging from food, fermentation, and textile to paper industries (Table_1). Although amylases can be derived from several sources, such as plants, animals, and microorganisms, the enzymes from microbial sources are generally used to meet industrial demands and have made significant contribution to the production of foods and beverages in the last three decades. The microbial amylases have almost completely replaced the starch hydrolyzing chemicals in starch processing industry.

Like most microorganisms, actinobacteria can also survive in both mesophilic and thermophilic conditions; they have the ability to degrade starch by hydrolysis [15]. The occurrence of amylases in actinobacteria has been a well-known phenomenon since it was established that several representatives of the genera *Nocardia* and *Streptomyces* display amylolytic activity when cultivated on media with maltose [16], although, amylolytic enzymes quite often occur in mesophilic actinobacteria. Unfortunately, only a few of them have been studied in detail, as their enzymes are similar to bacilli amylases which are relatively thermolabile, thus limiting their value in biotechnological processes. On the basis of the literature survey, the more promising amylase producers are the strains of *Streptomyces hygroscopicus* [17], *S. limosus* [18], and *S. praecox* [19] as a result of extensive screening program from among others. To date, amylases from *S. hygroscopicus* and *S. praecox* have been used for the commercial preparation of high-maltose syrups [20]. More attention has been paid to thermostable amylases produced by the thermophilic actinobacteria namely *Thermomonospora curvata* [21] and *T. vulgaris* [22] and by a *Thermoactinomyces* sp. [23; 24]. The amylases from *Thermomonospora* species and *T. vulgaris* are highly active and stable at 60°-70°C and act at slightly acidic and neutral pH values (Table 2) [25].

Enzyme producers	Enzyme	Temperature (°C)	pH	Reference
T. fusca NTU22	α-amylase	60	7.0	Chao-Hsun and Wen-Hsiung, 2007
Streptomyces transformant T3-1	Cellulase	50	6.5	Hung-Der and Kuo-Shu, 2003
Thermoactinomycetes sp. HS682	Protease	70	11·0	Tsuchiya *et al.,* 1991
Streptomyces rimosus R6-554W	Lipase	50	9.0-10.0 (4-10)	Abrami *et al.,* 1999
Thermomonospora fusca	Xylanase	60-80	7.0 (6-8)	McCarthy *et al.,* 1995

Table 2. Industrially important enzymes from thermophilic actinobacteria

3.3. β-N-Acetyl-D-Glucosaminidase

β-N-acetyl-D-glucosaminidase (2-acetamido-2-deoxy-β-D-glucoside) is frequently encountered in microorganisms, higher plants, and mammalian tissues. This enzyme splits hydrolytically chitobiose, N,N'-diacetylchitobiose moieties of asparagines-linked oligosaccharides of various glycoprotein and hydrolyzes N-acetyl-β-D-galactosaminidases, yielding oligosaccharide chains from glycoproteins [30]. Thus, it has been found to be very useful for the structural determination of the carbohydrate moiety of several glycoproteins and for studying their biochemical functions and biosynthesis [31]. Generally, actinobacteria producing enzymes are synthesized at extracellular region including endo-β-N-acetyl-D-glucosaminidase H [32] and endo-β-N-acetyl-glucosaminidase L [33] isolated from *Streptomyces griseus* (formerly *S. plicatus*). The enzyme were designated as endo-β-N-acetyl-D-glucosaminidase L [34; 35] and appear to be extremely useful for structural determination of ovoalbumin and several other glycoproteins [30; 36].

3.4. 1, 3-α and 1, 3-β Glucanase

Endo-l, 3-α-D-glucanases (1, 3-α-D-glucan glucanohydrolase) hydrolyzes fragments of polysaccharides that contain consecutive 1, 3-linked α-D-glucosyl residues. Consequently, 1,3-α -D-glucanases are useful in detection of 1,3- α -D-linkages sequences in dextran as well as provide a route for 1,3-α-D-glucans study in fungal cell walls [37; 38]. These enzymes are produced by fungi and bacteria and are quite common among actinobacteria. Therefore, these enzymes may be useful as protective agents for odontological purposes. The presence of mutan-hydrolyzing enzymes was detected in *S. chartreusis* and *S. werraensis* [39]. Endo-1,3-β-D-glucanases (1,3-β-D-glucan glucanohydrolase) occur in bacteria, fungi, higher plants, and actinobacteria. Investigations of 1,3-β glucanases isolated from actinobacteria have been mainly carried out for their ability to degrade the cell walls of yeasts and fungi [40]. Several species of actinobacteria excrete 1, 3-β-glucanases together with chitinases. A laminarinase system consisting of three different types of l,3-β glucanases and chitinases was isolated from *S. rimosus* [41]. When laminarin (1,3- β glucan) was used as substrate, laminaritriose was obtained as the major product of one type of endo-1,3-β glucanase in addition to oligomeric breakdown products. The second laminarin-degrading (exo-splitting) enzyme yields predominantly laminaribiose. Another exo-l,3-β glucanase liberates glucose but not oligosaccharides from the nonreducing end of laminarin. The mycolase system produced an extracellular complex when the strain was grown on media with crab-shell chitin and fungal mycelia. δ1,3-β glucanases have also been isolated from *Streptomyces* (*Actinomyces*) *cellulosae* [42] and *Streptomyces* sp. [43]. Some of them, such as 1,3-β glucanase isolated from *S. murinus*, are used in wine preparation [44]. The preparations of 1,3-β glucanases have also been used to obtain some saccharides. The enzymatic preparation of laminaribiose (3-0-β gluco-pyranosyl-D-glucose) was achieved by the hydrolysis of curdlan (1,3- β- D-glucose) with the l,3-β-glucanase system from *Streptomyces* sp. K.27-4 [45]. The hydrolysate obtained consisted mainly of glucose and laminaribiose in an approximate ratio of 1:1 by weight. The application of the yeast *Schizosaccharomyces pombe*, which selectively metabolized all the glucose present in the hydrolysate, resulted in crystalline laminaribiose at 30% of yield.

3.5. Cellulase

Cellulose, which forms almost half of the dry weight of the earth's biomass, is an unbranched polymer consisting of D-glucose units linked by 1,4-β glycosidic bonds. This macromolecule has a complex crystalline structure, is insoluble in water, and is quite resistant to depolymerizing enzymes and chemical reagents. Under natural conditions, cellulose is almost always combined with hemicellulose and lignin [46], which makes its degradation by microorganisms even more difficult.

Investigations on the mechanism of cellulose degradation and its possible applications have been carried out for many years [47]. Recently, the enzymatic hydrolysis of cellulose for D-glucose production has aroused an ever-increasing interest [48; 49]. Cellulose-degrading enzymes are excreted by microorganisms into the surrounding environment and as with most enzymes-degrading biopolymers they constitute a multicomponent lytic complex that acts synergistically on the cellulose. The cellulolytic system consists of three major components: 1,4-β glucan glucanohydrolase acting as endoglucanase, l,4-β-D-glucan cellobiohydrolase displaying exoglucanase activity, and β-glucosidase, which splits cellobiose. The enzymatic system of cellulases operates synergistically, i.e., endoglucanases make random scissions of the cellulose chain yielding glucose and oligosaccharides; exoglucanases attack the nonreducing end of cellulose forming cellobiose; and finally cellobiases hydrolyze cellobiose to glucose [5042]. Members of several mesophilic and thermophilic actinobacteria have been studied for their ability to degrade cellulose. *Thermomonospora* species have been found to be highly cellulolytic [51-55]. Cellulases produced by representatives of *Thermomonospora* species are multiple, extracellular exoglucanases and endoglucanases at pH of 6.0 and temperature ranging from 60° to 70°C; they also display considerable heat stability. The mesophilic actinobacteria known to produce cellulolytic complexes include *Streptomyces antibioticus* [56], *S. flavogriseus* [57-62], and *S. viridosporus* [63]. Mesophilic streptomycetes also produce complex cellulases at pH between 5 and 7; they show their highest activity at 40°-55°C (Table_2). Both mesophilic and thermophilic actinobacteria produced cellulolytic complexes when cultivated on media supplemented with powdered cellulose.

3.6. Protease

Proteases, generally classified into exopeptidases (cleave off peptide bonds from the ends of the protein chain) and endopeptidases (cleave peptide bonds within the protein) (Figure 2.), are the major industrial enzymes and fulfill more than 65% of the global market need [64]. These enzymes are extensively used in the food, pharmaceutical, leather, and textile industries [64; 65]. Among the extremophilic sources, thermostable proteases have been reported from certain haloalkaliphilic bacteria and actinobacteria [66; 67]. With the increasing demand of the enzymes, there will be an ever-increasing need for stable biocatalysts capable of withstanding extreme conditions of operation. Proteases generally activate a nucleophile, which will in turn attack the carbon of the peptide bond. The electrons in the carbon-oxygen double bond migrate onto the oxygen as the nucleophile attaches itself. This tetrahedral intermediate is a highly energetic intermediate, and the protease will stabilize this intermediate. The intermediate will then decompose, usually releasing the two peptide fragments.

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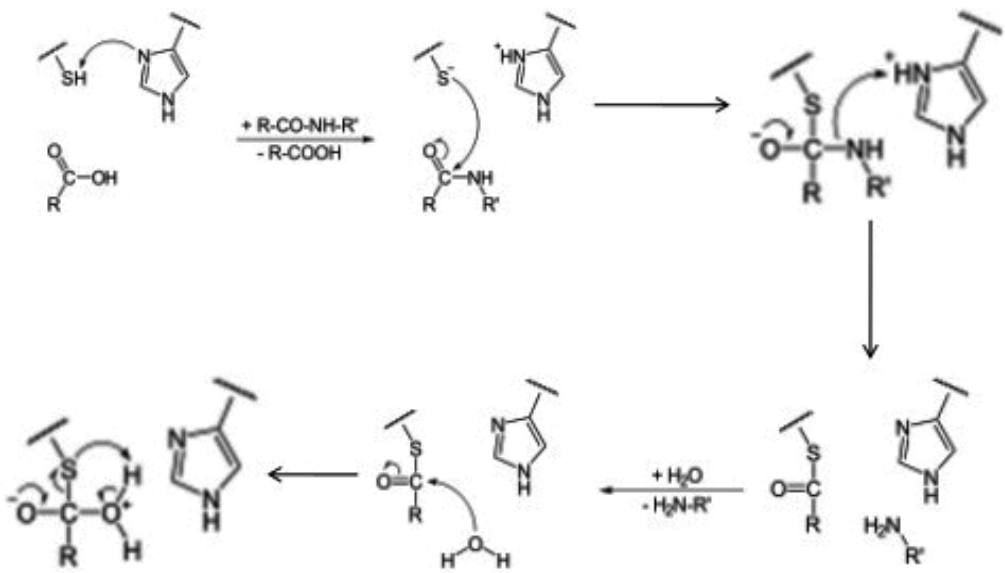

Figure 2. Catalytic mechanism of protease

The ability to produce a variety of proteolytic enzyme is a well-known phenomenon in mesophilic actinobacteria; *Streptomyces* protease including "pronase 7M" (*S. griseus*) and "fradiase 7M" (*S. fradiae*) are commercially useful. While alkaline proteases from bacteria are extensively characterized, similar attention has not been paid to alkaliphilic actinobacteria namely *S. nigellus, S. albidoflavus,* and other genus *Nocardiopsis*, *Thermomonospora,* and *Thermoactinomyces* [68].

