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Yeasts in Biotechnology

Edited by Thalita Peixoto Basso

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Meet the editor

Thalita Peixoto Basso received her Bachelor's degree in Agriculture Engineering (2008) from Londrina State University (PR-Brazil). During this period, she studied fermentation characteristics of *Saccharomyces cerevisiae* isolated from ethanol industrial processes. She obtained her Master's degree in Science from the Agri-food Industry, Food and Nutrition Department of the University of Sao Paulo (ESALQ/USP, SP-Brazil) in 2010. During this time, she isolated and selected fungi with high cellulose activity for the enzymatic hydrolysis of sugarcane bagasse.

She received her PhD in Science from the Soil Science Department (Agricultural Microbiology Program) at ESALQ/USP (2015), with a period of one year as a visiting scholar at the University of California Berkeley and Energy Bioscience Institute. Meanwhile, she worked on the improvement of *S. cerevisiae* by hybridization for increased tolerance toward inhibitors from second-generation ethanol substrates. Currently, she is a postdoctoral working with metabolomics and proteomics of fermentation processes in the Genetics Department at ESALQ/USP.

Contents

Preface

Yeasts are one of the most important eukaryotic microorganisms due to their role as model organisms for eukaryotic biological studies with a focus on understanding and interpreting human DNA. Recently, researches have been working with several yeast genera and species such as *Saccharomyces cerevisiae*, *Pichia pastoris*, and *Schizosaccharomyces pombe* as model organisms for cancer biology investigation, including tumorigenic mechanism studies, development, and production of anticancer drugs.

Many thousands of years ago, Egyptian, Babylonian, and Greek civilizations used these microorganisms to produce fermented foods and beverages, such as bread, beer, and wine. Since then, yeasts have been applied to the food industry and their utilization has been increasing over the last few decades. On the other hand, new yeast platforms such as cell factories have been developed for the production of biochemicals such as fuels and drugs.

This book offers a broad understanding of yeast applications such as cell factories for the production of biofuel, food, and bioproducts with high value. Additionally, the book provides an overview of yeast utilization as probiotics in animal nutrition. The book starts with an introduction chapter on the advances of yeast utilization in human history up to current biotechnology approaches.

The six chapters are grouped into three sections: Animal Nutrition, Food Industry, and Industrial Bioproducts. The Animal Nutrition section comprises two chapters dealing with the application of yeast as a probiotic in animal nutrition. In these chapters, the reader is guided through the action mechanisms of indigenous and commercial *S. cerevisiae* yeasts on ruminant gut microbial balance and can identify the ideal probiotic yeast for animal feeding.

In the Food Industry section, the advantages of nonconventional yeast strains on the food and beverage industry such as wine making, brewing, coffee and cocoa fermentation, and xylitol production are presented. It also covers the biotransformation process and analytical procedures, such as mechanisms of accumulation, extraction, and quantification of organically bound selenium by *S. cerevisiae.*

The Industrial Bioproducts section deals with the application of yeast as a platform for the biosynthesis of biochemicals such as flavonoids, alkaloids, terpenoids, and saponins used in the agriculture industry. It also covers the application of *Rhodotorula/Rhodosporidium* as a platform cell factory for biofuel feedstock, carotenoids, enzymes, and biosurfactant production.

This book intends to present a broad understanding of several biotechnology applications of yeasts in industrial bioprocesses. It will cover a multitude of issues such as the development of a platform cell factory, the use of nonconventional yeasts, and the diverse application of yeasts on bioprocesses. Other aspects related

to bioprocesses will also be analyzed, such as biofuels and bioproduct production and yeast application in wine making and brewing.

Thalita Peixoto Basso, Ph.D.
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Section 1

Introduction

Chapter 1

Introductory Chapter: Yeasts in Biotechnology

Thalita Peixoto Basso, Luiz Carlos Basso and Carlos Alberto Labate

1. Introduction

Yeasts are very important for many reasons. These microorganisms were the first species to be domesticated by man, although not intentionally. For millennia they were used in fermented beverages and foods without knowing their existence. Biochemistry as a science was born when physiologists looked deeper in sugar fermentation in the final of nineteenth century.

Today yeast takes a place in several fields of science and technology. As long as yeast genes and mammal cells encode very similar proteins, these microorganisms are useful as a model to understand and interpret human DNA sequences. Indeed, yeast genetic manipulation is much easier and cheaper than mammalian systems. So yeast has turned out to be a useful model for eukaryotic biology [1, 2].

Furthermore yeasts such as *Saccharomyces cerevisiae*, *Picchia pastoris*, and *Schizosaccharomyces pombe* have been used as model organisms to study cancer biology, including research and development of tumorigenic mechanisms and production of anticancer drugs [3].

Particularly, *Saccharomyces cerevisiae* is a model organism to study epigenetic traits that can be characterized as a stably heritable phenotype resulting from changes in a chromosome without alteration in the DNA sequence. As a result of yeast small eukaryotic genome, short generation time and easy genetic manipulation [4].

Additionally yeasts are very important players in many economical relevant bioprocessing as bakery, brewery, distilling, food industry, and biofuel, leading yeasts to be considered the most explored and studied eukaryotic microorganism.

2. Yeast application

Since 8000 years ago in our history, humans have been using microorganisms to produce fermented foods and beverages. More recently chemicals and fuels have been produced by bioprocesses. The development of cell factories has been incentivized for the industrial production of new chemicals. However the development of new yeast platform cell factory is costly and time-consuming. The difficulty to develop new cell factories to produce a specific metabolite is due to metabolism which has evolved to allow cell growth and maintenance to keep homeostasis [5].

Yeasts from phyla of ascomycetes and basidiomycetes have diverse biotechnological application on food industry. They are responsible for a wide range of fermented products such as alcoholic beverages (e.g., beer, wine, and "cachaça"), fermented milk, cheese, bread, and so on. Yeast also has an application in the functional food industry as probiotics and nutraceutical products [6].

Saccharomyces cerevisiae has been metabolically engineered for the production of first-generation and second-generation bioethanols, advanced biofuels, and chemicals [7–11].

Recently, new tools for genome editing as CRISPR-Cas9 technology have the advantage to allow introduction of many genes into any chromosome location [12, 13]. On the other hand, high-throughput methods as transcriptomic, proteomic, and metabolomic analyses support the introduction of metabolic pathway over cellular physiology metabolism. Indeed next-generation sequencing allows the identification of any genome modification responsible for desirable phenotype [5].

3. Conclusion

In conclusion, we believe that the yeasts are a nearly ideal model system for eukaryotic biology at the cellular and molecular level. Additionally their use in increasingly technological applications will augment the importance of yeast for human well-being.

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The authors would like to acknowledge CAPES and Inter-unit Bioenergy Post Graduation Program (USP/UNESP/UNICAMP) for PNPD/CAPES postdoctoral fellowship (process number 88882.317582/2019-01), Max Feffer Laboratory from Genetic Department (ESALQ/USP), and Yeast Physiology and Fermentation Laboratory from Biological Science (ESALQ/USP).

Conflict of interest

The authors confirm there is no conflict of interest.

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Section 2

Animal Nutrition

Chapter 2

Common Methods to Understand and Develop Indigenous Probiotics Yeast for Ruminant

Shakira Ghazanfar, Aayesha Riaz, Ghulam Muhammad Ali, Saima Naveed, Irum Arif, Sidra Irshad, Naeem Riaz and Khanzadi Nazneen Manzoor

Abstract

Probiotic yeast enhanced the ruminal gut microbial balance by producing intercellular effectors and important metabolites. The impact of yeast addition on animal health is influenced by different interlinked factors including animal genomics, its gut microbiota, and environment. Therefore, all factors should be considered regarding achieving the maximum outputs from animal probiotic yeast. In the situation of a high feeding cost, microbial feed supplements provide a suitable nutritional approach, which allows increased nutrient digestion rate and accordingly improves animal performance. Many yeast products are commercially available, but their efficiency as probiotic dietary addition in a particular breed is mostly questionable. Therefore, identification of ideal probiotic yeast strain is of great interest in this context. Innovative methods in relation to develop new probiotic are mainly focused on the exploring novel microbial strains from indigenous sources. It has been noted that for the identification of best probiotic strain for the host, a linkage between culture-independent and culture-dependent methods is a functional step. In this chapter, we will discuss the mode of action of probiotic yeast on animal lower gut microbiota and identification of ideal probiotic yeast by using advanced molecular methods.

Keywords: indigenous probiotic yeast, lower gut, microbiota, molecular methods

1. Introduction

Over the past decades, the livestock industry has been revolutionized toward the use of microbial feed additives due to an increasing awareness of the stockholders on the beneficial role of probiotics in production and gut health status [1, 2]. There are several probiotic products that are commercially available and marketed for animal use [3]. Most probiotic products at the moment do not go through pre-market approvals and are commonly used for a much wider range of scenarios in which their efficacy is not well established. Similarly, latest molecular methods such as gene sequencing and phylogenetic analysis are not used to identify the probiotic strains as feed supplements. For the selection of best probiotic product, it is highly important to determine the real probiotic potential of the microbial strain by using latest molecular methods. In this contract, locally isolated and validated probiotic strains

will be better than any unauthorized local available strain. The competitive advantage and adaptability to local microbial ecosystem will allow local probiotic strain to grow and adhere well in the local animal breed. Literature showed that probiotic strains should specifically prepare according to purpose and function related to the milk enhancement in local breed [4, 5]. Nowadays, it is highly accepted that probiotic yeast is highly productive in terms of milk and meat for large animals [6, 7]. Probiotic yeast improves the ruminal gut microbiota which may increase the nutrient digestibility and leads to improve animal productivity [8]. In large animals, ingested feed digested by numerous microbial species is present along the gastrointestinal tract [9]. This microbial community consists of 1014 members, mainly composed of fibrolytic bacterial species [10]. Literature highlighted that gut microbiota plays important role in the feed digestion and utilization. The gut microbial populations in cow have been identified in almost 90% of the total microbial community [11]. On the other hand, a certain fraction of the GI tract bacterial community has yet to be identified due to less knowledge of the microbial community in gut microbial ecosystem because majority of the 16S rRNA gene sequences from feces are taken from unidentified species, and many modern methods of genomic analysis of communities to determine changes in microbiota have been used by many scientists [12]. Studies have utilized culture-independent sequencing techniques, 16S rDNA bacterial tag-encoded FLX amplicon pyrosequencing and many more have added a new era to determine the microbial diversity of the GI tract [13]. Research noted that the culture-independent methods deliver a comprehensive assessment of the microbial community composition, while the culture-dependent methods provide the structural and functional diversity of the microbial strains [14]. In this chapter, a detailed discussion on the effects of probiotic yeast in ruminant's well being, production performance, uses of different omics methodologies for the discovery of ideal animal probiotic strains and development of indigenous probiotic yeast for ruminant will be employed.

2. Yeast: an ideal microbial feed supplement for ruminants

The *Saccharomyces cerevisiae* (baker's yeast) is the first eukaryotic sequenced genome. The sequencing of first whole eukaryotic genome was a challenging task for the scientists, but the efforts of more than 600 scientists from Europe, North America, and Japan made it possible. The entire sequence of the yeast was released in 1996. The size of the baker's yeast genome is 12.1 Mb containing 16 chromosomes and 5400 coding genes approximately. The sequence information of yeast is available at *Saccharomyces* Genome Database (SGD), Yeast Protein Database (YPD), and Munich Information Center for Protein Sequences (MIPS) [15] (**Table 1**).

Ruminant nutritionists have been pondering to improvise new methodologies for ameliorating the roles of microflora in ruminants and enhance processes of

Yeast genome	
Genome size	12.1 Mb
Chromosomes	16
Genes	5300–5400
Base pairs	12 million base pairs
Databases	SGD, MIPS, YPD

Table 1.
Details of first eukaryotic sequenced genome (yeast).

digestion and fermentation along with augmented nutrients usage and bioavailability using feed supplementation. One of the commonly used methods was the use of growth promoters (antibiotics) to restrict the pathogenic effect on productivity of ruminants [16]. Nevertheless, antibiotics have been reported to cause serious health challenges to consumers and environmental implications. Thus, their usage has been banned in 2006 due to emerging antibiotic resistance. In the light of these concerns, consumer preferred more natural product. A super alternate of feed additives was the use of probiotics [17]. Probiotics are living microorganisms confined in animal feed that affect the host by improving the digestion [18]. Other definition includes probiotics as microorganisms (viable) that functions in gaining weight and feed conversions along with reducing diarrheal incidence [19]. Probiotics have been deployed as one of the recent exploited proposals in ensuring efficiency of production systems and safety to both consumers and environment [20, 21]. In ruminant nutrition, yeast probiotics are commonly being used because of their efficient roles in rumen stabilization and maintaining microbial communities specifically fibrolytic bacteria [22]. The yeast cells function in maintaining throughout viability of the digestive tract [23]. Yeast supplementation as probiotics enhanced feed conversion, efficient fermentation, and fiber digestion in the rumen, maintained ruminal pH, increased milk production [24, 25] and feed intake and production of organic acids and vitamins to activate the growth of the lactic acid bacteria (LAB) [26]. The commonly used yeast probiotic is *Saccharomyces cerevisiae*. Numerous literatures on *Saccharomyces cerevisiae* as supplement are available that dated back to the 1950s and continued under study till today [27]. Significant role of yeast supplementation (live) in diet has been stated for lactating and growing ruminants. Recent studies confirmed that they increase the ruminant's milk production early lactation period by altering the fermentation of food inside the GIT of ruminants[28]. Latest beef and dairy production systems demand active muscle growth and high milk yield via feeding animal at high ruminal ferment ability rates. This would result in increased risk of metabolic disorders such as acidosis due to dysbiosis in ruminal microbial environment resulting in abnormal functioning in rumen which further leads to poor feed intake, health, and decreased productivity [29]. Therefore, yeast supplementation in ruminant diet is beneficial in the ruminal functioning and overall animal health and maintenance. The ameliorating functions of yeast probiotic on digestibility of high forage diets also underscore the potential use of yeast supplementation to optimize the use of lower quality feeds.

3. Understanding of the ruminant microbial community for development of ideal probiotic yeast for ruminants

Rumen microbial manipulation by using the probiotics to improve the ruminant feed digestion is a promising production improvement strategy. A better understanding of the rumen microbiology is an important step to select and prepare a new yeast strain affecting on functional specific microbes. Latest molecular techniques have provided the opportunity to study the rumen microbiota in detail for development of the ideal probiotic.

3.1 Digestive system of ruminants

Digestive system of ruminant is composed of four parts: reticulum, rumen, omasum and abomasums. The rumen is that part of the digestive system in which fermentation is carried out [30]. The rumen can also be defined as a complex ecosystem in which nutrients consumed by different microorganisms are digested

anaerobically. Microbial biomass and volatile fatty acids are most common end products of fermentation which are then used by ruminant host. Interaction of host animal and microorganisms is a symbiotic relationship that helps the ruminant hosts in digestion of fiber-rich and protein-low diets. Rumen microorganisms provide enzymes that are necessary for fermentation processes, which in turn allow ruminants to obtain energy contained in forage [31]. Growth and activity of ruminal microorganisms are influenced by different factors including pH, temperature, osmotic pressure, buffering capacity, and redox potential. These factors are determined by environmental factors. Temperature of the rumen is in the range of 39–39.5°C. But when animal eats, fermentation occurs that generates heat due to which temperature increases up to the limit of 41°C [32, 33]. Short-chain fatty acid generation along with their absorption, saliva production, feed intake level and type, as well as exchange of phosphates and bicarbonates through epithelium of the rumen are the factors that affect pH [34]. In the reticule ruminal environment, these factors determine the buffering capacity as well as pH. There is a constant change in pH but mostly it remains in the range of 5.5–7.0 [35]. When there is an acidic environment in the cell, bacterial intracellular pH decreases. Microbial enzymes are very much sensitive to pH, i.e., bacterial growth is inhibited when there is an acidic pH. This is due to the disproportion of intracellular hydrogen ions [36]. In the rumen, ions and molecules affect osmotic pressure due to which gas tension is created. Fermentation process in the rumen depends upon the environmental factors and the diet due to which these factors also affect rumen osmotic pressure [37] (**Figure 1**).

3.2 Microbial community of GIT

Bacteria are more in number than any other microbes. It is noted that there are five groups of rumen bacteria: (1) free-living in liquid phase, (2) loosely attached with feed, (3) firmly attached with feed, (4) attached with rumen epithelial lining, and (5) attached with protozoa/fungi. The bacterial species inside the rumen are 99.5% obligatory anaerobic. Mostly rumen bacteria are involved in the fermentation of fibers, starch, and sugar present in the feed and converted into volatile fatty acid, H_2, and CO_2 [38]. Most of the bacteria are responsible for degradation of different types of dietary components [39] (**Table 2**).

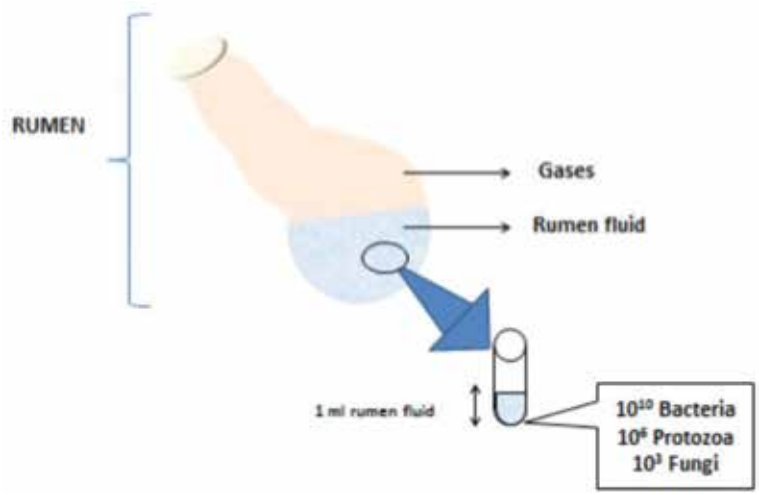

Figure 1.
Rumen ecosystem: different types of microbial flora present inside the rumen. The most abundant microbes are bacteria.

Majority of anaerobic rumen fungi is from order *Neocallimastigales* within the *phylum Neocallimastigomycota*. On the phylogeny basis, six genera have been identified, which are *Piromyces*, *Neocallimastix*, *Caecomyces*, *Anaeromyces*, *Orpinomyces*, *and Cyllamyces* [40]. In fiber digestion, fungi play a very important role because of the vegetative thallic rhizoids. The main functions of the rumen fungi are the lignin and fiber degradation by producing different types of enzymes [41] (**Table 3**).

3.3 Mechanism of action of probiotic yeast in the rumen

The rumen is the first part of the ruminant stomach which has a well-developed microbial ecosystem containing different types of microbes (bacteria, fungi, protozoa, and bacteriophages). These microbes coexist in ecological equilibrium

Bacteria	Species
Carbohydrate-utilizing bacteria	*Fibrobacter succinogenes* *Ruminococcus flavefaciens* *Ruminococcus albus* *Clostridium cellobioparum* *Clostridium longisporum* *Clostridium lochheadii* *Eubacterium cellulosolvens* *Cillobacterium cellulosolvens* *Butyrivibrio fibrisolvens* *Prevotella ruminicola* *Bacteroides ruminicola* *Eubacterium xylanophilum* *Bacteroides uniformis*
Nitrogen-utilizing bacteria	*Prevotella ruminicola* *Ruminobacteramylophilus* *Clostridium bifermentans*
Lipid-utilizing bacteria	*Anaerovibriolipolytica*

Table 2.
Bacterial diversity of the rumen microbial ecosystem.

Microbial species	Rumen	Fecal
Bacteria	*Bulleidia* *Roseburia* *Prevotella* *Ruminococcus* *Acidaminococcus* *Megasphaera* *Succiniclasticum*	*Bacteroides* *Bifidobacterium* *Clostridium* *Collinsella* *Blautia* *Dorea* *Lactobacillus* *Peptostreptococcus* *Treponema* *Succinivibrio* *Faecalibacterium*
Fungi	*Caecomyces* *Orpinomyces* *Piromyces*	*Caecomyces* *Orpinomyces* *Piromyces*
Archaea	*Methanobrevibacter* *Methanosphaera*	*Methanobrevibacter* *Methanosphaera*

Table 3.
Bacteria, fungi, and archaea present inside the rumen and feces of dairy cows.

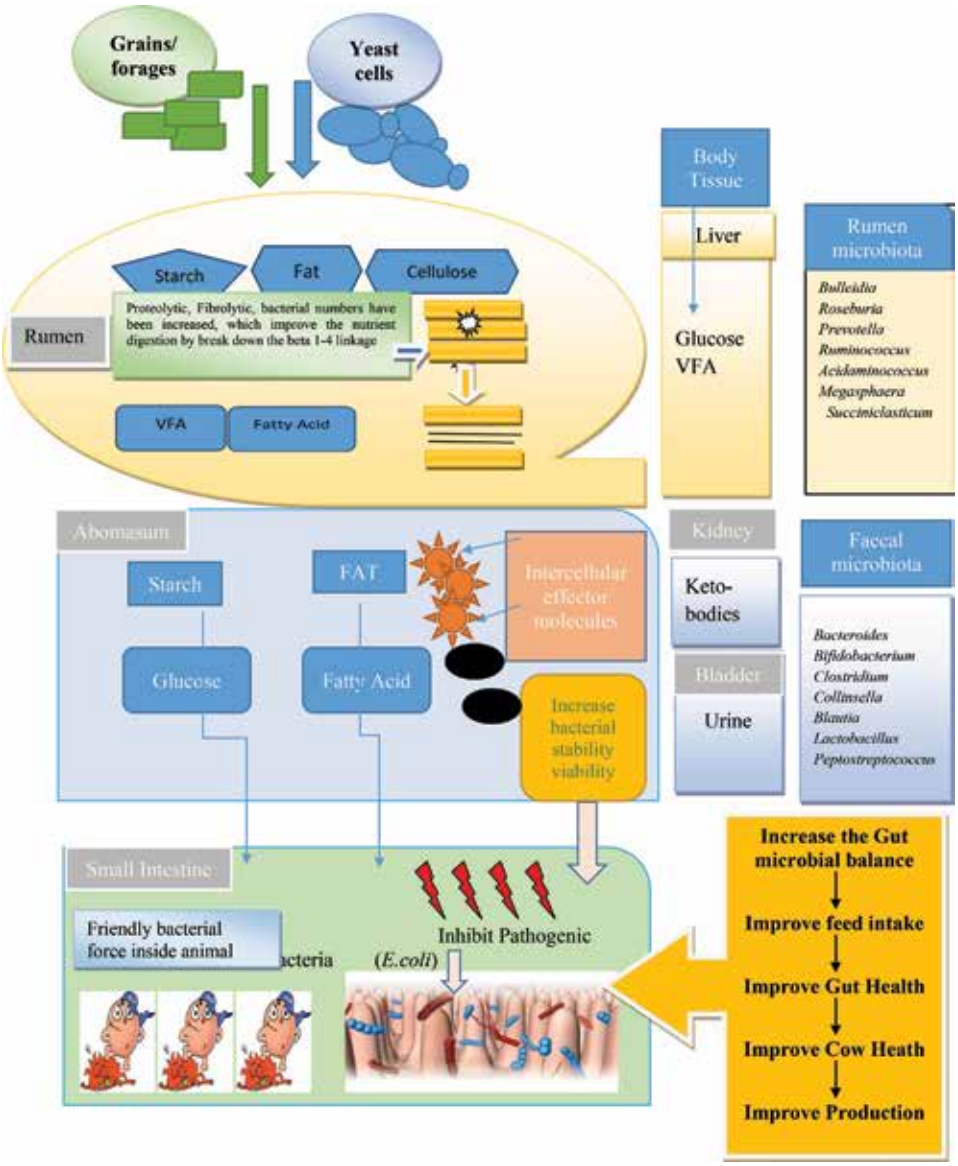

Figure 2.
Representative scheme of effect of live yeast on the microbial flora of the gastrointestinal tract in ruminants: live yeast improves carbohydrate, protein, and lipid digestion rates by improving the production of cellulolytic, hemi-cellulolytic, and proteolytic and lipolytic bacteria and fungi.

in unique symbiotic relationship between cows and rumen microbes. The cows supply food to the rumen microbes which in turn digest the feedstuff to provide cows the essential nutrients in the form of microbial protein as organic acid energy sources. The microscopic view of rumen ecosystem showed that it is consisted of a number of bacteria, protozoa and fungi [42]. Bacteria make the largest population in this diverse microbial world. Their function is to digest the fibers, starch, sugar acids, and protein to give useful compounds and elements necessary for the growth and productivity of the cows. The role of protozoa and fungi is less clear. However, these microbes do provide help in digestion of feed. The structure and function of microbial community are influenced by feed composition

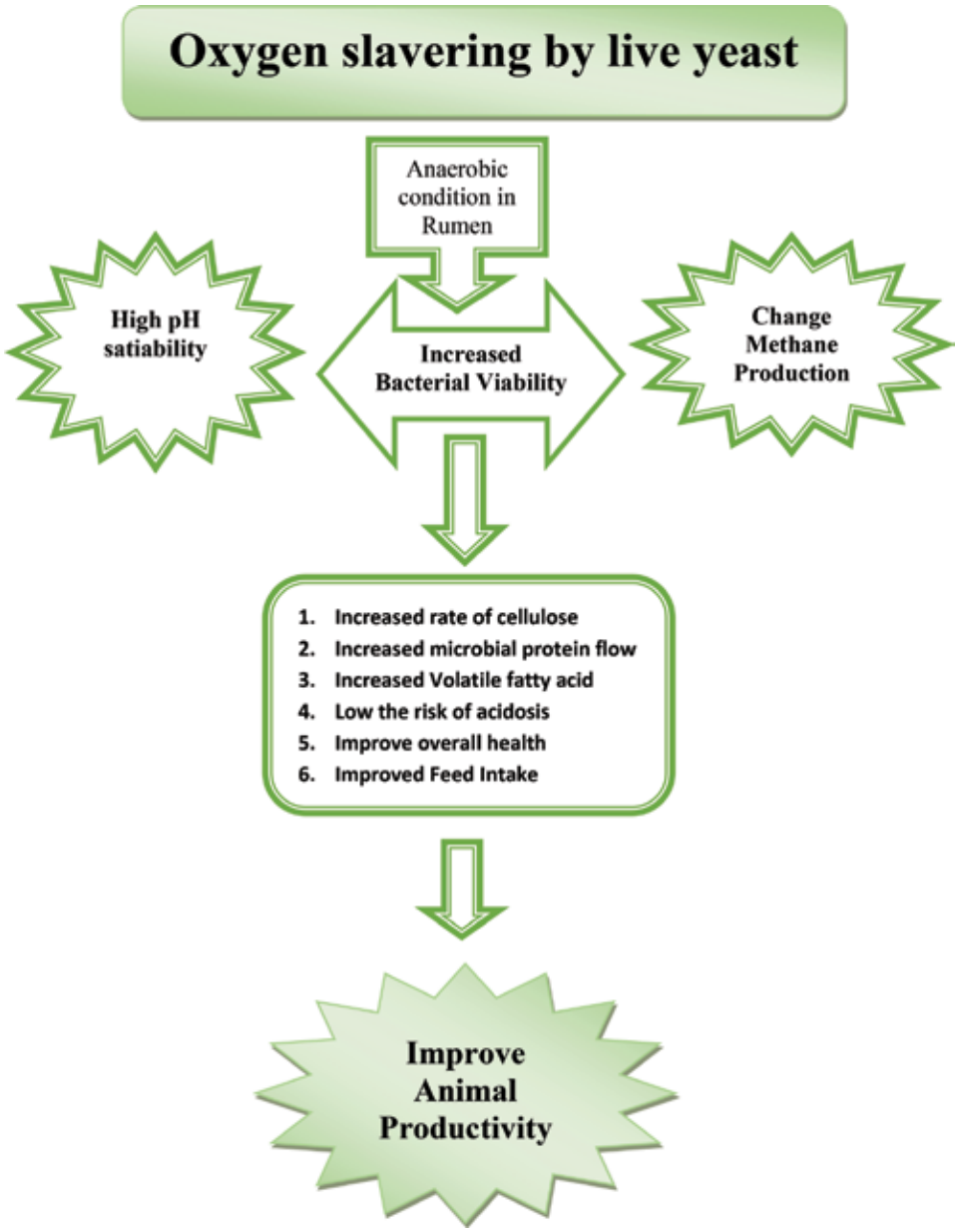

Figure 3.
A scheme describing the mode of action of yeast culture: improved the gut microbial balance is related to the O_2 slavering by live yeast cells.

and mainly by the host genetic potential. *Prevotella* and *Succinivibrionaceae* are the dominated rumen bacterial communities, cellulolytic and fibrolytic genera; *Neocallimastigaceae* are the dominant fecal and rumen fungal communities; and *Methanobrevibacter* are the dominant fecal and rumen archaeal communities in the adult ruminants. *Bacteroidetes* and *Firmicutes* are the dominant phyla of bacterial communities. *Bacteroidaceae*, *Lachnospiraceae*, *Prevotellaceae*, *Ruminococcaceae*, *Succinivibrionaceae*, and *Veillonellaceae* are the most abundant bacterial families in adult ruminant [43]. The term "yeast" is originally derived from the Dutch word

gist, which basically refers to the foam that formed during beer fermentation. A variety of roles is played by yeast in veterinary practices, livestock feeding, and medicine as well as in biomedical and pharmaceutical industries [44]. Hayduck first discovered the inhibitory activity of yeast. Probiotics such as yeast or fungi have been extensively used in ruminant feed for the improvement of growth, health, and lactation due to their impact on rumen pH, intake of dry matter, and digestibility of nutrients [45]. Probiotic yeast has potential beneficial effects on the rumen. In the cattle, the ability of live yeast for enhancement of milk yield as well as weight gain is due to the fact that yeast is responsible for stimulating bacterial activity in the rumen [46]. Mechanism of action of yeast mainly stimulates the growth of cellulatic and hemicellulatic bacteria [47]. Increase in the number of bacteria in the rumen is due to the reproducible effects of probiotic yeast. Yeasts remove oxygen from the rumen due to which bacterial performance improves in the rumen. To maintain the metabolic activity, yeast cells consume available oxygen on the surface of freshly ingested feed in the rumen. Few studies showed that there is a significant decrease in redox potential, up to -20 mV by providing yeast supplementation (**Figure 2**).

