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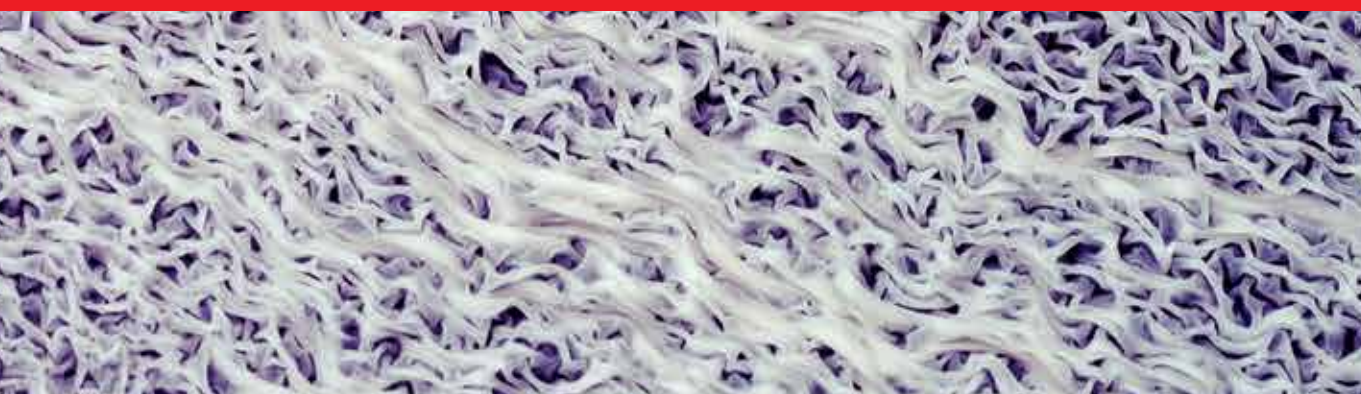
Fermentation Processes

Edited by Angela Faustino Jozala



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Notice

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Meet the editor

Angela Faustino Jozala is Professor at Universidade de Sorocaba. PhD in Fermentation Technology and Master of sciences in Food Technology, both at the Faculty of Pharmaceutical Sciences at USP. Has experience in industrial biotechnology and pharmaceutical microbiology, highlighting the production process and purification (up and dowstream) of biomolecules and biopolymers of different applications in the areas of food, medicine and pharmaceutics.

Contents

Foreword

Fermentation is a theme widely useful for food, feed and biofuel production. Indeed each of these areas, food industry, animal nutrition and energy production, has considerable presence in the global market. Fermentation process also has relevant applications on medical and pharmaceutical areas, such as antibiotics production. The present book, *Fermentation Processes*, reflects that wide value of fermentation in related areas. It holds a total of 14 chapters over diverse areas of fermentation research.

This book includes a chapter about the importance and application of biosensor in fermentation process helping to control the process. Two chapters deal with the application of fermentation for feed, focused on high-quality silage production and factors that affect rumen fermentation. Notably, these two chapters reveal the importance and advantage of fermentation process has to keep the animals healthy. Three chapters mention biofuel production, such as bioethanol and biogas. One of these reports kinetic model design to describe 1-G ethanol fermentation process and this model also has application for second-generation ethanol process. Two of these describe about biogas production by anaerobic digestion process using microorganism for energy. The first one uses waste as substrate. One chapter presents the microbial population optimization for control and improvement of dark hydrogen fermentation. Another one shows the importance of redox potential during fermentation. It has two chapters on the medical and pharmaceutical areas. The first one shows the production of monoclonal antibodies by fermentation. The second one is about the production of lipopeptides by fermentation processes. Three chapters mention enzyme production, such as cellulase, xylanases and laccase, by solid-state and/or liquid fermentation by mono- and/or coculture. One chapter refers to the application of lactic acid bacteria in food.

I sincerely hope that all areas covered by those chapters in this book will be interesting for researchers involved with fermentation process. I also hope new microorganisms could be applied in more unique fermentation processes to produce advanced bioproducts.

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Section 1

Fermentation Process

Chapter 1

Importance of the Fermentation to Produce High-Quality Silage

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Additional information is available at the end of the chapter

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Abstract

The objective of this chapter was to discuss the importance of the fermentation processes for silage making and how it affects the final quality of the silage. The preservation of the forage crops as silage is based on a fermentation process that lows the pH and preserves the nutritive value of the fresh crop. The main principle is the production of lactic acid by the lactic acid bacteria from the metabolism of the water-soluble carbohydrates in the fresh crop. However, different fermentations may occur into the silo environment and it depends on the availability of substrate, the microbial populations, the moisture content, and the buffering capacity of the crop at the ensiling. The fermentation is quite important in the ensiling process because it affects the nutritional quality of the silage and the animal performance. If the fermentation does not occur as recommended and the undesirable fermentations will take place, which will result in a total spoiled feed that is potentially risky for animals and human's health. Well-fermented silage can be used in diets for ruminant animals without any risk for their health and without compromise the productive performance.

Keywords: additives, ammonia nitrogen, mycotoxins, lactic acid, organic acids, pH

1. Introduction—silage production and utilization

Grazing is the most common and economical way to feed cattle; however, it is cannot be done over the entire year, due to the climatic conditions that limit the grasses growth. The availability of pastures in livestock systems depends on the seasons because the factors that affect plant

growth (e.g., temperature, luminosity, and rainfall) are different for each season, which leads to periods with high forage production and periods of its shortage. In the winter, for example, there is no forage production enough to feed the animals [1].

The choice of suitable forage conservation process to provide constantly feed, essentially depends of the climatic conditions at harvest. In hot areas with dry seasons, probably the haymaking is the best choice for forage preservation, because it is a simple technology, where the fresh crop is dehydrated after cutting and the material is stable and preserved after reach an adequate moisture content.

In tropical regions with hot and humid climates, it is difficult to produce high-quality hay, due to high humidity and frequent rainfall at the optimum stage of maturity for crop with better nutritional value. In this context, ensiling is an important method of forage preservation because it is not too dependent on weather as the haymaking. In addition, in many parts of world, the silage is the major source of energy in the total mixed rations of ruminants [2, 3]. Thus, the objective of this chapter was to describe the fermentation processes for silage making and its manipulation and how it affects the final quality of the silage, which includes the effects on animal performance and health.

2. Importance of the fermentation for silage making

According to [2], in short, the silage is made by keeping chopped crop air-tight in a silo, as follows: (1) the crops are harvesting and chopping in a specific length at the better nutritional value and proper moisture content; (2) application of continuously heavy weights to pack at adequate densities; (3) and complete sealing. The preservation of the forage crops as silage depends of anaerobic environment, because it is based on a lactic acid fermentation that decreases the pH and associated with high osmotic pressure that inactivates the microorganisms preserving the nutritive value of the fresh crop (**Figure 1**). Even the presence of some mycotoxins in the fresh crop may be denatured due to the acid pH of silage.

The main principle of silage is anaerobic environment and fermentation of the water-soluble carbohydrates in the fresh crop by the epiphytic lactic acid bacteria (LAB) and production of lactic acid. However, different fermentation pathways may occur into the silo environment, depending on the availability of substrate, the predominant microbial populations, the dry matter (DM) content, and the buffering capacity of the crop at the ensiling (**Figure 2**). In addition, the fermentation must be limited to a certain extent, because it alters the chemical composition of the feed. This process may last for days or months, which may result in silage containing high levels of alcohols, butyric acid, ammonia, amines, and acetic acid that represent the major silage losses. Generally, the epiphytic microbial populations found in growing crops include pseudomonas, actinomycetes, listeria, and mainly the LAB that we expect to dominate the fermentation process to produce high-quality silage (**Table 1**) [4].

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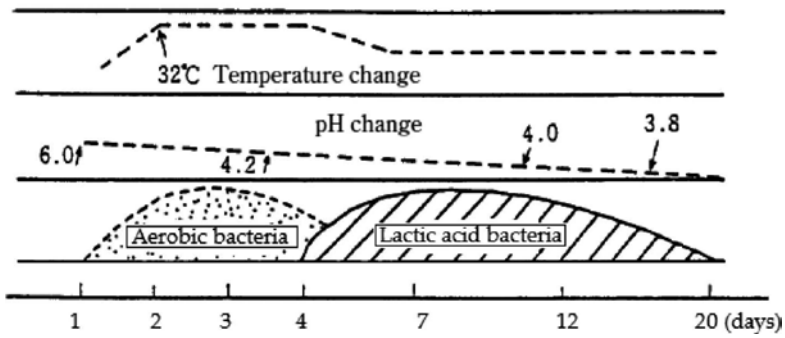

Figure 1. Diagram of silage fermentation process [2].