3.7. Chitinase

Chitin, a polymer occurring in crustaceans, fungi, and insects, is a fibrillar 1,4 linked 2-acetamido-2-deoxy-β-D-glucan with acetyl groups attached to nitrogen to various extents. It is found in three polymeric forms with various degrees of crystallinity. Fully deacetylated chitin is known as chitosane [69]. Enzymatic hydrolysis of chitin, liberating free N-acetyl-D-glucosamine, is caused by the chitinolytic complex which consists of chitinase (polyβ-1,4- (2-acetamido-2-deoxy)-D-glucose glycanohydrolase) and chitobiase (β-N-acetyl-D-glucosaminidase) (Figure 3.). As a result of the action of chitinase complex, chitobiose and chitotrose are released. Chitinases are specific to linear polymers of N-acetylglucosamine, but they do not split chitobiose. They hydrolyze chitin to chitobiose and to a lesser extent to chitotriose [70].

Chitinolytic complexes commonly occur in bacteria, fungi, and especially in actinobacteria. The chitinase have been isolated from culture filtrates of *S. griseus* [71], *S. antibioticus* [70], *Amycolatopsis* (*Streptomyces*) *orientalis* [72], and several strains of *Streptomyces* spp. [73-75].

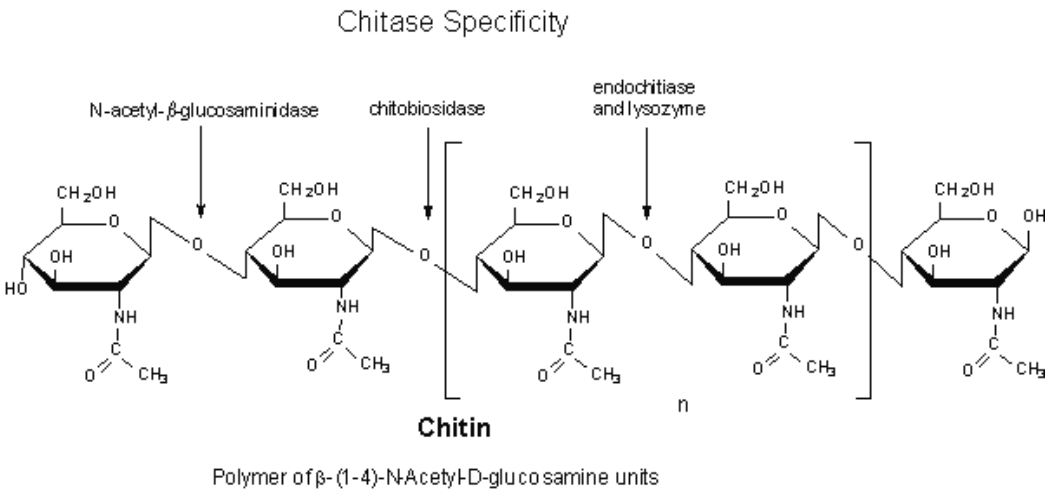

Figure 3. Polymer of β- (1-4)-N-acetyl-D-glucosamine units

Chitinases are produced in abundance where strains are cultivated on chitin-supplemented media. Purified chitinases are more active at pH 5.0 but they are not heat-stable. Their properties have been considered from the view of fungal cell wall degradation [76] and utilization of chitin wastes [77].

3.8. Lipase

Cholesterol esterase, which converts cholesterol esters into free cholesterol, is used in clinical tests for determining the cholesterol level in blood serum [78]. Until now, little is known about the properties of cholesterol esterases. These enzymes differ not only in their optimum pH for production but also in their substrate specificity. For example, cholesterol esterase isolated from *S. lavendulae* had lipolytic activity [79]. A lipase of the arylesterase group able to hydrolyze specifically phthalate esters to a free phthalic acid and simple n-alcohols was isolated from a *Rhodococcus (Nocardia) erythropolis* [80]. The enzyme, which was most active at pH 8.6 and at 42°C, hydrolyzed several phthalate esters and to a lesser extent olive oil and tributyrin. The lipase production by several *Streptomyces* strains was reported by Chakrabarti *et al.* [81] but details were not given on substrate specificity. There is an increasing interest in lipases, especially those which display high stereo-specificity and may be useful for resolution of racemic acids and alcohols applied as chiral substrates in organic synthesis [82]. More attention has been paid to lipases from actinobacterial origin since these microorganisms are known for their ability to produce various secondary metabolites and hence provide a potential source of enzymes with substrate specificity.

3.9. Phospholipases

Phospholipases, the enzymes capable of selective cleavage of ester bonds in glycerophosphatides, occur widely in both animal and plant kingdoms. Because of their high specificity, they

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are used for the analysis of phospholipid components of biological membranes as well as for clinical diagnostic tests. Phospholipases are classified into four groups, A, B, C, and D (Table 3). Serum choline phospholipids are hydrolyzed by phospholipase-D and the amount of liberated choline can be estimated quantitatively. Phospholipase-D from streptomycetes has been found useful for the determination of serum choline-phospholipids and in clinical diagnostic tests [83; 84].

Enzyme	Name of the producer	Optimum pH	Leading references
Phospholipase - A_2	*Streptomyces cinnamomeus*	7.0	Okawa and Yamaguchi (1976a)
Phospholipase - B	*S. hiroshimensis*	9.0	Walker and Walker (1975)
Phospholipase - C	*S. griseus*	7.5	Verma and Khuller (1983)
Phospholipase - D	*S. chromofuscus*	8.0	Imamura and Horiuti (1979)

Table 3. Practical significance of phospholipases isolated from actinobacteria

3.10. Xylanase

Xylan, a hemicellulose, is composed of l,4-β-linked D-xylose units that form a linear backbone to which 4-O-methyl-D-glucuronic acid and L-arabinose are attached as side chains (Figure 4). This polymer, which occurs together with cellulose, is degraded by xylanases. Xylanases can be found in large amounts in both microorganisms as well as several invertebrates [85]. Together with other carbohydrases, xylanases play an important role in the degradation of terrestrial biomass [86]. Like cellulases they occur in microorganisms in the form of extracellular complexes, which consist of endo and exoxylanases that differ in substrate specificity. Xylanases produced by mesophilic actinobacteria belong to the endotype (l,4-β-D-xylan xylanohydrolase). They have been isolated and purified from several species of streptomycetes, such as *S. flavogriseus* [67], *S. lividans* [87], and *Streptomyces* sp. [88]. These enzymes were produced by microorganisms grown on media with xylan or its hydrolysates as a carbon source and/or in the presence of nonmetabolizable inducer [89]. The isolated xylanases exhibited their higher activity at pH 5.0-7.0 and at 40-60°C. Little attention has been given to xylanases produced by thermophilic actinobacteria [90; 68]. Thermostable xylanases isolated from *Thermomonospora* strains are heat-stable and most active at 60-70°C and at pH from 5.0 to 8.0. Apart from attempts to apply them to biodegradation of hemicelluloses and xylanases, they have also been used in the food industry for the production of D-xylose. Since this is not assimilated by mammalian organisms, it is used as an artificial sweetener in dietetic preparations [86]. An original method of obtaining xylobiose was developed by Kusakabe *et al.* [91] who prepared a pure xylobiose using a xylan hydrolysate from corncobs and rice straw and xylanase produced by *Streptomyces* sp. E-86. D-xylose, formed during the hydrolysis, was eliminated by the yeast *Candida parapsilosis,* which utilized xylose as a carbon source.

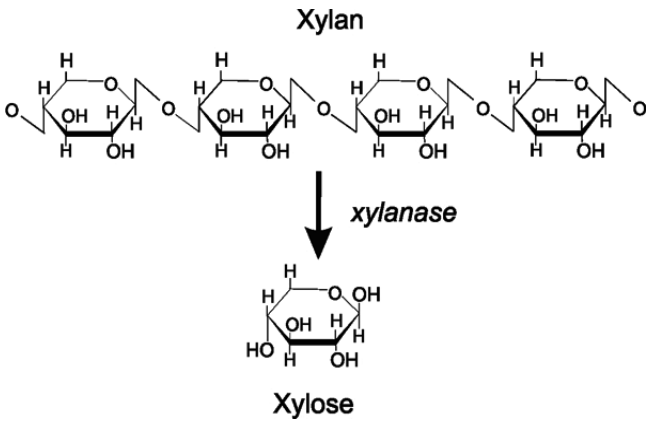

Figure 4. Structure of xylan and digestion of xylose by using xylanase

3.11. N-Acetylmuramidase

N-acetylmuramidase, an enzyme resembling lysozyme in action, cleaves the N-acetyl-muramyl-β,4-N-acetylglucosamine bonds of the polysaccharide chain of peptidoglycan, liberating free-reducing groups of N-acetylmuramic acid. N-acetylmuramidase (mucopeptide N-acetylmuramoyl-hydrolases) belongs to the group of bacteriostatic enzymes comprising glycosidases that hydrolyze peptidoglycan (murein), which is basic component of the bacterial cell wall. Murein, composed of glycan strands consisting of alternating acetylated amino sugars, N-acetylglucosamine and N-acetylmuramic acid linked by β (1-4) glycosidic bonds mutually cross-linked by peptide chains, forms a mono- or multilayer net covered with lipopolysaccharides, phospholipids, and lipoproteins. The peptide moiety of murein is composed of short chains of unbranched aliphatic amino acids and/or amino acids that form stem peptides linked to the carboxyl group of N-acetylmuramic acid and cross-linked by interpeptide bridges [92]. Peptidoglycans, especially available high in Gram-positive bacteria, are highly diversified. The determination of the primary structure of peptidoglycans has revealed differences between the bacteria and it provides significant taxonomic tools [93]. The enzyme was isolated from *Streptomyces globisporus* [94; 95], and is also found in other streptomycetes, including *Streptomyces* sp. [96] *S. griseus* [97], *S. erythraeus* [98], *S. (Actinomyces) levoris* [99], and *S. rutgersensi* [100].