Better conditions have been created by this change for the growth of anaerobic cellulolytic bacteria which in turn stimulates their attachment to forage particles as well as increases the initial rate of cellulolysis. Recalcitrant plant lignocellulosic material is not degraded by ruminants on its own. They rely on rumen microbial flora for its degradation [48]. The main components of the fiber are cellulose, hemicellulose, and lignin. It has been estimated that 20–70% of the ruminant feed is composed of the cellulose and hemicellulose [49]. The most abundant carbohydrate in plant cell wall is the cellulose which makes up to 40% of the plant cell wall. The microbial cellulolytic enzymes have the capability to digest the β-1,4 links present inside the cellulose, glucose molecules [50] (**Figure 3**).

3.4 Mechanism of action of probiotic yeast in the lower gut

The lower gut microbial population is affected by dietary supplementation of the probiotic yeast. The probiotics provide a desirable microbial balance due to shift in the balance of friendly and pathogenic microbiota. The GIT having healthy microbial populations are often related with improved host performance and its immune system. In the lower gut, the pathogenic microbial species reduces due to the production of the antimicrobial material (bacteriocin) and the attachment of the friendly microbes to the gut wall, via the competitive exclusive method. The most common modulation of the GIT microflora is provided by probiotics [51].

4. Modern methods to understand and develop fibrolytic probiotics for ruminants

Latest researches have improved our understanding related to the mode of action of probiotic yeast inside the rumen. Well-designed animal studies have verified that target-specific probiotic strains have health and production benefits in the ruminants. These studies have made the livestock industry to accept and understand the probiotic concept [52]. On the other hand, current probiotic has not been chosen for definite purposes in the animal feed. Therefore, some unique molecular methods are needed for selection and characterization of target-specific probiotic strains [53]. It has been noted that during stress conditions, some portion

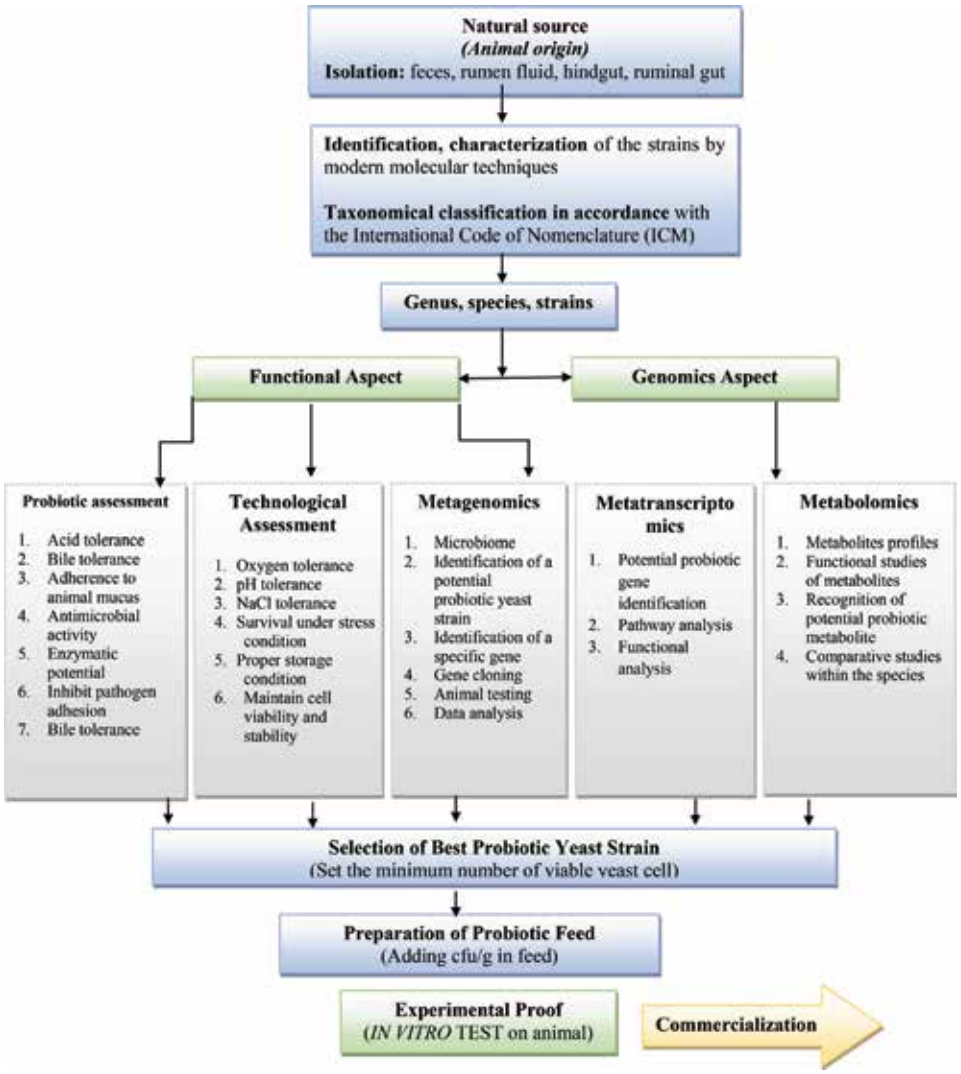

Figure 4.
Probiotic preparation: general steps for the isolation and characterization of probiotic yeast strains for local animal breed.

of the live probiotic microbial strain enters in the dormant but metabolically active state called viable but nonculturable (VBNC) state. These microbial cells have an ability to replicate when acclimated to a favorable condition inside the host [54]. Uses of molecular techniques have changed the study of the rumen ecosystem. First is the PCR which is more sensitive than growth on traditional selective media in determining small differences in population sizes in response to dietary changes or upon the inclusion of an additive to the diet and thus may identify changes or shifts within levels of the microbial population which may have been previously overlooked [55] (**Figure 4**).

In response to various feeding sources, changes within the microbial population can be studied by DNA fingerprinting (DGGE, TTGE, and TGGE). Probiotic can be classified into three different types, like mono-probiotic, poly probiotics, and combined probiotics depending on the probiotic strain function [55] (**Figure 5**).

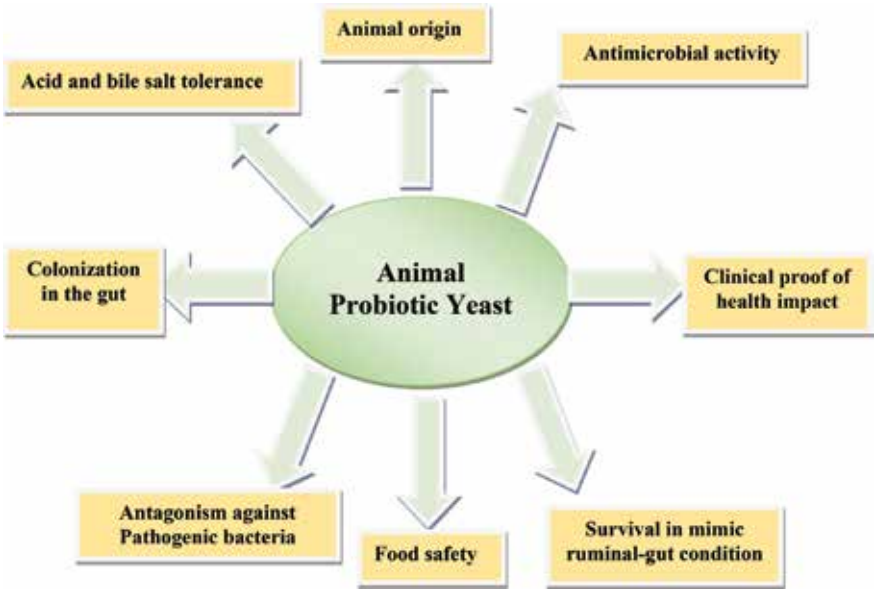

Figure 5.
Potential characteristics of typical animal probiotic yeast.

5. Common methods used to identify indigenous probiotic yeast

Yeasts and fungi are the ideal organisms and have been used in vast genetic studies and comparative genomic studies in eukaryotes because of their small and compact genomes.

We have sketched sampling approaches and finalized the protocols that will guide researchers in identifying the most ideal probiotics for animal use. Livestock is under increasing threat of antimicrobial resistance genes; therefore, continued optimization of protocols is urgently needed so that these threats can be reduced through the use of probiotics. Two sequence-based methods are commonly used for the identification of yeast. The first and the most common method used for the identification is PCR amplification of internal transcribed spacer (ITS) of nuclear ribosomal variable region that has been recognized as the universal barcode for the identification of fungi. The second and the advanced approach to identify fungal species or strains is shotgun metagenomics [56]. Microbes are very vital to life present on the earth. Their significance is increasing day by day as their beneficiary potential has been recognized in the field of health and medicine. There are two methods which have been utilized till now for the identification of the microorganisms present in microbial community.

- Culture-dependent method
- Culture-independent method

Both approaches have their own significance. Culture-based methods are considered effective for the morphological, physiological, and functional characterizations of a particular strain, while culture-independent technology is preferred to unravel the microbial diversity along with genomic and genetic identification of microbial communities. Studies have also indicated that there is a loss of 99% microbes in the laboratory-dependent culturing methods. Culturing-independent

method has been recognized as an effective and efficient method to isolate the DNA of a number of microbes from an environmental sample which seems impossible using the cultural methods. The linkage of culture-dependent and culture-independent data has been recognized as a crucial step for the identification of probiotics [57]. For identification of the potential probiotic strains, researchers should use the latest molecular methods, and the probiotic strains should be deposited in some recognized microbial culture collection. Proteomics and metabolomics may also be used for choosing the best yeast species [58]. By utilizing strain's proteome and metabolome, which are argued to yield a positive influence upon ruminal fermentation, it may be possible to identify specific traits, characteristics, and secondary growth metabolites that play a potential role to enhance the growth of target-specific microorganisms inside the rumen. Even accounting for the potential bias of latest molecular methods, it is obvious that these methods are the dominant tools recently accessible for monitoring the gut for bacterial diversity of dairy animals and developing new yeast strain [59]. Extensive use of molecular methodologies may give insights into the new era where such microbial studies are no longer limited to a handful of laboratories with an abundance of funding and labor. It is noted that the specific yeast strains of known origin act more precisely and efficiently as compared to the yeast strain obtained from any unknown origin [60]. As we note all ruminates live in different parts of the world; therefore, upon the ruminal fermentation different yeast strains may exhibit markedly different effects. Therefore, we should identify new yeast strains for getting best results on the rumen fermentation. Uses of molecular techniques have changed the study of the rumen ecosystem. First is the PCR which is more sensitive than growth on traditional selective media in determining small differences in population sizes in response to dietary changes or upon the inclusion of an additive to the diet and thus may identify changes or shifts within levels of the microbial population which may have been previously overlooked. In response to various feeding sources, changes within the microbial population can be studied by DNA fingerprinting (DGGE, TTGE, and TGGE). To select best yeast strains, proteomics and metabolomics may also be used. By characterizing the proteome and metabolome of microbial isolates endowed with the ability to have a positive impact on the rumen fermentation, it may be possible to identify specific traits, characteristics, and secondary growth metabolites which play genuine role in the improvement of the growth of some important microbial species [61] (**Figure 6**).

5.1 Culture-dependent techniques

Cultural approach is the widely used method in microbiology to grow a microbe in a laboratory. Sampling is the basic and the crucial step for the identification of the indigenous probiotic yeast. The second step is isolation of the pure yeast strain under laboratory conditions which requires a series of inoculation steps of the microbes on the selective media. After purification of the yeast isolate on the OGA media, the biochemical tests are performed to identify the distinct features of the pure isolates. Morphological features of the isolate are determined by using electron microscope. The next step is the molecular identification of the yeast via 18S rRNA gene sequencing. The probiotic characterization is usually performed according to the standards defined by the WHO [62]. The best probiotic strain is retrieved among all the selected potential candidates, and in vivo experiments are performed using an animal model. After functional testing, all technological and safety measures are accessed, and the probiotic yeast strain is ready for probiotic product and packaging [63].

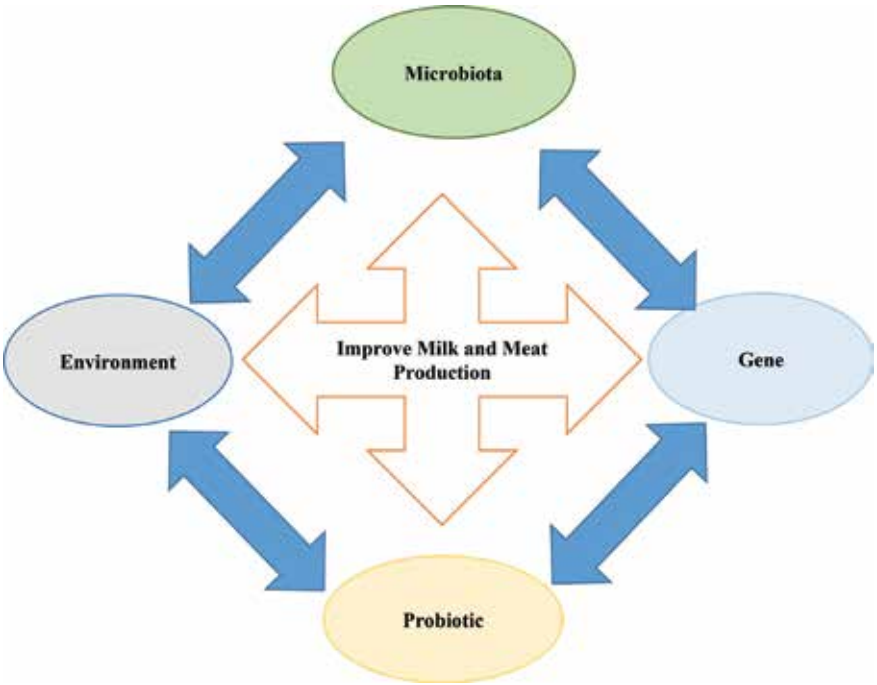

Figure 6.
Interlinked factors involved in the application of probiotic in the ruminant nutrition.

5.2 Culture-independent techniques

The use of omics approach has been emphasized to study the microbiome of microbes. To identify the potential probiotic strains among the microbial community present in any environment, it is very important to identify all the microorganisms in microbiota and determine their structural and functional differences at genomic level. Below are the currently available omics approaches for the identification, screening, and selection of probiotic strains of indigenous yeast [64] (**Figure** 7).

5.2.1 18S amplicon sequencing

Amplicon sequencing refers to the sequencing of a specific fragment of interest of a microbe using high-throughput sequencing technique. 18S amplicon sequencing is specifically used to determine the most prevalent fungal yeast species present in microbiota [65]. The methodologies used in the recent researches for the identification of bacterial probiotics can be applied in the recognition of indigenous probiotic yeast strains. The comparative and detailed analysis of 18S amplicon sequencing data can help the scientists in the isolation of potential probiotic after the identification of functional and structural characteristics of the indigenous yeast in microbiota. Further experiments and testing would be required to maximize the production and ability of probiotic yeast in the gut of an animal [66]. Furthermore, the 18S amplicon sequencing does not only help in the indigenous yeast identification, but it also reveals the diversity of microeukaryotes when 18S rRNA gene is sequenced [67].

5.2.2 Shotgun metagenomics

Shotgun metagenomics is one of the most advanced techniques of sequencing in which the entire microbiome of microbiota is sequenced. The data generated

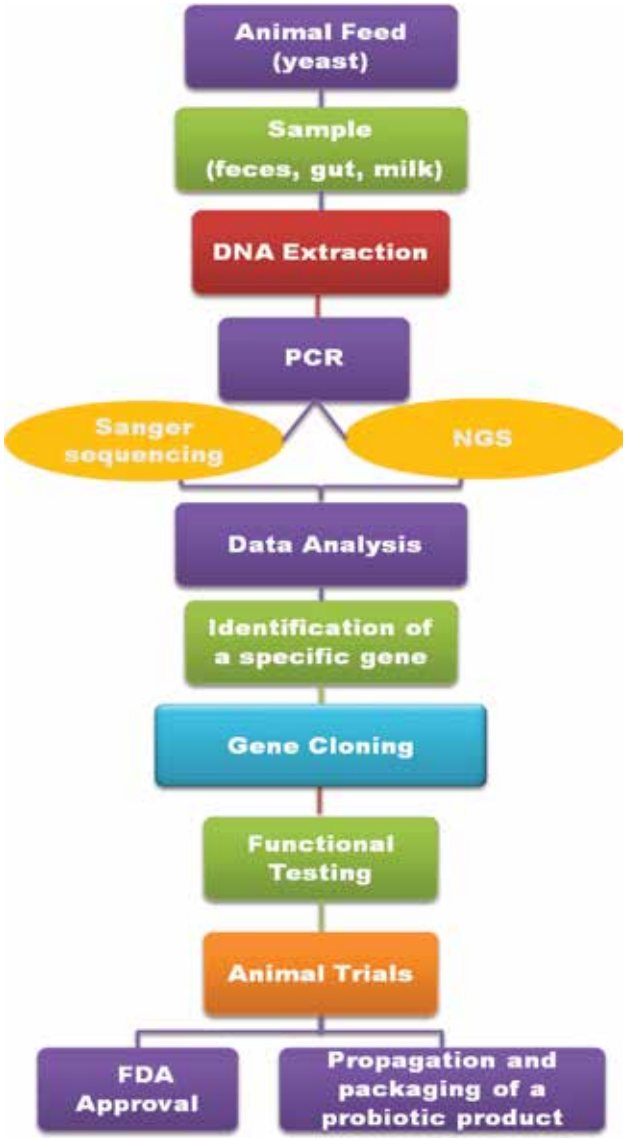

Figure 7.
Omics approaches to identify the probiotic.

using this method provides all the information about the genome of an organism [68]. Metagenomics information unravels the composition of microbial community and also indicates the genes, their functions, and associated genetic pathways. The identification of the indigenous yeast and their probiotic potential and capabilities can also be determined using the metagenomics data. Their relationship within the microbial community and their effect on the host can also be studied on the basis of the retrieved information [69].

5.2.3 Metatranscriptomics

Scientists and researchers are using metatrancriptomics to study and analyze the expression profiles of mRNA in a microbial community. The identification of genes, genetic pathways and their regulation, host-microbe interaction, and the symbiotic relation among microbes can easily be determined by using the mRNA

expression data. Metatranscriptome approach can be pursued in the identification of indigenous probiotic yeast within the microbiota of an animal. For this purpose the sampling methods and molecular techniques should be improved [70].

5.2.4 Metabolomics

Metabolomics refers to the study of the metabolites or final cellular products. This is also considered one of the useful and efficient methods for the identification of probiotic potential of a microorganism within a microbiota of an animal or selected biological sample [71]. Indigenous probiotic potential of yeast can also be determined using this technique. Studies are still needed to fully understand the function of metabolites in context of probiotic potential and other inhibitory functions of metabolic compounds. As metabolites vary in structure and function, so they could be used in the comparative studies of species and populations. A number of species with high probiotic potential could be approached using metabolomics [72].

6. Challenges in preparation of suitable probiotic yeast

- Yeast probiotics not only help to improve the performance factor of cattle, but it also enhances nutrient digestibility. However, the effectiveness of yeast-supplemented products is variable. Therefore, future studies are required to estimate the potency of these diet products as supplements for finishing beef cattle, with an objective to have healthier and productive animals without negotiating their efficiency and costs.

- The animal body is a "supraorganism" and refers to the gastrointestinal tract as a virtual organ of the human body. The ongoing research is mainly on probiotics that are used chiefly for the GI tract, whereas there is an impetus need to evaluate the progress on other regions of the body as well.

- Yeast supplementation is an effective strategy; thus, it is vital to ensure the stability and viability of yeast-supplemented diet products by developing practicable and cost-effective technologies (e.g., storage, microencapsulation, etc.), which poses marketing and technological challenges for producers at industrial level. Polysaccharides, lipids, and proteins are chiefly used for encapsulation materials in food industry. However, cost-effective production remains a challenge for production of future probiotics and formulation technologies.

- Role of yeast probiotics in combating antibiotic-associated diseases has been extensively reported through control trials and ingestion of yeast probiotics (*Saccharomyces boulardii*) and has positive therapeutic effects specifically in preventing antibiotic-associated diarrhea (ADD), but validated biomarkers for numerous target diseases are probiotic or antibiotic deficient. Therefore, in the field of probiotic investigation, the defining of validated biomarkers needs to be advanced.

- There is a dire need to understand the composition and relationship of microbial community within an animal gut for improving the production of dairy products. Advances in the high-throughput technologies, computational tools, and omics approaches give insights into the molecular and genetic potential of an organism. Studies in the omics arena are still needed to fully understand the genetic mechanisms and pathway analysis.

7. Conclusions and future research

Every living organism is different in terms of their genetic makeup. The current progresses in sequencing and functional omics techniques have delivered better understandings into the precise mechanisms underlying probiotic functionality. The emerging understanding of the animal gut microbiota allowed accurate characterization of probiotic effects on the commensal microbiota of animal in vivo. Identification of genes vital to probiotic functionality is providing scientists the capacity to genetically tailor probiotics to encounter the requirements for precise applications. The livestock sector has a larger proportion of land consumption than agriculture keeping in view both grain feed intake and grazing. This trend is expected to rise, putting pressure and competencies on land resources in the agriculture sector. Moreover, there is a high demand for quality production which Cannot be attained by traditional practices for feeding ruminants. Quality cereal feed costs high and is uneconomical for large production. Consequently, this creates an imbalance in nutrition which drastically reduces dairy production. Probiotic yeast can overcome dairy production disparity. It augments nutrient uptake and increases Immunity, overall better health and production. Utilization of probiotic yeast for health and production is influenced by many different factors including probiotic strains, age, and breed of cattle. Essentially, yeast probiotics enhance assimilation by balancing the microflora of the rumen. It facilitates fiber digestion via inducing fermentation and stabilizing high pH. Facilitating an environment that flourishes rumen microbes is one factor. Other avenues need to be explored for probiotic yeast. More probiotic yeast strains are needed to be identified. For the preparation of probiotic feed, a complete nutritional profile generation is required. Furthermore, the amino acid profile of milk produced by dairy heifers fed on yeast probiotic should be analyzed.

8. Recommendations

The recommendations are outlined as follows:

- Sampling source should be indigenous for isolation of the probiotic strains.
- The identification of the probiotic strains must be based on the international validated molecular methods.
- The identified strain name should be deposited in validated microbial culture collection.
- The probiotic as well as genetic properties of the probiotic strains should be studied. Good manufacturing practices must be applied with quality assurance and shelf-life conditions established and labeling made clear to include minimum dosage and verifiable health claims.

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Chapter 3

Yeasts as Dietary Additives to Manipulate Ruminal Fermentation: Effect on Nutrient Utilization and Productive Performance of Ruminants

Oscar Ruiz Barrera, Jaime Salinas-Chavira and Yamicela Castillo Castillo

Abstract

There is considerable interest in the use of microbial additives such as yeasts in the nutrition of ruminants. The prohibition of the antibiotics as growth promoters in animal feeds increased the interest to investigate the effects of yeasts as natural additives on the gastrointestinal ecosystem and animal productive behavior. The effect of yeast-based preparations on the rumen environment and on the growth performance of ruminants has been well documented and has generated considerable scientific attention in the last two decades. However, the precise action modes by which the yeast cultures improve nutrient utilization and livestock production are still under study. Therefore, the objective of this chapter is to deepen into the action mechanisms of the yeasts at the ruminal level and at the productive level for their use as additives in animal feeding.

Keywords: ruminants, probiotics, natural additives, feeding, growth

1. Introduction

The yeasts are not part of the ruminal ecosystem, and their presence is mainly due to the ingestion in feeds and water. Marrero [1] and Castillo-Castillo et al. [2] showed that supplementing ruminant feeds rich in yeasts, they survived for longer time in the rumen and improved the conditions that favored the dry matter (DM) use by microorganisms that inhabit in it. These results corroborate the approach made by Galves [3] that when certain allochthonous microorganisms are deposited in a new habitat with nutrients, they survive and are able to use part of the resources of the environment in which they were deposited; in this case, the yeasts use the little oxygen existing in rumen and favor the conditions of anaerobiosis [4] that facilitate or potentiate the growth of other anaerobic microorganisms as the cellulolytic bacteria.