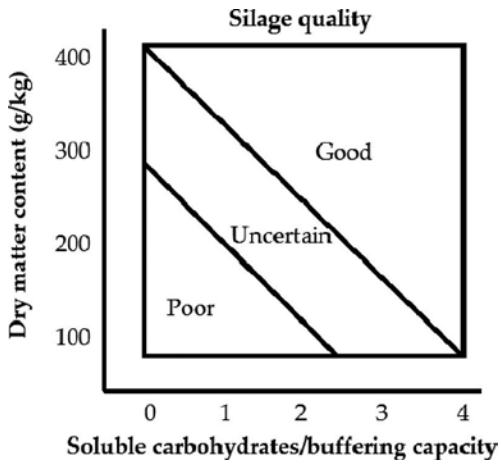

Figure 2. Effects of dry matter content and water-soluble carbohydrates: buffering capacity on silage quality [5].

High-quality silages are resulted of a fast and efficient fermentation preserving the crop nutrients, which depends if the fresh crop has high nutritional value and good characteristics for the ensiling process, as described before. In addition, the fermentation process cannot improve the crop nutritive value, but in some cases occur an increase on digestibility, always with energy losses. Efficient fermentation ensures a more palatable and digestible feed, which improves the animal performance. As noted above, the most important factors related to the crop characteristics to ensiling are adequate dry matter content, sufficient water-soluble carbohydrates for fermentation, and low buffering capacity.

The dry matter content affects directly the microbial activity, specific density, and effluent losses. Crops with dry matter content below 25% at ensiling show high effluent losses and high activity of undesirable microorganisms such as the genus *Clostridium* [6]. In addition, the LAB are more tolerant to low moisture conditions (low water activity) than other undesirable anaerobic microorganisms. However, dry matter content above 45% difficult the process of

forage packing, resulting in high porosity, which may cause losses by the development of aerobic microorganisms [7].

Group	Population, colony-forming units/g of fresh forage
Total aerobic bacteria	>10,000,000
Lactic acid bacteria	10–1,000,000
Enterobacteria	1000–1,000,000
Yeasts	1000–100,000
Molds	1000–10,000
Clostridia	100–1000
Bacilli	100–1000
Acetic acid bacteria	100–1000
Propionic acid bacteria	10–1000

Table 1. Typical microbial populations on crops before ensiling [4].

About the amount of water-soluble carbohydrates, they present a narrow range of optimum values (60–80 g/kg of dry matter), because they are readily available substrates for the LAB and other microorganisms [6]. Furthermore, the excess sugar can stimulate the growth of anaerobic yeasts that are not fully inhibited by the low pH, as occurs in sugarcane silage, which results in high DM losses because the fermentation goes to the ethanol pathway [8].

The silage resistance to the pH lowering is named buffering capacity. This is exerted by compounds present in the crop, as the crude protein, inorganic ions, organic acids, and others. The greater buffering capacity needs more water-soluble carbohydrates content for an effective fermentation by reducing pH and inhibiting undesirable fermentations [9].

The fermentation coefficient (FC) was developed to predict if the crop is suitable to ensiling or not, as follows [10]:

FC = DM (%) + 8 WSC/BC

where FC = fermentation coefficient, DM = dry matter content, WSC = water-soluble carbohydrates, and BC = buffer capacity.

The forage crops with FC < 35 can result in undesirable fermentations and high dry matter losses, requiring additive application to control silage fermentation. If the FC ≥ 35, sufficient fermentable substrates are available. However, in high DM, crops are used microbial inoculants to ensure the presence of osmotolerant LAB to dominate the fermentation process.

3. Silage fermentation processes

The ensiling process, didactically, is divided into four principal phases [4]:

1. Initial aerobic phase since the harvest to the oxygen exhaustion in the silo. This phase is characterized by crop respiration and activity of all obligate and facultative aerobic organisms such as molds, yeasts, and some bacteria until finish up all the oxygen (**Figure 1**). In addition, the plant enzymes such as proteases and carbohydrases remain active. This phase must be short, because the sugars are converted to CO_2 and water with heat release, representing dry matter losses, increased Maillard products, and drops in the silage quality. This phase is also important because of CO_2, hydrogen peroxide, and other compounds that are produced with antimicrobial effect.

2. The main fermentation phase started with a short lag phase followed by rapid growth of facultative and obligate anaerobic microorganisms. The undesirable microorganisms as enterobacteria, clostridia, and yeasts compete with the desirable genera of LAB by the substrates. The main genera of LAB commonly associated with silage are *Lactobacillus, Pediococcus, Leuconostoc, Enterococcus, Lactococcus, Streptococcus*, and *Weissella*. The lactic acid production and the rate of pH decline are responsible for the disappearance of enterobacterial and clostridial secondary fermentations. Obtaining a well-fermented silage depends on the fresh crop characteristics as the adequate dry matter content (300–500 g/kg of fresh matter), water-soluble carbohydrates (60–120 g/kg of dry matter), and low buffering capacity. In addition to speed of harvesting, length of chop and silage distribution and compaction will be responsible for the successful conservation of feed nutrients.

3. Stable phase: In the acidic environment and without oxygen, the activity of microorganisms decreases substantially, and only acid-tolerant enzymes keeps a slow hydrolysis of carbohydrates and protein. The final pH of the ensiled forage depends on the ensiled crop. Theoretically, under ideal conditions, silage can be stored indefinitely if those conditions are maintained, because the losses are minimal. However, in the farm, it is usually stored for a maximum of 1 year or until the next harvest season. In arid and semiarid regions, farmers can store silage for longer periods because the dry period can comprehend two or more years.

4. Feed-out phase is very critical, because the undesirable microorganisms consume the compounds that make the silage stable in the silo (lactic acid) in the presence of oxygen and can produce many compounds decreasing the silage quality. Well-fermented silage with high lactic acid content and residual carbohydrates are more susceptible to aerobic deterioration, because they are the main substrates for the yeasts that initiate the deterioration process. The molds, yeasts, and acetic acid bacteria consume the acids, sugars, and protein for growth releasing heat and can cause considerable changes in the chemical composition in addition with rise in pH other microorganisms that were inhibited can proliferate and lead to a massive spoilage. Because this, care must be taken of removing a uniform layer of silage every day to not provide sufficient time for the undesirable microorganism's proliferation. Normally, silage can stay stable when exposed to air for approximately 30–40 h, but it depends of environmental conditions and silage characteristics.

3.1. Substrates

The most important substrates for the fermentation are the water-soluble carbohydrates and various amino acids and vitamins of the crop. In addition, after chopping the enzymes, plants can hydrolyze starch and hemicelluloses providing more hexoses and pentoses to microbial growth. Hexose monosaccharides, oligosaccharides, and polysaccharides, such as glucose, fructose, sucrose, and fructans, are the main water-soluble carbohydrates readily available for fermentation. Other important carbohydrate is the starch, which is the main storage polysaccharide in some crops, but it is practically not used in the fermentation because it is insoluble in water [9].

3.2. Types of fermentations

In silage fermentation, several pathways occur simultaneously; the fermentation type depends on the environmental conditions, microorganism species, and substrate availability. The LAB show two basic types of hexose fermentation to lactic acid. The most efficient pathway in energy conservation is the obligate homofermentative, which produces almost exclusively lactic acid (>85%). The facultative heterofermentative lactic acid bacteria show besides the homolactic pathway; they present ability to ferment pentoses, because they have both enzymes aldolase and phosphoketolase. The obligate heterofermentative lactic acid bacteria present DM loss from hexose fermentation due the CO_2 production as well as lactic acid, and acetic acid or ethanol [4]. The acetate or ethanol production depends on the fermentation substrate: if the fermentation substrate is a hexose, the end-product is acetic acid, and if it is a pentose, the end-product is ethanol [6]. Although heterolactic pathway causes DM loss, a partial increase in acetic acid concentration improves the aerobic stability of silage, because the acetic acid inhibits the activity of yeasts during the feed-out phase [11]. The end-products of well-fermented silages are presented in **Table 2**.

Item, % of dry matter	Silage and dry matter contents				
	Alfafa, 32.5%	Alfafa, 50.0%	Grass, 30.0%	Corn, 37.5%	High moisture corn, 75.0%
pH	4.3–4.5	4.7–5.0	4.3–4.7	3.7–4.2	4.0–4.5
Lactic acid	7.0–8.0	2.0–4.0	6.0–10.0	4.0–7.0	0.5–2.0
Acetic acid	2.0–3.0	0.5–2.0	1.0–3.0	1.0–3.0	<0.5
Propionic acid	<0.5	<0.1	<0.1	<0.1	<0.1
Butyric acid	<0.5	0	<0.5	0	0
Ethanol	0.5–1.0	0.5	0.5–1.0	1.0–3.0	0.2–2.0
Ammonia N[1]	10.0–15.0	<12	8.0–12.0	5.0–7.0	<10.0

[1]% of total nitrogen.

Table 2. Amounts of common fermentation end products in various silages [12].