3.12. Neuraminidase

Neuraminidase (acylneuraminyl hydrolase) splits 2,3-, 2,6-, and 2,8- and 2,9-glucosidic linkages which join terminal nonreducing N- or O-acetylated neuraminyl residues present in oligosaccharides and glycoprotein. Neuraminidases or sialidases occur widely in bacteria, viruses, animal tissues, and biological fluids [101]. These enzymes, isolated from various sources and differing in their substrate specificity, are applied in a wide area of biological and immunological research, particularly in cell surface and clinical studies [23; 102]. In actino-bacteria, neuraminidases have been found in representatives of the genera *Corynebacterium,*

Mycobacterium and *Nocardia* [101], *Streptomyces griseus* [103; 104], *Actinomyces naeslundii,* and *A. viscosus* [105]. Neuraminidases isolated from *Streptomyces* strains showed optimum activity at pH 3.5-5.0 and at 50°-60°C. They differed notably in their properties, including substrate specificity. Neuraminidase from *Clostridium perfringens* and *Vibrio cholerae* are able to split all types of sialic acid linkages.

3.13. Peptide hydrolase

Proteolytic enzymes of microbial origin were classified by Morihara [106], on the basis of their catalytic mechanism, into serine, thiol, metallo, and acid proteases according to the general systematic scheme introduced by Hartley [107]. The ability to produce a variety of proteolytic enzymes is a well-known phenomenon in mesophilic actinobacteria [106]. There is also an increasing interest in proteases derived from thermophilic actinobacteria including members of the genera *Thermoactinomyces* (*Micromonospora*), *Thermomonospora,* and *Streptomyces*. These actinobacteria are still not fully exploited as a potential source of thermostable enzymes acting not only over a wide range of pH but also great number of proteases with wide spectrum of substrate specificity. An increasing interest is observed in the application of actinobacterial proteases in bioorganic chemistry. For commercial purposes, they are routinely obtained as by-products formed during biosynthesis of antibiotics in the logarithmic phase of growth [107], from the fermentation broths of *Streptomyces fradiae* [109], *S. griseus* [110], and *S. rimosus* [108]. The preparations obtained are enzymatic complexes that contain a mixture of endo- and exopeptidases; as commercial preparations they are known as pronase (*S. griseus*) or fradiase (*S. fradiae*). Actinobacterial proteolytic complexes provide an excellent source of protease in various substrates specificity. Of the actinobacterial protease complexes available, the most attention has been given to pronase obtained from *S. griseus*.

Pronase like enzymes are produced not only by *S. griseus* but also by members of several other species of streptomycetes. A trypsin-like serine protease was isolated from *S. erythraeus* [111] and *S. fradiae* [112]; carboxypeptidase T, which has a mixed specificity compared with pancreatic carboxypeptidase A and B, is produced extracellular by a *Thermoactinomyces* strain [113]. Aminopeptidases have been isolated from culture filtrates of *S. rimosus* grown under conditions conducive to the industrial biosynthesis of oxytetracycline [114] and from other *Streptomyces* sp., including *S. mauvecolor* [34], *S. peptidofaciens* [115], and *S. sapporonensis* [116].

3.14. L-Asparaginase

The important application of the L-asparaginase enzyme is in the treatment of acute lymphoblastic leukemia, Hodgkin's disease, acute myelocytic leukemia, acute myelomonocytic leukemia, acute and chronic lymphocytic leukemia, lymphosarcoma treatment, reticulosarbom, and melanosarcoma [117]. L-asparaginase broadly distribute among the plants, animals, and microorganisms. The microbes are a better source of L-asparaginase, because they can be cultured easily and the extraction and purification of enzyme from them is also convenient, facilitating large-scale production [118]. L-asparaginase has been arousing considerable interest as it displays an antineoplastic activity against a variety of murine neoplasms. As an antineoplastic agent, L-asparaginase from *Escherichia coli* has been the most widely available

[119], but the enzyme also occurs in actinobacteria. It has been isolated from several actinobacteria including *Mycobactserium bovis* [120] and *M. tuberculosis* [121]. These enzymes were active at pH 8-9. L-asparaginase obtained from *Streptomyces karnatakensis* [122], like others, was also intracellular and showed stereospecificity not only toward the L-isomer but was also able to hydrolyze D-asparagine to a smaller extent. The L-asparaginase production was reported from various actinobacteria namely *Psedonocardiae endophytica* VUK-10 [123]; *Streptomyces* sp. WS3/1 [124]; *Streptomyces* sp. (SS7) [125]; *S. halstedii* [126]; *Streptomyces acrimycini* NGP [127].

3.15. Penicillin amidase

Penicillin amidase (penicillin amidohydrolase) is an enzyme that hydrolyzes penicillins to 6-aminopenicillanic acid (6-APA) and carboxylic acid. The cleavage of penicillin into 6-APA and side chain is a reaction in which the penicillin nucleus, the basis for the production of semisynthetic penicillins, is obtained. Penicillin amidases in the form of immobilized preparations are applied in the production of 6-APA on an industrial scale [128]. Penicillin acylases are classified into three groups on the basis of their substrate specificity: the first group includes the enzymes that hydrolyze phenoxymethyl-penicillin (penicillin V); the second, those that act on benzylpenicillin (penicillin G); and the enzymes of the third group display specificity with respect to D-a-amino-benzylpenicillin, ampicillin [129]. The penicillin hydrolysis reaction procedes in an alkaline medium and at lower pH values and is reversible. This property was exploited to synthesize semisynthetic penicillins and cephalosporins by the application of penicillin amidase preparations [128] occur in bacteria and actinobacteria and to a minor extent in yeasts and moulds [129-131]. The majority of those found in actinobacteria such as *Mycobacterium*, *Nocardia*, and *Streptomyces* [131; 132] are able to hydrolyze phenoxymethyl-penicillin [133]. These enzymes are mainly intracellular and they display optimum penicillin hydrolysis at pH 7.0 to 8.0. Similarly to acylases isolated from the other sources, they catalyze penicillin synthesis in an acidic medium at pH from 4.0 to 5.5 [131]. So far they have not been reported to be of commercial significance.

4. Conclusion

Enzymes are considered as a potential biocatalyst for many biological reactions. Particularly, the microbial enzymes have extensive uses in industries and medicines. The microbial enzymes are also more active and stable than plant and animal enzymes. In addition, the microorganisms, particularly actinobacteria, represent an alternative efficient source of enzymes because they can be cultured in large quantities by fermentation and owing to their biochemical diversity and susceptibility to gene manipulation. Industries are looking for new microbial strains in order to produce different enzymes to fulfill the current enzyme requirements. Hence, the actinobacteria as biofactory of potential enzyme as well as secondary metabolites production, fulfill the requirements of several industrial enzymes. In a world with a rapid increasing of population and approaching exhaustion of many natural resources, enzyme technology offers a great potential for many industries to help meet the challenges they will face in the years to come.

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Chapter 15

Advanced Oxidation Process Applied to Actinobacterium Disinfection

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Additional information is available at the end of the chapter

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Abstract

Actinobacteria, such as *Mycobacterium*, that constitute one of the main phyla within the bacteria and some genus of this phylum are reported to be a pathogen of or associated with nosocomial infections and pseudoinfections promoting health risks for immunocompromised people, particularly AIDS patients. They are also related to lower quality of surface water due to their odor production (*Actinomycineae* and *Streptomycetaceae*). These bacteria have been isolated from hospital water distribution systems, municipal drinking water, freshwater, and among other environmental samples. Their biofilm formation, amoeba-associated lifestyle, and resistance to chlorine/ozone have been recognized as important factors that contribute to persistence of these bacteria in water distribution systems. Research for new disinfection methods that are able to promote complete inactivation of these bacteria has currently increased. Among them is the use of advanced oxidation process that has demonstrated promising results; the production of ·OH radicals with high oxidizing power are capable to kill bacteria and can also destroy the products generated from lyse cell. The goal of the present work is to review the main processes based on advanced oxidation process that are able to promote actinobacterium disinfection. The fundaments of this process are also reviewed. Special emphasis was done for the photocatalysis and photoelectrocatalysis methods and the phenomena occurring at the electrode/electrolyte interface.

Keywords: Actinobacteria, photocatalysis, Mycobacterium, disinfection, TiO_2

1. Introduction

Actninobacteria or actinomycetes constitute a vast phylum within the domain Bacteria and is formed by bacteria with high content of guanine and cytosine in the DNA [1–3]. Actinobacteria

have some similarities with the microorganisms of the domain Fungi, such as the formation of hyphal and spore dissemination. However, the absence of nuclear membrane, the presence of the typical bacterial flagellum, and sensitivity to antibiotics provided the migration of actinobacteria to the domain Bacteria [4].

There are various different lifestyles among *Actinobacteria*, and the phylum includes pathogens (e.g., *Mycobacterium spp*., *Nocardia spp*., *Tropheryma spp*., *Corynebacterium spp*., and *Propionibacterium spp*.), soil inhabitants (*Streptomyces spp*.), plant commensals (*Leifsonia spp*.), nitrogen-fixing symbionts (*Frankia*), and gastrointestinal tract inhabitants (*Bifidobacterium spp*.) [3].

They are widely distributed in terrestrial [5] and freshwater environmentals [6]. The initial hierarchical classification system of *Actinobacteria* embraced more than 222 genera belonging to 53 families and 23 suborders. A wide variety of morphologies are exhibited by actinobacteria, from coccoid (*Micrococcus*) or rod-coccoid (e.g., *Arthrobacter*) to fragmenting hyphal forms (e.g., *Nocardia spp*.) or permanent and highly differentiated branched mycelium (e.g., *Streptomyces spp*.) [7].

Mycobacteria are a genus belonging to the class of Actinomycetes and are among the most important microorganism pathogens, causing diseases such as leprosy and tuberculosis. Although it has been more than 100 years since the discovery of its etiological agents, these diseases are still associated with high levels of morbidity and mortality, respectively [7, 8]. These bacteria usually occur as straight or slightly curved rods, although they may also appear in the form of hyphae that are fragmented in coccoid elements or rods. Although considered as Gram-positive bacteria, studies confirm the presence of an external membrane in the mycobacterial envelope, resembling in this respect a Gram-negative bacteria [9]. Another outstanding feature for mycobacteria is the significant presence of mycolic acids in the cell wall, forming a waxy, water resistant layer, making for a great resistance to adverse conditions such as dryness. In addition, mycolic acids are also responsible for alcohol- and acid-resistance, another outstanding feature of this genus [10].

The two major human mycobacteriosis are leprosy and tuberculosis, caused by *Mycobacterium leprae* and *Mycobacterium tuberculosis,* respectively. However, other species of the same genus known as atypical mycobacteria is capable of causing opportunistic infections not only in humans but also in animals [11]. For example, the mycobacteria of the *M. avium* complex, due to their varied mechanisms, allow this group to efficiently infect macrophages, monocytes, and epithelial cells. These pathogens can cause various lung damage in immunocompromised patients or even in immunocompetent persons [11]. In contrast to the much opportunistic mycobacteria, these bacilli are able to join, and later invade host cells, suppress the local inflammatory response, and translocate through the wall of the respiratory tract [12]. This translocation ability through the epithelial cells has been investigated, as it represents a form of bacillus that spread to other body organs [13]. The isolation of *M avium, M. kansasi*, and *M. xenopihave*, known as nontuberculosus mycobacteria (NTM), in drinking water and water distribution systems in a hospital has been reported in the literature. Factors such as biofilm formation, amoeba-associated lifestyle, and resistance to chlorine may have contributed to the survival and colonization of these species in these environments [10].