Research reports where yeasts are a natural alternative to manipulate ruminal microbial fermentation and animal productivity are shown below. Yeasts have

shown to improve the digestibility of DM and neutral detergent fiber (NDF) [5], feed consumption [6], milk production [7], and live weight gain [8]. However, not all yeast cultures have been shown to modify ruminal metabolism or improve animal productivity [9, 10]. These inconsistencies could be related to the type of yeast strain used [11], the specificity of the different commercial additives [12], or to the diet composition [13]. The objective of this review was to deepen into the mechanisms of action of the yeasts as well as their effects at the ruminal level and at a productive level for their use as animal feed additives.

2. Yeasts

Yeasts are unicellular microorganisms that ferment carbohydrates, and they are reproduced by budding. Most commercial yeast-based products contain a mixture of varying proportions of living cells of *Saccharomyces cerevisiae* and dead cells. Products with a predominance of living cells are sold as live yeasts, while those containing more dead cells with the growth medium are sold as yeast cultures [14]. Examples include Yea-SACC (Alltech Inc.), Levucell SC-20 (Lallemand Animal Nutrition), and Yeast Cultivation Diamond V (Diamond V, Mills Inc.). Yeast cultures are foods generally considered as safe (GRAS) denomination given by the Food and Drug Administration (FDA) (USA).

3. Mechanisms of action of yeasts in the rumen

One of the proposed action modes is that living yeasts through their aerobic respiration allow the elimination of the small percentage of oxygen (1%) that enters to the rumen when the animal ingests their feeds, thus facilitating the growth of the most anaerobic microorganisms as cellulolytic bacteria and fungi [4, 15].

Another proposed mechanism of action is that yeast cultures provide vitamins (specifically thiamin), glucans, mannano-proteins, and organic acids, which stimulate the growth of microorganisms that digest fiber and use lactic acid [16–18].

An additional effect is that yeast cultures are rich in organic acids (mainly malic acid) that stimulate the growth of *Selenomonas ruminantium*. This ruminal bacterium consumes the lactic acid produced in the rumen and therefore contributes to the stabilization of the pH in rumen, which favors the growth of cellulolytic microorganisms [17].

Yeasts also produce changes in the bacterial flora by competition and growth stimulation, increasing the growth, and activity of the acetogenic populations that compete with the methanogenic ones by the use of the metabolic hydrogen [16]. This decrease the energy losses in the animal caused the methane formed in rumen, which decreases the negative effect of this greenhouse gas on the environment [19].

4. Effect of the addition of yeasts in the ruminal fermentation

Yeast cultures based on *Saccharomyces cerevisiae* have been widely used in the diet of ruminants to improve digestibility and DM utilization [14].

The yeast products available in the market vary widely depending on the strain of *S. cerevisiae* used and the number and viability of the cells. Some products guarantee a high number of live yeast cells, while other products are sold as yeast cultures, which contain living cells and the medium where they grew [14].

Interestingly, it has been observed that not all strains of *S. cerevisiae* are capable to stimulate digestion in the rumen [11, 12]. For example, yeast culture "Diamond-VXP" and "A-Max Concentrate" generated different values of ruminal pH and molar concentration of total and individual VFA [12]. These differences were not related to the number of viable cells in the preparation or to the differences in metabolic activity [11].

In vitro and in vivo studies found no effect of yeasts on ruminal pH [4, 20–24]. However, results where pH decreased were reported by Williams [6] when they supplemented *S. cerevisiae* (10 g/d) in steers consuming barley hay. Lynch and Martin [25] also found a decrease in pH at adequate values for ruminal cellulolysis when they studied the in vitro effect of a *S. cerevisiae* culture on the fermentation of Bermuda hay and alfalfa hay.

On the other hand, regarding to the concentration of ammonia-nitrogen ($N\text{-}NH_3$), Lila et al. [22] and Erasmus et al. [20] showed that the inclusion of yeast does not affect the levels of this compound. Contrary to the above, Lattimer et al. [21] reported a decrease in the $N\text{-}NH_3$ concentration when they evaluated the effect of a yeast culture on the in vitro fermentation of a high fiber diet. Also, Moallem et al. [26] reported a decrease in ruminal $N\text{-}NH_3$ from dairy cows supplemented with yeast.

Other effects of yeasts include increase in metabolites as VFA [12, 18], decrease in lactic acid concentration [22], reduction of methane production [22, 25], increase in growth of cellulolytic bacteria and fungi [4, 16, 22] as well as acetogenic bacteria [16], and increase in fiber degradability [12, 22].

5. Use of yeasts in animal feeding

The use of feed additives is important in the feeding of ruminants. Yeast proteins have a high nutritional value, characterized by a balanced amino acid profile with a high content of lysine and threonine, which gives it an extraordinary potential for use as a supplement to animal diets, since these could be deficient in these amino acids [27].

The yeast of *Saccharomyces cerevisiae* as an additive in animal nutrition has been extensively investigated; however, the results obtained are variable and not very repeatable, possibly due to the great diversity of diets offered to the animals in study, the different strains of yeasts, and the different doses supplied to the animals. It is pointed out that *S. cerevisiae* increases feed consumption, milk production, feed conversion, and daily gain of weight, in response to increases in the amount and activity of the total anaerobic and cellulolytic bacteria that modify the concentration of volatile fatty acids, ruminal pH, and ammonia-nitrogen; however, the results are not consistent, so it is recommended to differentiate *S. cerevisiae* yeast strains that promote the use of the neutral detergent fiber of the ration [28].

Baiomy [29], in a study carried out with lactating sheep, mentioned that supplementation with live yeasts of *S. cerevisiae* has a significant effect on the development and metabolism of animals during the lactation period. In view of the above, it is recommended to include live yeast (Yea Sacc1026) in the animal diet in an amount of 6 g $animal^{-1}$ day^{-1}. On the other hand, Sotillo et al. [30] in dairy goats found that the addition to the diet of 0.08 g kg^{-1} of dry matter of the additive Yea-Sacc® TS (*S. cerevisiae*) causes a 7% increase in milk production and an increase in the percentage of fat in it. In turn, there is an increase in the level of urea in milk and an improvement in body condition. There was also a decrease in somatic cell count values, indicative of a better health status of the animals. It is necessary to consider that the effects of the yeasts can be variable, depending on several factors related to the animal (species, physiological

stage, consumption, etc.) and/or diet (composition of the diet, mode of distribution, etc.) [31, 32]. However, the concentration of viable cells, the type of yeast, and the used dose are also of great importance in this variability [33]. In this regard, little research has been conducted to determine the effects of different doses of the microbial additive, and the doses used differ only in a narrow range, from 2 to 5 times [33, 34].

6. Other non-*Saccharomyces* strains of yeasts with potential commercial use as probiotics

A joint project carried out by Cuban and Mexican researchers has developed additives based on native yeasts adapted to their local conditions, showing a good potential to be utilized as growth enhancers in ruminants and could be economically competitive in the international market. Several studies have been conducted to examine the potential use of these non-saccharomyces yeasts on animal nutrition [35, 36]. Additionally, Marrero et al. [37] studied the effects of the addition of two strains, Levazoot 15 (*Candida norvegensis*) and Levica 25 (*Candida tropicalis*), and found that both yeasts stimulated ruminal fermentation of oat straw and alfalfa, although it was better when the Levazoot 15 was inoculated. In recent study, Castillo-Castillo et al. [2] demonstrated the fermentative capacity of Levazoot 15, which showed greatest gas production than the yeast-free control and positively affected the in vitro ruminal fermentation parameters of alfalfa and oat straw. Based on these results, the Levazoot 15 yeast strain could be potentially used as an additive for ruminants consuming high-fiber diets. In another study, Ruiz et al. [38] showed that the same strain of yeast did not affect the ruminal cellulolytic bacterial counts. However, the treatment with yeast cultures positively influenced the cellulolytic fungal populations in rumen after 4 h of incubation. Methane production did not exhibit any trends across the fermentation times. A significant reduction in methane production was only observed at 8 h by the yeast treatment; at other time points, there were noticeable numerical decreases, although not statistically significance. In addition, Levazoot 15 strain also increased volatile fatty acid (VFA) concentration such as acetic, propionic, butyric, valeric, and isovaleric acids and enhanced the in vitro dry matter digestibility (IVDMD). In accordance, Ando et al. [39] with different strains of *Candida utilis* studied the in vitro degradation of grain and forage; the results showed that the microorganisms not only increased fibers degradability but also improved the utilization of the lipids. Finally, in a related study reported by Angulo-Montoya et al. [40], *C. norvegensis* Levazoot 15 strain preferred glucose as an energy source than other carbohydrates such as sucrose and lactose for its growth. In this study, only manganese was used as trace element, and the addition of vitamins in the culture medium was not required. This yeast used tryptone as a nitrogen source and did not denote sodium requirements.

7. Conclusions

The results in the use of yeast as feed additives are not conclusive; however, in rumen, they stimulate the growth and activity of total and cellulolytic bacteria, improving fiber digestion, synthesis of microbial protein, enhances feed intake and growth performance of ruminants. Yeasts also use ruminal oxygen, facilitating the growth of obligate anaerobes. In addition, yeasts may stimulate the bacteria that consume the lactic acid produced in rumen and may contribute to modulate the pH in rumen, reducing the risks of acidosis, and it can contribute significantly to the reduction of production of methane in the rumen.

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Section 3

Food Industry

Chapter 4

Wild Yeast and Lactic Acid Bacteria of Wine

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Abstract

Wine is an ancient and popular alcoholic beverage made from fermented grapes. Different yeasts and bacteria strains produce different styles of wines. Over time, the inoculation of *Saccharomyces cerevisiae* strains to produce wine has been the common practice in the wine industry, and the other species of yeasts have been considered undesirable for the alcoholic fermentation. However, in the last decades, the use of wild or indigenous yeasts and lactic acid bacteria strains has significantly increased. Wild yeasts and lactic acid bacteria are interesting microorganisms that contribute to differentiate the wine character of a region. The production of wines by spontaneous or inoculated fermentations by selected wild microorganisms may be an interesting tool to improve the quality of wines. This chapter summarizes relevant aspects of these microorganisms related to this scientific field.

Keywords: biotechnology, fermentation, flavor, microbiota, *Saccharomyces*

1. Introduction

Wine is an ancient and popular alcoholic beverage made from fermented grapes. The wine quality is determined by many factors, including the climate, the soil characteristics, the grape variety and the production processes, such as the viticultural practices, the winemaking techniques and the aging period. Among these factors, the fermentations carried out during winemaking, mainly alcoholic and malolactic fermentation, strongly influence on the wine composition. Different yeasts and bacteria strains produce different styles of wines derived from the biotransformation involved in both fermentations. Generally, the alcoholic fermentation (AF) is conducted by yeasts which convert sugars into ethanol, carbon dioxide and other minor metabolites. On the other hand, the malolactic fermentation (MLF) is conducted by lactic acid bacteria (LAB), which mainly convert malic acid into lactic acid and carbon dioxide. These metabolic processes are complex and sophisticated and sometimes may induce undesirable metabolite production pathways. Accordingly, an adequate selection of the yeast and bacteria strains is an important task for winemakers.

Over time, the inoculation of *Saccharomyces cerevisiae* strains to produce wine has been the common practice in the wine industry, and the other species of yeasts have been considered undesirable for the AF. However, in the last decades, the use and the inoculation of wild, native, autochthonous or indigenous yeasts and LAB strains to conduct the AF and the MLF have been significantly increased.

The isolation, selection and inoculation of the indigenous strains are useful tools to avoid sluggish and stuck fermentations and to increase the microbial diversity, enhancing the wine character [1]. In this way, this chapter pretends to summarize the most relevant aspects of these microorganisms and showing results derived from the studies related to the wild yeast and LAB strains on the wine properties.

2. Wild microorganisms associated to wine fermentations

The biotransformation of grape juice into wine is a complex ecological and biochemical process involving the sequential development of several microbial species such as yeasts, bacteria and fungi. Yeasts are the most important microorganisms involved in this process, being *S. cerevisiae* the main responsible of the AF. Although there are other genera and species present during winemaking, *Saccharomyces* possess a range of singular characteristics that are not found in other genera, such as the high capacity to ferment sugars, the high alcohol tolerance and the great ability to compete with other species and to colonize the wine medium [2]. The non-*Saccharomyces* yeasts are also commonly known by winemakers and wine microbiologists. This term includes many different yeast species. The current taxonomy recognizes around 149 yeasts genera and 1500 species, and more than 40 species have been isolated from grapes and grape juices [3]. *Dekkera* (anamorph form of *Brettanomyces*), *Candida* (anamorph form of *Metschnikowia*), *Cryptococcus*, *Debaryomyces*, *Hanseniaspora* (anamorph form of *Kloeckera*), *Kluyveromyces or Lachancea, Pichia*, *Rhodotorula*, *Saccharomycodes*, *Schizosaccharomyces*, *Torulaspora* and *Zygosaccharomyces* are the well-known non-*Saccharomyces* genera [4]. Generally, the non-*Saccharomyces* yeasts are commonly known as wild yeasts, because they are mostly present in grapevines, grape clusters and berry surfaces. The wild microbiota found in grapes and, therefore, in musts is affected by many external factors, such as the geographical location, the climatic conditions, the grape variety, the stage of maturity, the age of vines, the use of fungicides, the berry physical damages caused by fungi and even the presence of insects and birds [5–7]. Within a winemaking environment, the diversity of yeast species can be influenced by the population of cellar habitats, such as wall surfaces, equipment and oak barrels, among others. Thus, the cleaning and the cellar hygienic practices influence the winery microbiota affecting their diversity, composition and evolution. Nowadays, the actual hygienic practices used in the modern cellars seem to minimize the contamination by the resident cellar flora and, therefore, its diversity [8, 9]. In general, the non-*Saccharomyces* wine-related species have a low fermentation activity and a low SO_2 resistance [3]. However, they have the ability to colonize non-inoculated musts and to start the AF. They play an important role in the wine aroma complexity mainly due to their interesting enzymatic activities (proteases, β-glucosidases, esterases, pectinases and lipases) [10–12]. Many reports showed that the enzymatic activity of yeasts is conditioned by the pH, temperature, as well as the presence of inhibitors (sugars and ethanol) [13]. It seems that approximately 80% of the wild yeasts possesses one or more enzymes with biotechnological interest, being polygalacturonase the most common enzyme, followed by proteases (casein, gelatin) [14]. The β-glucosidase activity was linked to *Metschnikowia pulcherrima* species. A proteolytic activity was observed in *Pichia membranifaciens* and also in *Metschnikowia pulcherrima*. Furthermore, *Hanseniaspora* and *Torulaspora* genera are reported good producers of β-glucosidases, pectinases, proteases and enzymes involved in the xylan degradation [15–20]. *Lachancea thermotolerans* exhibited the activities of four carbohydrolases and three aminopeptidases. So, this strain could be an excellent candidate for improving the color and the turbidity of the red wines. Furthermore,

this strain could even increase the acidity due to its ability to produce lactic acid during the AF [21, 22]. However, the secretion of each enzyme is not characteristic of a particular genus or species and depends on the strains. It is important to notice that although non-*Saccharomyces* populations were not detected at the end of the vinification, their secreted enzymes remained in the fermenting media [16].

LAB are the second important group of wine microorganisms, which are also present in grapes. The LAB of wines, musts and grapes belong to the genera *Oenococcus, Pediococcus, Lactobacillus* and *Leuconostoc* [23]. LAB can be homofermentative and producing exclusively lactic acid and CO_2 from sugars (glucose and/or fructose) or can be heterofermentative and also producing ethanol, acetic acid and CO_2. Generally, the MLF is conducted by *O. oeni*, which presents a heterofermentative metabolism. Other species of the mentioned genera, such as *P. pentosaceus* and *P. damnosus*, have a homofermentative metabolism, while *Lactobacillus casei* and *Lactobacillus plantarum* have been described as facultative heterofermentative. Other *Lactobacillus* species, such as *brevis* and *hilgardii*, are strictly heterofermentative [23, 24]. The acetic acid bacteria are considered spoilage microorganisms during winemaking. Their metabolism is strictly aerobic, and their principal property is that they can oxidize ethanol into acetic acid by the acetaldehyde pathway. Finally, the fungi found in vines, such as *Botryotinia*, *Uncinula*, *Alternaria*, *Plamapara*, *Aspergillus*, *Penicillium*, *Rhizopus*, *Oidium* and *Cladosporium*, can infect and colonize grapes prior to harvest and to be present in musts [25]. *Botryotinia fuckeliana* (or its anamorph form *Botrytis cinerea*), *Aspergillus* spp. and *Penicillium* spp. are able to produce metabolites that can delay the growth of yeasts during the fermentation. Furthermore, the fungi growth on grapes may contribute to the growth of some acetic acid bacteria on the grape surface.

3. Population dynamics

The main important microorganisms present in grapes are yeasts and in a minor proportion LAB, acetic acid bacteria and fungi. The content and diversity strongly depend on the sanitary status of grapes. Although grape musts are relatively complete in nutrients, its low pH and its high sugar content convert them in a selective media in which only a few bacteria and yeasts species can grow. The number of yeasts on the grape berry just before harvest varies from 10^3 to 10^6 cells/mL depending on the abovementioned factors [26]. The predominant wild species on the surface of grape berries are *Candida*, *Hanseniaspora*, *Hansenula*, *Metschnikowia* and *Pichia*. The *S. cerevisiae* population is very low in grapes [27], while the non-*Saccharomyces* could proliferate up to reach about 10^6–10^7 cells/mL populations, although it declines at mid-fermentation. *S. cerevisiae* species are the most alcohol-tolerant yeast and can reach populations of at least 10^7–10^8 cells/mL [26]. Thus, at the last stage of fermentation, they become predominant and complete the process. Besides, some species of *Brettanomyces*, *Kluyveromyces*, *Schizosaccharomyces*, *Torulaspora* and *Zygosaccharomyces* may also be present in wine during fermentation. Some of these species are considered spoilage microorganisms because they produce metabolites with an undesirable impact in wine [8].

Regarding LAB, the population and behavior mainly depend on the pH and the SO_2 content, and they can reach 10^2–10^4 cells/mL populations after grape crushing. In general, an increase on the pH involves higher LAB populations and diversity. At this initial fermentation stage, the four genera abovedescribed can be commonly identified, although the greatest diversity of LAB species is mainly detected during the AF. During the first days of the AF, the LAB population generally increases to a maximum of 10^4 cells/mL and then decreases until 10^2 cells/mL.

At the end of the AF, *O. oeni* is commonly the only species identified and remains in a latent phase waiting to the proper conditions to start the MLF. The MLF starts when their population achieves values around 10^6 cells/mL and the environment conditions are adequate (pH, ethanol, temperature and SO_2 content) [28]. As soon as the malic acid is completely degraded, the bacterial population begins to decline [24, 29].

4. Spontaneous and inoculated fermentations

There are a lot of different species in grapes that can participate on the wine fermentations. In general, the AF is conducted by a mixture of yeasts species [5]. The AF can be conducted spontaneously without inoculating any yeast strains or by the inoculation of the specific strains, commercial or wild. The most common worldwide practice is the use of commercial starters from *S. cerevisiae* to ensure a reproducible, predictable and controlled fermentation. The use of commercial wine yeasts can influence the natural microflora of musts and often leads to its removal. Wines produced under this practice show low variability, complexity and typicity with analytical and sensory properties often similar [1]. In contrast, the spontaneous fermentations have some problems to predict their evolution, due to the variability on the microbiota that comes from the grapes. However, wines produced under this kind of fermentation have greater complexity and present higher differentiating notes and character [30]. In the inoculated fermentations, *S. cerevisiae* is the most common active dry wine yeast (ADWY) used as starter culture since it offers a great control on the fermentation evolution. Currently, a wide commercial ADWY yeast strains and species are available for cellars.

The MLF is not always successful even if it is conducted by inoculated commercial *O. oeni* strains. Some reports showed the presence of different species in spontaneous MLF, although, as mentioned, *O. oeni* has been described as the principal species. The evolution of the MLF and the diversity species of LAB implicated in this process may modulate the composition of wine (pH, the ethanol content, etc.), the fermentation temperature, the winemaking technology used, the geographical region and also the yeast strains employed during the AF [31–35]. As in the case of yeasts, to develop a correct spontaneous MLF, a wild bacterial starter is needed, which is well adapted to the specific producing area and to the cellar conditions. LAB inoculation is recommended in modern and industrial wineries in order to control the evolution of the MLF. Fast and reliable fermentations are essential to obtain a high-quality wine [36]. However, the use of commercial starters shows some controversies because of the homogeneity and standardization of wines, limiting their organoleptic properties [37].

In summary, the use of wild yeast and LAB can be used to define the typicity of the wines of a region. Some authors stated that the microflora diversity is characteristic of a given area and could be considered its microbiological fingerprint [38, 39]. The inoculation of selected wild yeast and LAB species could help to control the development of the AF and MLF and to improve the complexity and could typify the wine of a region [1].

5. Fermentation end-products and wild microorganisms

Numerous fermentation end-products contribute to the aroma and flavor characteristics of wines, which determine their quality and final complexity. As it is known, wine is made up of thousands of aromatic compounds, and a large part

of them are produced or transformed during the AF and MLF [40]. As mentioned above, these processes are carried out by wild or commercial strains. The use of wild strains allows us to obtain wines with a unique expression with representative characteristics of each variety and area. The aromatic profile of wines is determined by varietal aromas (from grapes), fermentative aromas (produced by yeast and LAB during fermentations) and post-fermentative aromas (associated to the aging period). The fermentative aromas clearly influence the final quality of wines, and the strains used during winemaking are responsible for the presence or absence of some flavors and other non-volatile metabolites [41]. Ethanol, carbon dioxide and glycerol are the main fermentative products. Ethanol is the main volatile product of yeasts metabolism, followed by diols, higher alcohols and esters. The ethanol content influences the wine viscosity and contributes on the aroma fastening. Other important metabolites derived from AF, such as pyruvic acid, participate on the formation of secondary products namely diacetyl, keto acids, succinic acid and butanediol [23]. Succinic acid and glycerol are two of the most important by-products affecting the "body" of the wine. Succinic is the main acid produced by yeasts, and its formation is strain dependent. The tartaric acid experiment slightly changes during fermentation, and the malic acid usually decreases during MLF, although the yeasts metabolism can also modify its concentration during the AF. Regarding acetic acid, this compound may reach more than 90% of the volatile acidity, and it is one of the most important by-products that negatively affect sensory profile of wine. This acid is mainly synthetized by acetic acid bacteria but also may be synthetized by yeasts and LAB.

Other volatile acids, such as propionic and hexanoic acids, are also produced by yeasts and bacteria, as a result of the fatty acid metabolic pathway [42]. Another important, but not always desirable, secondary metabolite of wine fermentation is the acetaldehyde. This compound is the product of the decarboxylation of the pyruvate during the AF. The higher alcohols represent another group of secondary products influencing the sensory profile of wines. The concentrations of higher alcohols are influenced by factors such as the yeast strains, the concentration of amino acids (the precursors of higher alcohols), ethanol concentration, fermentation temperature, pH, composition of grape must, aeration, etc. The higher alcohols are also important precursors for the ester formation; both of them are associated with pleasant aromas, although at high concentrations they can be undesirable.

LAB modulate the flavors of wines by modifying their chemical composition and, therefore, its sensory properties. LAB are responsible to maintain the result of the yeasts metabolism and to increase the complexity and microbial stability of the wine [24]. LAB decrease the wine acidity by the decarboxylation of malic acid to lactic acid, and they also contribute to the aroma by metabolizing other acids such as citric acid. The degradation of citric acid produces acetic acid and diacetyl, both of which have an important and undesirable effect on the wine flavor. Other metabolites affected by LAB metabolism, which have an impact on wine flavor, are alcohols, such as glycerol and mannitol, or carbonyls such as acetaldehyde and diacetyl. Finally, esters can also be modified for LAB species, including ethyl acetate, ethyl hexanoate, ethyl lactate and ethyl octanoate [42]. Depending on the species or even on the strains, LAB may be beneficial or detrimental to wine quality [29]. Meanwhile, acetic acid bacteria, as mentioned above, are only spoilage microorganism because they lead to the formation of such major oxidized aromas (acetaldehyde, acetic acid and ethyl acetate).

During the spontaneous AF, the development of many aromatic compounds occurs, mainly those belonging to the families of alcohols, ethyl esters, fatty acids, acetates and carbonyls. Aliphatic esters and alcohols seem to be more influenced than acids and carbonic compounds. In addition, terpenes and norisoprenoids, well-known primary aromas, can be provided by the wild yeasts during

fermentation [43, 44]. The main aromatic descriptors of all of them are the fruity and floral notes, always appreciated in wines. The use of wild yeast to conduct a spontaneous AF may produce higher concentration of alcohols (1- hexanol, phenyl-ethanol), terpenes and other aromatic compounds, such as β-phenyl acetate and γ-nonalactone, compared to wines produced by selected yeasts [45]. The selection of indigenous *S. cerevisiae* in red musts and its effect in their aromatic profile have been studied. The results showed that the produced wines had greater content of aromas and color intensity. These native yeasts synthesized higher content of linalool and citronellol, which exceeded their sensory limits [46].

The most related aromas of the inoculated MLFs are commonly associated with butter, yogurt, sulfur and toasted notes. Moreover, during spontaneous MLFs, the formation of many aromatic compounds is affected. Some studies have demonstrated the biosynthesis of the aromatic compounds produced during this kind of fermentations and its sensory repercussion. A reduction of herbaceous and vegetable aromas has been highlighted, and the appearance of fruity and floral aromas has been reported [47]. The changes produced in the aromatic composition of Tempranillo wines during spontaneous MLF by using wild LAB have been reported, showing significant increase in esters, lactones, terpenes, norisoprenoids and volatile phenols, such as vanillin and furfural [48].

6. Selection of wild yeast and lactic acid bacteria

For the selection of wild yeasts and LAB species of a specific wine region, first, it is needed to conduct a biodiversity study, knowing which species are present in grapes. After that, knowing the species at the different stages of a spontaneous fermentation, at the beginning, middle and end of fermentation, is essential. The first stage is to conduct a spontaneous fermentation. Then, isolate different colonies at each stage of the fermentation to obtain a collection of the different microorganisms implied. The second stage is to identify and typify each colony. The recovery and the molecular characterization of a high number of yeasts and LAB strains should be considered to establish a strain collection of oenological interest.

For the yeast species identification, different techniques could be applied. The restriction analysis of ribosomal gens is the simplest technique, reliable and extended [10, 49]. Nevertheless, several available techniques, such as microsatellites (SSRs), Rapid Amplification of Polymorphic DNA (RAPD-PCR), Pulsed-Field Gel Electrophoresis (PFGE) and DNA array technology, have been used to typify yeast strains. Between all these techniques, the more usual techniques for their simplicity and reproducibility are the restriction analysis of mitochondrial DNA [50] and the amplification of delta elements [51]. PCR-based methods have been already successfully used to identify LAB in different wines. To identify different LAB species, a good technique, fast and reliable, is the Restriction analysis of the amplified 16S-rDNA (ARDRA-PCR) [52, 53]. RAPD-PCR (Random Amplified Polymorphic DNA) is considered to be a suitable method to typify *O. oeni* strains in winemaking [54], such as PFGE (Pulse Field Gel Electrophoresis) of DNA digested with *Sfi*I [24].