When the acidification is not fast and adequate and/or with high moisture content, undesirable secondary fermentations can occur by other microorganisms, which are able to compete for nutrients with the LAB. Enterobacterial fermentation pathway is similar to the heterofermen-

tative LAB and ferments glucose to acetic acid, formic acid, and alcohol. In addition, enterobacteria can decarboxylate and deaminate amino acids and reduce NO_3. Other undesirable is the clostridial fermentations, which derive their energy from organic compounds such as carbohydrates and proteins producing butyric acid, acetic acid, propionic acid, ethanol, biogenic amines, and CO_2. Those processes represent major losses that decrease silage quality and increase the production cost because of the low DM recovery. In addition, other smaller fermentations like the *Propionibacterium* can ferment glucose, fructose, glycerol, lactate, lactose, sucrose, xylose, and starch producing propionic acid, acetic acid, CO_2, and formic acid or isovaleric acid. The facultative anaerobic yeasts can ferment glucose, maltose, and sucrose with the main products such as ethanol, CO_2, and others compounds (alcohols, volatile fatty acids and lactate). The facultative anaerobic bacilli can ferment carbohydrates to organic acids or ethanol, 2,3-butanediol, and glycerol [4].

The secondary fermentations are undesirable because they preserve less energy in its end-products compared to the lactic acid fermentation, which is explained by the production of CO_2. These fermentations can also produce toxic compounds that impair the animal health and performance.

3.3. Efficiency of the fermentation process

The prevalent fermentation pathways in the ensiling process depend on several factors. They are related to the fresh crop and are basically the contents of DM and water-soluble carbohydrates. In addition, there are some characteristics related to the process techniques such as particle size, specific density, and especially the length time until the installation of anaerobic conditions in the silo. According to [13], the homofermentative LAB pathway results in only 0.7% of energy loss and it can be described as follows:

Glucose or fructose + 2 ADP + 2 Pi = 2 lactate + 2 ATP + 2 H_2O.

The heterofermentative LAB pathway from glucose results in 24% of DM loss and 1.7% of energy loss. When they ferment, fructose results in 4.8% of DM loss and 1.0% of energy loss, and it can be described as follows:

Glucose + ADP + Pi = lactate + ethanol + CO_2 + ATP + H_2O, or

Fructose + 2 ADP + 2 Pi = lactate + acetate + 2 mannitol + 2 CO_2 + 2 ATP + H_2O.

In the clostridial fermentations, DM loss is 51.1% and the energy loss is 18.4%, and it can be described as follows:

2 lactate + ADP + Pi = butyrate + 2 CO_2 + 2 H_2 + ATP + H_2O.

In the yeasts' fermentation, the DM loss is 48% and the energy loss is 0.2%, and it can be described as follows:

Glucose + 2 ADP + 2 Pi = 2 ethanol + 2 CO_2 + 2 ATP + 2 H_2O.

4. Manipulating silage fermentation

The knowledge about silage fermentation provides technology improvement to produce high-quality silages. In addition, crops that were once considered inappropriate to ensiling, mainly legumes, are routinely ensiled in many farms nowadays. Theoretically, all forage crops can be conserved as silage, if the ensiling techniques such as the finely chopped, well packed in the silo, and complete sealed through of plastic sheet are done carefully to promote adequate anaerobic condition. However, the crop intrinsic characteristics will direct the fermentation pathway and affect the final silage quality.

4.1. Changing the harvest time

Each crop, depending on environment, has the ideal stage of maturity for silage production considering the yield due to the profitability, dry matter, and fermentable sugar contents for bacteria and maximum nutritional value for livestock (**Table 3**). Practically, all factors involving the fermentation will change with crop maturity stage. In addition, the water-soluble carbohydrates have a diurnal fluctuation cycle, and their concentrations are highest at 18:00 h and lowest at 06:00 h. Generally, advancing crop maturity results in increases in dry matter, carbohydrates, and LAB population as well as total microorganism number. In addition, decreases in buffering capacity and crude protein concentration are observed, and some crops have showed a decrease in digestibility with advancing maturity [9].

Crop	Maturity	Dry matter (%)	Cut length (mm)
Corn	Milk line 1/2–2/3 down the kernel	28–37	9.5–12.7
Alfafa	Mid-bud 1/10 bloom, wilt to	30–40	6.4–9.5
Cereal	Milk or soft dough, wilt to	28–37	6.4–9.5
Grasses	When the first stems head out	28–37	6.4–9.5
Clover	1/4–1/2 bloom, wilt to	28–37	6.4–9.5
Sorghum	Grain medium to hard dough	30–35	9.5–12.7

Table 3. Harvest and dry matter recommendation for main crops conserved as silage [14].

4.2. Wilting

Some crops, like tropical grasses and some legume such as alfalfa and forage soybean (**Table 4**), have a quite low DM content at the same time when the nutritive value is high. Obtaining a good fermentation and eliminating the effluent losses must increase the dry matter content prior to chopping and ensiling. Generally, those crops need be wilted at harvest with a mower-conditioner to increase DM content and to enhance the lactic fermentation. Mowing and conditioning can increase the leaves losses and affect the microbial populations on the crop. The plant juice released can increase the nutrients losses and bacterial population and cause a shift in the microbial species present [15].

	Maturity stage			Wilting	
	R4	**R5**	**R6**	**20 h**	**28 h**
Crop dry matter, g/kg of fresh matter					
After cutting	244	266	282	–	–
Wilted	449[c]	471[b]	529[a]	438	528[*]
Ensiled	454[c]	485[b]	518[a]	444	528[*]
pH	5.19	5.23	5.10	5.11	5.24
Chemical composition, g/kg of dry matter					
Ammonia-N	3.3	2.5	2.4	2.9	2.5
Lactic acid	32.7	29.9	29.3	32.0*	29.3
In vitro rumen degradability					
Fiber	0.319[c]	0.388[b]	0.465[a]	0.399	0.382
Crude protein	0.391[c]	0.503[b]	0.548[a]	0.495[*]	0.466

[a–c,*] $P < 0.05$.

Table 4. Effect of crop vegetative stage and preharvest wilting time on ensiling parameters and *in vitro* rumen degradability of forage soybean silage [16].

The wilting before ensiling is more common in regions with dry weather or with well-defined seasons, because the rainfall during the wilting period may cause significative losses than the ensiling wet crop. During the wilting, the crop remains metabolically active, and the cell respiration and proteolysis cause losses, the most important factor is the time until reaching the desired DM. The fast dehydration decreases plant carbon losses and protein degradation. The respiration loss is unavoidable, and its intensity depends on the oxygen, DM, and water-soluble carbohydrate contents. Depending on environmental conditions, the crop containing high level of crude protein may have high proteolysis during wilting, which decreases the silage quality [15].

4.3. Silage additives

In specific cases, when all ensiling techniques and fermentation process are understood and managed properly, the use of additives is necessary to regulate the fermentation process and to obtain high-quality silages. Silage additives can be used to help fixing some historic problems of the crops (low LAB epiphytic, and low DM and soluble sugars contents), oversized silos, silage storage for prolonged time, or silage moved from silo to another structure [17]. In addition, the additives are used to reduce heating and DM losses improving the silage fermentation quality and profitability. Most commercial additives contain more than one active ingredient in order to enhance efficacy and broad range of applicability [10]. According to [18], the additives, basically, have five functions (**Table 5**). Once again, it is important to emphasize that the use of additives will never correct or fix failures from poor management of the silage-making process.

Functions	Examples
Fermentation stimulators	Homofermentative lactic acid bacteria
	Glucose, sucrose, molasses, cereals, wheat, citrus pulp, and enzymes
Fermentation inhibitors	Formic acid, acetic acid, lactic acid, benzoic acid, acrylic acid, citric acid, and sorbic acid
	Formaldehyde, sodium nitrite, sodium metabisulfite, sodium chloride, antibiotics, and sodium hydroxide
Aerobic deterioration inhibitors	Heterofermentative lactic acid bacteria
	Propionic acid, caproic acid, sorbic acid, and ammonia
Nutrients	Urea, ammonia, biuret, and limestone
Moisture absorbents	Citrus pulp, ground corn, cassava meal, straw, and coffee hulls

Table 5. Silage additives [18].

4.3.1. Fermentation stimulators

The additives that promote the desirable lactic acid fermentation are called fermentation stimulators, by either providing additional fermentable sugars or increasing the LAB population in the ensiled crop.

Additives containing water-soluble carbohydrates will improve the fermentation in crops containing low sugars such as some legumes and tropical grasses. The use of molasses in the ensiling process was a practice widely used in the past to accelerate and increase the lactic acid fermentation. However, it was recommended to be used in relatively high concentrations (40–50 g/kg of fresh matter) and crops containing low DM content showed increase in effluent losses. Due to the high cost and viscosity, which are difficult to apply the molasses, today it is not too used in the farms. Other products or by-products can also be used for the same purpose, but attention should be paid to the availability and cost [19].