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It is important to note that not all actinobacteria are pathogenic. It is highlighted that the genus *Streptomyces*, which as previously mentioned, is grown as filaments, similar to fungi. They are Gram-positive and have the ability to produce bioactive secondary metabolites such as antifungals, antivirals, anti-tumor, anti-hypertensives, and mainly antibiotics and immunosuppressives [14].

The presence of *Actinomycineae* and *Streptomycetaceae* in drinking water may produce odors, geosmin, and 2-methylisoborneol (MIB), which contribute to the decrease of the quality of surface water when used for drinking [15]. In this form, the presence of actinobacteria has been related to a lot of environmental issues. The abundance of the above-mentioned microorganisms in water could stem from their resistance of conventional disinfection methods such as chlorination and/or ozonation [5]. Research for new disinfection methods has been increasing and among them, the use of an advanced oxidation process has been giving promising results.

2. Occurrence of actinobacterium in water samples

The nature of the microbiology of tap water delivered to consumers via public drinking water distribution systems has been studied by Holinger et al. [16]. The authors studied tap water from 17 different cities between the headwaters of the Arkansas River and the mouth of the Mississippi River. The occurrence of Actinobacteria was detected in 24% of the samples, of which 85% were *Mycobacterium spp.* Other bacteria such as Proteobacteria (35%), Cyanobacteria (29%, including chloroplasts), Firmicutes (6%), and Bacteroidetes (3.4%) were also obtained.

The presence of mycobacteria has also been reported in municipal drinking water distribution systems, hospital water systems, and in ice machines, swimming pools, and whirlpools [17–21].

Klanicova et al. [22] have analyzed 124 samples of four drinking water supply systems in the Czech Republic, 52 dam sediments, 34 water treatment plant sludge samples, and 38 tap water household sediments. Actinobacteria of 11 different species were isolated by culture from 42 (33.9%) samples; the most prevalent were *M. gordonae* (16.7%), *M. triplex* (14.3 %), *M. lentiflavum* (9.5%), *M. avium* (7.1%), *M. montefiorenase* (7.1 %), and *M. nonchromogenicum* (7.1%). The presence of *M. paratuberculosis* in water treatment stations for potable water production is also described [23]. They have found *M. paratuberculos* in the final treated water and concluded that the public might be exposed through water supplies.

The analysis of freshwater lakes in China showed the bacterial diversity and were identified as Proteobacteria (40.9%), followed by Actinobacteria (15.9%), Cyanobacteria (11.4%), Verrucomicrobia (11.4%), Plantomycetes (6.8%), Bacteroidetes (4.5%), and Chloroflexi (4.5%) [24]. The investigation of the composition and diversity of biofilm bacterial community present in real drinking water distribution systems (DWDSs) with different purification strategies (conventional treatment and integrated treatment) show that actinobacteria is the major component of the biofilm bacterial community [25]. The abundance, identity, and activity of uncultured actinobacterium present in a drinking water reservoir (North Pine Dam, Brisbane,

Australia) has also been shown [26]. The structures and dynamics of bacterial communities from raw source water, groundwater, and drinking water before and after filtration were studied in four seasons of a year. The bacterial communities of different seasons from the four sampling sites were clustered into two major groups: water before and after filtration and source water and groundwater; representatives of the phyla Actinobacteria were found at all four sampling sites [27].

Analysis of bacterial communities associated with different drinking water treatment processes (e.g., coagulation, sedimentation, sand filtration, and chloramine disinfection) and from distantly piped water showed a large proportion of the phyla Actinobacteria and Bacteroidetes. The piped water exhibited increasing taxonomic diversity, including human pathogens such as the *Mycobacterium*, which revealed a threat to the safety of drinking water. In addition, actinobacteria was relatively consistent throughout the processes [28].

The presence of NTM was also reported in waters of a hemodialysis center [29]. Analysis of 210 samples of water from the hydric system of the unit (post-osmosis system, hemodialysis rooms, reuse system, and hemodialysis equipment) and from the municipal supply network was studied. The results showed that 24.3% of the collected samples tested positive for NTM; both the municipal supply network (2 samples, 3.2%) and the hydric system of the hemodialysis center (49 samples, 96.1%) contained NTM. Among the mycobacteria isolated, the authors highlight the presence of *Mycobacterium lentiflavum* (59.0%), *M. kansasii* (5.0%), *M. gordonae* (24.0%), *M. gastri* (8.0%), and *M. szulgai* (4.0%). The results show the need of more effective water disinfection procedures in this unit.

The incidence of NTM was also obtained in hot water systems of hospital settings with disinfection using hydrogen peroxide and silver; thermal disinfection or chlorine dioxide showed the persistence of NTM in 47% of the samples, equivalent to the remaining concentration between 10–1625 CFU L^{-1}. Among the NTM species that were isolated are *M. kansassi*, *M. xenopi*, and *M. fortuitum*. The lowest incidence was observed in the system treated by thermal disinfection and chlorine dioxide [30].

It was confirmed that drinking water supply systems (watershed–reservoir–drinking water treatment plant–household) might be a potential transmission route for actinobacteria, where mycobacteria are most reported. Some of these mycobacteria can cause various disseminated infections, tuberculosis-like illnesses, lymphadenitis, osteomyelitis in animals and in immunocompromised humans, paratuberculosis (Johne's disease), and chronic enteritis in ruminants [28].

The abundance of the above-mentioned microorganisms in water could stem from their biofilm formation, amoeba-associated lifestyle, and resistance to chlorine, which have been recognized as important factors that contribute to the survival, colonization, and persistence of actinobacteria in water distribution systems. The presence of actinobacterium in tap water, mainly mycobacteria, has been linked to nosocomial infections and pseudoinfections and provides a health risk for immunocompromised people, particularly AIDS patients. The research for new disinfection methods has been increasing and among them, the use of advanced oxidation process has been give promising results.

3. Advanced Oxidation Processes (AOPs): Basic concepts

Chemical oxidation may be defined as a reaction in which electrons are removed from a substance, increasing their oxidation state [31]. The reactions involving oxidizing agents, such as H_2O_2 and O_3, are thermodynamically favored. However, they can be kinetically slow. In the presence of highly-oxidizing free radicals, such as hydroxyl radicals (·OH), could be found reaction rates of a million to a billion times faster compared with the oxidizing agents mentioned above [32]. Thus, the generation of hydroxyl radicals (·OH) is an essential step in the efficiency of advanced oxidation processes [33]. They are formed via the oxidation of water on the anode surface, as shown in Equation 1.

$$H_2O \rightarrow \bullet OH + H^+ + e^- \quad (1)$$

These chemical species are effective in destroying organic chemicals because they are reactive electrophiles (electron preferring), which react rapidly and are non-selective in relation to all electron-rich organic compounds [33, 34]. Furthermore, these react unselectively with a wide range of recalcitrant organics, often at diffusion-limited rates [35–38].

Table 1 lists the standard reduction potential of some oxidants. It is observed that the radical ·OH is a strong oxidant, only lower than fluoride, surpassing O_3 and H_2O_2.

Chemical Specie	Standard reduction Potential
F_2	+3.06
●OH	+2.77
O_3	+2.07
H_2O_2	+1.78
ClO_4-	+1.43
Cl_2	+1.36
O_2	+1.23

Table 1. Reduction potential standard for different chemical species [36–38].

For the generation of hydroxyl radicals in a reaction medium, there are different methods to classify them as homogenous and heterogeneous, and they may occur with or without light irradiation. Homogeneous systems are characterized by the absence of a solid catalyst utilized to initiate the reaction. In contrast, heterogeneous processes make use of solid catalysts to contribute to the formation of hydroxyl radicals [39]. Figure 1 summarizes the most common of AOPs systems.

Among the different AOPs systems, heterogeneous catalysis has received prominence over the years. Photocatalytic oxidation can be a good option for water disinfection, its performance is greatly restricted by fast electron-hole (e/h^+) recombination. The strategy to increase photocatalytic efficiency is photoelectrocatalysis (PEC), which consists of introducing a reverse

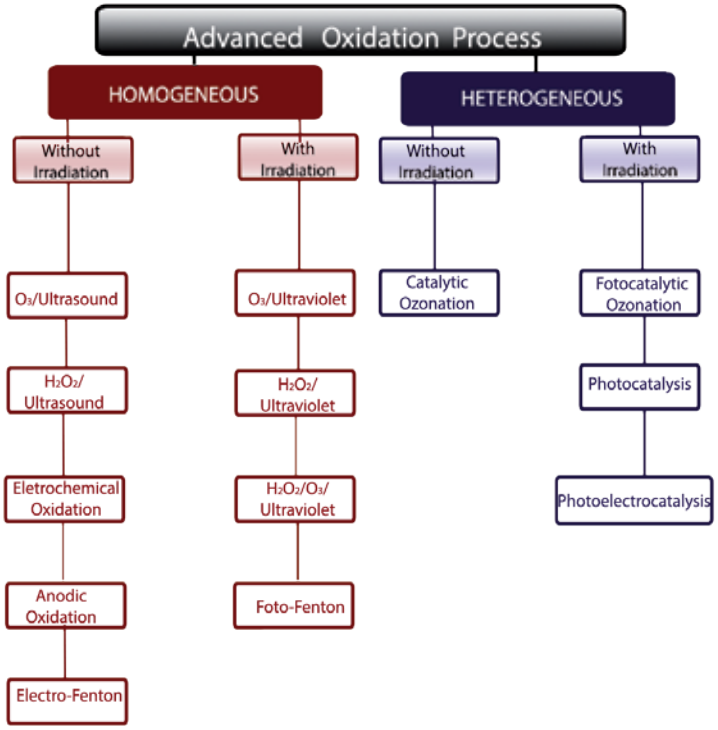

Figure 1. Schematic representation of different treatment based on AOPs

bias potential to the anode coated with the photocatalyst [40–42]. The PEC process minimizes recombination, this process exists under bias potential, creating the potential gradient on the anode that is enough to remove the electrons from the conduction band to an external counter electrode. This increases the availability of ·OH radicals and other reactive oxygen species (ROS), which can attack the bacterium cell wall and organics compounds when the bacteria gets in contact with the catalyst [42].