Once the wild species and strains are identified and typified, the next step is to characterize each isolated strain, which has different genetic profile between them and between commercial yeast. Performing micro-fermentations with pure inoculations of all the strains with the specific characteristics of wine region must in order to test relevant species starter kits. With a better understanding of the different yeasts properties, the yeast selection procedure can be adapted to acquire strains that could improve the wine quality [55]. The AF and MLF performance by selected strains at winery conditions is the last step of selection.

The wild starter kit can be a single strain of *S. cerevisiae* or a mixture of *S. cerevisiae* and non-*Saccharomyces* species. The main trends in wine biotechnology is the use of different non-*Saccharomyces* species as starter cultures, such as *Torulaspora delbrueckii*, *Pichia kluyveri*, *Lachancea thermotolerans*, *Metschnikowia pulcherrima*, *Hanseniaspora uvarum*, *Schizosaccharomyces pombe*, etc. This practice combines the advantages of recovering features from traditional spontaneous fermentation, with a control of the vinification process, decreasing the risk associated with the microbial spoilage. The different species of non-*Saccharomyces* yeast starters are generally used in either sequential or simultaneous inoculation with *S. cerevisiae* [56]. Several studies have shown that the mixed inoculation starter kit with *S. cerevisiae* and non-*Saccharomyces* species can contribute positively to wine flavor. *M. pulcherrima* decreases volatile acidity [57], *H. uvarum* increases ester content in wine [58] and *Schizosaccharomyces pombe* deacidifies musts and increases the synthesis of glycerol and pyruvic acid [59]. In mixed fermentation, the interactions between the different yeasts composing the starter culture can led the stability of the final product and the analytical and aromatic profile [60].

The LAB selection as starters requires an ecological study and the characterization of useful technological and physiological features of the isolated strains in order to select the ones that are potentially more suitable for industrial applications. The selection of LAB for wine inoculation is essentially based on the survival of this strain and the consumption of malic acid. However, there are other important properties that are required to study the ability to produce biogenic amines and different enzymatic activities related to the final aroma profile. *O. oeni* is the preferred starter species because of its resistance to the alcohol, pH and SO_2 content. The ability to resistance the harsh wine conditions is strictly strain dependent. Furthermore, *O. oeni* ensures control of the time and the rate of MLF, reducing the potential for spoilage microorganisms and, finally, giving positive effects on flavor and aroma [61]. The development of the *Lactobacilli* and *Pediococci* in wine samples was linked to the decrease in wine quality [23]. González-Arenzana et al. demonstrated a high diversity *O. oeni* in spontaneous MLF and the complexity of the ecology involved [24]. They suggested a successful adaptation to winemaking conditions for some strains and also their potential utility for the selection of wild LAB starter cultures as individual or mixed strains.

The selection of microorganisms has been successfully used to improve the technological properties of different wines as well as their sensorial profiles helping in the production of wines without sulfites, reducing the levels of ethanol, increasing the glycerol content, varying the acidity of the must and realizing different aromatic components. In summary, the selection of wild yeast and LAB offers the best way to obtain different species and strains, which could improve the oenological characteristics and sensorial profile of wines, giving tools to the oenologist to direct their wine fermentation process. The exploitation of the microbial diversity that exists in the vineyards and in the cellars with the selection of wild yeast and LAB strains has been considered an interesting approach to overcome the distinctive peculiarities of wines produced in different regions [57].

7. Wild yeasts and lactic acid bacteria from viticultural Spanish regions

This section summarizes several studies carried out in VITEC (Wine Technology Center) from different grape varieties and Spanish regions. The isolation, identification and selection of wild yeasts and LAB were performed in 2016 and 2017 vintages, from grapes and spontaneous fermentations. The grape varieties studied were Verdejo from D.O. Rueda, Albariño from D.O. Rías Baixas and Tempranillo from D.O. Ribera del Duero and D.O.Q. Rioja. In all the cases, different species of

S. cerevisiae, non-*Saccharomyces* species and LAB were identified (**Tables 1** and **2**). **Table 1** shows the different non-*Saccharomyces* species found for each grape variety and region. The results showed that a great variety of yeasts species and strains present in grapes during spontaneous fermentation has been reported. Up to fourteen different strains of *S. cerevisiae* and seven species of non-*Saccharomyces* were identified in Verdejo. Seventy strains of *S. cerevisiae* and nine species of non-*Saccharomyces* were identified in Albariño, only at 2017. Seventy-eight strains of *S. cerevisiae* and ten non-*Saccharomyces* species were identified from Tempranillo in both regions. The non-*Saccharomyces* species were isolated in order to be inoculated together with a selected *S. cerevisiae* strain in mixed cultures. As mentioned, some of the used species are described as interesting wild yeasts, since they are able to led desirable compounds and metabolites to improve the wine quality (*Torulaspora delbrueckii*, *Pichia kluyveri*, *Lachancea thermotolerans*, *Candida/Metschnikowia pulcherrima* and *Hanseniaspora* species). It has been reported that *T. delbrueckii* can produce lower levels of volatile acidity than *S. cerevisiae*. *M. pulcherrima* can produce high concentrations of esters, especially ethyl octanoate; *Starmerella bacillaris* can produce high levels of glycerol and *Hanseniaspora* can improve the aromatic composition [30].

In order to study the influence of fermentation mixtures from wild selected yeasts on the wine properties, several studies were carried out in VITEC. The behavior of all yeast strains were studied, conducting fermentations at laboratory scale and at semi-industrial scale, in pure (*S. cerevisiae*) and mixed inoculations (*S. cerevisiae* and non-*Saccharomyces*). In the case of pure inoculations, significant differences were obtained in all the analyzed parameters (alcoholic degree, volatile acidity, total acidity, sulfur dioxide, glycerol and malic acid), except in the lactic acid content (data not shown). In these studies, firstly, the non-*Saccharomyces* species were inoculated and later the *S. cerevisiae* strains. In these mixed inoculations, the differences obtained depended on the time of the inoculation of the *S. cerevisiae* strain. As later the inoculation of *S. cerevisiae* is done, more differences were obtained. The inoculation time

Non-*Saccharomyces* species	**Verdejo Rueda**	**Albariño Rías Baixas**	**Tempranillo R. del Duero**	**Tempranillo Rioja**
Metschnikowia pulcherrima	√	√		√
Hanseniaspora vineae			√	√
Torulaspora delbrueckii	√	√	√	
Lanchacea thermotolerans			√	√
Hanseniaspora guillermondii	√	√		
Issatchenkia orientalis	√			
Pichia kluyveri	√			
Hanseniaspora uvarum	√	√	√	√
Aureobasidium pullulans			√	√
Rhodotorula glutinis		√		
Cryptococcus flavescens		√		
Cryptococcus magnus		√		
Starmerella bacillaris		√		
Pichia membranifaciens		√		

Table 1.
Identification of non-Saccharomyces species at different grape varieties and Spanish regions.

LAB species	Tempranillo Ribera del Duero	Tempranillo Rioja
Lactobacillus delbrueckii	√	
Lactobacillus helveticus	√	√
Lactobacillus hilgardii	√	√
Lactobacillus fermentum	√	
Lactobacillus pentosus	√	
Lactobacillus collinoides		√
Pediococcus acidilacticii		√
Oenococcus oeni	√	√

Table 2.
Identification of LAB species in Tempranillo grapes.

affected the basic oenological parameters and the aromatic fermentative compounds, including higher alcohols, esters, acetates and acids.

Concerning LAB, the highest diversity was found at the beginning of the AF. Up to eight different wild LAB species and fourteen *O. oeni* strains were identified. The fermentative characteristics of different *O. oeni* strains were studied. The results showed that some of these strains were able to conduct MLFs when the alcoholic degree did not exceed 14.5 vol., both in low and high pH wines (pH ranged from 3.3 to 4).

8. Conclusions

Wild yeasts and lactic acid bacteria are interesting microorganisms that contribute to differentiate the wine character. The suitable use of numerous wild species and strains during winemaking favors the improvement of the complexity and the organoleptic properties of wines. The production of wines by spontaneous or inoculated fermentations using selected wild microorganisms is a remarkable practice for wineries. Further studies should be done in order to deep into the knowledge of the wild microflora of grapes and wines to better understand their behavior and importance. Even more, some drastic physical and chemical winemaking techniques, increasingly used in wineries, could be replaced taking advantage of the biological properties of these microorganisms. Above all, wild yeast and lactic acid bacteria may help to produce modern and new wine styles in a climate change viticultural environment.

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Conflict of interest

We have no conflict of interest to declare.

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Chapter 5

Biotechnological Applications of Nonconventional Yeasts

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Abstract

Yeasts not belonging to species of the *Saccharomyces* genus, called nonconventional yeasts, have gained prominence recently in the biotechnological scenario. For many years, they have been generally characterized as undesirable contaminants in fermentative processes. However, several studies pointed them as useful for many biotechnological applications. This chapter will cover some of these applications, highlighting the most widely employed nonconventional yeasts. The use of non-*Saccharomyces* strains in (I) xylose fermentation for the production of ethanol and xylitol, (II) brewing industry, (III) improvement of coffee and cocoa fermentation, and (IV) plant growth promotion will be presented.

Keywords: nonconventional yeasts, xylose fermentation, brewing industry, coffee/cocoa fermentation, plant growth promotion

1. Introduction

Yeasts have been extensively exploited by humanity for the production of bread, alcoholic beverages, bioethanol, biomass (as human or animal protein supplement), and glycerol [1–4]. They are suitable models for studies of molecular genetics, biotechnology, evolutionary biology, genomics, and eukaryotic cell biology due to their ease of culture, simple and relatively fast life cycles, and small genomes [5]. The most known species of yeasts belong to the subphylum Saccharomycotina, including *Saccharomyces cerevisiae* (eukaryotic model system), *Candida albicans* (common human commensal and opportunistic pathogen), and over 1000 other species. These eukaryotic single-celled microorganisms are found in every biome and continent and are more genetically diverse than chordates or angiosperms [5].

The different applications of yeasts represent one of the oldest uses of biotechnology by mankind. Although *S. cerevisiae* is the most domesticated and widely used industrial yeast, many other yeast species that evolved over half-a-billion years have been overshadowed, but could also show a high biotechnological potential [5–7]. A variety of other yeast genera and species than *Saccharomyces*—called nonconventional yeasts—may support experimental studies and favor the biotechnological generation of value-added products [8, 9].

The wide variety of nonconventional yeasts has gained attention for numerous applications in the areas of biocatalysis (pharmaceuticals, chemical intermediates, and biotransformations), biofuels, alcoholic beverages (enhancement of desirable flavors), fundamental biological research (molecular and cellular biology, genomics, functional genomics, pathway engineering, and system biology mechanisms), biomedical research (drug discovery, drug resistance and metabolism, and elucidation of disease mechanism), biocontrol (crop protection, food and feed safety, and probiotics), environmental biotechnology (bioremediation and pollutant degradation), and heterologous protein production (protein pharmaceuticals, enzymes, hormones, vaccines, and toxins) [10–14].

In this chapter, we mined the literature in order to present some applications of nonconventional yeasts. First, a metabolism not present in *S. cerevisiae*, the fermentation of the pentose sugar xylose, is addressed whose process can generate both ethanol and xylitol as the final products. The metabolic pathway, the main nonconventional yeast species able to perform this activity, and the challenges of the process are discussed. Then, the potential of nonconventional yeasts for the production of new flavors and aroma attributes in the production of craft beer, coffee, and chocolate is described. The chapter is finished with a review regarding an alternative biotechnological application of yeasts, the use of nonconventional strains able to perform many activities associated with plant growth promotion.

2. Some applications of nonconventional yeasts

2.1 Xylose fermentation

The world energy scenario urgently needs alternative sources for replacing fossil-based fuels, mainly because of the damage that they cause to the environment [15]. Petroleum-based fuels are the most widely used, a scenario that involves high costs and climate concerns [16, 17]. The burning of fuels such as gasoline and diesel contributes to the release of most of the gases causing the greenhouse effect [17, 18]. For these reasons, biofuels are attractive alternatives to the finite fossil fuels [19]. Bioethanol is a promising example of clean and renewable energy source. It is the most widely used biofuel worldwide, both in pure form or as a gasoline additive [20].

Brazil is the world's second largest ethanol producer, whereas USA is the leader using corn starch as the fermenting substrate [21]. However, the main raw materials used for obtaining fermentable substrates, *i.e.* corn, sugarcane, and sugar beet, also show food destinations that raise a recurrent discussion about new alternatives for ethanol production [22]. In addition, the conventional production of biofuels faces several obstacles to achieve the desired levels, encouraging the use of advanced technologies, such as ethanol produced from lignocellulosic biomass, also known as second-generation ethanol [23].

Lignocellulose has a great biotechnological value and comprises most part of the plant dry weight, composed of 35–50% of cellulose, 20–35% of hemicellulose, and 10–25% of lignin. It is the largest source of renewable organic material, highly generated through agricultural and forestry practices [24, 25]. The polysaccharide components of the lignocellulosic biomass (cellulose and hemicellulose) can be processed through a hydrolysis reaction to release sugar monomers, such as hexoses and pentoses (especially glucose and xylose, respectively) that can be used as substrates in fermentation processes [26, 27]. Xylose has a high biotechnological potential; it is the most abundant sugar of the biosphere after glucose. D-xylose can be used by nonconventional yeasts for the bioconversion to ethanol or xylitol [28, 29], while *Saccharomyces cerevisiae*—the most widely employed yeast species in

fermentative processes—is not able to metabolize it [30–32]. In this context, exploring the biodiversity of yeast strains not belonging to *Saccharomyces cerevisiae* species for xylose fermentation is of fundamental importance.

Xylitol has a sweetening power similar to sucrose and is found in nature, fruits, and vegetables. It is effective in sucrose substitution, since its metabolism is independent of insulin, which allows its utilization in the treatment of diabetes [33–36]. Due to its physical and chemical properties, xylitol is a compound of high added value, attracting high interest of the pharmaceutical, food, and dentifrice industries. Moreover, its proven efficiency in reducing the incidence of tooth decay promoted application in the oral health field [37–39]. The concentration of xylitol in plants is relatively low; thus, it is not economically feasible to extract it. On the other hand, bacteria, filamentous fungi, and non-*Saccharomyces* yeast species are microorganisms capable of performing an efficient bioconversion of D-xylose to xylitol. Yeasts belonging to the genus *Candida* are especially known for the capacity to perform this bioconversion. *C. guilliermondii* and *C. maltosa* are species that have been identified with high rates of D-xylose consumption and xylitol production in the fermentation under microaerophilic conditions [40, 41].

The discovery of D-xylose-fermenting yeasts began in the 1980s [42], and since this decade, we can list some species of nonconventional yeasts studied for ethanol production: *Pachysolen tannophilus* [43], *Kluyveromyces cellobiovorus* [44], *Scheffersomyces* (*Candida*) *shehatae* [45], and *Scheffersomyces* (*Pichia*) *stipitis* [45–47]. Most recently, *Spathaspora arborariae* [48, 49], *Sp. passalidarum* [50–53], and *Sp. piracicabensis* are other species described as able to perform this activity [54]. *Spathaspora passalidarum*, *Sp. arborariae*, *Sp. piracicabensis*, *Sp. gorwiae*, *and Sp. hagerdaliae* produce ethanol mostly from D-xylose, while the remaining species within this clade are considered xylitol producers. Among the set of *Spathaspora* species able to ferment D-xylose, *Sp. passalidarum* is the highest ethanol producer under oxygen-limited or anaerobic conditions, showing rapid D-xylose consumption and also the ability to ferment glucose, xylose, and cellobiose simultaneously. *Sp. passalidarum* is a potential candidate for use in the fermentation of sugars from lignocellulosic biomass [55].

Many other *Candida* species are also reported as capable of fermenting xylose for ethanol and/or xylitol bioconversion: *C. tenuis* (*Yamadazyma tenuis*), *C. tropicalis*, *C. utilis* (*Cyberlindnera jadinii*), *C. blankii*, *C. friedrichii*, *C. solani*, and *C. parapsilosis*. Also described as xylose fermenters are species of the genera *Debaryomyces*, *Brettanomyces*, *Clavispora*, and *Schizosaccharomyces* [56–58].

The hydrolysis reaction of the lignocellulose biomass is conducted first through a pretreatment that can be mechanical, chemical, or heat-based. It is performed in order to reduce the amount of lignin and enable the following enzymatic hydrolysis of the polysaccharides [59]. Although there are many studies with particular species able to ferment D-xylose, the conversion of this pentose into ethanol still faces a high number of challenges, such as (I) metabolic repression due to a fermentation involving a mix of pentose and hexose sugars present in hydrolyzed substrate, (II) the presence of toxic substances generated by the pretreatment and hydrolysis reaction (furfural and acetic acid), (III) the unbalance between coenzymes required by the enzymes associated to the early stages of xylose metabolism, and (IV) the need of an oxygen supply for ethanol production [32]. Therefore, ethanol production from D-xylose has not yet reached commercial levels, which requires deeper studies aiming to achieve an appropriate conversion process [60]. The exploitation of yeasts for D-xylose fermentation is also limited by the low tolerance of cells to ethanol, slow fermentation rates, and also the difficulty in controlling the rate of oxygen supply at an optimum level during fermentation. The combined presence of sugars (hexose and pentose) present in the hydrolysate also introduces difficulties as

mentioned above [61], because hexoses can inhibit D-xylose metabolism by repression and inactivation of the D-xylose transport systems or catabolic enzymes [62].

Throughout the biosphere, microbial diversity is poorly known and represents a huge potential source for the discovery of new species [63, 64]. This untapped biodiversity probably contains species able to ferment D-xylose and other sugars efficiently. Bioprospection and identification of new yeasts capable of fermenting sugars from lignocellulosic biomass are of particular interest for the development of technologies aiming to produce biofuels in a useful and viable way. D-xylose fermenting yeasts have been isolated for example from the gut of wood-boring insects such as passalid beetles (*S. stipitis, S. shehatae, Sp. passalidarum, C. jeffriesii,* and *Sp. allomyrina*) [51, 65, 66], rotting wood (*Sp. arborariae* and *Sp. piracicabensis*) [50, 54], mushrooms, bromeliads (*S. shehatae*) [67, 68], larval gut of the silkworm (*Blastobotrys bombycis*) [69], and peat in a tropical swamp forest in Surat Thani province of Thailand (*C. kantuleensis*) [70]. The prospection of species in Brazilian ecosystems has focused mainly on Atlantic Rainforest, and other few studies have been conducted on Cerrado ecosystem and Amazonian Forest sites [71]. The draft or complete genomes of the D-xylose fermenting yeasts *S. stipitis*, *Sp. arborariae*, and *Sp. xylofermentans* were already sequenced and have been studied accurately. The genes involved in the mechanisms of regulation of xylose metabolism are very important for engineering xylose metabolism in *S. cerevisiae* [72–74].

The uptake of D-xylose by yeast cells occurs through membrane-carrying proteins. D-xylose transport occurs by a facilitated diffusion mechanism; thus, the only driving force is the sugar gradient. It also occurs by an active transport system (co-transport of H^+ and sugar) [32, 75]. The metabolism of D-xylose in yeast occurs according to the descriptive scheme of **Figure 1** [29, 42, 61, 76, 77]:

1. D-xylose is initially transported into the cell;

2. With the presence of coenzymes NADH and NADPH, the enzyme xylose reductase (XR) converts xylose to xylitol, releasing NAD^+ and $NADP^+$, respectively;

3. The xylitol formed can be either excreted in the medium or oxidized to xylulose by the enzyme xylitol dehydrogenase (XDH) in the strict presence of the NAD^+ coenzyme, releasing NADH;

4. The produced xylulose is phosphorylated by the enzyme xylulokinase (XK) and forms xylulose-5-phosphate;

5. Xylulose-5-phosphate is metabolized by pentose phosphate pathway (PPP);

6. The pentose phosphate pathway (PPP) has two phases: the oxidative phase and the nonoxidative phase (regeneration phase);

7. Metabolites resulting from the pentose phosphate pathway (fructose-6P and glyceraldehyde-3P) are metabolized in glycolysis (Embden-Meyerhof route) and converted to pyruvate;

8. Pyruvate can be oxidized by the Krebs cycle and recovers the coenzymes through the respiratory chain, or it can also be fermented into ethanol by the action of pyruvate decarboxylase and alcohol dehydrogenase enzymes (in this process NADH is reoxidized as a result of glyceraldehyde-3P oxidation).

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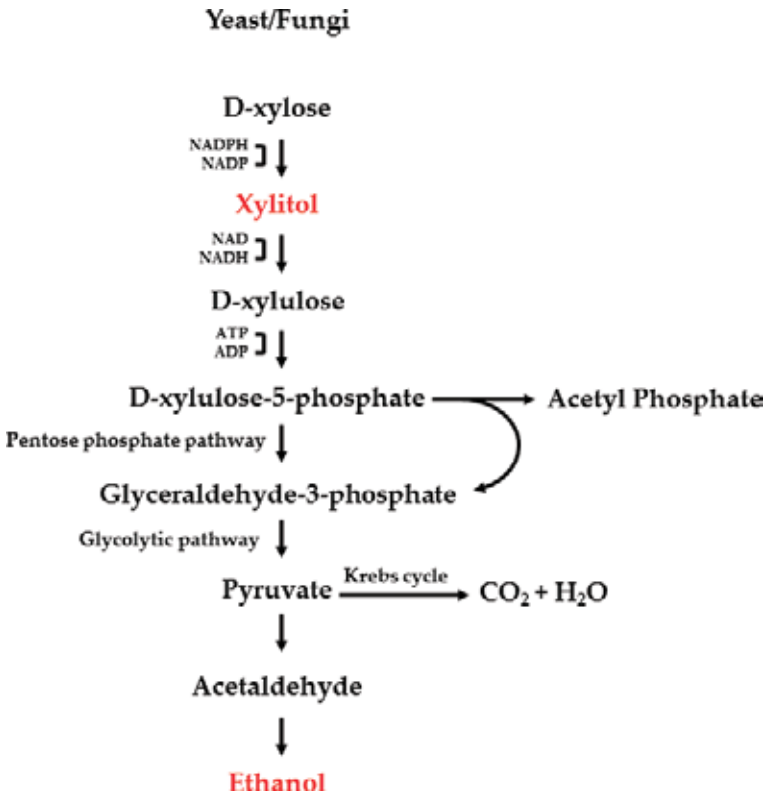

Figure 1.
Schematic diagram of D-xylose metabolism.

Understanding xylose metabolism is important for obtaining high yield fermentation. In some yeast strains, the enzyme xylose reductase (XR) is only dependent on the NADPH coenzyme, releasing $NADP^+$. In others, the XR enzyme can use both coenzymes (NADPH and NADH), releasing $NADP^+$ and NAD^+, respectively. In the following step, xylitol dehydrogenase (XDH) is strictly dependent on the NAD^+ coenzyme, causing the unbalance of coenzymes, which limits the fermentation process of D-xylose to ethanol, since this specific dependence can cause xylitol accumulation [78].

The coenzymes used by the main enzymes involved, xylose reductase (XR) and xylitol dehydrogenase (XDH), proceed with even more unbalance of coenzymes under anaerobic conditions, since the coenzyme $NADP^+$ can be reduced via fructose-6-phosphate, and the coenzyme NADH cannot be oxidized in the absence of oxygen. In this way, xylitol accumulates, and hence, ethanol production decreases [77]. Therefore, oxygenation control at the optimal level is one of the most important physiological factors. Aeration determines the division of xylose carbon flux between cell growth and bioconversion. The enzyme XR that has activity in the presence of both coenzymes can favor the production of ethanol under anaerobic conditions [79, 80].

Industrial xylitol is produced by chemical reduction of xylose. However, its biological production would be more attractive due to the low costs associated to its production and better organoleptic characteristics. The chemical production requires more energy supply, which increases the price of the product and makes it less competitive compared to other sweeteners. The biotechnological process is carried out under conditions of moderate temperature and pressure, and therefore

has the lowest energy requirements. This bioconversion is highly specific, resulting in higher yields and lower costs of product separation and purification, as well as cleaner effluents [37, 81, 82]. The biotechnological production of xylitol is associated with the ability of microorganisms to synthesize the enzyme xylose reductase (XR). As mentioned above, XR catalyzes the reduction of xylose to xylitol with the participation of the NADPH or NADH. Xylitol can be either excreted from the cell or oxidized to xylulose by the enzyme xylitol dehydrogenase (XDH), whose activity requires the NAD^+ cofactor. Briefly, the production of xylitol depends on a high activity of the XR enzyme or a low activity of the enzyme XDH. The degree of activity of these enzymes is a criterion used to identify the best producers [78, 82–86].

The enzyme xylose isomerase (XI) is able to transform xylose directly into xylulose. The xylose isomerase (XI) pathway does not produce xylitol and is performed by some prokaryotes, fungi, and plants. However, such natural ability in yeasts has not yet been described. There are genetically modified strains with this enzymatic capacity [87]. Research also reports the ability of genetically modified *S. cerevisiae* strains to ferment xylose into ethanol, but these studies also raise questions about the fermentation viability [30, 32].

2.2 Beer fermentation

The most widely used yeasts for beer fermentation are of the genus *Saccharomyces*. Yeast strains belonging to the species *S. cerevisiae* and *S. pastorianus* are used for the production of the two main families of beers: ale and lager, respectively. The selection and use of *Saccharomyces* spp. (mainly *S. cerevisiae*) for controlled fermentations over time is related to several attractive traits, such as high fermentation performance, tolerance to ethanol and other stressors, production of desirable flavor and aroma compounds, and the safe use for fermented foods and beverages, *i.e.*, the absence of production of toxic metabolites [88].

Despite the widespread use of such traditional starter yeasts for brewing, the use of nonconventional yeasts, especially non-*Saccharomyces* species, has been gaining attention with the increasing demand for product innovation and diversity [89]. The growth of the craft beer segment has boosted the search for strategies to bring differentiation to the beverages, and the role of different yeasts in defining the beer traits has been exploited and gained special attention [90].

Generally, non-*Saccharomyces* yeasts present lower fermentative efficiency when compared to *Saccharomyces* spp., exhibiting reduced ethanol yield [91], and they usually have been seen as contaminant agents of the brewing process due to associated problems and negative influence on beer quality [92]. However, these yeasts may display distinct physiologic and metabolic activities, which can all contribute to secondary metabolite production, impacting on the sensorial complexity of the beer [89, 91].