Enzyme additives usually are active enzyme combination (cellulases, hemicellulases, and amylases) used to break down the crop fiber and starch to release water-soluble carbohydrates, which could be fermented by LAB. The best results are improvements in silage fermentation and decreases in fiber content. However, the enzymes require certain conditions for maximum activity such as the pH, temperature, surface area, dry matter content, and crop proteases may inhibit enzyme activity. In addition, their positive effects also depend on the LAB initial population, crop characteristics, and application rate. The most suitable role for enzymes may be in combination with microbial inoculants [17, 19].

Inoculants containing homofermentative LAB are used with the purpose of increasing the initial population of this bacteria ensuring efficient fermentation to produce lactic acid. In addition, the use of homofermentative inoculants may accelerate pH reduction because the lactic acid is a stronger acid (p*K*a 3.86) than acetic acid (p*K*a 4.76) [4]; improving the lactic acid:acetic acid ratio consequently reduces dry matter losses. Homofermentative inoculation would also limit degradation and deamination of crop proteins and reduce ammonia produc-

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tion, which increases silage quality [20]. It was observed by [21], when evaluating the effects of homofermentative inoculants in alfalfa silage; they observed that some of the evaluated inoculants, with faster growing and ability to dominate the epiphytic microflora, decreased the pH since the first day of fermentation (**Figure 3**).

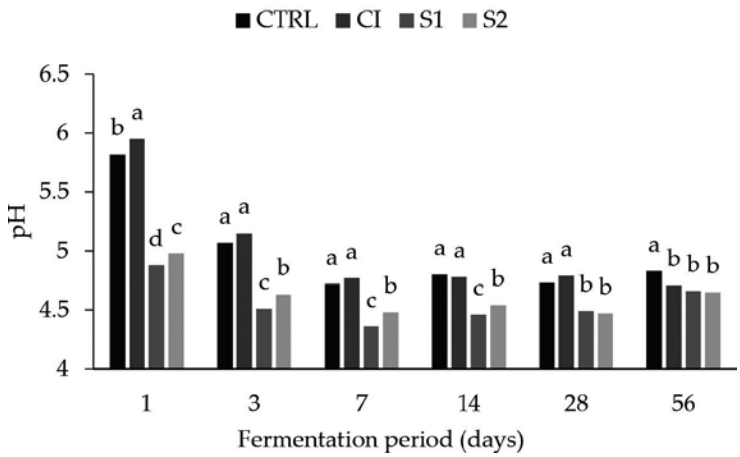

Figure 3. The pH (a), ammonia nitrogen (b) and lactic acid of alfalfa silages as a function of microbial inoculant within each fermentation period. [a–c]Means followed by different letters in bars are different according to the predicted difference ($P < 0.05$). CTRL = control (without inoculant); CI = commercial inoculant, Sil-All® 4 × 4 W.S. (Alltech, Sao Paulo, Brazil); S1 = *Pediococcus acidilactici*, Strain 10.6; S2 = *P. pentosaceus*, Strain 6.16 [21].

Microbial inoculants include one or more of these bacteria: *Lactobacillus plantarum, L. acidophilus, L. salivarius, Pediococcus acidilactici, P. pentacaceus, Enterococcus faecium*, and *Streptococcus bovis*. Some combinations are used in accordance with the LAB capacity and potential of synergistic actions. For example, the use of *Streptococcus*, which exhibit faster growth and simultaneous drop in pH, combined with *Pediococcus*, which are more tolerant to conditions of temperature, pH, and high dry matter content. However, *Lactobacillus plantarum* is the most common species used [17]. According to [22], the inoculant should be added at a rate that is at least 10% of the epiphytic population to fermentation improvement. For commercial inoculants, recommendation ranges from 1×10^5 to 1×10^6 colony-forming units (cfu)/g of fresh forage.

4.3.2. Fermentation inhibitors

These are all chemical additives that affect the undesirable fermentation and microorganism growth. Based on the same principle of food conservation, several substances are used for this purpose. However, the choice of a suitable additive depends on cost-efficiency and historical occurrence of silage with poor-quality fermentation. Generally, they are used in wet crops with low WSC content and/or high buffer capacity. In addition, in crops containing high WSC, the acid-tolerant yeast can proliferate and decrease the silage quality. Salts of acids have become

the most popular fermentation inhibitors, because they are easier and safer to handle [10], and they are effective on controlling yeast growth [23].

4.3.3. Inhibitors of aerobic deterioration

During the feed-out phase, when opening the silo, the presence of oxygen allows the development of molds, yeasts, and aerobic bacteria that consume the silage nutrients. The length of time that silage remains cool and does not spoil after it is exposed to air is called of aerobic stability. There are chemical and biological additives that are used to improve the aerobic stability by inhibit aerobic spoilage, mainly yeasts and acetic acid bacteria, because these microorganisms are responsible to initiate the aerobic deterioration. Generally, the chemical additives are more expensive and difficult to handle than are biological, and successful treatment depends on application rate. However, the variation in the effects when chemical additives are used is lower than the biological additives. Chemical additives with strong antimycotic activity are sorbic and benzoic acid [19, 23]. Besides the use of chemical additives, there is the possibility of using of biological additives based on heterofermentative LAB, such as *Lactobacillus buchneri*, which anaerobically degrade lactic acid to acetic acid and 1,2-propanediol causing a yeast inhibition [10, 23]. Yeast inhibition by organic acids is due to the undissociated form in acid pH. The inhibition effectiveness depends on the dissociation constant (pk) of organic acid; the acids with the highest pk are more effective in inhibiting. The ascending order of pk is formic acid, lactic acid, acetic acid, and propionic acid (3.75, 3.86, 4.76, and 4.87, respectively) [4].

4.3.4. Nutrients

The quality of crop can be improved by supplementation of dietary components that are essential for ruminants through of specific additives at the time of ensiling. In addition, despite of the buffering effect, the urea and ammonia can improve the aerobic stability of silage and increase crude protein content [6]. Grains can be added to increase levels of metabolizable energy in the silage. In other cases, some minerals can be added in order to meet a possible deficiency of the crop to better animal performance [19].

4.3.5. Moisture absorbents

Good results have been obtained in crops with a low DM content (<25%) at the ensiling to prevent excessive effluent losses and clostridial fermentations. Some additives can also improve the nutritive value and final silage quality [6]. Grains can be added to increase moisture absorbent to reduce silage effluent losses [19].

4.4. Using mixed crops

It can be used with several goals always taking advantage of a potential synergistic effect from improvement of soil tillage and fertilization and increased nutritive value, and/or supply the dry matter content and water-soluble carbohydrates to ensure a high-quality silage. Mixing legumes with cereal crops has been to increase grain yields and crude protein of crops while

improving soil fertility but can increase the buffering capacity, which can decrease the fermentation efficiency in drops in the pH [24].

5. How does the fermentation process affect silage quality?

All microorganisms present in the silo, crop epiphytic population, and possible contamination primarily consume energy of water-soluble carbohydrates and other compounds for their growth and proliferation. Theoretically, the homolactic fermentation recovers 99% of the energy from glucose. However, in the silage fermentation process, many pathways occur simultaneously with different extensions, beyond the initial cellular respiration and enzymes activity, which are decisive in the final silage quality. Reducing losses by effluent is also important because it contains cellular content with high nutritional value that can contaminate the environment [6, 25]. High-quality silage is the result of adoption of appropriate techniques, starting with soil preparation and fertilization. In addition, the crop must have high DM yield, adequate nutritional value, and good characteristics for fermentation at the ensiling. Actually, even if high-quality crops are harvested efficiently, significant losses in the quality can occur if the ensiling process is inadequate (**Table 6**).

Source	Management	
	Good	Poor
Respiration	0–4%	10–15%
Fermentation	4–6%	10–15%
Seepage	0–2%	5–15%
Aerobic storage	5–7%	10–20%
Total	9–17%	20–40%

Table 6. Dry matter losses in silage under good or poor management [26].

Forages should be harvested for silage making when they have high nutritional value and the DM content is between 30 and 35%. Therefore, the monitoring of dry matter content at harvest period is essential, because some crops are required to be wilted or ensiled with additives to reach the recommended DM content. The crop must be chopped to about 0.5–1.5 cm length so that the work of packing and taking out is carried out easily. The chopped forage must be well packed in the silo, so less air will be trapped inside the stack, and the peripheral area should have packed more intensely. Filling the silo as quick as possible (within 3 days) limits the forage exposure to air, but each night until it is filled, the stack should be covered. The last step is complete seal with plastic as soon as filling and compaction is completed. In addition, the plastic should be covered, usually with tires or soil to eliminate gases and to prevent damage of the plastic. The packing density at of a good silage should be about 650 kg of fresh silage per cubic meter.