The principle of photocatalysis is based on the activation of a semiconductor (commonly titanium dioxide (TiO_2)) by light irradiation. In order for the semiconductor to become a conductive material, charge carriers need to be created, usually by photoexcitation. The principle of the process is the generation of a pair of electron/hole (e^-/h^+) by promotion of an electron from the valence band (VB) to the conduction band (CB) (Equation 2). A semiconductor material is characterized by the presence of a VB and a CB separated by a band gap energy (E_g) [36]. Figure 2 shows a schematic representation of a photoexcited semiconductor.

$$Semiconductor \xrightarrow{hv} Semiconductor\,(e^-_{CB} + h^+_{VB}) \quad (2)$$

In a semicondutor, the Fermi level (Ef) is a very important parameter and can be defined as the electrochemical potential of an electron in a metal or semiconductor, which can be

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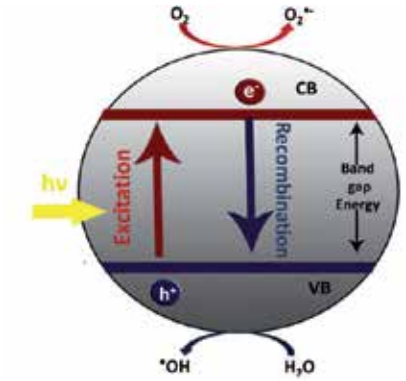

Figure 2. Schematic representation of a photoexcited semiconductor

modulated by the applied external potential. In order to promote electrical conduction in a semiconductor, the charge transport should be obtained by one of the following mechanisms: thermal generation, doping, or photoexcitation [43]. Thus, metals and semiconductors are distinguished by the Fermi level position, which is related to the concentration of charge carriers [43–44]. In the n-type semiconductor, the Fermi level is closer to the band conduction, while for the p-type semiconductor it is close to the valence band.

The presence of an essentially filled energy band, a separate almost empty band, leads to semiconductor photosensitivity. The absorption of photons with energy greater than the energy of the "band gap" results in the promotion of electron from the valence band to the conduction band with concomitant generation of a hole (h^+) in the valence band. This can be visualized in the Equation (2).

The hole formed on the surface of the catalyst (n-type for instance) can usually promote oxidation of adsorbed species on the semiconductor surface and/or water oxidation leading to the formation of hydroxyl radical, and both can act on organic material degradation. Different mechanisms can be proposed:

a. The organic matter (OM) from the actinobacteria cell wall, for example, adsorbs on the surface of the photocatalyst and can be oxidized by the holes (h^+) in the valence band, forming a cation that can react rapidly with oxygen [45].

$$OM_{ads} + h^+_{VB} \rightarrow OM^{\bullet+}_{ads} \tag{3}$$

b. Depending on the chosen semiconductor and the pH of the system, the water adsorbed on the surface is oxidized to hydroxyl radical through the hole from the valence band [45]. As previously mentioned, the hydroxyl radical has a high oxidation power and can promote the oxidation of the species present in the system, subsequently.

$$Semiconductor - h^+_{VB} + H_2O_{ads} \rightarrow Semiconductor - OH_{ads} + H^+ \tag{4}$$

c. Other radical oxygen species formed by the capture of photogenareted electrons can promote the oxidation process [24, 25].

$$e^- + O_2 \rightarrow O_2^{\bullet -} \tag{5}$$

$$O_2^{\bullet -} + H^+ \rightarrow HO_2^{\bullet} \tag{6}$$

The efficiency of photocatalysis depends on the competition between the effectiveness of the electron removal from the semiconductor surface and the recombination process involving electron/h_{vb}^+ generating heat as a product [36, 46, 47]:

$$Semiconductor\left(e_{CB}^- + h_{VB}^+\right) \rightarrow Semicondutor + heat \tag{7}$$

Therefore, there is high demand for methods that are able to minimize the recombination process of electrons returning from the conduction band to the valence band. Studies reported in the literature have shown that the photocatalytic oxidation can be improved by an anodic bias potential [19]. In the under bias potential, there is a formation of a potential gradient in the interface substrate/electrolyte and the electrons are removed, leading to the decrease in recombination of the pair electron/hole [36, 42]. Figure 3 illustrates the mechanism of PEC.

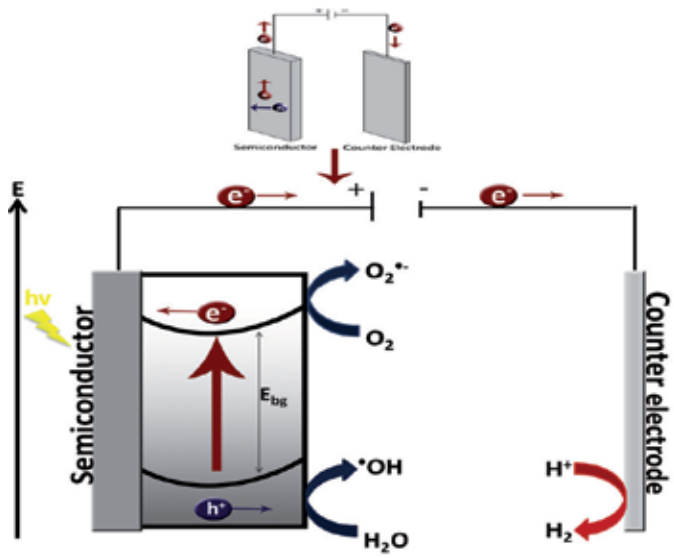

Figure 3. Mechanism of photoelectrocatalysis and charge separation in a photoelectrochemical system, where a gradient of potential is created.

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Understanding the efficient charge separation mechanism in PEC is essential. The moment a semiconductor is in contact with the electrolyte, a junction semiconductor/electrolyte interface is formed in charge of determining the electron hole separation kinetics. Due to the different potential present in the interface, there is a change in the potential Fermi of the semiconductor. Thus, the equilibrium of this interface requires a power flow between the phases, providing the creation of band-bending in the semiconductor phase. The space charge layer (SCL) is the region where there is bending and is characterized by the presence of electrons or holes in the surface [36, 48, 49].

The application of a bias potential also contributes in controlling the Fermi level [48]. Flat band potential is conceptualized as an exact potential for which the potential drops between the surface and the bulk of the electrode is zero. Thus, applying a greater potential than the flat-band potential will provide an increase in band-bending in an n-type semiconductor. In this situation, electrons are depleted and holes are enriched at the surface. The electron that has been excited in the conduction band flows through an external circuit to the counter electrode where reduction reactions may occur such as the reduction of H^+ to H_2. Figure 4 shows an energy diagram of an n-type (TiO_2) semiconductor in different situations: before semiconductor-electrolyte contact, equilibrium established after semiconductor-electrolyte contact, and applying anodic bias.

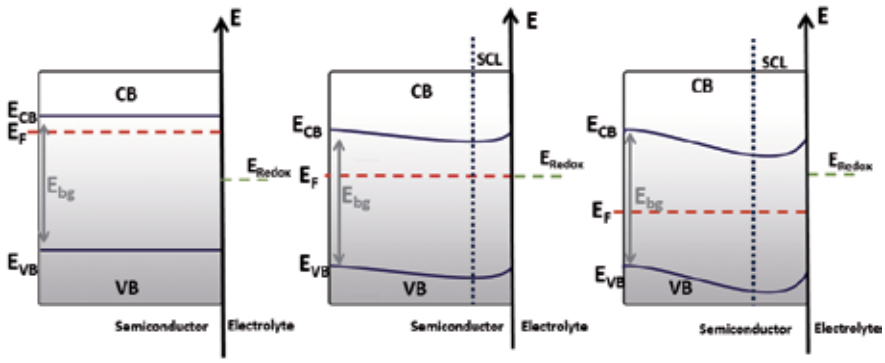

Figure 4. Energy diagram of n-type (TiO_2) semiconductor in different situations: before semiconductor-electrolyte contact, equilibrium established after semiconductor-electrolyte contact and applying anodic bias.

Thus, the high oxidizing power of hydroxyl radicals formed during photocatalytic and photoelectrocatalytic processes has gained attention in the disinfection process. Their applications in promoting actinobacterium disinfection is reported in the literature and a revision of this contribution is shown in the following sections.

4. Main advanced oxidation process applied to actinobacterium disinfection

Considering the resistance of actinobacterium to conventional disinfection treatments and the promised results of disinfection processes based on advanced oxidation process, the aim of

this work is to describe the efficiency of the advanced oxidation process applied to actinobacterium disinfection.

Among the advanced oxidation process applied to actinobacterium disinfection, the main reported are photolysis, photocatalysis, and photoelectrocatalysis specially applied to mycobacteria disinfection. Some of the works related in the literature are show below.

4.1. Photolysis

The inactivation by photolysis using mainly ultraviolet (UV) irradiation has been reported by many researches using different UV sources applied specially for *Mycobacterium* inactivation in the environmental samples. The kinetics of inactivation of *M. tuberculosis*, *M. fortuitum*, and *M. marinum* has been reported since 1971. The inactivation reached 90% for *M. tuberculosis* and *M. marinum* cells after irradiation of 7 to 22 s, respectively. However, *M. tuberculosis* and *M. marinum* are shown to be capable of photoreactivation [50]. The use of UV at 20 mJ/cm^2 inactivate more than 3 log of *M. avium*, *M. intracellulare*, and *M. lentiflavum*, whereas *M. fortuitum* required a dose of more than 50 mJ/cm^2. The study showed that mycobacteria were found 2–10 times more resistant to UV than *E. coli* [51]. The efficiency of low-pressure UV light for *Mycobacterium* complex inactivation in water is also reported. Using low-pressure UV light, the results showed greater than 4 log inactivation at fluencies of less than 20 mJ/cm^2 [52]. The 405 nm light (10 mW cm^{-2}) generated from an array of 405-nm light-emitting, inactivated 4-5 log CFU mL^{-1} *M. terrae* in liquid suspension and on exposed surfaces using a dose between 144 and 288 J cm^{-2} [53]. The use of microwave with UV lamps (peak emissions at 253.7 nm) that also generate ozone, have been tested for its ability to eradicate high populations of microbial vegetative cells and spores (of bacteria and fungi) artificially added to filter surfaces. *M. phlei* deposited on the surfaces as clumps required 30 min treatment to affect complete killing. It has been considered that longer irradiation periods are required to facilitate the complete destruction of surface microorganisms, ozone, and other oxidizing species produced by microwave UV lamps that act to enhance microbial destruction [54]. The method has been also proposed for the treatment of secondary effluents and metal working contaminated with microorganisms such as mycobacteria.