The use of non-*Saccharomyces* yeast strains in the production of beers has the potential to provide distinctive flavor and aroma attributes [93]. In food and beverages, the production or enhancement of flavor compounds via (micro) biological systems is referred to as bioflavoring [94], and several non-*Saccharomyces* yeasts species have been pointed out as feasible agents to enhance, improve, and diversify the beer sensorial characteristics [95]. Besides bioflavoring, other applications have been highlighted, such as the production of low-/no-alcohol beers and light (low calorie) beers [89, 93, 95]. Non-*Saccharomyces* yeasts are commonly encountered in spontaneous and uncontrolled fermentation processes, e.*g.,* in the production of sour beers, such as Belgian lambics and American coolship ales [92]. During fermentation, a succession of autochthonous yeasts and bacteria are observed along the process, which lasts for 1–3 years in oak barrels. Among non-*Saccharomyces,* primarily *Debaryomyces* has been detected in lambic, and *Rhodotorula* in coolship ale

at the first month of fermentation. Both yeasts are replaced by *Saccharomyces* spp., which dominate the main alcoholic fermentation for 3–4 months. Subsequently, the latter is gradually outcompeted by *Brettanomyces* (teleomorph *Dekkera*), mainly *B. bruxellensis*, which presents high tolerance to ethanol and remains the predominant non-*Saccharomyces* yeast until the end of fermentation (along the acidification-maturation phase) [96, 97]. During this phase, *Dekkera/Brettanomyces* contributes to a higher attenuation of the wort, due to the consumption of oligosaccharides that *Saccharomyces* is not able to metabolize, and to the production of several aroma compounds, such as caprylic and capric fatty acids and their ethyl esters, ethyl phenol, ethyl guaiacol, isovaleric acid, acetic acid, and ethyl acetate, which together characterize those particular beer styles [88, 93, 95]. Other non-*Saccharomyces* yeasts of the genera *Kluyveromyces, Torulaspora, Candida, Hanseniaspora, Pichia, Meyerozyma, Wickerhamomyces, Cryptococcus*, and *Priceomyces* have already been found in such spontaneous fermentations [92, 97], but not many of them have been evaluated to be used under controlled beer fermentations [98].

Nowadays, the most commonly used nonconventional yeast in craft and specialty beers is *Dekkera/Brettanomyces* [99]. The ability to produce a large diversity of aroma compounds, most notably phenolic compounds, ethyl esters, and (fatty) acids, has made this yeast an interesting bioflavoring agent to bring specific sensory characteristics to the beers [88, 99]. A lot of aromatic descriptors, both positive and negative, have been associated to *Dekkera/Brettanomyces* spp. ferments, including floral, fruity, citrus, spicy, clove, leather, barnyard, smoky, plastic, mousy, phenolic, medical, and/or "band-aid", which are usually called "Brett flavor" [100]. Some common beer styles in which those species can contribute to with their specific flavors include Lambic, Gueuze, and American Kettle sour (marked by sour character); Kriek, Berliner Weisse, and Brett IPA (fruity character); Saison Farmhouse ale, Belgian Trappist ale, and Old English ale (phenolic character); and Cask Brett barley wine, Old stout, and Flanders Red ale (woody character) [99].

Regarding the production of volatile phenolic compounds, the interest in some *Dekkera/Brettanomyces* spp. for use in craft and specialty beers also relies on their ability to produce ethyl phenols [99]. Their syntheses are related to hydroxycinnamic acid (ferulic, p-coumaric, and caffeic acids derived from cereal grains used for mashing) metabolization, in which two consecutive reactions catalyzed by specific enzymes are involved, phenylacrylic acid decarboxylase and vinylphenol reductase, the last one being exclusive to *Dekkera/Brettanomyces* [100, 101]. Some strains of *D/B anomala* and *D/B bruxellensis* have showed efficient conversion of ferulic acid into 4-ethyl guaiacol, making them suitable for beers in which spicy, clove-like and vanilla flavors are desired [102]. Despite the ability to metabolize hydroxycinnamic acids is strain specific, many strains suitable for brewing prefer to consume ferulic acid among other hydroxycinnamic acids [103].

Another common property of *Dekkera/Brettanomyces* is its ability to metabolize complex sugars, such as dextrins, which are not assimilable by *Saccharomyces* and account for the main residual sugars in the beer. This metabolization capacity enables its use in the production of super-attenuated and low-calorie beers [100]. These yeasts were also shown to present β-glucosidase activity, a strain dependent feature that enables the hydrolysis of glycosides present in hops, fruits, flowers, and woods, releasing more aromatic molecules, *e.g.* terpenes derived from hops [95, 99]. Furthermore, one *D. bruxellensis* expressing high β-glucosidase activity was shown to be able to produce resveratrol, a molecule with antioxidant and antiaging actions [104]. Future researches could consider such ability for the production of functional beers, which can provide health benefits for consumers.

Besides *Dekkera/Brettanomyces, Torulaspora delbrueckii* (anamorph *Candida colliculosa*) has been pointed out as a feasible agent to enhance beer bioflavor,

displaying a great contribution to produce specialty beers with different flavors and aromas [89, 95, 102, 105]. These microorganisms have shown ability to produce esters and higher alcohol compounds both in pure and mixed fermentations with *Saccharomyces* spp., positively impacting on the overall analytical and sensory profile of beer [106–108]. Its co-inoculation with *S. cerevisiae* seems to raise the concentration of esters, such as both ethyl decanoate and ethyl dodecanoate as well as citronellyl acetate [105], phenyl ethyl acetate, ethyl hexanoate and ethyl octanoate [106], and ethyl acetate and isoamyl acetate [107], which can overall contribute to fruit-like flavors. Increased levels of higher alcohols, mainly amylic and isoamylic alcohol, in *S. cerevisiae/T. delbrueckii* mixed fermentations [107], and 2-phenylethanol and amyl alcohol in some *T. delbrueckii* pure fermentations [108] have also been described. The ability of some strains to augment the concentration of 4-vinyl guaiacol (clove-like descriptor), a desirable flavor in some wheat and blond beer styles, has been stood out in fermentations sequentially inoculated with *S. cerevisiae* [102] and, furthermore, pure culture of some *T. delbrueckii* has already been used to produce Hefeweizen beers (a German wheat beer style), imprinting rose, bubblegum, banana, and clove-like aromas [109]. Also, its ability to convert some monoterpenoids of hops to linalool, terpineol, and geraniol, which can stamp floral and fresh aromas to beers [110], has been reported.

Pichia kluyveri is another yeast species that may increase the levels of fruity acetate esters in beer [102]. In sequential fermentations with *S. cerevisiae*, a considerable enhancement was observed in the isoamyl acetate (banana-like flavor) concentration in a wheat beer production [111]. Another yeast species, *Wickerhamomyces anomalus*, whose characteristics have been further studied for winemaking, seems to have potential to be used for beer fermentations. Several strains were shown to present a wide range of enzymatic activities, including β-glucosidase activity, and they were shown to be good producers of esters, mainly ethyl acetate, and other fruity acetate esters, such as isoamyl acetate, contributing to improve aromatic complexity of fermented wort, likewise in co- or sequential inoculations with *S. cerevisiae* [112].

Regarding the efficiency in producing lactic acid and ethanol during wort fermentation, and the sensory characteristics, some isolates of the species *Hanseniaspora vineae, Lachancea fermentati, Lachancea thermotolerans, Schizosaccharomyces japonicus*, and *Wickerhamomyces anomalus* have been pointed out to be used for the production of sour beers in a single fermentation step, without the need of lactic acid bacteria for souring [113, 114]. This process was called primary souring, and the resulting beers showed both lactic tartness and fruity aromatic and flavor notes [114].

Several strains of nonconventional yeast species have been demonstrated to be useful in producing beers with reduced ethanol levels, besides contributing on improved sensory profile. The inability to consume the main brewery wort fermentable sugars, maltose and maltotriose, makes them less efficient at producing ethanol, enabling the production of low-alcohol (0.5–1.2% v/v) and alcohol-free (<0.5% v/v) beers [89, 115]. Pure cultures of several strains of *T. delbrueckii* were shown to ferment beers with an ethanol content varying from 0.9 to 2.6% (v/v) and characterized by rich fruity flavors [106, 108]. In the same way, strains of the species *Saccharomycodes ludwigii* stand out as suitable candidates, producing beers containing low alcohol concentration and higher amount of esters, besides lower diacetyl levels, contributing to mask the wort-like flavor, which is commonly identified in these kind of beers [115, 116]. Moreover, this yeast is already being used commercially to produce alcohol-free beers with increased fruity notes [115]. On the other hand, in a study aiming at screening basidiomycetous yeasts of *Mrakia* spp. for low alcohol beers, one strain of *M. gelida,* a psychrophilic one, was revealed to produce a

more aromatic beer, judged to be fruitier with apricot, grape, and litchi descriptors, when compared with that produced by a commercial starter of *S. ludwigii* [117].

Other yeast strains of the species *Pichia kluyveri,* able to consume only glucose of brewing wort, were also shown to produce low-alcohol and alcohol-free beers with enhanced amounts of ester compounds and with a flavor profile similar to commercial standard beers of around 4% alcohol (v/v) [118]. One strain of *Williopsis saturnus* var. *mrakii* was demonstrated to have application in fermenting extra-fruity low-alcohol beers, due to the ability to increase the levels of acetate esters and to retain the terpenes and terpenoids of hopped wort, positively stamping fruity and floral flavors, besides preserving the hop aromas [119]. *Candida shehatae, C. tropicalis*, and *Zygosaccharomyces rouxii* are other species that have already been considered for the purpose of producing beers with low or no alcohol [89, 115].

The use of nonconventional yeasts in the brewing processes, especially considering the growth in the craft beer sector, stands out as natural and innovative choices to improve and bring differentiation to the beers. The potential of different yeast species to enrich and diversify the flavors and aromas, bringing sensory complexity to the beverages, recently comes out and should be more explored and studied as these abilities can result in peculiar beers, with unique features.

2.3 Coffee and cocoa fermentation

For satisfactory conduction of industrial fermentative processes, yeasts are applied in order to (I) reduce fermentation time, (II) generate end-products with high productivity, and (III) standardize processes. Concerning coffee and cocoa, yeast fermentation has not been well established. Recent studies pointed out that yeast fermentation can control cocoa and coffee fermentations and produce beans with improved quality. However, to reach a uniform level of high quality, similar to those obtained by fermentation processes of wines, beers, dairy products, and meat, the prerequisite is to know the different yeast groups that actively participate in these fermentation processes to draw a correlation of the final quality. The focus of this section is to describe the main nonconventional yeasts associated with the fermentation of coffee and cocoa beans and their role for each process.

During fermentation, depulped coffee beans are exposed to a diversity of microorganisms, such as yeasts, filamentous fungi, and bacteria that find favorable conditions for their development. The microbial activity generates a range of metabolites that can influence the final quality of the beverage [120, 121]. Yeasts are considered to be important due to pectin degradation and formation of flavor metabolites such as ethanol, organic acids, and esters. Due to these characteristics, many yeasts have been used as starter cultures for cocoa and coffee [122–124]. Different studies have demonstrated a high diversity of nonconventional yeasts during the coffee processing stage, including *Pichia kluyveri, P. anomala, P. guilliermondii, Kloeckera apiculata, Hanseniaspora uvarum, S. marxianus* (*Kluyveromyces marxianus*), *S. bayanus, Debaryomyces hansenii, D. polymorphus, Torulaspora delbrueckii, Torulopsis famata, Candida guilliermondii, C. parapsilosis, C. pelliculosa, C. famata, C. tropicalis, C. fermentati, C. membranifaciens, Rhodotorula mucilaginosa, Arxula adeninivorans, Cryptococcus albidus*, *Schizosaccharomyces* sp. and *Kloeckera* sp. [120–122, 125–128].

During coffee processing, the use of pectinolytic microorganisms is made to remove the mucilage layer from the coffee beans. This activity can significantly reduce fermentation time from 80 to 20 hours by enzymatic treatment. Thus, the production of pectinases has been the main attribution of yeasts and bacteria during coffee processing. Nonconventional yeasts, such as *P. anomala, P. kluyveri, P. Caribbean, P. guilliermondii*, and *H. uvarum,* have been associated with the production of pectinases during coffee fermentation [126]. The ability of yeasts

belonging to the species *P. anomala, P. kluyveri*, and *H. uvarum* to produce pectic enzymes suggests that these species may act in the degradation of pectin during fruit fermentation [129].

Yeasts as starter cultures for the fermentation industry have been widespread by the ability to generate uniform and safe products, as well as the ability to modify various constituents related to organoleptic properties and nutritional, chemical, and microbiological characteristics. Most research on starter cultures for coffee fermentation has focused on selection of pectinolytic yeasts. Nonconventional yeasts, such as *P. guilliermondii* and *C. parapsilosis*, have revealed important pectinolytic potential [130]. In addition, yeasts such as *C. parapsilosis* and *S. cerevisiae* are coffee starters due to differentiated aroma production [131].

Researches with nonconventional yeasts in coffee fermentation have revealed the production of different volatile organic compounds. Different yeast species isolated from the dry and semi-dry processes of coffee produce significant amounts of aroma compounds (*e.g.*, acetoin, furfural, butyric acid, 2-phenyl-ethanone, 1,2-propanediol, hexanoic acid, decanoic acid, and nonanoic acid, among others), suggesting the strains *P. guilliermondii* UFLACN731 and *C. parapsilosis* UFLACN448 as promising candidates for coffee fermentation [130].

The importance of fermentation in the contribution of chocolate quality has been recognized for more than 90 years. Several studies and research have been conducted in different countries to determine the species of microorganisms associated with this process. A succession between yeast, lactic acid bacteria, and acetic acid bacteria is generally observed. This succession begins when high concentrations of sugars, low pH, and oxygen tension favor the growth of yeasts that convert the carbohydrates of the pulp into ethanol, dominating the process for approximately 48 hours. Lactic acid bacteria also ferment the sugars and utilize citric acid from the pulp; its growth is favored by the scarcity of oxygen and slight elevations of pH and temperature. With disintegration of the mucilaginous pulp surrounding the cocoa beans and together with the pH and aeration of mass, the citric acid present in the pulp is reduced by action of the yeasts, which favors the growth of acetic bacteria. These bacteria promote the oxidation of ethanol, initially produced by the yeast, to acetic acid in an extremely exothermic reaction, raising the temperature of the fermentative mass to levels of 45–50°C. The high temperature is important to enzymatic reactions, necessary for the development of the aroma and flavor of chocolate. Acetic acid, when penetrating cotyledonous tissues, promotes the death of the bean embryo (48–72 hours), and, together with ethanol, they act synergistically causing the diffusion of polyphenols in cotyledonary tissues. These reactions are important in the generation of well-fermented cocoa beans. Other microbial groups, such as *Bacillus* and filamentous fungi, develop in the final stages of fermentation, but the role of these microorganisms has not yet been fully elucidated [132–135].

To date, cocoa and coffee fermentations have been carried out with wild microorganisms present in the raw material and equipment used. Some studies have been carried out for the selection of cocoa and coffee cultures, where the main objective is to develop a faster fermentation process through the use of microorganisms producing pectinolytic enzymes. A study carried out cocoa fermentation inoculated with *Saccharomyces chevalieri* (now classified as *Saccharomyces cerevisiae*), *Candida zeylanoides*, and *Kluyveromyces fragilis* (now classified as *Kluyveromyces marxianus*) [136]. These yeasts were selected as being part of the natural flora of the cocoa fermentation environment and for producing pectinolytic enzymes. The fermentations inoculated with these yeasts were faster and produced chocolates with similar sensory characteristics in relation to spontaneously conducted fermentations [136].

The use of aromatic nonconventional yeasts, *P. kluyveri* and *K. marxianus,* during the cocoa fermentation process is able to alter the flavor profile of the chocolates

compared to a spontaneously fermented control. The chocolates obtained after being submitted to this fermentation process can obtain higher grades in relation to the fruity flavor, cocoa aroma, and general taste. Some patents have been developed with the objective of establishing methods to optimize the cocoa fermentation process [137]. A method consisted of the addition of aromatic substances during the cocoa fermentation process with the aim of obtaining modified cocoa almonds. These substances comprise fruit pulp, aromatic leaves, and wood parts, among others [138]. *Pichia kluyveri* strain is a starter culture during cocoa fermentation adjusting the contents of isobutyl acetate and isoamyl acetate in obtained cocoa nibs. In addition, *P. kluyveri* demonstrated the ability to liquefy the cocoa pulp completely, due to its high pectinolytic activity [139].

2.4 Plant growth promotion

Throughout this chapter, the biotechnological potential of yeasts from other genera than *Saccharomyces* for activities and products commonly performed and generated by *Saccharomyces* spp. was discussed and exhibited. These activities are mainly associated with the industrial utilization of yeasts for the production of food, bioethanol, and alcoholic beverages through the fermentative metabolism. Nonconventional yeasts were initially shown to be able to generate high levels of a compound conventionally produced by *S. cerevisiae* (ethanol), but fermenting an alternative sugar (xylose) whose *Saccharomyces* spp. are naturally unable attracts interest in the bioenergy industry. Many of these strains are also able to generate a compound of industrial interest that *Saccharomyces* spp. cannot produce, *i.e.*, xylitol. Then, several strains of nonconventional yeasts able to replace *Saccharomyces* spp. in the industry of beer, coffee and chocolate were presented, which can improve aroma and flavors of these products. Yeasts are being unintentionally used by mankind for such purposes since the primitive beginning of microbial biotechnology. Therefore, food and bioenergy are the traditional fields of application of these unicellular fungi. However, the application of nonconventional yeasts is beyond the fields where *Saccharomyces* spp. are traditionally used. In this section, the potential application of nonconventional yeasts in a nonconventional field: agricultural biotechnology will be shown.

Many strains of nonconventional yeast species have been demonstrated as plant growth-promoting (PGP) microorganisms. PGP microorganisms live in the soil surrounding plant roots (rhizosphere), inside plant roots, stems and leaves (endophytes), or externally attached to plant surfaces (epiphytes) [140, 141]. The set of microbes inhabiting these many plant compartments is organized in microbial communities comprising the plant microbiome [140, 141]. The rhizosphere harbors a microbiome with higher diversity, abundance, and activity than the other plant compartments and is enriched in PGP microbes [142]. PGP microbes perform many activities that support plant growth and health, helping plants against biotic and abiotic stressors, besides directly acting in plant nutrition and growth regulation [142].

Inoculation of PGP microbes has the potential to replace the agrochemicals normally used in cropping systems, which can be expensive and environmentally harmful, and from nonrenewable sources [143]. The studies and applications of plant growth-promoting yeasts have lagged behind those of bacteria and other fungi [144, 145]. However, some studies showing beneficial activities of yeasts for plant productivity indicate that they can be as important as other microbes in agricultural biotechnology [146–148]. Some PGP features, as well as the main nonconventional yeast species able to perform these activities are discussed below.

Among the PGP activities performed by yeasts, biosynthesis of indole-3-acetic acid (IAA) is the most reported in the literature, found in many distinct yeast species and genera [144, 145, 147, 149–155]. IAA is a plant hormone of the auxin class, associated

with phytostimulation by increasing root growth [156, 157]. In the *Rhodotorula* genus, two species were identified as able to produce IAA in many studies. *R. graminis* is a yeast species isolated from the internal tissues of poplar tree and able to produce high amounts of IAA [149]. This capacity results in the growth promotion of distinct plants, such as poplar itself, as well as pepper and maize [150–152]. A genome survey did not detect the genes involved in the conventional pathways of IAA biosynthesis and suggested putative genes for alternative pathways in this species [154]. Strains of the species *R. mucilaginosa*, isolated from poplar stems or from soils cropped with legumes, were also found as high IAA producers [149, 155]. Experiments using this species indicated its growth promotion activity in poplar and tomato [150–152].

Candida tropicalis is another important yeast species isolated from the rhizosphere of rice and maize, whose ability to synthesize IAA besides other PGP activities was shown to promote growth of these mentioned crops [145, 153]. Other examples of IAA biosynthesizing yeast species are *Aureobasidium pullulans*, *Cryptococcus flavus*, *Hannaella sinensis*, *Rhodosporidium paludigenum*, *Torulaspora globosa*, and *Williopsis saturnus* [144, 147, 155].

Another important PGP activity performed by some yeast species is phosphate solubilization and interaction with mycorrhizal fungi able to mobilize phosphate to plants [153, 158, 159]. Phosphorus (P) is a macroelement required by plants in high levels. Plants cropped in soils with low availability of phosphate are highly benefited from the activity of P-solubilizing microbes for their nutrition. These PGP microbes produce organic acids that release the phosphate attached to mineral surfaces, making this nutrient available for root absorption [160].

The yeast species *Yarrowia lipolytica*, *Torulaspora globosa*, and *Candida tropicalis* were shown as able to solubilize inorganic phosphate in *in vitro* tests [147, 153, 160]. Some strains of the species *Candida railenensis* and *Cryptococcus flavus* were not able to directly solubilize phosphate, but exhibited a microbial interaction with arbuscular mycorrhizal fungi resulting in higher P-solubilization and promotion of maize growth [159]. On the other hand, another study observed that yeast strains from the genera *Rhodotorula* and *Cryptococcus* were able to both interact with the mycorrhizal fungus *Glomus mosseae* and solubilize phosphate [158].

The agricultural productivity of many crops is highly impaired by plant diseases, mainly caused by fungal pathogens. Use of PGP microbes that perform biocontrol of these fungi is a promising alternative to the use of expensive and bioaccumulative fungicides [143]. The capacity of pathogen biocontrol is present in many nonconventional yeast species that perform different types of antagonism, including competition for space and nutrients, antibiosis, fungal cell wall degradation, mycoparasitism and induction of host resistance [146].

Strains of the species *Candida valida*, *Rhodotorula glutinis*, and *Trichosporon asahii*, isolated from the sugar beet rhizosphere, were shown to control the fungus *Rhizoctonia solani* that causes root damping-off, promoting growth and health of this plant [161]. Another strain of *R. glutinis* was isolated from the inner tissues of the apple fruit and was able to promote growth of apple tree by controlling the pathogen *Botrytis cinerea* [162]. The species of the genus *Rhodotorula* commonly produce a siderophore called rhodoturulic acid, which inhibits *B. cinerea* spore germination. Utilization of *R. glutinis* in combination with the application of rhodoturulic acid resulted in a more efficient control of *B. cinerea* [162]. Other yeast species like *Hannaella sinensis* and *Rhodosporidium paludigenum* produce different siderophores, but the analyzed strains of these species showed no antifungal activity [147].

An epiphyte yeast strain of the species *Torulaspora globosa* isolated from rice leaf surface is antagonistic to many fungi causing plant diseases, including *Fusarium moniliforme*, *Helminthosporium oryzae*, and *Rhizoctonia solani* [147]. In addition to efficient biocontrol, this strain synthesizes IAA, indicating its high potential to be used

as an agricultural inoculant [147]. Strains of the species *Aureobasidium pullulans* and *Rhodotorula mucilaginosa*, isolated from soils cropped with legumes, showed antagonism to the fungi *Phytophthora infestans* and *Fusarium graminearum*, respectively [155].

In addition to the three mentioned PGP activities previously shown, *i.e.* IAA biosynthesis, P-solubilization and biocontrol, many others are present in nonconventional yeasts. For example, yeasts can help plants against several types of stress. The compound 1-aminocyclopropane-1-carboxylic acid (ACC) is a precursor in ethylene biosynthesis. High ethylene levels are induced in plants facing many types of stress [163]. PGP microbes that produce the enzyme ACC deaminase decrease the levels of ethylene, alleviating plant stress and indirectly promoting their growth [163]. Despite this activity is more present in bacteria, the yeast species *Candida tropicalis*, also capable of P-solubilization and IAA biosynthesis, was shown to produce ACC deaminase, which possibly contributed to rice growth promotion in pot experiments [145]. The biotechnological potential of the strain *C. tropicalis* HY was validated with the inclusion of this strain in the commercial biofertilizer product BioGro, which improves paddy rice yield [145].

Other type of common stress in plants is the oxidative. Reactive oxygen species such as hydrogen peroxide (H_2O_2) can damage plant tissues. The accumulation of these compounds can be mitigated by the activity of the enzyme catalase. Although this PGP function is also more observed in bacteria, some yeasts that use methanol as carbon and energy sources contains catalases, since H_2O_2 is a by-product of that metabolism [147]. Strains of *Cryptococcus flavus*, *Hannaella sinensis*, *Rhodosporidium paludigenum*, and *Torulaspora globosa* produce catalase and thus can potentially help plants against the oxidative stress, in addition to the PGP activities already described for these species [147].

The biotechnological utilization of PGP microbes in agriculture is in its infancy, since much knowledge and technology must be developed in order to efficiently replace agrochemicals. Nonconventional yeasts were shown to perform many beneficial activities to plants and need to be explored more to increase their utilization together with PGP bacteria and filamentous fungi for a more sustainable agriculture.

Conflict of interest

The authors declare that they have no competing interests.

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Section 4

Industrial Bioproducts

Chapter 6

The Oleaginous Red Yeast *Rhodotorula/Rhodosporidium*: A Factory for Industrial Bioproducts

Mathew Lyman, Salustra Urbin, Cheryl Strout and Bonnee Rubinfeld

Abstract

Rhodotorula genus, amended in 2015, is polyphyletic and contains *Rhodotorula* species that grow as single-cell yeast (monomorphic) and reproduce asexually via budding/fission (anamorphic); it also contains *Rhodosporidium* species that reproduce sexually (teleomorphic) and alternate between a yeast phase and dikaryotic filamentous phase (dimorphic). Several species of these "red yeast" produce industrial bioproducts, namely biofuel feedstocks, carotenoids, enzymes, and biosurfactants. This chapter highlights the biotechnology areas that *Rhodotorula/Rhodosporidium* contributes to and the future market value of those industries. The primary yeast species to be discussed include *Rhodosporidium toruloides, Rhodotorula glutinis, Rhodosporidium diobovatum, Rhodosporidium kratochvilovae, Rhodotorula graminis, Rhodotorula babjevae, and Rhodotorula taiwanensis.*

Keywords: *Rhodotorula*, *Rhodosporidium*, red yeast, oleaginous, carotenoids, biofuels, biodiesel, antioxidants, DAAO enzyme, PAL enzyme, PEFA, surfactants, antagonistic yeast

1. Introduction

The aim of this chapter is to discuss the *Rhodotorula* genus in the context of biotechnology; it is not meant to be an academic "deep-dive" into all things known about the topic (their discovery and classification in the early 1900s could be a separate chapter unto itself [1]). The word "oleaginous" used in the chapter title is important, and it means "oil producing" or "rich in oils." Much of the interest in these yeast stems from the fact that they often store excess carbon as triacylglycerols (TAG), not as polysaccharides [2]. These lipids can account for up to 70% of the cells dry mass depending on growth conditions, and these oils can be harvested and used as raw material in second-generation biodiesel production. Some *Rhodotorula* species also produce glycolipids, known as polyol esters of fatty acids (PEFA), which have broad interest in the biosurfactant industry. Several emerging publications and patents have been reported in this area. In addition, these yeasts are indeed "pinkish red." These hues are carotenoid compounds produced by the yeast; these natural dyes can be extracted from *Rhodotorula/Rhodosporidium* and used in the food and vitamin industries. This chapter will further expand upon these intriguing facts and illustrate other examples that are in the scientific and patent literature, all while highlighting the future market potential of these "industrious" yeast.