5.1. Chemical composition and nutritive value

Changes are inevitable in chemical composition during the ensiling process; it is due the conversion of soluble carbohydrates into organic acids, as well as degradation of fiber and protein of fresh crop. First, changes in the composition start immediately after cutting, still in an aerobic environment. Early in this phase, enzymes break down fructans, starch, and hemicellulose, releasing simple sugars, and also degrade protein to peptides, amino acids, amides, and ammonia. In addition, during the respiration, soluble carbohydrates are converted to CO_2 and water by releasing heat. If the respiration period is extended, it can increasing losses due the development of molds and yeasts. Also, the heat released by respiration may decrease the digestibility due to the Millard reaction. The heat binds amino acids to the hemicellulose increasing the indigestible fiber and undegradable protein [4].

During LAB fermentation, the soluble carbohydrates are converted to lactic acid, acetic acid, ethanol, CO_2, and water, which represents slight losses of DM and energy. However, if there is a clostridial fermentation, which causes major problems in the silage quality, it converts the soluble carbohydrates and amino acids to organic acids, glycine, biogenic amines, ammonia-nitrogen, H_2, and CO_2. The fermentation length is important in the crop preservation. When the fermentation length is extensive, the losses and changes in nutritional value are greater [4].

Another major problem about the silage chemical composition is at the silo opening. With air exposure, the microorganisms, which were inhibited, can proliferate and consume the silage energy. Heating and spoilage during feed-out is one of the greatest contributors to DM losses. In addition, it can produce some substances, like mycotoxins, that may pose risks to animals fed with this silage [26].

5.2. Animal performance

The feed intake is the key constraint limiting performance of ruminant animals fed diets containing forages. Regulation of feed intake in ruminants involves multiple mechanisms and complicated interactions between animal and feed characteristics. Evaluating factors that affect the silage intake of dairy cows, [27] concluded which silage intake can be predicted based on the silage digestibility, total acids, and DM content. Silage intake increased with increasing silage digestibility which was influenced by stage of maturity at harvest. The same authors showed that the total organic acids produced by silage fermentation process depress the silage intake, but it will depend on the proportion of the silage included in the diet. In addition, a positive association between DM content and silage intake, and DM content independently affects the silage fermentation and animal performance.

Feeding spoiled silage can be a big problem, because the deterioration decreases silage digestibility and intake in cattle. In addition, molds in spoiled silage can produce mycotoxins that cause serious health problems in the animals and farmers [26]. Silage additive is one of the ways to try to ensure efficient fermentation and thus obtain high-quality silage. When studies from North America evaluating the effects of silage additives on animal responses were summarized, [28] showed that although not replace good techniques of the ensiling process, the microbial inoculation can improve the silage quality and animal performance. This activity

in animal performance is not well understood and might inhibit detrimental microorganisms in both silage and rumen to enhance the animal health and performance [29].

5.3. Animal health

The microorganisms in the microbial epiphytic population are usually nonpathogenic. However, the contamination, especially with the soil, may increase the presence of enterobacteria and spores of clostridium and bacillus in the silage. Therefore, in some cases, the silage can be a contamination source of animal products, such as meat, milk, and cheese, besides affecting the animal health [4]. During the silage fermentation process, a succession of microorganisms and denaturation and production of several compounds occur. However, the main problem is the occurrence of undesirable fermentations, which reduces the nutritive value of silage. Furthermore, the presence of some microorganisms or compounds produced may be a risk to the animal health [26].

Enterobacteria present in the crop may have a small positive effect on the hygienic quality of silage because during the first stage of ensiling, they reduce the nitrate (NO_3^-) to intermediates as nitrite and nitric oxide which inhibit clostridial fermentations. However, enterobacteria are undesirable because they have an endotoxin, which can reduce the silage intake and increase cases of mastitis, besides the less effective fermentation than LAB [4].

The anaerobic environment into the silo is essential for high-quality silage and inhibition of molds that produce mycotoxins. Generally, the mycotoxins in silage are related to molds with high tolerance to CO_2 concentrations. Feeding spoiled silage results in reduced intake, increased abortions, hormonal imbalances, and suppressed immune function. In addition, good ensiling conditions reduce the most of the population of potential pathogens such as *Listeria monocytogenes*, *Escherichia coli*, and several *Salmonella* species, because they are strongly inhibited by acid pH (<4.5). Actually, the biggest problems are caused by clostridia and bacilli due the ability to form endospore and their presence later in food production systems, requiring special treatment for their elimination [4].

6. Future trends

With the knowledge of the silage process, some techniques are being developed to improve the efficiency of the preservation and production of high-quality silage. The development of monitors for the DM content of the crop at harvest will help farmers to know the crop quality and ensiling characteristics to choice of additives when needed at accurate rates. In addition, the development of specific additives for each culture that are used in the world with ample effect, since the silage fermentation until the animal performance. Furthermore, today many researches are aimed to developing plastic films more resistant and impermeable to oxygen. Through improvements in the plant, breeding is possible to obtain suitable crops for the most different environmental conditions with high quality and productivity, besides the suitable characteristics for silage production and animal performance.

7. Conclusions

Despite being a well-known technique, it is not easy to produce high-quality silage. Starting with the crop containing high nutritional value that usually is expensive and requires much care. In addition, the ensiling process needs specific machinery, physical structure (silos), and plastic sheets for the coverage. Moreover, the farmers cannot afford the risk of losing the entire crop with a poorly made silage.

Some crops at better nutritional value also have good ensiling characteristics such as corn and sorghum. However, to ensure a high-quality silage is often necessary to use techniques such as crop wilting and application of additives, which can become the process more expensive. A quick and efficient fermentation in reducing the pH is the most desired at ensiling. It depends on the anaerobic environment, water activity, and substrate for LAB fermentation. The homofermentative LAB are the most efficient in preserving the crop characteristics. However, some heterofermentative LAB are also desirable because of its effect on the aerobic stability of silage.

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Chapter 2

Fermentation and Redox Potential

Chen-Guang Liu, Jin-Cheng Qin and Yen-Han Lin

Additional information is available at the end of the chapter

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Abstract

Redox potential, known as oxidation–reduction or oxidoreduction potential (ORP), not only indicates the reduction and oxidation capacity of the environment but also reflects the metabolic activity of microorganisms. Redox potential can be monitored online and controlled in time for more efficient fermentation operation. This chapter reviews the enzymes that modulate intracellular redox potential, the genetically engineered strains that harbor specific redox potential–regulated genes, the approaches that were used to manipulate and control redox potential toward the production of desired metabolites, the role of redox potential in metabolic pathway, and the impact of redox potential on microbial physiology and metabolism. The application of redox potential–controlled ethanol fermentation and the development of three redox potential–controlled fermentation processes are illustrated. In the end, the future perspective of redox potential control is provided.

Keywords: redox potential, ORP, fermentation, bioprocess, ethanol

1. Introduction

The fermentation industry has a long history since human ancestor occasionally produced alcohol, yogurt, and pickled food. Most of these fermentation products are related to the pathways of glycolysis and TCA cycle, which required microaerobic or anaerobic conditions to avoid the desired products being oxidized by oxygen.

Precisely controlling microaerobic or anaerobic states is a challenge when using a general dissolved oxygen electrode because of the detection limit of the probe. Therefore, the measurement of redox potential (aka oxidoreduction potential, ORP) is considered as an ideal alternative approach because of its rapid response and high sensitivity to oxidation reaction.

What's more, redox potential also correlates to metabolic network, involving the genes, proteins, and metabolites. Since maintaining intracellular redox potential balance is a basic demand of cells, either intracellular or intercellular redox potential control could be the effective methods to redistribute metabolic flux toward targeted products. This idea has been applied to make a broad range of fermented products.

In this chapter, the basic principle of redox potential and its intracellular influence on genes, proteins, and metabolites are reviewed. Furthermore, redox potential control by metabolic modification and process engineering on the various metabolite fermentations are illustrated, specifically for ethanol production as an example.

2. Basic theory of redox potential

Chemically, the oxidation–reduction potential (aka ORP or redox potential) is defined as the tendency for a molecule to acquire electrons. It involves two components known as redox pair during the electron transfer process, of which the oxidizing one (Ox) attracts electrons and then becomes the reducing one (Red). This relationship is illustrated below:

$$Ox + ne^- = Red\ 1 \tag{1}$$

Electrons are exchanged during a redox reaction, in which a pair of oxidation reaction and reduction reaction must be involved. As an illustration, when oxidizing iodide by ferric iron to form iodine, the iodine ion loses two electrons to from iodine (known as oxidation), concurrently ferric ion receives the same amount electrons to form ferrous ion (known as reduction). As a result, a complete redox reaction is established.

Oxidation: $2I^- = I_2 + 2e^-$

Reduction: $2Fe^{3+} + 2e^- = 2Fe^{2+}$

Redox reaction: $2Fe^{3+} + 2I^- = 2Fe^{2+} + I_2$

In an aqueous system, the redox potential is related to the capacity of releasing or accepting electrons from all redox reactions. Similar to pH where it indicates the availability of hydrogen ions, the overall redox potential portrays a relative state of gaining or losing electrons. However, the net changes of redox potential are caused by all oxidizing and reducing agents in the aqueous system, not just alkalis and acids that determine pH values.