Secondary effluents spiked with *Mycobacterium terrae* filtered through nylon filters (100, 41, and 20 μm) to partition aggregate sizes were exposed to UV light doses and free chlorine; rapid inactivation with 2.5 log reduction at 14 mJ/cm^2 and 56 mg min/L for UV and free chlorine, respectively, were obtained. Mycobacteria are acid-fast and their outer waxy layer may contribute to their relative resistance to UV and chlorine disinfection. Furthermore, mycobacteria tend to aggregate (clump) and accumulate in biofilms, which may further increase their resistance to disinfectants. It has been reported that a dose of 4.2 mJ/cm^2 was required to achieve 1 log inactivation of *M. terrae*, which is higher than 1 log inactivation of any other vegetative bacteria (for example: *E. coli* 1.5 mJ/cm^2) [55]. The efficacy and rapidity of UV (192 μW/cm^2, 55 W) radiation for disinfection has been reported for the inactivation of 10^4–10^7 CFU/mL *Mycobacterium chelonae*; 74% reduction was observed after 10 min of exposure [56]. The photoinactivation of *Arthrobacter nicotinovorans* and of *Streptomyces griseus* were tested by irradiation at 222 nm and 254 nm reaching a 4-log reduction [57]. The effect of different fractions

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of natural UV radiation doses on two Antarctic marine bacteria (*Arthrobacter* UVvi and FCB-related UVps strains) were evaluated by six treatments: DARK, PAR (with UVR shielded off), UVA360, UVA320, UVB305, and UVB280 [58]. In all UVR treatments, strains showed significant losses of viability under high and moderate irradiance and no differences were observed between UV treatments. Under high UVB dose (15.0 kJ m^{-2}) received in only two hours, the effect of UVB treatments was significantly higher than that observed under UVA. However, for both strains, UVA wavelengths are sufficient to suppress bacterial viability significantly in marine situations, principally UVA (λ< 360 nm). When exposed to UVB wavelengths, both strains lose viability exponentially with the dose. The efficiency of the photolysis in the inactivation of the actinobacterium has been based mainly on the damage caused to the nucleic acids (DNA/RNA) of the cell. However, the photolytic treatment has not showed promising results, thus, a lot of alternatives have been proposed to improve the efficiency of disinfection, especially considering the possibility of photoreactivation of actinobacterium next to a dark period. Among the alternatives, photocatalysis has shown promising results.

4.2. Photocatalysis

Photocatalytic treatments are based on an irradiation source and a photocatalyst as a semiconductor. Among them, the TiO_2 is one of the most powerful semicondutor materials used for photocatalysis due its high activity, strong oxidizing powers, and long-term stability. TiO_2 can generate strong oxidizing power when illuminated with UV light at wavelengths lower than 385 nm. The photon energy generates an electron hole pair on the TiO_2 surface. The hole in the valence band can then react with water or hydroxide ions adsorbed on the surface to produce hydroxyl radical (·OH), and the electron in the conduction band can reduce O_2 to produce superoxide ions (O_2^-). Both holes and ·OH are extremely reactive upon contacting organic compounds and microorganisms [59]. However, others semiconductors have been proposed for actinobacterium disinfection and some of the main works are described in this section.

The photocatalysis with immobilized TiO_2 and UV irradiation has been proposed as seawater disinfection technology. The method was evaluated using marine bacteria assigned as actinobacteria *Corynebacterium stationis*. The photocatalytic treatment promoted faster disinfection kinetics for both bacteria species, between 30% and 33% less UV dose to achieve the same level of disinfection [60]. Shintani et al. [61] have shown TiO_2 membrane as a photocatalyst irradiated by UV lamp to sterilize airborne microorganisms in health care. A statistical difference was observed between UV and the photocatalyst sterilization ($p<0.01$) when humidity was increased to 60–70%.This indicates that maintaining high humidity levels gives more effective sterilization results due to the higher production of OH radicals. Among representative airborne microorganisms found in the dialysis room are *Micrococcus spp.*, *Bacillus spp.*, and *Corynebacterium spp*.

The anti-bactericide activity of TiO_2 nanofilms deposited onto polyvinyl chloride and glass substrates was evaluated by monitoring *Micrococcus lutes* (odor-causing bacteria) disinfection. *M. lutes* bacteria were completely killed in 20 min over TiO_2 films under the UV solar simulator (150 W). On the other hand, about 30% of bacteria were destroyed in 20 min under UV black

light (100 W) [62]. The antibacterial activity against *Micrococcus luteus* has also been reported using apatite-coated TiO_2. Its antibacterial performance was observed under black light, visible light, and dark conditions. The number of viable bacteria decreased with irradiation time and became most prominent at 24 hours. The black-light irradiation demonstrated higher antibacterial activity than either visible-light irradiation or dark conditions. Around 50% reduction within 24 h using apatite-coated TiO_2 under black light has been observed [59].

Hetero-nanostructured film of titania nanosheets and lysozyme have been proposed as a semiconductor-like antibacterial agent for *Micrococcus lysodeikticus* with high inhibition rate. In the presence of UV light, a significant high inhibition rate was observed when the multilayer film is present in turbid bacterial suspension [63]. The photocatalytic kill of *Micrococcus* has also been reported using visible light-induced sulfur-doped TiO_2 [64].

The impregnation of TiO_2 with different metals has been proposed to improve the photocatalytic actives, such as Ag, Cu, and Pt. Su et al. [65] proposed the use of TiO_2/Ag nano-antibacterial materials prepared at low temperature using polyethylene glycol (PEG-600) as reducing and stabilizing agents. The antibacterial activity study was carried by growth inhibition rates against *E. coli* and *Streptomyces*. The growth inhibition rates against *Streptomyces* was 97.9% and the concentration of TiO_2/Ag was 10 ppm, but changes of inhibition rates are not very obvious with the decrease of concentration of TiO_2/Ag. Under photocatalytic conditions, it is expected that the energy of the photons absorbed by Ti/TiO_2-Ag will generate charges. The separation of these charges after reduction of O_2 can produce hydroxyl radicals (·OH) in the valence band, due to water oxidation and superoxide ions (O_2^-) in the conduction band. The decoration with Ag nanoparticles can trap the photogenerated electrons, increasing the lifetime of the holes that are able to produce hydroxyl radicals. Consequently, the frequency of charge recombination is lower than the usual value observed for similar processes conducted without Ag, improving the efficiency of the disinfection [66].

The efficiency of undoped TiO_2 and platinized sulfated TiO_2 (Pt/TiO_2) to photocatalytic oxidation was investigated with microorganisms loaded over photocatalyst films from aerosols to load *Mycobacterium smegmatis*, *Bacillus thuringiensis*, vaccinia virus, and influenza A (H3N2) virus. About 90% inactivation is reached in 30 min UVA irradiation on TiO_2 and from 90% to 99.8% on Pt/TiO_2. It has been proposed that Pt/TiO_2 showed increased rate of mineralization, as well as increased inactivation likely due to a better charge carrier separation in the doped semiconductor photocatalyst. The results demonstrate that photocatalytic filters with deposited TiO_2 or Pt/TiO_2 are able to inactivate aerosol microorganisms and completely decompose them into inorganic products and Pt/TiO_2 provides higher disinfection and mineralization rates [67]. Pt/TiO_2 nanoarchitecture activated by a 365 nm UV light has also been proposed for the photo-driven killing of *Micrococcus lylaecells* [68]. Significant cell death is observed only for the photoactivated Pt/TiO_2 film, with which 70% of the M. *Lylaecells* were killed in 60 min. The authors proposed that oxidative species can easily diffuse out of the porous matrix to attack the cells. However, considering that the photocatalyst can not directly attack the cells that were protected by an outer peptidoglycan layer [69], the oxidative species are believed to be responsible for killing *M. lylae* cells. The detailed bactericidal mechanism of these photocatalytically-induced oxidative species is not unambiguously clear. However,

based on previous studies, the authors proposed that the plasma membrane can be first attacked by the oxidative species penetrating the outer layer of the bacteria. These reactive species can then oxidize coenzyme A and the plasma membranes. The oxidation of coenzyme A would inhibit cell respiration and directly cause cell death [70]. Meanwhile, the oxidation of the plasma membrane can break the main permeability barrier of the bacteria. This would result in the slow leakage of the intracellular materials, including RNA, protein, and minerals, leading to the subsequent death of *M. lylae*.

The use of Cu-doped TiO_2 nanoparticles (NPs) for the inactivation of *Mycobacterium smegmatis* was investigated under three light conditions (complete dark, fluorescent light, and UV light) [71]. The survival rate of *M. smegmatis* (in a minimal salt medium for 2 h) exposed to the NPs varied depending on the light irradiation conditions as well as the dopant concentrations. When TiO_2 NPs were doped with copper (>3wt% dopant), their inactivation potential was promoted and the UV-resistant cells were reduced by over 99%. Limbach et al. [72] have proposed that Cu NPs may enter the cytoplasm of the cell through a Trojan-horse mechanism and that the inactivation capability of NPs is enhanced due to an increased uptake of ions into the cell structure, disturbing its function. However, Cu-doped TiO_2 NPs tend to agglomerate in the aqueous solution and their effective sizes were not much smaller than the bacteria itself [73]. Also, mycobacteria have a very thick and waxy cell wall. Therefore, the Trojan-horse mechanism discussed earlier may not play an important role here [72]. Instead, the leached Cu^{2+} ion, which electrostatically interacts with the negatively charged cell membrane, forms a concentrated ionic zone of copper, enhancing the microbial effects.

Another catalyst that has been reported is Ag/ZnO composite NPs. This photocatalyst was applied for textile treatment. Long-term stable sols of ZnO and Ag/ZnO NPs were prepared and applied as liquid coating agent for textile treatment in combination with an inorganic-organic hybrid polymer binder sols prepared from the precursors 3-glycidyloxypropyltrimethoxysilane (GPTMS) and tetraethoxysilane (TEOS) [74]. The antimicrobial activity of the NPs applied on textile fabrics was tested against the Gram-negative bacterium *Escherichia coli* and Gram-positive *Micrococcus luteus*. It was observed that bacterial susceptibility improved considerably with the increase in the silver contents.

The mycobactericidal properties of an iron-based novel heterogeneous-modified polyacrylonitrile (PAN) catalyst in combination with hydrogen peroxide were examined against *Mycobacterium chelonae*. The 0.5% w/v hydrogen peroxide and 2-g catalyst system resulted in average log reductions of >5.80 for *M. chelonae* at 30 min exposure at room temperature. This was a significant increase in activity ($P<0.01$) compared to 0.5% w/v hydrogen peroxide alone [75].

There are many photocatalytic actinobacterium disinfection processes based on different semiconductors, mainly TiO_2 (pure and impregnate). Although photocatalytic oxidation can be a good option for water disinfection, its performance is greatly restricted by fast electron–hole (e^-/h^+) recombination. A strategy to increase photocatalytic efficiency is PEC, which consists of introducing a reverse bias potential to the anode coated with the photocatalyst. The PEC process minimizes recombination because the system exists under bias potential, creating a potential gradient on the anode that is enough to remove the electrons from the conduction band to an external counter electrode. This increases the availability of ·OH radicals and other

reactive oxygen species (ROS) that are able to attack the bacterium cell wall where the bacteria gets in contact with the catalyst. There are few researches about the actinobacteria photoelectrocatalytic inactivation and they are only described for *Mycobacteria* inactivation. These works are shown in the section below.

4.3. Photoelectrocatalysis (PEC)

The photoelectrocatalytic inactivation using TiO_2 nanotubular array electrodes of 10^3 CFU (Colony-Forming Units) mL^{-1} M*ycobaterium fortuitum*, *M. chelonae*, and *M. abscessus* was achieved after 3 min treatment. The metabolites released during cellular lysis were also degraded with 240 min of photoelectrocatalytic treatment, the monitoring was carried out by mass spectroscopy measurements. Mineralization was greater than 70% under optimum conditions. The photoelectrocatalytic method gave better results than the photolytic and photocatalytic techniques [76].