1.1 A brief introduction to the *Rhodotorula* genus

There was a major revision of the subphylum *Pucciniomycotina* (Phylum *Basidiomycota*, Kingdom *Fungi*) published by Wang et al. in 2015; therefore, this chapter will focus solely on species that are categorized under the revised *Rhodotorula* genus in the Sporidiobolaceae family. Species to be discussed include *Rhodosporidium toruloides, Rhodotorula glutinis, Rhodosporidium diobovatum, Rhodosporidium kratochvilovae, Rhodotorula graminis, Rhodotorula babjevae, and Rhodotorula taiwanensis*. Species that were removed from the *Rhodotorula* genus in 2015, for example, *Rhodotorula bogoriensis* (reclassified as *Pseudohyphozyma bogoriensis*), will not be discussed in detail, even though it produces sophorolipid biosurfactants and is of industrial interest [3–5].

It is noteworthy that the *Rhodotorula* genus is polyphyletic. It contains *Rhodotorula* species that grow as single-cell yeast (monomorphic) and reproduce asexually via budding/fission (anamorphic) [6]. It also contains *Rhodosporidium* species that reproduce sexually (teleomorphic) and alternate between a yeast phase and dikaryotic filamentous phase (dimorphic). For *Rhodosporidium*, sexual reproduction begins with the fusing of compatible haploid yeast cells. They then grow as dikaryotic hyphae/mycelium, during which diploid teliospores are produced. Teliospores germinate forming basidium (where karyogamy and meiosis occur) and then extrude haploid basidiospores. Germination of basidiospores then restores the yeast phase of growth [7, 8]. Thus, the yeast phases of *Rhodotorula* and *Rhodosporidium* species are virtually indistinguishable. It is notable that "all known *Rhodosporidium* species [have been] isolated as haploid yeasts and have a bipolar mating behavior, i.e., their strains belong to either one of two complementary mating types, designated *A1* and *A2* or *A* and *a*" [9]. Due to this mating behavior, *Rhodosporidium toruloides* is being developed as an alternative biotechnology platform to *Saccharomyces cerevisiae* [10] with unique biochemical pathways for the production of biofuels, carotenoids, and industrial enzymes.

1.2 The industrial markets of the genus *Rhodotorula*

The most relevant question for the purposes of this chapter, regardless of the details of any given species, is "what biotechnology markets are impacted by the *Rhodotorula* genus?" **Figure 1** summarizes the five major industrial markets where *Rhodotorula* yeast is utilized (or will likely be utilized in the future). These include biofuels, carotenoids, enzyme production (e.g. D-Amino acid oxidase (DAAO) and L-Phenylalanine ammonia lyase (PAL)), biosurfactants, and antagonistic yeast.

1.2.1 Biofuels

Biofuels continue to grow as a global industry, despite the challenges in production capacity and automotive engine compatibility [11]. The biofuels market size is expected to reach USD ~218 billion by 2022 [12]. For example, United Airlines announced in 2018 that it would begin blending more biofuel with conventional fuel, with a goal to reduce greenhouse emissions by 50% on all flights by 2050. It is expected that other airlines will follow suit, further increasing biofuel demand. Thus, there continues to be a need for microbial factories to produce biofuels [13, 14], especially given the market competition of using vegetable oils in both the food and biodiesel sectors [15]. Microorganisms may contribute to biofuel production in several ways; bisabolene (a diesel alternative) can be produced through bioengineering the isoprenoid pathway [16], and alkanes and alkenes can be produced through fatty acid biosynthesis inside the cell [15, 17]. Furthermore, microbial triglycerides,

Figure 1.
The primary biotechnology industries impacted by the Rhodotorula genus. "Rhodotorula in Industry" was written in Rhodotorula taiwanensis MD1149 on a Hommel minimal salt (HMS) agar plate supplemented with glucose.

fatty acids, and other lipids can be converted to fatty acid methyl esters (FAME) by transesterification with methanol; FAME molecules are the primary component in biodiesel fuel.

The biofuels industry is largely divided between two types of fuel: biodiesel and bioethanol. These biofuels are further separated as "first generation, second generation, third generation, or fourth generation." First-generation biofuels are produced using feedstocks that can also be used for food (e.g. soy, canola, or sunflower oils). These oils are transesterified into FAME by reacting a triacylglyceride with a short alcohol—usually methanol—in the presence of a catalyst (strong base) and heat. By contrast, second-generation biofuels are produced using *non-food/non-edible* sources such as wood, organic waste, and food crop waste. Second-generation biofuels are preferred as they do not utilize food crops for fuel. Third-generation biofuels are produced by engineered algae; fourth-generation biofuels are aimed at using biomass that captures CO_2 while producing lipids that can be converted into biofuel. In this context, *Rhodotorula/Rhodosporidium* species that are used in biodiesel production are classified as "second generation" biodiesel producers that accumulate single-cell oils (SCOs); they will be discussed later in this chapter.

1.2.2 Carotenoids

In addition to lipids, these yeasts also produce carotenoids, which are of industrial interest. A recent report entitled "Global Carotenoid Market – Growth, Trends, and Forecast (2018–2023)" predicted a global carotenoid market of USD 2 billion by 2023 [18]. Carotenoids are yellow, orange, and red pigments produced by microorganisms, algae, higher plants, and some animals; these compounds are responsible for giving *Rhodotorula* species their "Red Yeast" moniker. Common examples of carotenoids include alpha-carotene, beta-carotene, beta-cryptoxanthin, lutein,

lycopene, torulene, and torularhodin. Carotenoids are effective "quenchers" of reactive oxygen species (ROS), and the protective health effects of dietary carotenoids (as antioxidants) are of intense interest to the industrial research community [19]. Some carotenoids found in fruits and vegetables can be converted into vitamin A, namely beta-carotene, and to a lesser extent alpha-carotene and beta-cryptoxanthin (note: these compounds are often referred to as "provitamin A"). The impact of Vitamin A on human health is nicely summarized by Ulbricht et al. [20]. Carotenoids may also be used as natural coloring agents in the food, cosmetic, and pharmaceutical industries [21].

1.2.3 Enzymes

The *Rhodotorula/Rhodosporidium* enzymes, D-Amino acid oxidase (DAAO) and L-Phenylalanine ammonia lyase (PAL), have notable utility within industry. DAAO is a "FAD-dependent oxidoreductase that catalyzes stereospecifically the oxidative deamination of d-amino acids to α-keto acids, ammonia, and hydrogen peroxide" [22]. Even though DAAOs have been identified in a wide range of living organisms (e.g. bacteria, fungi, humans) [23], DAAO from certain *Rhodotorula* strains—discussed later in this chapter—has advantages over others, mainly due to a higher turnover rate and their increased stability of FAD binding [24]. This makes them ideal for the enzymatic deamination of cephalosporin C to 7-(5-oxoadipoamido)-cephalosporanic acid, an important intermediate for the production of cephalosporin [25]. Cephalosporin antibiotics are the largest selling class of antibiotics in a market, which is expected to reach USD 57 billion by 2024 [26, 27]. The PAL enzyme is also a major contributor to the enzymatic synthesis of "industrially relevant biomolecules," namely pure L-phenylalanine (L-Phe), L-phenylalanine methyl ester (L-PM), and para-hydroxycinnamic acid (p-HCA) [28]. L-Phe, being an essential amino acid, is used in food formulations, feed for livestock, dietary supplements, and nutraceuticals [29]. L-PM is a precursor in the production of the sweetener aspartame [28], and p-HCA has utility in the cosmetic, health, and flavoring industries [30].

1.2.4 Biosurfactants

The biosurfactant market is estimated to be worth over USD 2.7 billion by 2024, primarily driven by their usage in the personal care and cosmetic industries [31]. Biosurfactants are small molecules that contain both hydrophilic and hydrophobic moieties and reduce the surface tension between oil and water mixtures (colloquially they are known as "green detergents" or "bio-soaps"); they are produced by several microbial species, viz., bacteria, yeast, and fungi [32, 33], and they are utilized extensively in multiple industries: cosmetics, food, explosive, pharmaceutical, detergents, and paints [34, 35]. The primary market barrier for biosurfactants is the vast library of existing chemical surfactants produced from petroleum feedstocks; end users can choose from hundreds of synthetic surfactants to fulfill their industry need. A major drawback is that chemical/synthetic surfactants can accumulate in the environment and are toxic to microbes, plants, aquatic life, and higher vertebrates including humans [36]. Therefore, because biosurfactants are biodegradable and petroleum-independent, their value will likely increase as pollution increases.

1.2.5 Antagonistic yeast

Rhodotorula species also play a role in the market of biocontrol agents, also known as antagonistic yeast. Significant losses in harvested fruit occur from decay by filamentous fungi such as *Botrytis cinerea* and *Penicillium expansum* [37]. In order to

replace or augment chemical fungicides, biological control agents have been studied heavily the past two decades, with some yeast-based biocontrol products commercially available for specific commodities [38]. The ability to control different rots, on different fruits/vegetables, is still an important goal when developing postharvest biocontrol products. Therefore, antagonistic yeasts continue to be an active area of research, and several *Rhodotorula* species have shown promise in this field.

2. Species utilized in biotechnology

2.1 *Rhodosporidium toruloides*

Rhodosporidium toruloides has tremendous potential as a workhorse for multiple industrial applications [39]. It can grow on a variety of carbon sources associated with modern waste streams, and its bioproducts can be used in antibiotic manufacturing, biofuel synthesis, and the food industry.

R. toruloides is a nonpathogenic, aerobic, oleaginous red yeast that has been isolated from a variety of sources, e.g. conifers, soil, wood pulp, dry leaves, and a salt farm [2]. It is able to accumulate lipids to more than 70% of its dry cell weight [40–43]; this occurs when carbon is in excess during growth, and other key nutrients such as nitrogen are sparse [44]. This lipid production can occur on a variety of different carbon sources, e.g. sugarcane juice, crude glycerol, lignocellulosic hydrolysates, vegetable market waste, and Jerusalem artichoke plants [45–49].

Due to the ability of *R. toruloides* to use different types of carbon sources for growth and lipid production, several studies have been carried out on growing this organism using "cheap" carbon sources that are the waste streams from other large industries, namely food waste and crude glycerol waste. Food wastes are an appealing nutrient source, because they are cheap, abundant, and decrease the environmental impact of disposing of these materials using traditional consumer and agricultural waste infrastructure [50, 51]. Crude glycerol is of interest as a "waste" source as the biofuel industry generates ~10% w/w of glycerol for every batch of biodiesel produced; *R. toruloides* has been shown to use crude glycerol as a carbon source for the production of microbial lipids of interest, even in the presence of impurities [52, 53]. In addition, it has been reported that *R. toruloides* has the ability to degrade/utilize hydrocarbon fuels [54]. This opens the possibility of using this yeast as a bioremediation tool for oil contaminated soil.

The bioproducts produced by *R. toruloides* are also of intense interest, namely lipids, enzymes, and carotenoids. Wild-type and engineered strains of *R. toruloides* are truly oleaginous. They produce lipids at high titer making them promising organisms for the production of lipid-based chemicals such as biofuels, lubricants, surfactants, solvents, waxes, creams, and adhesives [2, 55, 56]. It has been shown that under low nitrogen conditions, growth on simple sugars, like glucose, fructose, xylose, or the carbohydrate glycerol, can increase TAG production [55]. To this end, several groups are working to further increase lipid production using engineered strains of *R. toruloides,* primarily for biofuels [45, 56, 57]. As a practical example, scientists in Brazil have performed a successful diesel engine test using biodiesel manufactured from lipids produced by *R. toruloides* when the organism was grown in sugarcane juice (carbon source) and urea (nitrogen source) [48].

R. toruloides is also a "gold mine" for industrial enzymes. It produces a high titer of esterase enzymes, which makes this organism of immense interest to the drug industry. For example, the process of making antibiotics in the cephalosporin class of compounds requires 3-acyloxymethyl cephalosporins to be enzymatically deacylated to the more stable intermediary 3-acyloxymethyl cephalosporins. This can be achieved

by the esterase enzymes produced by *R. toruloides*. Another enzyme produced by *R. toruloides* is phenylalanine ammonia-lyase (PAL), which is a major contributor to the enzymatic synthesis of pure L-phenylalanine (L-Phe), L-phenylalanine methyl ester (L-PM), and para-hydroxycinnamic acid (p-HCA) [28].

R. toruloides has also played an important role in the development of an enzyme substitution treatment of Phenylketonuria (PKU) [58, 59], a genetic mutation manifesting in the inability to metabolize L-Phe. In the human diet, L-phenylalanine is found naturally in protein foods, such as eggs, meats, fish, cheese, and soybeans, and is also produced as a product that can be added to foods, in the case of aspartame. The buildup of L-Phe in the blood is extremely toxic and impacts neurological function in the form of seizures, tremors, and loss of muscle coordination, especially in the extremities [58]. Newborns are screened for PKU at birth to increase the chances of early discovery and attempt to limit the effects of PKU. A new drug, Palynziq™, was developed based on some of the research that involved *R. toruloides* along with other PAL producing species; it was approved by the FDA in May 2018 as an enzyme substitution therapy for PKU [59].

2.2 *Rhodotorula glutinis*

Rhodotorula glutinis, like *R. toruloides*, is of high industrial importance as it also synthesizes numerous valuable compounds: lipids (SCO, single-cell oils), enzymes (in particular, PAL), and carotenoids (lycopene, β-carotene, torulene, and torularhodin). An example workflow of harvesting these bioproducts from these species is shown in **Figure 2**.

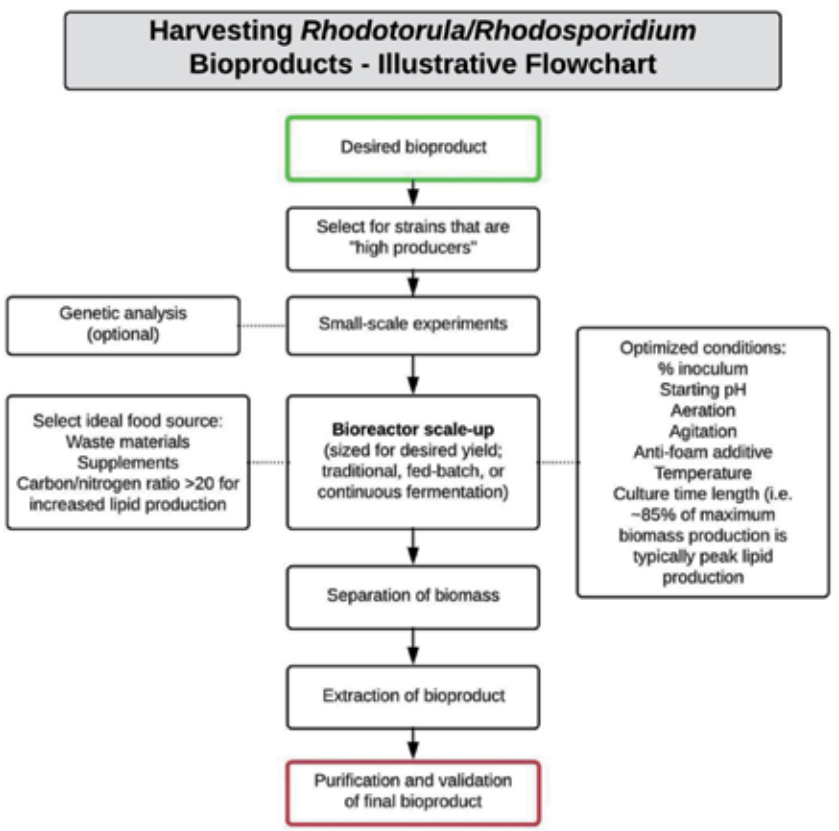

Figure 2.
An example workflow of harvesting bioproducts from members of the Rhodotorula genus.

R. glutinis isolates have been isolated from air, fruit, seawater, soil, grass, milk, and cheese products [60, 61]. Previously, 370 collection strains were assigned to this species based upon color and growth properties; however, additional methods—namely genetic sequencing—have pared this list to a few dozen strains [61]. The majority of these strains are spherical, ellipsoidal, or elongated in shape; aerobic; and mesophilic, although some can thrive under lower temperatures. They use different sources of carbon for growth: glucose, galactose, sucrose, maltose, trehalose, ethanol, glycerol, and hexadecane. *R. glutinis* lack the capacity to perform sugar fermentation and can grow in salt (10% NaCl) but cannot tolerate high sugar (>50% glucose). Depending on strain and growth conditions, their colonies are smooth, moist, and mucoid. As with most species in the *Rhodotorula* genus, color can be creamy, yellow, salmon, pink, orange coral, and blood red due to the production of carotenoids [60].

R. glutinis strains also produce several microbial oils—dominated by oleic, linoleic, palmitic, and stearic acid [62, 63], and the lipid content in their biomass can reach up to 72% [64]. Numerous factors can affect the lipid content and distribution including choice of strain, carbon sources (molasses, glucose, sucrose, glycerol, or waste materials), nitrogen sources (ammonium sulfate or chloride, yeast extract, or monosodium glutamate wastewater), C/N ratio, and cultivation times. These factors result in a wide lipid content distribution between 18 and 66% [60]. Additionally, investigators have worked to increase microbial oil production by either improving culture medium (Chinese patent CN102559788A) or by genetically engineering *R. glutinis* (Chinese patent CN102796675B).

It is noteworthy that several conditions were evaluated for the co-production of both lipid and carotenoids; variables tested included irradiation (as a stressor), temperatures, and C/N ratios. This allowed for the development of a two-stage cultivation strategy where the first stage maximized biomass and carotenoid production under irradiation/high temperature and then maximized lipid content when switched to dark/low temperature [63]. Additional growth studies have evaluated the effect of pH with potato wastewater and glycerol [65], modulating air flow rates using glycerol with yeast extract as the nutrient supply without pH control [66] and using lignocellulosic biomass [67]. Other parameters for scale-up were explored using an airlift bioreactor with mixed carbon sources [68]. *R. glutinis* has also been genetically engineered by introducing both the beta-carotene biosynthesis genes and cellulase genes to increase yields and co-production [69].

Per other industrial applications, *R. glutinis* has been evaluated as a biocontrol agent for post-harvest microbial diseases of fruit (US patent US5525132A). To this end, it has been shown to significantly reduce the incidence of the gray mold, *Botrytis cinerea* on strawberries and apples [70, 71] possibly due to the attachment capability of the antagonistic *R. glutinis* to *B. cinerea* [72]. *R. glutinis* was also used in combination with rhamnolipids to be more efficacious against *Alternaria alternata* infection in cherry tomato fruit than either agent alone [73].

It is also interesting that *R. glutinis* has been isolated during the production of olive oil and during the fermentation and storage of green and black olives in the USA [74, 75]. Olive processing results in the generation of large quantities of olive mill wastewater (OMW) high in phenol. Advantageously, *R. glutinis* has been utilized to treat OMW by dephenolization [76].

2.3 *Rhodosporidium diobovatum*

Rhodosporidium diobovatum has been identified as being a top-tier lipid producer among a group of 69 varied oleaginous yeast strains when glucose is used as the sole carbon source; it is also amenable to scalability [77, 78]. However, biofuel research has also focused on *R. diobovatum* as a second-generation biodiesel producer due

to its ability to consume the impure glycerol waste that is created as a byproduct of first-generation biodiesel production [77]. For example, it was recently reported that *R. diobovatum* could be an "effective strain for production of neutral lipids" given its high yields of oleic, palmitic, and linoleic acid [79], although growth on glucose produced more TAGs than glycerol. Importantly, a glycerol consumption strategy would allow for the continued use and optimization of the first-generation biodiesel production pipelines, while simultaneously allowing investment in the development of second-generation approaches.

Interestingly, it has been reported that up to 70% of the cost to make biodiesel could be accrued *after* the production of the cell biomass, when chemical methods of extracting TAGs are used [80]. This is a costly process that requires the complete drying of biomass, full chemical diffusion of solvent into the cells [81], yeast cell wall disruption, and lipid extraction [2]. The *direct* conversion of wet biomass to biodiesel is the ideal solution, but current methods for that are not scalable, require high temperatures, have lengthy reaction periods, or utilize expensive catalysts [82]. Recent advances with microalgae utilized an ionic liquid for wet cell disruption and lipid extraction in less than 1.5 hours [83]. This same method was effective for *R. diobovatum* and was optimized to produce 97.1% conversion of maximum FAME yields in 2.5 hours at 65°C. They did note a loss of ~40% of their KOH catalyst but conclude that switching from a homogenous to a heterogeneous catalyst could mitigate this [80]. Therefore, research is ongoing to develop *R. diobovatum* as top biofuel production species.

R. diobovatum is also being examined in several novel applications. These include as a way to produce the vitamin supplement glutathione (GSH) [84], which has reported antioxidant properties, and also can be utilized as an anti-toxicant, as a cell metabolism modulator, and potentially as a neuromediator [85]. GSH is synthesized by canonical yeasts but was found in *R. diobovatum* using high performance liquid chromatography (HPLC). Consequently, the *R. diobovatum* synthase genes have been identified and characterized, with the expectation that it could be a valuable industrial producer of GSH in future [84]. This species also has potential as a bioremediation agent for fertilizer pollution. It efficiently assimilates nitrogen at higher rates than other yeasts and may become a useful tool for treating agriculture wastewater [86].

2.4 *Rhodosporidium kratochvilovae* **and** *Rhodotorula graminis*

Rhodosporidium kratochvilovae cultures have been used to create single-cell oil biodiesel when cultured on cane molasses, a sugar refinery waste product [87]. This biodiesel meets standard specifications in Europe and the USA for quality and purity (ASTM D6751, EN14214). The strain was originally discovered in a screen for oleaginous yeasts in Ethiopia. Of the 340 yeast isolates screened, 18 tested positive for oil production, and a *R. kratochvilovae* strain was one of three chosen as best candidates for further optimization based on productivity [88]. Multiple parameters of its cultivation were optimized for lipid production [89] prior to being used to create SCO.

Rhodotorula graminis has also been developed for biodiesel using crude glycerol and undetoxified lignocellulosic hydrolysate (hydrolyzed corn stover; aka, the stalks, leaves, and cobs that remain in fields after harvest) [90]. This strain produced a high lipid content (34% w/w) on hydrolysate and increased this production on waste crude glycerol (54% w/w) [90]. Further optimization of this production pipeline was discussed in patent US9322038B2 granted in 2016 to Washington State University for "Simultaneous saccharification and fermentation (SSF) of lignocellulosic biomass for single-cell oil production by oleaginous microorganisms," with details of strains and culture conditions therein.

R. kratochvilovae and *R. graminis* also produce carotenoids (specifically beta-carotene, torularhodin, and torulene) in order to mitigate cellular damage done by ROS [91]. The benefits of beta-carotene have been understood for some time (antioxidant), but those of torularhodin and torulene only been elucidated recently, namely their potent anti-cancer [92] and anti-microbial activity [93]. Importantly, *R. graminis* and other *Rhodotorula* species have the ability to produce these rare carotenoids at high levels [94] and represent viable options for industrial production of these compounds.

R. kratochvilovae and *R. graminis* are also involved in the biological control of plant pathogens [95]. In a screen for yeasts that control pathogenic fungal strains, *R. kratochvilovae* LS11 was found to have high antagonistic activity [96]. Further development of this strain is hoped to be achieved, and its detoxification pathways are currently being examined in detail [97]. *R. graminis* was also been examined for this application, alone and in combination with fungicide [98].

The PAL enzyme of *R. graminis* (RgrPAL) has industrial interest as it is highly stable in comparison to that of other species, and the organism has flexible culturing requirements. Mutagenesis was employed to create mutants that expressed higher levels of PAL, including an isolate that showed a fourfold increase in production [99]. RgrPAL is also useful to pharmaceutical production, as it accepts analogues of its substrate L-Phe, which can be included in peptidomimetic drugs. The enzymatic activity of RgrPAL was recently increased 28-fold using directed evolution methodology in a recent study [100].

2.5 *Rhodotorula babjevae* and *Rhodotorula taiwanensis*

It was recently published that strains of *Rhodotorula babjevae*, and other *Rhodotorula* species, produce polyol esters of fatty acids (PEFA) [101, 102], similar in composition to extracellular glycolipids reported in the 1960s [103]. The authors stated that "discovery of these PEFA-secreting yeasts may aid in improving production of renewable, sustainable, environmentally friendly surfactants for use in household and industrial cleaning products, as well as many other applications" [102]. To this end, a full patent application was submitted in 2017 by the University of California for "Methods of producing polyol lipids," with details of *Rhodotorula* strains and culture conditions that optimize PEFA production (WO2017184884A1).

It is important to note that the general utility of surfactants in industry—whether they be chemical surfactants or biosurfactants—is determined by their hydrophilic-lipophilic balance (HLB). The HLB concept was first published in 1948 brochure by Atlas Powder Company, along with a follow-up journal paper by William Griffin (an Atlas chemist) [104]. Griffin described HLB as follows: "emulsifiers consist of a molecule that combines both hydrophilic and lipophilic groups and the balance of the size and strength of these two opposite groups is called HLB. For the purpose of convenience, the effective balance of these groups is assigned a numeric value [105]." Griffin then went on to develop the HLB number system, on a scale 0–20, based on polyoxyethylene (POE)-type surfactants. Although the correct classification of surfactants in the HLB system is still being refined and debated [104, 106, 107], the concept of different surfactants having different HLB values remains the benchmark for surfactant science. An example of different surfactant HLB values, their solubility, applications, and use in industry is provided in **Table 1**.

Perhaps the greatest market barrier that biosurfactants face is their limited coverage of the HLB scale. Therefore, they can only be used in very targeted commercial applications. By contrast, hundreds of petroleum-derived chemical surfactants

Surfactant HLB	Solubility	Applications	Industry examples
1.5–3	Oil soluble	Antifoaming agents (defoamers)	Hydraulics, paper, oil drilling, machine tools
3–6	Oil soluble	Water-in-oil emulsions	Cosmetics, sunscreen, margarine
7–9	Oil soluble	Wetting and spreading agents	Herbicides, fertilizers
12–16	Water soluble	Oil-in-water-emulsions	Mayonnaise, cosmetics, dispersants
13–15	Water soluble	Detergents	Laundry and dishwashing detergents
15–18	Water soluble	Solubilizing agents	Pharmaceuticals

Table 1.
The hydrophilic-lipophilic balance (HLB) scale covers 0–20, with different industrial applications depending on the HLB value.