In 1889, Walter Hermann Nernst (1864–1941; Nobel Prize: 1920) developed an equation to interpret the theory of galvanic cells by taking the changes of Gibbs free energy (ΔG) and the mass ratio into account. The Gibbs free energy is a thermodynamic potential, a reduction of G is a necessary condition for the spontaneity of processes at constant pressure and temperature. The chemical reaction can occur only if the ΔG is negative.

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$$E_h = E^0 + \frac{RT}{nF} \ln \frac{[Ox]}{[Red]} 2 \quad (2)$$

$$\Delta G = -nF\Delta E_h 3 \quad (3)$$

E^0 is the standard redox potential of a system obtained at standard state. Every chemical pair has its own intrinsic redox potential. The greater affinity for electrons, the higher standard redox potential could be. Generally, NAD^+/NADH, $NADP^+$/NADPH, GSSG/2GSH, ubiquinone (ox/red), and oxygen/water are some of the most common chemical pairs in cells, whose E^0 were −320, −315, −240, +100, and +820 mV, respectively.

R is the universal gas constant; T is the absolute temperature; F, Faraday constant (96,485 C/mol), is the number of coulombs per mole of electrons, and n is the number of transferred electrons. The equation implies the concentration of species and temperature plays the key roles for redox potential change.

For instance, the reaction of NADH oxidized by oxygen is the final step of electron transport chain during aerobic respiration in mitochondrion. Usually, the reaction involves two redox pairs, just like oxygen/water (+820 mV) and NAD^+/NADH (320 mV), thus ΔE_h = 820 mV – (−320 mV) = 1140 mV, ΔG = −125 kJ/mol, which indicates that this process occurs spontaneously due to the negative value of ΔG.

Although the Nernst equation has been broadly used in biological systems because of the involvement of electron transfer chain, one fact should be noticed that the redox potential measured by a platinum electrode is not a thermodynamically calculated value. It measures the redox state in an aqueous system as voltages. Although a living biosystem centers on cell growth and metabolism, it is an open system where the intracellular equilibrium state is not always established. Nevertheless, the significance of redox potential on functioning biological systems was predicted nearly one century ago by two prestigious British scientists at the University of Cambridge [1]. Many scientists, since then, have successfully explored various correlations between extracellular redox potential measured by an electrode and intracellular biological properties.

3. Extracellular redox potential

The extracellular redox potential is different from intracellular redox state due to cytomembrane separation and cell redox homeostasis. Environmental factors are critical to indirectly shift the cellular redox potential. Based on Nernst Equation, the redox potential is simply determined by the ratio of oxidative state to reductive state at a fixed temperature, which is always a constant parameter in most biological processes. **Figure 1** illustrates three general approaches to control extracellular redox potential in biological devices.

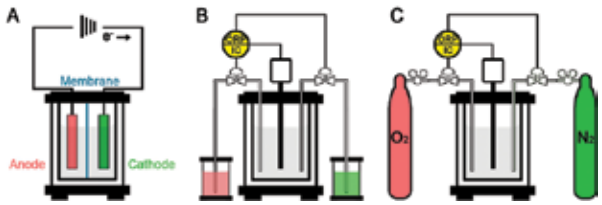

Figure 1. Approaches to control extracellular redox potential. (A) energy input, (B) redox reagents, and (C) gas sparging.

3.1. Control extracellular redox potential by energy input

Bioelectrical reactors (BERs), equipped with anodic and cathodic electrodes, were developed to regulate extracellular redox state in the medium through an external power source. It was used to replace chemical electron donor and acceptor in biosystem. BERs control redox potential at a certain level as easy as tuning a radio. It has been applied to microorganism cultivation and metabolites production [2]. Nevertheless, BERs have been implemented in a laboratory setting or for the production of high-value products in order to compensate for its complicated equipment requirement and extra electrical energy consumption.

3.2. Control extracellular redox potential by redox reagents

Numerous chemicals with higher or lower standard redox potential than common metabolic components are supplemented into fermentation broth in order to alter environmental redox potential. Some commonly used reductants and oxidants to control extracellular redox potential include $FeCl_3$, Na_2S, potassium ferricyanide, dithiothreitol, cysteine, methyl viologen, neutral red, H_2O_2, and even directly NADH and NAD^+ as additives. Unlike BERs requiring the design of a specific reactor, supplementing redox reagents can be employed in any type of bioreactor. However, the disadvantages are obvious: (a) extra chemicals added in media potentially interfere with intended bioprocessing and (b) some chemicals are too costly for industrial fermentation.

Those problems could be solved using substrates with different reducing degree. Girbal and Soucaille [3] used mixed substrates (glucose, glycerol, and pyruvate) to interfere with the intracellular NADH/NAD^+ ratio in *Clostridium acetobutylicum*. Snoep et al. [4] chose some energy source substrates, such as mannitol, glucose, and pyruvate, to govern cellular redox potential in *Enterococcus faecalis*.

3.3. Control extracellular redox potential by gas sparging

Oxygen and nitrogen are commonly used in aerobic and anaerobic fermentation, respectively. Thus, sparging pure or mixed gases into fermentation broth is one of the desired approaches to avoid unwanted reactions caused by redox salts. Generally speaking, oxygen elevates redox potential and hydrogen depresses it, whereas nitrogen and helium as inert gases remove dissolved oxygen or hydrogen from the medium. Furthermore, by adjusting the ratio of mixed

gases, a different redox potential level can be maintained. Carbon monoxide and SO_2 were also utilized to reduce the redox potential sometimes [5]. However, aerating a fermenter during fermentation is considered cost-effective only when air is used. As a mix of nitrogen, hydrogen and helium were applied to regulate redox potential in the above settings, these methods become too luxurious for industrial applications.

3.4. Extracellular redox potential and dissolved oxygen

Controlling the level of dissolved oxygen in a fermenter is essential for microorganisms to propagate under optimum physiological condition, not only because oxygen is involved in maintaining cell membrane integrity and function by synthesizing unsaturated fatty acid and sterol, but also for keeping metabolic flux channeling toward the production of desired products.

A number of bioreactions toward the syntheses of intended metabolites requires maintaining dissolved oxygen at a proper level. For most microaerobic and anaerobic fermentations, conventional oxygen probe has trouble in distinguishing trace level dissolved oxygen from background noise, and its response time is not sufficient for the purpose of regulating dissolved oxygen level. Even for aerobic fermentation, redox potential still offers much more details about gaseous conditions than that collected from dissolved oxygen measurement [6]. The standard redox potential for the O_2/H_2O pair has the highest value among typical metabolites related to microbial metabolism during fermentation. If electrons were transferred to acceptors, oxygen must be the preferable choice even though its concentration is lower than other metabolites. Therefore, redox potential is much more sensitive in monitoring the presence of a trace amount of dissolved oxygen under microaerobic and anaerobic conditions.

4. Intracellular redox potential

Currently, advanced technologies, such as a nanosensor that can embed into individual cells, have been developed to measure intracellular redox potential directly for in-depth understanding on intracellular redox balance and its impact on cell physiology and metabolism. However, the indirect approaches, such as the measurement of NAD(P)H pools, $NAD(P)^+$/NAD(P)H, GSH/GSSG, and the total oxidization power, are still commonly adopted to monitor the distribution of intracellular redox potential.

4.1. Universal redox pairs in a cell

A conjugate pair that constitutes a complete redox reaction is the fundamental of metabolic network in a cell. Many metabolic functions are realized through keeping intracellular redox balance with the main redox pairs, such as glutathione (GSH)/glutathione disulfide (GSSG), thioredoxin ($TrxSS/Trx(SH)_2$), nicotinamide adenine dinucleotide (NAD), and nicotinamide adenine dinucleotide phosphatase (NADP). These redox systems, such as $NADP^+$/NADPH, GSSG/2GSH, and $TrxSS/Trx(SH)_2$, are not isolated systems. Both the Trx and GSH systems use

NADPH as a source of reducing equivalents; thus, they are thermodynamically connected to each other. The role of NAD(P)$^+$/NAD(P)H in redox reaction is illustrated in **Figure 2**.

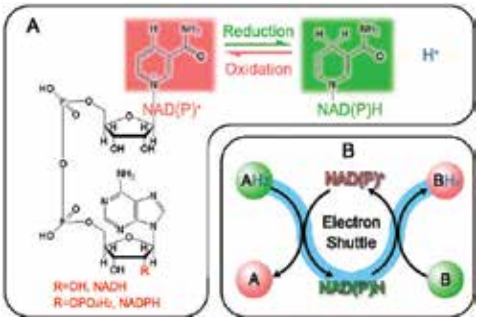

Figure 2. The structure (A) and function (B) of NAD(P)H.