TiO_2 nanotubular array electrodes coated with 16% (w/w) Ag NPs (Ti/TiO_2-Ag) have shown excellent performance in the disinfection of water containing *Mycobacterium smegmatis* [77]. Photoelectrocatalytic disinfection of *M. smegmatis* 5.1×10^3 CFU mL^{-1} in 0.05 M Na_2SO_4 (pH 6), applied potential of +1.5 V versus Ag/AgCl and UV irradiation promoted 100% inactivation after 3 min of treatment. In addition, analytical methods such as TOC removal, sugar release, chromatography, and mass spectroscopy measurements confirmed that there was complete inactivation of mycobacteria and degradation of the by-products generated during cellular lyses by the proposed method.

The irradiation of Ti/TiO_2–Ag with visible irradiation as photoelectrode promoted 99.6% inactivation of *M. smegmatis* after 30 min. In addition, the analysis of silver in the solution along with the reaction shows a negligible lixiviation and a high stability of the surface silver deposit [78]. Ti/TiO_2-Ag photoanode has also been proposed to inactivate *Mycobacterium kansasii* and *Mycobacterium avium* for a mycobacteria population of 5×10^8 CFU mL^{-1}; it achieved 99.9% and 99.8% inactivation after 240 min. Using a mycobacteria population of 7.5×10^4 CFU mL^{-1}, total mycobacteria inactivation within 3 min of treatment was obtained. The presence of Ag nanoparticles in the electrode provided 1.5 larger degradation rate constant as compared with the Ti/TiO_2 anode [79]. An explanation for this is that in the photoelectrocatalytic process, there are reductions in the recombination of charges photogenerated since under bias potential there are formations of a gradient potential on the photoanode and flow of electrons from the conduction band to the external counter electrode connected to the photoreactor. This arrangement increases the availability of ·OH radicals and other ROS that are able to attack the bacterium cell wall where the bacteria gets in contact with the catalyst [80, 81]. This results to larger damage to all cell wall layers, allowing small components such as ions to leak. Damage at this stage may be irreversible and cell death ensues. Because peptidoglycan is a highly cross-linked molecule, damage may not be evident at this stage; indeed, visualization would require leakage of components with higher molecular weight such as proteins [82].

So, among the advanced oxidative process described until this moment, the photoelectrocatalytic treatment gave the best results, since it promotes the inactivation and also degradation of cell lyse components.

4.4. Others alternative advanced oxidation processes

Among the other advanced oxidation processes, we can highlight the use of ozone, Engineered Water Nanostructures, peroxide, and direct electrolysis.

The effect of ozone on cariogenic microorganisms has been reported for actinobacteria *Actinomyces viscosus* and other bacteria such as *Streptococcus salivarius* and *Streptococcus mutans*. The microorganisms were exposed to power levels 2, 3, or 4 of an ozone device for 20, 40, 60, or 120 s. CFU numbers of bacteria were counted after the ozone application; as a result, the number of bacterial cells decreased. In addition, the bacterial cells were evaluated with a fluorescence microscope and showed that some bacterial cells were killed instantaneously in the ozone [83].

Pyrgiotakis et al. [84] have been the first to report the use of Engineered Water Nanostructures (EWNS) for mycobacteria inactivation. The method is based on the transformation of atmospheric water vapor into EWNS. Electron paramagnetic resonance (EPR) showed that EWNS contain a large number of ROS, primarily ·OH and superoxides. *M. parafortuitum* was inactivated on surfaces eight times faster than the control. It was demonstrated that the EWNS effectively deliver the ROS encapsulated during the electrospray process to the bacteria, oxidizing their cell membrane and resulting to inactivation. Overall, this is a method with the potential to become an effective intervention technology in the battle against airborne infections.

The disinfection of *Streptococcus mutans*, which generates acid that causes mineral loss of the teeth and the so-called dental caries, has been also evaluated by hydroxyl radicals generated by photolysis of H_2O_2 [85]. Laser irradiation of suspensions in 1M H_2O_2 resulted in a >99.99% reduction of the viable counts within 3 min of treatment. Treatment of a biofilm composed by *S. mutansin* resulted in a >99.999% reduction of viable counts within 3 min.

Finally, disinfection using electrolyzed strongly acidic water (ESW) against *Mycobacteria* and the recovery of the disinfection potential of inactivated ESW by re-electrolysis was reported. The ESW was carried out using positive and negative platinum-coated titanium electrodes and 0.1% NaCl electrolyzed at a constant voltage of 24 V. ESW containing 10, 20, and 30 ppm free chlorine was applied in a solution with *M. bovis* cells (10^5–10^8 CFU/mL) for 0–7 min. To clarify the recovery of the disinfection potential of inactivated ESW by re-electrolysis, it was mixed with ESW containing 10 ppm free chlorine with *M. bovis* cells (10^7 CFU/mL) for 1 min. The number of viable cells decreased to $1/10^3$, but the cells were still detected. After re-electrolysis for 7 min, viable cells were not detected. ESW (10 ppm) showed its complete bactericidal effect against 10^5 and 10^6/mL of bacterial suspension within 1 and 3 min, respectively. Against NTM, one strain each of *M. avium*, *M. intracellulare*, and *M. kansasii* ESW (10 ppm) decreased the number of viable cells by less than $1/10^5$ within 1 min of contact [86].

Then, considering the abundance of the actinobacteria in water mainly due their resistance to conventional water treatment, such as chlorine, the advanced oxidation process is a promising alternative to the water treatment, especially to water used in therapeutic applications.

5. Final consideration

The advanced oxidation process has been reported as an excellent alternative for actinobacterium disinfection. Good results have been obtained by photolysis treatment, however, it requires longer time and there is the possibility of photo-reparation next to a dark period. The use of photocatalysis gave the best results using different photoanodes such as TiO_2 (bare and impregnated) with metals such as Au, Ag, and Pt, which can improve the photocatalytic activated. The most promising technique seems to be photoelectrocatalysis as it is able to promote inactivation at a short period of illumination and higher mineralization of products generated from the cellular lyse. Nevertheless, the technique deserves further test to improve the economic aspects involved in using the combination of bias potential and UV irradiation.

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Chapter 16

Green Nano Actinobacteriology – An Interdisciplinary Study

Mrinalini J. Singh

Additional information is available at the end of the chapter

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Abstract

Green nano actinobacteriology has been considered as a novel field of study in order to develop least expensive and highly qualitative strategies for production of eco-friendly beneficial products with diverse applications. The uniqueness of bioactive actinomycetes has turned the attention of scientists worldwide in order to explore its potentiality as effective "micronanofactories". This chapter provides a brief overview of the synthesis, characterization, and application of actinobacterial nanoparticles with an added note to actinobacterial detoxification.

Keywords: actinomycetes, micronanofactories, synthesis, characterization, application, detoxification

1. Introduction

Nanotechnology is a rapid, upcoming, multidisciplinary promising area that has an influence in medical, agricultural, and industrial fields, where they manufacture materials at the nano scale (one billionth of a meter or 10^{-9} in size). These nanomaterials are synthesized by physical, chemical, and biological methodologies. Physical processes include mechanical smashing, solid phase reaction, laser ablation, melt mixing, high-energy ball milling, physical vapor deposition, freeze drying, spread drying, ion sputtering, solvothermal synthesis, sol-gel technique, and precipitation [16, 17]. Chemical processes include aqueous and nonaqueous chemical reduction, electrochemical reduction, template method, ultrasonic-assisted reduction, photocatalytic reduction, microwave-assisted synthesis, biochemical reduction, irradiation reduction, and micro-emulsion method [32]. Both these physical and chemical processes need high temperature and high pressure to execute. These processes were costlier and produced highly toxic compounds along with consumption of high energy.

Therefore, scientists employ inexpensive, eco-friendly, easier scale-up biological synthesis methodology by utilizing both plant extracts [6, 10, 21, 26, 28] and microbes. The phytochemicals and enzymes present in plants and microbes reduce the metal compounds into corresponding nanoparticles [1]. Initially, among microbes, bacteria was utilized for green synthesis, which was later overcome by the use of yeast, algae [7, 25] and fungi [5, 8, 20, 27]. But the use of actinomycetes (filamentous bacteria) was comparatively less reported, though they act as valuable resource for numerous diverse bioactive metabolites [2]. Compared to bacteria, fungi and actinobacteria secrete more proteins which in turn increase biosynthesis production. Compared to fungi, actinobacteria comes under prokaryotes group. Hence, they can be manipulated genetically in order to achieve better control over size and polydispersity of the nanoparticles [14, 36]. This biogenic process takes place at ambient temperature and pressure conditions and no toxic chemical is involved [37].

In this chapter, green synthesis of nanoparticles by actinobacteria and their application in varied fields were studied under green nano actinobacteriology, which acts as the interface between "nanotechnology" and "actinobacteriology".

2. Biosynthesis of Nanoparticles

Actinomycetes are helpful in the biosynthesis of nanoparticles with good surface and size characteristics showing wide range of bioproperties. Actinobacterial production of metallic nanomaterials was mediated by extracellular and intracellular methodologies. Extracellular synthesis has got more commercial advantages compared to intracellular synthesis since polydispersity plays a significant role. In the case of intracellular synthesis, the accumulated nanoparticles are of particular dimension with less polydispersity [4]. In extracellular synthesis, steps such as fermentation, filtration, followed by enzyme substrate complex formation in dark condition were involved. Whereas in intracellular synthesis, additional steps like ultrasound treatment of enzyme substrate complex or its reaction with suitable detergents were required [4]. Hence due to the above-mentioned drawbacks, unlike bacteria and fungi, only less number of intracellular actinobacterial nanoparticle production was reported.

The basic principle is actinobacteria, when exposed to metal ions, releases enzymes that reduce the metal ions to yield highly stable nanoparticles. Usually, silver ions in the form of silver nitrate ($AgNO_3$) solution [9] or gold ions in the form of chloroauric acid ($AuCl_4$) solution were used as substrate to the enzyme secreted from microbes. Both gold and silver are nonallergic, biocompatible with slow oxidation rate, and effective antimicrobial agents. Several researches are conducted by using other metals like zinc, manganese, and copper for nanoparticle formation.

Most of the nanoparticle biosynthesis work was carried out in *Streptomyces* sp., except the first novel work in *Rhodococcus* sp where Ahmed et al. [38] reported the intracellular synthesis of gold nanoparticle of the dimension 5-15 nm. They were concentrated more on cytoplasmic membrane than on cell wall. Using *Streptomyces* sp., intracellular synthesis of gold nanoparti-

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cles [39, 40], silver nanoparticles [41, 42], zinc and manganese nanoparticles [33] were reported till date.