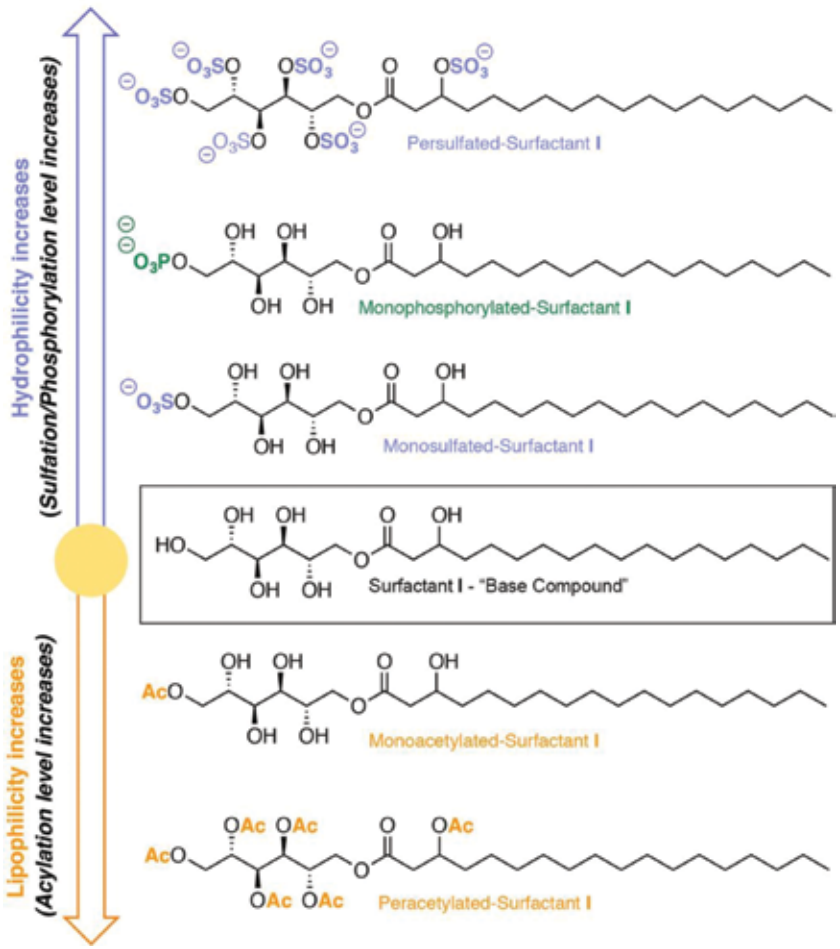

Figure 3.
A "tunable" PEFA biosurfactant. Rhodotorula taiwanensis would be genetically engineered to produce a single PEFA "base compound" and then be systematically modified to move up and down the HLB scale.

conveniently cover the entire HLB scale, to such an extent that their massive usage is now poisoning the environment [36]. Biosurfactants offer a more environmentally friendly option (biodegradable and petroleum-independent); however, there are a limited number of commercially available biosurfactants, and each one covers only a small portion of the HLB scale.

Therefore, in order to expand PEFA coverage on the HLB scale, it was recently published that a new strain of *Rhodotorula taiwanensis* produced hypoacetylated PEFA compounds compared to those produced by *Rhodotorula babjevae* [108, 109]; the difference in their acetylation profiles resulted in different surface tension of the growth medium, i.e. a different hydrophilic-lipophilic balance. It was noted in a subsequent patent filing that although the current *Rhodotorula taiwanensis* strain produced a complex mixture of acetylated and non-acetylated biosurfactants, it could be genetically engineered to produce an unmodified (non-acetylated) "base compound." This base compound could then be "tuned" to produce the full range of biosurfactants that could more extensively cover the HLB scale (**Figure 3**).

This "tunable" biosurfactant approach, using a single species of PEFA compound that can be systematically modified, provides a viable option for competing directly with all types of chemical surfactants, across all market sectors. The biosurfactant's chemical topology, highlighted by the carbohydrate unit's hydroxyl groups, offers further opportunity for chemical modifications that have a direct impact on the physical properties of the surfactant (e.g. antifoaming, emulsifying, wetting, detergent, and solubilizing). Each hydroxyl (OH) group on the molecule has the ability to be acetylated, sulfated, or phosphorylated, leading to modified versions of the parent molecule with different physicochemical properties. Therefore, modification of the hydroxyl groups by chemical and/or biochemical means will enable scientists to produce various biosurfactant products best suited for their unique industrial applications—serving markets not currently served by existing biosurfactants.

3. Conclusions

The *Rhodotorula* genus currently plays a significant role in yeast biotechnology and is poised to expand into various industrial markets: biofuels, carotenoids, biocontrol agents, enzymes, bioremediation, cosmetics, and others. Several advanced genetic systems are currently being developed for these red yeast, and they will likely become an alternative biotechnology platform to *Saccharomyces cerevisiae* [10]. The future looks bright (red) for these fascinating, diverse, and versatile oleaginous microbes.

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Conflict of interest

The authors declare no conflict of interest.

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Chapter 7

De Novo Synthesis of Plant Natural Products in Yeast

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Abstract

Plant natural products possess versatile biological activities including antiviral, anticancer and hepatoprotective activities, which are widely used in pharmaceutical and many other health-related fields. However, current production of such compounds relies on plant culture and extraction, which brings about severe concerns for environmental, ecological and amount of agricultural lands used. With the increasing awareness of environmental sustainability and shortage of lands, yeasts are engineered to produce natural products, for its inherent advantages such as the robustness, safety and sufficient supply of precursors. This chapter focused on the recent progress of yeast as a platform for the biosynthesis of plant natural products.

Keywords: natural products, flavonoids, alkaloids, terpenoids, terpenoids saponins, biomanufacturing, heterogeneous synthesis, yeast

1. Introduction

Plant natural products were a kind of active compounds including flavonoids, alkaloids, terpenoids and saponins etc. As the main composition of plants secondary metabolites, these compounds play an important role in plant communication and defensing, so these compounds have been widely used as herbicide and pesticide in the agricultural industry [1]. For example, oleanane saponins isolated from *Bellis sylvestris* exhibit strong phytotoxic activity against Aegilops geniculate, and saponins from alfalfa can cause a decrease of food metabolized by *Tenebrio molitor* [2]. These compounds were also the major bioactive constituents of some traditional herbal medicines, such as ginsenosides from ginseng and glycyrrhizin from licorice. In addition, some natural products possess special flavors. For instance, camphor alcohol has distinctive aroma, while glycyrrhetinic acid monoglucuronide and mogroside V have a strong sweetness which are approximately 941-fold and 300-fold of sucrose, respectively [3]. These sweet tasting compounds have been widely used in the food industries for weight loss. As a kind of amphiphilic compounds, saponins can also act as robust foaming agent in aqueous solutions. Based on this trait potential, they have been explored in the cosmetics and detergent industries.

Currently, the production of natural products are mainly based on extraction from plants, which is a low yield, time consuming, labor intensive and environment unfriendly supply way [4]. The inefficient approach could not match the huge demands of market, and further limit the application of these compounds in the pharmaceutical, agricultural, food, cosmetic and detergent industries. Thus, developing novel approaches are of great significance to replace the traditional method.

Producing natural products via microorganism cell factories turned out to be a promising solution. Compared with plants, microbes exhibit many advantages, including fast growing, land saving and controllable. Yeast especially *Saccharomyces cerevisiae* (*S. cerevisiae*) has become a widely used host for producing these products because of its similar intracellular structure with plant cells, such as the inherent endomembrane system for microsomal enzymes to stand on. Moreover, the generally recognized as safe (GRAS) yeast as a robust industrial strain is widely used in food and alcohol production and has a clear genetic background. Typically, nearly 25 g/L artemisinic acid has been produced in *S. cerevisiae*, which indicated that producing natural products by yeast is a potential approach to substitute the traditional supply way.

In this chapter, we systematically illustrate the decoded biosynthetic pathway of flavonoids, alkaloids, terpenoids and terpenoids saponins assisted by yeast. Then briefly summarize the progress of yeast to produce plant natural products. Furthermore, novel strategies and tools used to boost their production were discussed.

2. Synthetic pathways of natural products

Flavonoids and alkaloids are usually derived from the shikimate pathway, which exists in prokaryotic, eukaryotic, and archaeal microorganisms. The synthesis pathway start from the stereo-specific condensation catalyzed by 3-deoxy-Darabino-heptulosonate-7-phosphate synthase to generate 3-deoxy-Darabino-heptulosonate-7-phosphate (DAHP), which is further catalyzed to form chorismate a common precursor for various aromatic compounds, including aromatic amino acids, then these aromatic compound will be converted to flavonoids and alkaloids **Figure 1(a)**.

Terpenes and saponins are usually synthesized from the common five-carbon building blocks, 3-isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), which are synthesized through mevalonic acid (MVA) or 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway [5]. These five-carbon units are condensed to geranyl pyrophosphate (GPP), geranylgeranyl pyrophosphate (GGPP), and farnesyl pyrophosphate (FPP). These precursors are then diverted to specialized terpenes by terpene synthases. GPP is converted to monoterpene by

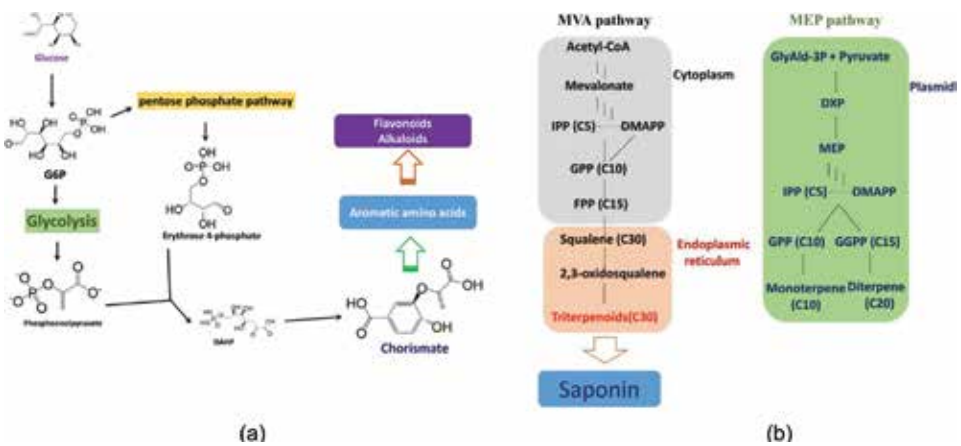

Figure 1.
(a) Scheme for flavonoid and alkaloid biosynthetic pathways. (b) Scheme for terpene and saponins biosynthetic pathways.

monoterpene synthases, GGPP were used to synthesize diterpenes which can be further converted to tretaterpenes, and FPP is the precursor for triterpenes which can be converted to saponins by UGTs **Figure 1(b)**.

In order to produce flavonoids, alkaloids, monoterpene, diterpene and tretaterpene heterogeneously, *E. coli* is widely used for enzyme identification, because of its ease genetic manipulations, whereas the yeast platform was mostly used in triterpene synthesis for its endogenous 2,3-oxidosqualene supplement and inner membrane structure for membrane located plant CYP450 to stand on, which is intensively used and studied in natural products synthesis.

During triterpenoids synthesis, FPP is condensed to 2,3-oxidosqualene, which is subsequently cyclized to polycyclic triterpenoid skeletons by oxidosqualene cyclases (OSCs). These molecules are oxidized by CYP450s forming aglycones, which are further glycosylated to triterpenoid saponins by UGTs.

Cyclization of 2,3-oxidosqualene by OSCs, was the first step in triterpenoid biosynthesis. Because of the efficient 2,3-oxidosqualene supplement, most OSCs was verified by directly expressing in yeast [6]. Plants OSCs always possess promiscuous activities, and could cyclize 2,3-oxidosqualene to different conformations simultaneously. For example, amyrin synthase from *Glycyrrhiza uralensis* can produce not only α-amyrin but also β-amyrin [7]. Due to the multiple-activity of OSCs, more than 100 kinds of triterpenoid skeletons could be generated during cyclization. This was considered as a part of plant defense, for many defensive substances could be produced by one process, which can alleviate the metabolic burden of the plants. However, the promiscuity of OSCs would lead to undesired structural analogue [8].

The triterpenoid skeletons could be further oxidized by CYP450s, introducing active groups such as hydroxyl, carboxyl or epoxy groups [9]. The CYP450s decoding process was complicated, for the identification of CYP450s need much chemical and bioinformatics information, and the membrane located plant CYP450s is hard to express in *E. coli*. Hence, *S. cerevisiae* together with chromatography mass spectrum and NMR play an important role in the decoding of plant biosynthetic pathways involving CYP450s especially for the biosynthesis of triterpenoids and their saponins. To date, 48 CYP450s have been identified involved in triterpenoid saponins biosynthesis. They were summarized in **Table 1**.

No.	Name	Accession number	Plant species	Substrate	Loci
1	CYP51H10	ABG88965.1	*Avena strigosa*	β-amyrin	C-12, 13, 16β
2	CYP71D353	AHB62239.1	*Lotus japonicus*	lupeol	C-20
3	CYP72A61v2	BAL45199.1	*Medicago truncatula*	24-OH-β-amyrin	C-23
4	CYP72A63	BAL45200.1	*Medicago truncatula*	β-amyrin	C-30
5	CYP72A67	ABC59075.1	*Medicago truncatula*	oleanolic acid	C-2
6	CYP72A68v2	BAL45204.1	*Medicago truncatula*	oleanolic acid	C-25
7	CYP72A69	BAW35014.1	*Glycine max*	β-amyrin	C-21
8	CYP72A154	BAL45207.1	*Glycyrrhiza uralensis*	β-amyrin	C-30
9	CYP87D16	AHF22090.1	*Maesa lanceolata*	β-amyrin	C-16α
10	CYP88D6	AQQ13664.1	*Glycyrrhiza uralensis*	β-amyrin	C-11
11	CYP93E1	BAE94181.1	*Glycine max*	β-amyrin	C-24
12	CYP93E2	ABC59085.1	*Medicago truncatula*	β-amyrin	C-24

No.	Name	Accession number	Plant species	Substrate	Loci
13	CYP93E3	BAG68930.1	*Glycyrrhiza uralensis*	β-amyrin	C-24
14	CYP93E4	AIN25416.1	*Arachis hypogaea*	β-amyrin	C-24
15	CYP93E5	AIN25417.1	*Cicer arietinum*	β-amyrin	C-24
16	CYP93E6	AIN25418.1	*Glycyrrhiza glabra*	β-amyrin	C-24
17	CYP93E7	AIN25419.1	*Lens culinaris*	β-amyrin	C-24
18	CYP93E8	AIN25420.1	*Pisum sativum*	β-amyrin	C-24
19	CYP93E9	AIN25421.1	*Phaseolus vulgaris*	β-amyrin	C-24
20	CYP716A12	ABC59076.1	*Medicago truncatula*	α-amyrin, β-amyrin, lupeol	C-28
21	CYP716A14v2	AHF22083.1	*Artemisia annua*	α-amyrin, β-amyrin	C-3
22	CYP716A15	BAJ84106.1	*Vitis vinifera*	α-amyrin, β-amyrin, lupeol	C-28
23	CYP716A17	BAJ84107.1	*Vitis vinifera*	α-amyrin, β-amyrin, lupeol	C-28
24	CYP716A44	–	*Solanum lycopersicum*	α-amyrin, β-amyrin	C-28
25	CYP716A46	–	*Solanum lycopersicum*	α-amyrin, β-amyrin	C-28
26	CYP716A47	AEY75213.1	*Panax ginseng*	dammarenediol-II	C-12
27	CYP716A52v2	AFO63032.1	*Panax ginseng*	β-amyrin	C-28
28	CYP716A53v2	AFO63031.1	*Panax ginseng*	dammarenediol-II	C-20
29	CYP716A75	AHF22088.1	*Maesa lanceolata*	β-amyrin	C-28
30	CYP716A78	ANY30853.1	*Chenopodium quinoa*	α-amyrin, β-amyrin, lupeol	C-28
31	CYP716A79	ANY30854.1	*Chenopodium quinoa*	α-amyrin, β-amyrin, lupeol	C-28
32	CYP716A80	ALR73782.1	*Barbarea vulgaris*	α-amyrin, β-amyrin, lupeol	C-28
33	CYP716A81	ALR73781.1	*Barbarea vulgaris*	α-amyrin, β-amyrin, lupeol	C-28
34	CYP716A83	AOG74832.1	*Centella asiatica*	β-amyrin	C-28
35	CYP716A86	AOG74831.1	*Centella asiatica*	β-amyrin	C-28
36	CYP716A140	AOG74836.1	*Platycodon grandiflorus*	β-amyrin, 24-OH-β-amyrin	C-28
37	CYP716A141	AOG74838.1	*Platycodon grandiflorus*	β-amyrin, 24-OH-β-amyrin	C-28
38	CYP716A180	–	*Betula platyphylla*	lupeol	C-28
39	CYP716A244	APZ88353.1	*Eleutherococcus senticosus*	β-amyrin	C-28
40	CYP716A254	–	*Anemone flaccida*	β-amyrin	C-28
41	CYP716AL1	AEX07773.1	*Catharanthus roseus*	α-amyrin, β-amyrin, lupeol	C-28
42	CYP716C11	AOG74835.1	*Centella asiatica*	oleanolic acid	C-2
43	CYP716E41	AOG74834.1	*Centella asiatica*	maslinic acid	C-6

No.	Name	Accession number	Plant species	Substrate	Loci
44	CYP716E22	–	*Solanum lycopersicum*	α-amyrin, β-amyrin	C-6
45	CYP716S5	AOG74839.1	*Platycodon grandiflorus*	β-amyrin, oleanolic acid	C-12, 13
46	CYP716Y1	AHF45909.1	*Bupleurum falcatum*	α-amyrin, β-amyrin	C-16α
47	CYP716A1	AED94045.1	*Arabidopsis thaliana*	β-amyrin	C-28
48	CYP716A2	BAU61505.1	*Arabidopsis thaliana*	α-amyrin	C-22

Table 1.
Overview of plant CYP450s in triterpenoid saponins biosynthesis.

Glycosylation is the last step of triterpenoid saponins biosynthesis that links hydrophilic sugar moieties to the hydrophobic aglycone by UGTs. By glycosylation, various monosaccharide units (including glucose, glucuronic acid, galactose, rhamnose, xylose and arabinose, etc.) could be linked to aglycone at the positions C-3, C-28, C-4, C-16, C-20, C-21, C-22 and/or C-23. The introduced of sugar moieties could improve triterpenoid saponins bioactivities. In view of the tremendous amounts of UGTs in plants, more than 120 genes encoding family 1 UGTs have been identified in *Arabidopsis thaliana* genome [10]. However, Decoding the specific UGT involved in target triterpenoid saponins biosynthesis was very difficult. So far, only 23 UGTs have been identified, which involved in triterpenoid saponins biosynthesis. They were summarized in **Table 2**.

No.	Name	Accession number	Plant species	Substrate	Loci
1	UGT71G1	AAW56092.1	*Medicago truncatula*	Medicagenic acid UDP- glucose	C-3, 28
2	UGT73AD1	ALD84259.1	*Centella asiatica*	Asiatic acid, Madecassic acid UDP- glucose	C-28
3	UGT73AE1	AJT58578.1	*Carthamus tinctorius*	Glycyrrhetinic acid UDP- glucose	C-3
4	UGT73AH1	AUR26623.1	*Centella asiatica*	Asiatic Acid UDP- glucose	C-28
5	UGT73C10	AFN26666.1	*Barbarea vulgaris*	Hederagenin, Oleanolic acid UDP- glucose	C-3
6	UGT73C11	AFN26667.1	*Barbarea vulgaris*	Glycyrrhetinic acid, Oleanolic acid UDP- glucose	C-3
7	UGT73C12	AFN26668.1	*Barbarea vulgaris*	Hederagenin, Oleanolic acid UDP- glucose	C-3
8	UGT73C13	AFN26669.1	*Barbarea vulgaris*	Hederagenin, Oleanolic acid UDP-glucose	C-3
9	UGT73F2	BAM29362.1	*Glycine max*	Saponin A0-gα UDP-xylose	C-22
10	UGT73F3	ACT34898.1	*Medicago truncatula*	Hederagenin UDP- glucose	C-28
11	UGT73F4	BAM29363.1	*Glycine max*	Saponin A0-gα UDP-xylose	C-22
12	UGT73F17	AXS75258.	*Glycyrrhiza uralensis*	Glycyrrhizin UDP-glucose	C-30

No.	Name	Accession number	Plant species	Substrate	Loci
13	UGT73K1	AAW56091.1	*Medicago truncatula*	Hederagenin, Soyasapogenols B and E UDP-glucose	C-3, 28
14	UGT74AE2	–	*Panax Quinquefolium*	Protopanaxadiol UDP-glucose	C-3
15	UGT74M1	ABK76266.1	*Vaccaria hispanica*	Gypsogenic acid UDP-glucose	C-28
16	UGT94Q2	–	*Panax Quinquefolium*	Ginsenoside Rh2 UDP-glucose	C-3
17	UGTPg1	–	*Panax ginseng*	Protopanaxadiol UDP-glucose	C-3
18	UGTPg100	–	*Panax ginseng*	Ginsenoside RF1, Protopanaxatriol UDP-glucose	C-6
19	UGTPg101	–	*Panax ginseng*	Ginsenoside RF1, Protopanaxatriol UDP-glucose	C-6, 20
20	Pg3-O-UGT1	–	*Panax quinquefolius*	Protopanaxadiol UDP-glucose	C-3
21	GmSGT2	BAI99584.1	*Glycine max*	Soyasapogenol B monoglucuronide UDP-galactose	C-3
22	GmSGT3	BAI99585.1	*Glycine max*	Soyasaponin III UDP-rhamnose	C-3
23	UDPG	–	*Panax ginseng*	Ginsenoside Rd. UDP-glucose	

Table 2.
Overview of plant UGTs in triterpenoid saponins biosynthesis.

3. Biosynthesis of natural products in yeast

3.1 Biosynthesis of flavonoids in yeast

Flavonoids are among the most extensively investigated natural products, which could be divided into several subgroups, including common flavonoids (e.g., galangin, eriodictyol, catechin, quercetin, luteolin, myricetin and cyanidin), isoflavonoids (e.g., genistein) and neoflavonoids (e.g., calophyllolide, isodispar B). Due to their physiological activity and decoded synthesis pathways, the heterologous biosynthesis of flavonoids and their derivatives, have been extensively studied in microbial hosts mostly in *E. coli*. However, high-level flavonoids could also be produced by yeast [11].

Based on yeast platform, 531 mg/L resveratrol production was achieved via the tyrosine pathway directly using glucose and ethanol as substrate in fed-batch fermentation. Through the subsequent pull-push-block strain engineering strategy, more resveratrol production formed via the phenylalanine pathway increased up to 800 mg/L directly from glucose which was the highest titer of resveratrol up to now.

The co-culture system was also developed for flavonoids production. In the collaboration system of *E. coli* and yeast to produce naringenin, *E. coli* provides precursors tyrosine and acetate for yeast to produce naringenin, and finally 21.16 mg/L naringenin was obtained from xylose.

3.2 Biosynthesis of alkaloids in yeast

Alkaloid compounds, especially plant-derived benzylisoquinoline alkaloids and monoterpene indole alkaloids are considered as a valuable source of pharmaceuticals for its anticancer, antiviral and antimalarial activities, et al. In order to replace plant-extracting method, the reconstruction of plant-derived alkaloid biosynthetic pathways in microbes are extensively studied. Though always achieve much lower titers than in *E. coli*, yeast platform was used to produce alkaloid with complex pathway.

By overexpressing 14 known monoterpene indole alkaloid pathway genes and an enhancement of secondary metabolism through overexpression of additional seven genes and deletion of three genes, strictosidine was produced in yeast with a production of 0.53 mg/L. (S)-reticuline (around 80 μg/L), baine (6.4 μg/L) and hydrocodone (0.3 μg/L) have been produced in yeast from simple sugars. Indeed, the titers was low; However, very recently, the total assembly and optimization of the noscapine biosynthetic pathway involving over 30 enzymes in yeast was realized, which was very hard to reconstruct in *E. coli*, and led to a final titer of 2.2 mg/L noscapine using sugars as substrate [12].

3.3 Biosynthesis of terpenes and saponins in yeast

Similar with flavonoids and alkaloids, some simple terpenoids skeletons such as monoterpenoid and diterpenoid, which were produced directly by terpenoid synthase, were mostly studied in *E. coli* for the high activity and easy expression of these terpenoid synthase in *E. coli*. However, in recent years, yeast hosts also attracted more and more attention in these fields.

By expressing geraniol synthase from *Ocimum basilicum*, 0.5 mg/L geraniol was obtained in the engineered yeast. The inefficient supplement of precursors GPP should be responsible for the low production. To improve the final geraniol concentration, an ERG20 mutant was used to block the competitive pathway and the production was improved to 5 mg/L. Based on the inherent GPP and the blocking of the competitive FPP synthesis pathway, other volatile monoterpenes, including sabinene and limonene, were also produced by expressing plant monoterpene synthase in yeast host.

Moreover, the heterologous biosynthesis of diterpenoid by yeast also became a new trend and gained more and more attention. Through introducing diterpenoids synthase and metabolic optimization, several plant diterpenoids including taxadiene and miltiradiene were produced in yeast. With the introducing of the taxadiene biosynthetic pathways, and the optimization of precursors supplement by strengthen the MVA pathway through overexpressing tHMG1 and UPC2-1 (a global transcription factor of MVA), the taxadiene was successfully produce in the engineered yeast and achieved a production of 8.7 mg/L, though the production was lower than that produced in *E. coli* (1 g/L). However, when it involves in more complex skeletons, such as ferruginol and carnosic acid which need further oxidation by CYP450s, yeast displays the huge potential. The membrane located plant CYP450s are hard to functionally expressed in *E. coli*. By co-expressing CYP76AH1 and plant cytochrome P450 reductase (CPR) genes heterogeneously in a miltiradiene producing yeast strain, ferruginol was successfully achieved with a titer of 10.5 mg/L. Based on ferruginol, carnosic acid, a C20 oxidations products of miltiradiene, was produced in yeast by introducing an extra CYP76AK6-8 and these compounds still cannot be produced by *E. coli*.

As an exception, the heterogeneous biosynthesis of triterpenoid and triterpenoid saponins were mostly studied in yeast, for the membrane located plant OSC and CYP450 are challengeable to correctly express in *E. coli*. By overexpressing the

codon optimized OSC from *Glycyrrhiza glabra* designated as β-amyrin synthase (βAS), Zhang et al. obtained a β-amyrin production as high as 138.8 mg/L in the engineered *S. cerevisiae* Sgib [13]. Through co-expressing the liquorice CYP88D6 and CYP72A154 with β-amyrin synthase and CPR genes in yeast, glycyrrhetinic acid was produced with a titer of about 20 μg/L. To boost the glycyrrhetinic acid titer, Zhu et al identified novel CYP450s uni25647 and CYP72A63 with higher activity than CYP88D6 and CYP72A154 by yeast host. With these two CYP450s and a novel liquorice CPR, Zhu et al. reconstructed the GA synthesis pathway in sgib and improved the glycyrrhetinic acid titer up to 18.9 mg/L, which is 1000 folds compared with that in the former study [14]. Zhao et al. overexpressed an efficient CPR (MtCPR1) gene, and reconstructed the galactose regulatory network by knocking out GAL1 and GAL80 while using strong inducible promoters GAL1 and GAL10 to operate CYPP450, MtCPR1, ERG1 and ERG9). Finally, 186.1 mg/L oleanic acid was achieved in yeast, which is the highest concentration in reported literatures [15]. More recently, Yu et al. introduced a high specific α-amyrin synthase in yeast leading to the production of α-amyrin, β-amyrin and lupeol at a ratio of 86:13:1 with the α-amyrin titer of 11.97 mg/L, 5.8-folds of the maximum production reported [16].