Both glutathione (GSH) and thioredoxin are important reducing agents in all organisms, involved in cell oxidative stress response where they play an antioxidant role. Glutathione is a tripeptide (glutamine, cysteine, and glycine) that prevents damage to cellular components caused by reactive oxygen species such as free radicals and peroxides, lipid peroxides, and heavy metals. Thioredoxin is another class of small redox proteins with thiol system in the cell, which appears in many crucial biological processes, including redox signaling.

The coenzymes are essential electron carriers in cellular redox reactions with the oxidized form NAD(P)$^+$ and the reduced form NAD(P)H. The reduction reaction requires an input of energy and the oxidation reaction is exergonic. During carbohydrate metabolism, NADH plays as a notable reducing substance in catabolism, whereas NADPH, the other reducing component connected to anabolism, favors formation of amino acids, fatty acids, and nucleic acids. There are 129 enzymes that need NAD$^+$ as cofactor in order to serve 931 redox reaction and 108 enzymes that require the involvement of NADP$^+$ as cofactor in order to catalyze 1099 redox reaction (KEGG, 2016-3).

4.2. Redox effect across the membrane

Cytosol is isolated from the extracellular environment by a selectively permeable cytomembrane, which not only prevents the main redox pair escaping from the plasma freely but also conditionally allows the external redox chemicals to enter into the cytoplasm. As shown in **Figure 3**, chemicals with different reduction degrees, such as dithiothreitol (DDT), diamine, hydrogen peroxide, and oxygen, can unrestrictedly cross the membrane bilayer, causing the changes to the intracellular redox potential. However, most of these chemicals are prohibited to across the membrane. In another scenario, membrane proteins, such as oxidoreductase, involved in electron transport will respond and change the extracellular redox potential. For example, ferric reductase assists ferrous iron transport across the cell membrane [7]. Hydrogenase facilitates electron flow through the membrane with the conversion of NADH and NAD$^+$ [8]. A low redox potential level results in the changes of thiol and di-

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sulfide balance on membrane proteins, making the membrane more permeable to protons [9]. A thiol-rich membrane protein transduces external GSH reducing power across the erythrocyte membrane, which can be explained as a thiol/disulfide exchange mechanism [10].

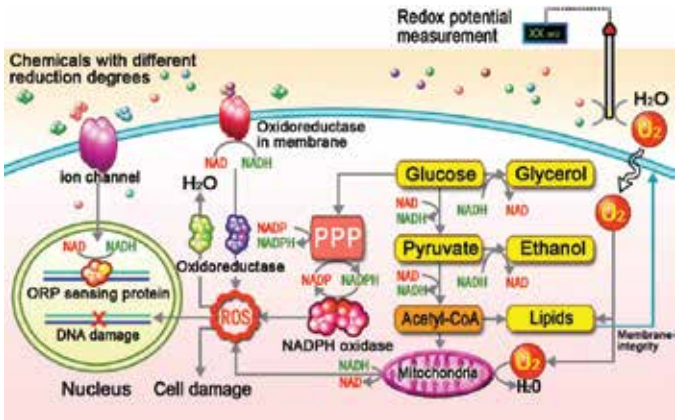

Figure 3. Intracellular redox response to extracellular redox potential and effects of redox potential on cellular metabolism and stress response.

4.3. Effects of redox potential on a cell

The influences of redox potential on enzymes activity have also been reported. Almost all enzymes related to oxidation–reduction reaction are redox potential sensitive, such as alcohol dehydrogenase, D-glyceraldehyde-3-phosphate dehydrogenase, quinone reductase (involved in quinone detoxification), NADH diphosphatase (involved in peroxisomal function), ubiquinone oxidoreductase (catalyzing the oxidation of NADH in the respiratory chain or in cytoplasm), mitochondrial NADH kinase (response to oxidative stress), and so on. The abovementioned proteins have been investigated in *Saccharomyces cerevisiae* in the past decades. Numerous proteins contain sulfhydryl groups (PSH) due to their cysteine content. In fact, the concentration of PSH groups in cells and tissues is much greater than that of GSH. These groups can be present as thiols (-SH), disulfides (PS-SP), or mixed disulfides; Hsp33 as a possible chaperone and cysteine protease in heat shock protein families is regulated by redox potential, whose conformation changes from reduced state to oxidized state with the exposure of hydrophobic surface [11]. Being a key regulator of glutathione and, in turn, of redox potential, the identification of GSTp as, a JNK regulator, provides an important link between cellular redox potential and the regulation of stress kinase activities [12].

Gene expression is controlled by redox states as well. It has been reported that overexpressing genes related to redox process in *Escherichia coli* resulted in the decrease of NADH/NAD^+ ratio, which improve the cell growth profiles, because sufficient NAD^+ is required to oxidize carbohydrate substrate during cell growth [13]. *GPD2* encodes NAD-dependent glycerol 3-phosphate dehydrogenase, the key enzyme of glycerol synthesis, and is essential for cell survival under osmotic and low redox potential conditions. Unlike its homologous gene *GPD1*

controlled by high osmolality glycerol response pathway, *GPD2* is regulated under anoxic conditions or, more accurately, oxygen-independent reducing environment [14]. *YAP1*, a transcription factor for sensing the high redox state (e.g. H_2O_2), usually exists in the cytoplasm but is transferred into nucleus to activate the transcription of antioxidant genes *SOD1*, *TWF*, *TRX2*, *GLR1*, and *GSH1*, when Yap1p C-terminal region with three conserved cysteine residues is oxidized in response to oxidative stress [15]. A redox sensing protein (RSP) binds transcriptional regulation regions located upstream from *adh*A, *adh*B, and *adh*E as a transcriptional repressor. The structure of RSP was changed from α-helix to β-sheet rich conformation when redox potential declined by adding NADH. Meanwhile, the repression of an alcohol dehydrogenase transcription caused by RSP was reversed [16]. Thioredoxin reduces cysteine moieties in the DNA-binding sites of several transcription factors and is therefore important in gene expression [17].

External redox potential correlates the net balance of intracellular reducing equivalents and the changes in the cellular redox environment can alter signal transduction, DNA and RNA synthesis, protein synthesis, enzyme activation, and even regulation of the cell cycle. Thus, monitoring and controlling environmental redox potential helps to elucidate cellular physiology and intracellular metabolic interaction.

5. Redox potential and metabolic flux

Strategies to control intracellular redox potential can be developed by altering intracellular redox potential pools, consequently resulting in redistribution of metabolic profiles. However, cells have a series of built-in mechanisms to adjust their own intercellular redox balance by cofactor regeneration through the oxidoreductase-harboring genes, including mitochondrial alternative oxidase (AOX), formate dehydrogenase (FDH), cytoplasmic H_2O-forming NADH oxidase (NOX), and mitochondrial NADH kinase (POS5). Therefore, modification of these genes is a promising strategy to "design" a robust strain subjected to redox regulation through extracellular manipulation, although such an alternation may result in unexpected outcomes.

5.1. Alternative oxidase

The alternative oxidase (AOX, EC: 1.10.3.11), also named ubiquinol oxidase, forms a part of the electron transport chain in mitochondria. The function of this oxidase is believed to dissipate excess reducing power. The reaction catalyzed by AOX oxidase (ubiquinol oxidase) is shown in Reaction (4).

$$\text{ubiquinone} + H^+ + \text{NADH} = \text{ubiquinol} + NAD^+ \quad (4)$$

When a cell subjected to increasing glycolytic fluxes under aerobic conditions, a decrease in respiratory capacity is caused by the presence of excess glucose that repressed respiratory pathways. Introducing a heterologous alternative oxidase into *S. cerevisiae*, increased metabolic

flux toward respiration and reduced aerobic ethanol formation [18]. In other investigation, the introduction of AOX pathway improved reactive oxygen species and pyruvate levels simultaneously under stressful conditions, such as suboptimal temperature and hyperosmotic pressure [19].

5.2. Formate dehydrogenase

Formate dehydrogenases (FDH, EC: 1.2.1.2) are a set of enzymes that catalyze the oxidation of formate to carbon dioxide (see Reaction 6), donating electrons to a second substrate, such as NAD^+ or cytochrome. NAD^+-dependent formate dehydrogenases are important in methylotrophic yeast and bacteria and are vital in the catabolism of C1 compounds, such as methanol.

$$\text{formate} + NAD^+ = CO_2 + NADH + H^+ \tag{5}$$

As the FDH gene from *Candida boidinii* was introduced into *Paenibacillus polymyxa*, highly expressed exogenous FDH increased NADH/NAD^+ and the titers of NADH-dependent products such as lactic acid and ethanol, while resulting in significantly decreased acetoin and formic acid [20]. In addition, the increased capacity of a FDH gene in *Bacillus subtilis* efficiently enhanced the production of 2,3-butanediol and decreased the formation of acetoin through increasing the availability of NADH [21]. In another case, an engineered strain for the conversion of D-fructose to allitol was developed by constructing a multienzyme coupling pathway and cofactor recycling system in *E. coli*. FDH gene was used to support the cofactor recycling system for the availability of NADH [22].