As mentioned above, compared to intracellular synthesis, more number of extracellular silver [2, 11, 14] and gold nanoparticles [49, 50, 51] were reported in *Streptomyces* sp. Rare actinobacteria like *Thermomonospora* [11, 48], *Nocardia farcinica* [47] were used to synthesize gold nanoparticles, whereas *Nocardiopsis* sp. MBRC-1 [23], *Rhodococcus* sp. [45], *Thermoactinomyces* sp. [30], and unnamed actinomycetes [18, 32, 44] were used to synthesize silver nanoparticles. Usha et al. [43] isolated *Streptomyces* sp. that was used for the biosynthesis of copper and zinc nanoparticles.

3. Characterization of Nanoparticles

Techniques used for characterizing nanoparticles and their respective applications are as follows.

a. Colour change test

The formation of nanoparticles was detected by the color change within 72 h. Change of color from pale yellow to brownish color shows formation of silver nanoparticles [19]. Change of color from pale yellow to pinkish color indicates formation of gold nanoparticles. Formation of whitish yellow to yellow color shows formation of manganese and zinc nanoparticles [33].

b. UV--Visible Spectroscopy

The reduction of metal ions was estimated by measuring the absorption level using UV-visible spectroscopy. Light wavelengths of 200-800 nm are considered for nanoparticle characterization. Absorption measurements in the wavelength range of 400-450 nm and 500-550 nm are used in characterizing silver and gold nanoparticles, respectively [4].

c. X-ray Diffraction (XRD)

The phase identification and characterization of nanoparticle crystal structure were studied using XRD. X-rays penetrate into the freeze-dried and powdered nanoparticle at the scan speed of 0.02/min and the resultant diffraction is compared with the standard in order to derive its structural information [2, 4].

d. Fourier Transform Infrared Spectrometer (FT-IR) Analysis

Dried nanoparticle sample was mixed with KBr in 1:100 ratio and scanned using IR rays of around 4000-400 cm^{-1} with reflectance mode at 4 cm^{-1} resolution. The chemistry and the variation in functional group attached to the nanoparticle surface are analyzed using FT-IR [4, 12, 30].

e. Dynamic Light Scattering (DLS)

DLS was performed to determine the size, surface charge, and distribution of nanoparticles suspended in a liquid [17].

f. Energy Dispersive X-ray (EDX)

EDX was performed to determine the elemental composition of metal nanoparticles [17].

g. Atomic Force Microscopy (AFM)

AFM was performed to determine the size and topological appearance of metal nanomaterials. Porosity, roughness, and fractal dimension are also analyzed with the help of AFM images. For AFM study, the sonicated metal nanoparticles were formed as a thin film on slide [29, 32].

h. Scanning Eelectron Microscope (SEM)

SEM was performed to determine the size and surface morphology of biosynthesized metal nanomaterials. For SEM study at an operating voltage of 15 kV, the nanoparticle solution was sonicated, centrifuged, and the resultant dried powdered nanoparticles were used as sample [29].

i. Transmission Electron Microscope (TEM)

Compared to SEM, TEM gives 1,000 fold higher morphological resolution including both size and shape. The ultrasonicated nanomaterial sample was placed on copper grid coated with 300 mesh palladium and carbon for TEM study at 80 kV [33].

4. Applications

Nowadays, biosynthesized nanoparticles, especially gold and silver, were used in diverse applications especially in diagnostic field due to its antibacterial, antifungal, larvicidal, antifouling, anticancerous, antioxidant properties. Green synthesis of nanoparticles using dextran as ligand was explored since dextran was considered as cheaper, nontoxic and biocompatible agent [31].

a. Antibacterial activity

Using *Streptomyces viridogens*, gold nanoparticles of spherical shape with 18-20 nm was synthesized through intracellular mode [39]. A considerable number of silver nanoparticles using *Streptomyces* sp. were reported to show antibacterial activity against varied human pathogens through extracellular mode [14, 22, 24, 29, 51, 53-57]. Unnamed actinomycetes mediated silver nanoparticles were also reported to produce antibacterial activity [13, 18, 44]. Rare actinobacteria such as *Thermoactinomyces* sp. produce spherical shaped 20-40 nm sized silver nanoparticles were reported by Deepa et al. [30]. Similarly, Usha et al. [43] reported the production of copper and zinc nanoparticles sized 100-150 nm using *Streptomyces* sp. showing antimicrobial activity. The mechanism behind this bactericidal effect is silver nanoparticles attach to the bacterial cell membrane and disturb both respiration and permeability. Then by penetrating deep into the DNA part, it causes further damage by disrupting DNA replication due to release of silver ions, which leads to target cell destruction [32, 34].

b. Antifungal activity

Biosynthesis of gold nanoparticles using *Streptomyces* sp. VITDDK3 showed antifungal activity against *M. gypseum* and *T. rubrum* by changing the membrane potential and inhibiting ATP synthase activity of the target cell [49].

c. Anti-biofouling property

Anti-biofouling is defined as the process of eliminating the microbes that aggregate on wetted surface forming biofilms leading to foul smell production. Shanmugasundaram et al. [42] reported the antibiofouling property of 5-50 nm spherical-shaped silver nanoparticles using *Streptomyces naganishii* MA7.

d. Antioxidant property

An antioxidant prevents the oxidation of other molecules by interfering with defense mechanism leading to ROS generation [46]. Antioxidant property of silver nanoparticles mediated by *Streptomyces naganishii* MA7 was reported by Shanmugasundaram et al. [42], showing positive DPPH scavenging activity.

e. Larvicidal property

Silver nanoparticles synthesized by using *Streptomyces* sp. GRD showed effective larvicidal activity against *Culex quinquefasciatus* and *Aedes aegypti*, which would be an effective bioprocess for mosquito control [35]. Nanoparticles penetrate through larval membrane into the intracellular space leading to denaturation of organelles and enzymes [35]. Karthik et al. reported the biosynthesis of gold nanoparticles with antimalarial activity using *Streptomyces* sp. LK3 [50].

f. Anticancerous property

Varied researches are going on in order to treat cancer and reduce its side effects worldwide. Manivasagan et al. reported the cytotoxic effect of silver nanoparticles mediated by *Nocardiopsis* sp. MBRC-1 [23]. Same way, an actinomycete PSBVIT-13 [18] and *Streptomyces naganishii* MA7 [42] were also reported to mediate silver nanoparticles with cytotoxic effect.

g. Others

Torres-Chavolla et al. [52] reported the production of gold nanoparticles using *Thermomonospora* sp. which can be utilized as biosensing enhancement analytical device meant for detection purpose in military field and pollution control field.

5. Drawbacks (Nanoparticle toxicity)

Exposure of cells to actinobacterial nanoparticles like silver nanoparticles alters mitochondrial functioning by collapsing proton-motive force across the cell membrane, which leads to increased membrane permeability. As a result, ROS (reactive oxygen species) will be generated, which seems to be the initiator for toxicity. Factors like breakdown of its unique surface plasmon resonance, magnetic, chemical, and optical properties are responsible for toxicity.

6. Actinobacterial detoxification

Actinomycetes are resistant to toxic heavy metals due to their chemical detoxification, solubility alteration, as well as energy-dependent ion efflux from the cell by membrane proteins that function either as ATPase or chemiosmotic cation or proton anti-transporters. Detoxification can be made by reduction, precipitation, biosorption, biomineralization, and bioaccumulation [4].

7. Future directions

Compared to physical and chemical approaches, biosynthesis of nanoparticles consumes extra time. Actinobacterial-mediated nanoparticle biosynthesis has got more advantages such as the possibility of scaling up the process, economic viability, and the possibility of covering a large surface area due to mycelial growth. [11]. Hence, several criteria like synthesis time, particle size, solubility, stability, monodispersity should be given utmost priority in order to obtain highly efficient actinobacterial nanoparticles. By optimizing parameters like type of microbe, growth stage, growth medium, synthesis conditions, source compound of target nanoparticle, etc., it is possible to obtain clean, nontoxic, and eco-friendly nanoparticles [15].

8. Conclusion

As discussed earlier, due to increased cognizance towards green chemistry approach, the need of the hour is to develop eco-friendly strategies for the synthesis of nontoxic nanoparticles. To serve that purpose, the least explored actinomycetes have to be further explored so that they can emerge as effective "micronanofactories" in future. Still, the exact mechanism of nanoparticle biosynthesis is not clear. By decrypting the never ending clues involved in its synthesis, actinomycetes would be considered as the efficient microbial group for harnessing nanoparticles.

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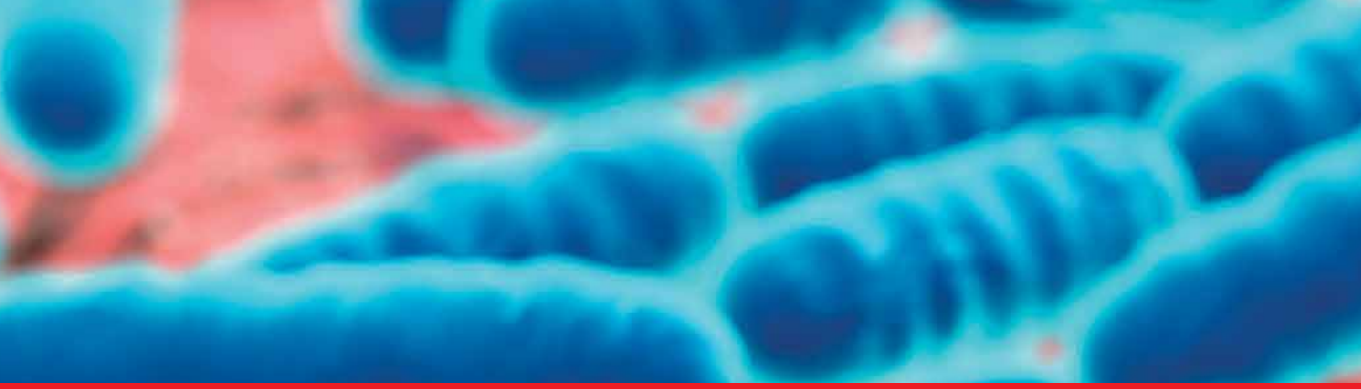

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This book presents an introductory overview of Actinobacteria with three main divisions: taxonomic principles, bioprospecting, and agriculture and industrial utility, which covers isolation, cultivation methods, and identification of Actinobacteria and production and biotechnological potential of antibacterial compounds and enzymes from Actinobacteria. Moreover, this book also provides a comprehensive account on plant growth-promoting (PGP) and pollutant degrading ability of Actinobacteria and the exploitation of Actinobacteria as ecofriendly nanofactories for biosynthesis of nanoparticles, such as gold and silver. This book will be beneficial for the graduate students, teachers, researchers, biotechnologists, and other professionals, who are interested to fortify and expand their knowledge about Actinobacteria in the field of Microbiology, Biotechnology, Biomedical Science, Plant Science, Agriculture, Plant pathology, Environmental Science, etc.

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