Benefit from the yeast hosts, the more complex saponins were also heterologously synthesized. By expressing *Barbarea vulgaris* UGT73C11 in a glycyrrhetinic acid producing yeast strain, Liu et al. realized the de novo synthesis of glycyrrhetinic acid-3-O-monoglucose starting from sample glucose [17]. With the decoding of plant biosynthetic pathways, many other triterpenoid saponins have been successfully synthesized in yeast factories and the high production of ginsenoside Rh2 (2.5 g/L) has highlighted the commercial feasibility of this approach.

Panax ginseng is a famous herb medicine widely used in Asia, which possesses various pharmaceutical activities including anticancer, anti-inflammatory and antiviral activities. These activities are mainly endowed by its triterpenoids saponins, known as ginsenosides [18]. Because of their low content and the long culture cycle of ginseng, the heterologous biosynthesis of ginsenosides in microbial cell factories is drawing more and more attention. To date, various ginsenosides and its aglycones have been successfully produced in *S. cerevisiae* (**Figure 2**), including protopanaxadiol, protopanaxatriol and oleanolic acid, the three main kinds of aglycones, and four kinds of ginsenosides, ginsenosides Rh2, Rg3, RF1 and Rh1. In addition, some unnatural ginsenosides such as compound K and 3β, 12β-Di-O-Glc-PPD have also been synthesized by combination various enzymes from different species.

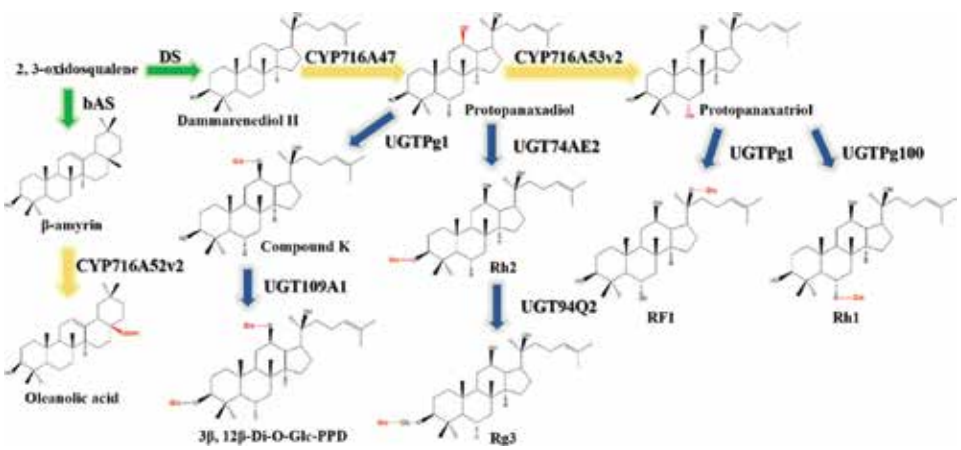

Figure 2.
Scheme for ginsenosides biosynthesized in S. cerevisiae. The green arrows represent OSCs, the yellow arrows represent CYP450s, the blue arrows represent UGTs.

Protopanaxadiol (PPD) is an important starting material for the biosynthesis of ginsenoside, which is synthesized from 2,3-oxidosqualene by dammarenediol-II synthase and CYP450s. Through expressing *P. ginseng* dammarenediol-II synthase (PgDS), *P. ginseng* CYP716A47 and *A. thaliana* CYP450 reductase 1 (AtCPR1), PPD was firstly produced in *S. cerevisiae* [19]. Subsequently, the more complex ginsenosides aglycone protopanaxatriol (PPT) was produced by engineered yeast carrying *P. ginseng* CYP716A53v2 and AtCPR1 which uses PPD as substrate. By the similar strategy of co-expression of *P. ginseng* β-amyrin synthase (PNY1) CYP716A52v2 and AtCPR1, oleanolic acid (OA), a oleanane-type pentacyclic triterpene, was synthesized in *S. cerevisiae* [20]. Although these three ginsenoside aglycones had been successfully synthesized in yeast, the titer was still too low. A truncated version of tHMG1 (3-hydroxyl-3-methylglutaryl-CoA reductase), ERG20 (farnesyl diphosphate synthase), ERG9 (squalene synthase) and ERG1 (2,3-oxidosqualene synthase) were overexpressed to improve the PPD production in *S. cerevisiae*. Together with a codon optimized *P. ginseng* CYP716A53v2, the production of PPD was increased by 262-fold and up to nearly 1.2 g/L. The efficient supplement of PPD made the strain an ideal platform for further tailored ginsenosides biosynthesis [21]. By similar strategies, the PPT production was increased by overexpressing *ERG9*, *ERG1*, *tHMG1* and corresponding CYP450 genes with codon optimization.

The widely studied ginsenosides Rh2 and Rg3 which are synthesized from PPD have been successfully biosynthesized in *S. cerevisiae*. By expressing PgDS, CYP450 system consisting of CYP716A47, AtCPR2 to supply PPD, and the co-expressing of PgUGT74AE2 and PgUGT94Q2 for glycosylation of PPD, Rg3 was heterologously synthesized in *S. cerevisiae*. Combine with the approach of replacing the native promoter of ERG7 with a methionine-repressible promoter (MET3), the production of Rg3 was increased up to 1.3 mg/L. [22] Other type of ginsenosides such as RF1 and Rh1 were synthesized through the co-expression of PgDS, CYP716A53v2, CYP716A47, PgCPR1 and UGTPg100 (or UGTPg1). The production of RF1 and Rh1 reached 42.1 and 92.8 mg/L in *S. cerevisiae*, respectively [23].

It is demonstrated that compound K (CK), generally considered as the metabolite of glycosidases [24], is the main functional form of oral administration of ginsenosides [25]. By the co-expression of PgDS, AtCPR2, CYP716A47 and UGTPg1, CK has already been synthesized in *S. cerevisiae*. Its production was further increased up to 1.4 mg/L by overexpressing tHMG1 and UPC2.1 as well as controlling heterogeneous genes via GAL promoters [26]. By combination of tailoring enzymes from various species, more unnatural ginsenosides could be synthesized in *S. cerevisiae*. Through the combination of plant PgDS, CYP716A47, AtCPR1 and UGT109A1 from *B. subtilis*, 3β, 12β-Di-O-Glc-PPD was produced in *S. cerevisiae*. To further optimize the production, overexpression of tHMG1 and the fusion expression PgDS was carried out, and ERG7 (encoding lanosterol synthase) promoter was replaced with an antisense one. Through these strategies, the production of 3β,12β-Di-O-Glc-PPD was increased up to 9.05 mg/L in the engineered *S. cerevisiae* [27].

Besides ginsenosides, the heterogeneous biosynthesis of other triterpenoids saponins with markedly physiological function also attracted much attention. The natural sweeter mogroside V from S. grosvenorii, which is nearly 300 times sweeter than sucrose, is widely used as a food additive in low-calorie sweet beverages [28]. Through analysis of S. grosvenorii transcriptome data and gene mining, the key genes involved in mogroside V synthesis including cycloartenol synthase (CAS) gene, epoxide hydrolases (EPH) gene, CYP102801, UGT94-289-3 and UGT720-269-1 have been identified. By introducing these enzymes together with squalene synthase (SQS), squalene epoxidase (SQE) and AtCPR1 in *S. cerevisiae*, mogroside V was successfully produced. These advances highlight the possibility to produce natural, noncaloric sweetener mogrosides by engineered yeast.

Saikosaponins are the major pharmaceutical constituents of *B. falcatum*, an important perennial herb widely used in traditional Chinese medicine which exert multiple bioactivities [29]. Currently, two saikosaponins aglycones, 16α-hydroxy α-amyrin and 16α-hydroxy β-amyrin have been synthesized in engineered *S. cerevisiae* through co-expressing CYP716Y1, AtCTR1, CaDDS (dammarenediol synthase from *Centella asiatica*) or GgbAS (β-amyrin synthase from *Glycyrrhiza glabra* in *S. cerevisiae*, respectively [30].

Moreover, by overexpressing lycopene synthetic genes from *Erwinia uredovora*, this ungroomed tetraterpene compound was synthesized by the engineered yeast strain, with a production of 3.3 mg/g CDW. To boost the lycopene production in yeast, Ma et al. overexpressed key genes associated with fatty acid synthesis, TAG production, and TAG fatty acyl composition, and deleted FLD1 to increase lipid-droplet size for more hydrophobic storage space of lycopene, leading to a production of 2.37 g/L, which weighed against that produced in *E. coli* [31]. This indicates the potential of yeast to produce simple terpenoid directly synthesized by terpenoid synthase.

All these progresses indicate the great potential of yeast for heterologous synthesis of natural products, especially for these with complex molecular structure and synthetic pathways such as terpenoids and their saponins. Recently, various kinds of terpenoids and saponins were heterologously produced in yeast, but most of them had a low final concentration far from to instead of plant extracting methods. To boost the production efficiency of engineered yeast strains, various strategies need intensively study.

4. Strategies for boosting biosynthesis of terpenoids in yeast

Although various kinds of natural products could be produced by yeast, many challenges still remain for this approach. The main bottleneck of building an efficient yeast cell factory was that the biosynthetic pathways are not totally elucidated and the poor or disappeared activity of plant enzymes when expressed in yeast. Moreover, the destabilization on the native metabolic flux caused by the heterogeneous pathways could lead to low cell growth and low final products concentration, and the cytotoxicity most of natural products also restrict the use of microbial hosts for producing natural products. Strategies and biotools focused on settling such issues to accelerate the microbial natural products biosynthesis in yeast host have been developed based on omics, metabolic engineering and protein engineering (**Figure 3**).

4.1 Strategies to redesign natural biosynthetic pathways

Unlike some prokaryotic biosynthetic pathway genes, which always locate on a gene cluster, the genes involved in triterpenoid saponins biosynthesis always distributes among the whole genome in plants. Moreover, the expression of these genes generally needs intricate inducible conditions, which increase the difficulty to elucidate triterpenoid saponins biosynthetic pathways.

Benefit from the rapid progress of sequencing technology, genome and transcriptome of many medicinal plants have been sequenced, and the information has been publicly available online (http://medicinalplantgenomics.msu.edu). The analysis of the genome and transcriptome date facilitates the prediction of genes involved in the targeted compound biosynthesis. Through the comparison of the transcriptome information between plants or tissues with high- and low-production of the target compounds, several key genes could be predicted. For example,

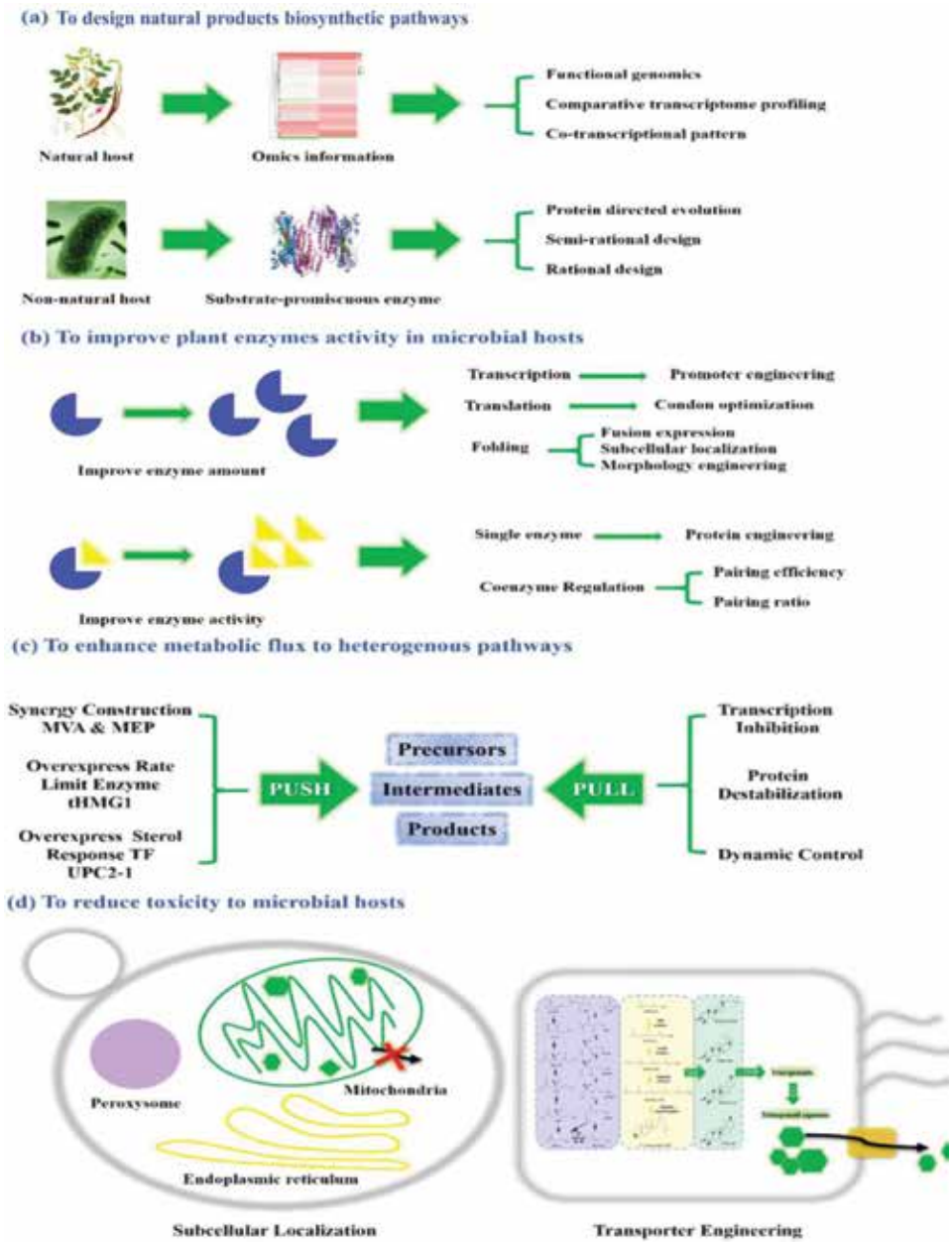

Figure 3.
Scheme for strategies used to advance biosynthesis of triterpenoid saponins in microbial hosts. (a) Strategies to design triterpenoid saponins biosynthetic pathways; (b) Strategies to improve plant enzyme activity in microbial hosts; (c) Strategies to enhance metabolic flux in microbial hosts; (d) Strategies to reduce toxicity to the hosts.

through comparing the transcriptome data between high- and low-producing varieties, the genes including bAS1, CYP716A79 and CYP716A78 that involved in quinoa saponins biosynthesis were targeted from *Chenopodium quinoa*. The predicted genes were then functionally identified by expressing in yeast [32]. By comparing the transcriptome data between ethyl alcohol and methyl jasmonate elicited conditions, key genes involved in maesasaponins biosynthesis including bAS, CYP716A75 and CYP87D16 from *Maesa lanceolate* were targeted and were subsequently identified by expressing in yeast host [33]. Depending on the comparison

of the transcriptome data between varieties with different phenotype, key genes involved in target compound biosynthetic pathways can be predicted, and be further identified by functionally expressed in yeast. Generally, the expression patterns of genes involved in the same biological process are strongly correlated. Thus, genes co-expression analysis was applied to predict the functions of unidentified genes. Based on this strategy, CYP716A12, CYP93E2, CYP72A61v2 and CYP72A68v2 from *M. truncatula* have been functionally identified [34].

Besides mining enzymes from native host, the application of the substrate-promiscuous enzymes turned out to be an alternative approach to reconstruct the target compounds biosynthetic pathways. For instance, the nonnative substrate-promiscuous glycosyltransferase Bs-YjiC from *B. subtilis* 168 was used for the synthesis of ginsenoside Rh1 and some unnatural ginsenosides in *S. cerevisiae* [35]. Other substrate-promiscuous enzymes like UDP-glycosyltransferase UGT109A1, was also used to produce the unnatural ginsenosides 3β, 12β-Di-O-Glc-PPD, 3β,12β-Di-O-Glc-PPT, 3β,20S-Di-O-Glc-DM, and 3β-O-Glc-DM.

The low final concentration of the synthesized compounds is always caused by the poor enzyme activity on the unnatural substrate. Protein evaluation, which could improve the catalytic characteristic involving "substrate specificity" of the substrate-promiscuous enzymes has been developed for the specific decoration of natural and unnatural substrates. One example is the engineering of the substrate-promiscuous UDP-glucose sterol glucosyltransferase UGT51 from *S. cerevisiae*. UGT51 can glycosylate a series of structural analogues, including pregnenolone, cholesterol, ergosterol, sitosterol, diosgenin, estradiol, PPD and PPT. Because of the wide substrate spectrum, UGT51 has been applied for the biosynthesis of ginsenosides Rh2 with the co-expression of PgDS, CYP716A47 and AtCPR1 in *S. cerevisiae*. However, only trace amount of Rh2 was synthesized. To improve the enzyme activity, a semi-rational design strategy was developed based on the crystal structure of UGT51 (PDB code: 5gl5). The best UGT51 mutant gained an 1800-fold higher catalytic efficiency (kcat/Km) in converting PPD to ginsenoside Rh2 in vitro and the Rh2 production reached up to 300 mg/L in vivo [35]. Using non-native promiscuous enzymes to reconstruct the natural products biosynthetic pathways is an efficient strategy to achieve the heterogeneous synthesis of triterpenoid saponins in yeast, especially for that without integrated illuminated pathways.

4.2 Strategies to improve plant enzyme activity

Plant CYP450s are indispensable enzymes for the C–H bounds oxidation of triterpenoid skeletons. However, heterogeneous expression of plant CYP450s in yeasts hosts usually exists problems as low expression level, poor catalytic efficiency or even incorrect folding structure. Plant CYP450s involved in triterpenoids synthesis are membrane-bound oxidase enzymes, which anchor in the endoplasmic reticulum (ER) of plants cells and requires electrons transferred by CYP450 reductase. Although CYP450 is essential for the hydroxylation of C–H bounds and can further oxidize the alcohol products to aldehyde and acid, it always shows poor activity on such substrates. As a result, it is challenging to establish high-yield triterpenoid saponins in yeast cell factories, and improving the plant CYP450s is essential to improve the situation. Currently, many strategies have been developed to improve the expression level and regulate the pairing efficiency of CPRs to plant CYP450s in microbial hosts.

Codon optimization and application of a strong promoter are the most common strategies to improve enzyme expression level in heterologous hosts and it is also effectively used to improve plant CYP450s expression level in microbial hosts [36]. Chimeric protein has been used to correct the folding of plant CYP450s in microbial

hosts. As plant CYP450s are anchored in ER membrane, the activity can be improved by replacing the native N-terminal sequence with ER-membrane bound proteins of yeast to facilitate correctly folding and anchoring. Protein directed evolution was also applied to enhance the activity of plant enzymes. In consideration of that the enlargement of ER would provide more room for the ER-located CYP450s and CPRs leading to higher protein abundance, a novel ER morphology engineering strategy is developed. Through the deletion of PAH1 gene encoding phosphatidic acid phosphatase, the ER membranes of *S. cerevisiae* was tremendously expanded, which accelerated the expression of CYP450s including CYP716A12, CYP72A67 and CYP72A68, and ultimately leading to increased production of triterpenoid and triterpenoid saponins. Approaches like codon optimization, Chimeric protein, protein directed evolution and ER enlargement have been used to improve CYP450s expression level in heterologous hosts.

The pairing efficiency of CYP450s and CPRs plays an important role for the catalytic activity of CYP450s. Mining novel CPRs is a straightforward way to improve the CYP450 activities as different CPRs has different pairing efficiency with CYP450s. For example, the CPR from *M. truncatula* was proved to be the most efficient one pairing with CYP716A12 among all the tested CPRs from *G. uralensis*, *Lotus japonicus* and *A. thaliana*, and boosted OA biosynthesis in yeast [15]. Moreover, the co-expression of CYP72A63 and/or uni25647 with GuCPR1 from *G. uralensis* showed higher activities than the co-expression with other CPRs from *M. truncatula*, *A. thaliana*, or *L. japonicus*. The using of GuCPR1 resulted in boosted GA production in yeast [14]. Besides the using different CPRs, ratios between CYP450 and CPR also contribute to the CYP450 activities. Fine-tuning the ratios between CYP450 and CPR is an important strategy to improve their pairing efficiency. For example, with the best ratio of 5:9 between CYP716Y1 and CPR1 from *A. thaliana*, the 16-α-hydroxy amyrin production was significantly increased [30]. These results indicate that improving the pairing efficiency by efficient CPRs and proper pairing ratio between CYP450 and CPR can improve the targeted compound production in yeast.

4.3 Strategies to enhance metabolic flux

The plant natural products biosynthetic pathways always include multiple steps. When introduced in yeast, the heterogeneous pathways would intensively interact with the native metabolic network, by means of competing substrates and co-factors as well as metabolites reverse influence. The disturbance will restrict the targeted compound production. Therefore, balancing metabolic flux distribution between heterologous pathways and native metabolic networks plays an important role in promoting the production of targeted compound.

Enhancing the precursors supplement to the targeted pathway is a straightforward strategy to enhance natural products production. As demonstrated, the five-carbon building blocks IPP and DMAPP are naturally synthesized through either eukaryotic MVA or prokaryotic MEP pathway in microorganisms. The combination of MVA and MEP pathways in one host could take advantages of both pathways and lead to more efficient precursor supplement for terpenoids. Through introducing a heterogeneous MVA pathway, 27.0 g/L amorphadiene was achieved in *E. coli*. Besides, the production of other terpenoids such as valerenadiene (62.0 mg/L), isoprene (24.0 g/L), and lycopene (47.0 mg/L) were also successfully increased by the synergy of the MEP and the MVA pathway in *E. coli*. In view of the availability of this strategy, the combination of MVA and MEP pathway have been developed in yeast. In addition to the cooperation of the MEP and the MVA pathways, taking full advantage of precursors synthesized in different organelles was helpful to boost

the terpenoids producing. By improving the utilization of acetyl-CoA through the simultaneously introducing dual MVA pathways located in cytoplasmic and mitochondrial of yeast, 2.5 mg/L isoprene was obtained [37]. Moreover, HMG-CoA reductase catalyzing the conversion of HMG-CoA to mevalonate is a rate-limiting factor of MVA pathway. The HMG-CoA reductase is feedback regulated due to its N-term transmembrane sequence, so the truncated version (N-term truncated) of HMG-CoA reductase (tHMG1) is intensively used to strengthen the precursor supplement. Globe transcriptional regulation by overexpressing UPC2-1, which is a global sterol regulatory element to induce sterol biosynthetic genes expression, is another effective method to improve the MVA flux globally [38].

Decreasing the metabolic flux of competing pathways is efficient to strengthen the flux to targeted pathway. However, in most cases, directly deletion of the enzymes involved in the competing could lead to lethality, for many of the genes are essential to the hosts. Therefore, decrease the metabolic flux to the competing pathways by down-regulation of key enzymes is a proper approach to strengthen the final production. Generally, the cellular protein concentration is regulated by transcription, RNA degradation, translation and protein degradation. The application of weaker promoters is the most commonly used strategy to down-regulate the transcriptional level of key genes. To decrease the sterol synthesis and redirect the metabolic flux to the β-amyrin synthesis pathway in yeast. ERG7 (lanosterol synthase gene) promoter was replaced by a methionine repressible promoter (PMET3). Moreover, in order to improve the α-santalene accumulation in yeast, the native promoter of ERG9 (squalene synthase gene) was replaced by PCRT3, the copper repressible promoter and PHXT1, a low concentration glucose repressible promoter which resulted in decreased metabolic flux to ergosterol synthesis and increased α-santalene production [39]. Recently, dynamic protein degradation was developed to weaken the competing pathways. Depending on the ER-associated protein degradation system, the cytosolic proteins can be degraded when attached by a PEST sequence. As a result, the strategy of using the G1 cyclin PEST sequence as a degradation degron to label the cytosolic term of squalene synthase was developed for the production of trans-nerolidol. Once labeled by degron, the squalene synthase will be degraded dynamically, resulting in enhanced sesquiterpene trans-nerolidol production. By the similar strategy, farnesyl pyrophosphate synthetase was labeled by a designed N-terminal degron on the N-terminus, which increased the titer of monoterpene linalool [40].

4.4 Strategies to reduce toxicity to the hosts

Natural products always exhibit cytotoxicity to the microbial hosts, leading to decreased cell growth and finally impair the production. In order to solve these problems, various strategies were developed including two-stage fermentation, pathway compartmentalization and transporters mediated compound secretion. In order to alleviate the negative influences on cell growth, the fermentation course is divided into two stages. In first fermentation stage, heterogeneous pathway keep silence and cells grow fast with precursor accumulated, while in the second stage, target pathway would be induced to produce the target compounds [41]. In addition to the traditional two-stage fermentation, the organelles including mitochondria, peroxisome and vacuole were also used to compartmentalize the heterogeneous pathways. Because the integrated membrane structure, these organelles are relatively independent from the cytoplasm, which could prevent the toxic precursors and products from distributing in cytoplasm to disrupt cell growth. Furthermore, subcellular compartmentalization of target biosynthetic pathways can concentrate the substrates, intermediates and enzymes in a more narrow space, which can improve the reaction efficiency of enzymes. Through locating the

amorpha-4,11-diene biosynthetic pathways in the mitochondria of yeast, the amorpha-4,11-diene production increased by 63% compared with locating in the cytosol. By this strategy, the precursor FPP was restricted in mitochondria by the membrane structure, which reduced the loss of FPP. Using similar strategy, the valencene biosynthetic pathway was reconstructed into the mitochondria of yeast resulting in eight-fold increase of valencene production. Besides the mitochondria, peroxisome was also used to compartmentalize the heterogeneous pathways, by introducing the lycopene synthesis pathway in peroxisome, lycopene production was improved up to 73.9 mg/L in Pichia pastoris [42]. Therefore, subcellular compartmentalization was a pioneering strategy to reduce products cytotoxicity to the microbial hosts.

Another strategy to reduce the inner cytotoxicity of natural products is to secrete these compounds outside the cell automatically. To achieve this goal, transporters were taken into account, for their significant contribution of transporting the products to the extracellular space. Due to the rarity of transporters that possess the ability to transport the complex natural products, transporter engineering has been developed to improve the situation. For example, through protein engineering, one variant of AcrB from AcrAB-TolC efflux pump can effectively improve the α-pinene efflux out of the *E. coli* cell. However, because of unclear of the transport mechanisms and lacking of crystal structures, the application of transporters in natural products is still limited.

In this chapter, the biosynthetic pathways of natural products and their reconstruction in yeast cell factories were systematically summarized. The strategies developed to increase natural products productivity in yeast were also discussed including protein engineering, metabolic engineering, subcellular localization and fermentation control. With these endeavors, the engineered strains can produce these compounds in different levels. These achievements indicate yeast a promising chassis for the heterogeneous biosynthesis of natural products.

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This book offers a broad understanding of several ways in which yeasts can be applied to the biotechnology industry. The seven chapters are grouped into three sections (apart from the "Introduction" section). The Animal Nutrition section comprises two chapters dealing with the utilization of yeast as a probiotic for animal nutrition. The Food Industry section addresses the utilization of yeast in food products. Finally, the Industrial Bioproducts section deals with the development of new yeast platforms as cell factories for biochemical production.

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