5.3. NADH oxidase

NADH oxidase (NOX, EC: 1.6.3.4) is a membrane-associated enzyme that catalyzes the production of superoxide, a reactive free radical, by transferring one electron from NADH to oxygen as the electron acceptor (see Reaction 7). It is considered one of the major sources of producing superoxide anions in humans as well as bacteria, subsequently used in oxygen-dependent killing mechanisms for invading pathogens.

$$2H^+ + 2NADH + O_2 = 2H_2O + 2NAD^+ \tag{6}$$

Glycerol is a main by-product in the 2,3-butanediol metabolic pathways. To minimize glycerol accumulation by an engineered *S. cerevisiae*, the *Lactococcus lactis* NOX gene was inserted and expressed, resulting in substantial decreases in intracellular NADH/NAD^+ ratio. As a result, the carbon flux was redistributed from glycerol to 2,3-butanediol [23]. NADH oxidase was also expressed with L-arabinitol dehydrogenase in *E. coli* to efficiently produce L-xylulose. Thus, the efficiency above 96% for the conversion of L-arabinitol into L-xylulose was achieved under optimized conditions [24].

5.4. NADH kinase

NADH kinase (like POS5, EC: 2.7.1.86) catalyzes the replacement reaction with two substrates ATP and NADH and two products ADP and NADPH (see Reaction 8). It provides a key source of the important cellular antioxidant NADPH.

$$ATP + NADH = ADP + NADPH \quad (7)$$

NADPH is a key cofactor for carotenoid biosynthesis. *Corynebacterium glutamicum* was always used for the production of amino acids, such as L-isoleucine. By implementing NADPH-supplying strategies based on NAD kinase (PpnK), NADH kinase, glucose-6-phosphate dehydrogenase (Zwf), and PpnK coupling with Zwf, the expression of all genes increased both the intracellular NADPH concentration and the L-isoleucine production [25]. Researchers constructed the NADPH regenerators of heterologous NADH kinase to increase the availability of NADPH and resulted in a superior S-adenosylmethionine production in *E. coli* without requiring L-methionine addition [26]. When a *S. cerevisiae* strain-producing carotenoid was constructed by overexpressing glucose-6-phosphate dehydrogenase and NADH kinase individually, the final product β-carotene yield increased by 18.8% and 65.6%, respectively. Thus, NADPH supply improved by overexpression of NADH kinase is more important than glucose-6-phosphate dehydrogenase [27].

6. Application of redox potential to fermentation processes

Controlling redox potential at a desired level alters the intracellular metabolic flow in order to favor the formation of desired product(s). Many researches have been conducted in this regard with a large number of examples for enhanced production of metabolites under redox potential–controlled conditions. Most studied metabolites using redox potential–controlled approaches are hydrogen, pyruvate, 1,3-propanediol, butanol, and 2,3-butanediol, and the following metabolites are reviewed but provided with references: acetoin [28], succinic acid [29], xylitol [30], and so on.

6.1. Hydrogen

Hydrogen, as a clean and high-combustion energy in widespread areas, can be generated by fermentative anaerobes. Hydrogen production from anaerobic fermentation by bacteria demands reducing level because the standard redox potential of H_2/H^+ is low. Zhang et al. [8] showed that the addition of NAD^+ during hydrogen fermentation by *Enterobacter aerogenes* resulted in the increase of overall hydrogen. Nakashimada et al. [31] investigated *E. aerogenes* for its hydrogen production under different intracellular redox state through the utilization of different substrates bearing various reduction degrees. Low redox potential accelerated the NAD(P)H-dependent hydrogenase activity in membrane and favors high H_2 evolution capability. Ren et al. [32] assessed H_2 production during butyric acid fermentation, propionic

acid fermentation, and ethanol fermentation by controlling redox potential and pH simultaneously. Besides, the NAD^+ synthetase encoded by nadE gene was homologously overexpressed in *E. aerogenes* to decrease the NADH/NAD^+ ratio and thus enhanced hydrogen yield [33].

6.2. Pyruvate

Pyruvate, a product of glycolysis, serves as an effective starting material for the synthesis of many drugs and agrochemicals and is presently used in the food industry. By combining adaptive evolution and cofactor engineering, a series of engineered yeasts that can produce pyruvate using glucose as the sole carbon source was obtained. Consequently, the constructed strains were able to produce 75.1 g/L pyruvate, increased by 21% compared with the wild strain. The production yield of this strain reached 0.63 g pyruvate/g glucose [34].

6.3. Propanediol

1,3-propanediol, made from glycerol under anaerobic condition, is a monomer for producing various industrial polymers. Du et al. [35] demonstrated that controlling redox potential at −190 mV was preferable for *Klebsiella pneumoniae* to ferment glycerol into 1,3-propanediol. They further developed a redox potential–based strategy for screening high productivity strain using the correlation between redox potential level and growth rate [36]. Zheng et al. [37] regulated redox potential under low levels (−200 and −400 mV) during 1,3-propanediol fermentation in order to avoid the accumulation of by-product. Wu et al. [38] engineered the pathways of 2,3-butanediol and formic acid in a recombinant *K. pneumonia* to improve 1,3-propanediol production. The intracellular metabolic flux was redistributed pronouncedly by shrinking all nonvolatile by-products and supplying the availability of NADH. Jain et al. [39] established novel metabolic pathways for 1,2-propanediol in *E. coli* by disrupting the major competing pathways for acetate production as well as the ubiquinone biosynthesis pathway that conserved more NADH.

6.4. Butanol

Butanol attracts public attentions due to its favorable physicochemical properties for blending with or for directly substituting for gasoline. Fermentation of butanol by *C. acetobutylicum* is generally a biphasic process consisting of acidogenesis and solventogenesis. It has been reported that an earlier initiation of solvent genesis under redox potential control at −290 mV could increase solvent production by 35% [40]. Li et al. [41] supplemented nicotinic acid, the precursor of NADH and NADPH, into the growth medium, and led to a significant increase of NADH and NADPH levels for a wild-type *Clostridium sporogenes* strain. As a result, the metabolic pattern was shifted toward the production of more reduced metabolites, in which butanol production was then enhanced. Bui et al. [42] constructed the recombinant *K. pneumoniae* by overexpressing the genes *kivD*, *leuABCD*, and *adhE1*, with several NADH regeneration strategies to overcome redox imbalance, including the introduction of NAD^+-dependent enzymes or elimination of the NADH competition pathway (1,3-propanediol synthesis). The NADH/NAD^+ ratio was increased resulting in butanol titer increase [42].

6.5. Butanediol

2,3-butanediol (2,3-BD) is a promising bulk chemical with extensive industry applications. In order to enhance the production of 2,3-BD, various strategies for increasing the NADH availability were developed through regulation of low dissolved oxygen, supplement of reducing substrates and gene modification. An *udhA* encoding transhydrogenase was introduced and more NADH from NADPH was provided to allow the enhancement of production [43]. For the same reason, two NADH regeneration enzymes, glucose dehydrogenase and formate dehydrogenase, were introduced into *E. coli* with 2,3-butanediol dehydrogenase, respectively [44]. In other case, an engineered *S. cerevisiae* harboring NADH oxidase gene (noxE) from *L. lactis* minimized glycerol accumulation, because intracellular NADH/NAD^+ ratio was decreased substantially and carbon flux was redirected to 2,3-BD from glycerol [23].

7. Redox potential process design: a case study of ethanol fermentation

Fuel ethanol, the most successful renewable energy so far, is produced worldwide and applied in transportation as alternative to fossil fuel. However, the high cost associated with bioethanol production urges researchers to innovate new fermentation technologies like redox potential–controlled ethanol fermentation. In this section, the role of redox potential in *S. cerevisiae* pathways, the correlation between yeast growth and redox potential, and the application of redox potential to very high gravity fermentation will be reviewed.

7.1. The role of redox potential in yeast pathway

S. cerevisiae has been considered as a model microorganism, whose genome, proteome, and relevant pathway information are almost unveiled. As illustrated in **Figure 4**, glucose is converted into small molecules through the coupling of redox reactions, in which NADH plays an essential role in key metabolites production such as ethanol, glycerol, and lactate. In this process, glucose is oxidized by NAD^+ to make pyruvate and NADH. The surplus of reducing power is then balanced by the formation of glycerol and ethanol, where NAD^+ is restored. When the growth environment favors the production of acetic acid, the implementation of redox potential control can alter the trend, leading to a more reduced state toward ethanol production.

Compared with other control parameters, such as temperature, pH, and the ingredients of medium, redox potential has less influence on improving fermentation results. Hence, the implementation of redox potential control in ethanol fermentation was not popular until the new concept of "very high gravity (VHG)" was proposed. VHG is generally regarded as the final ethanol concentration is greater than 15% (v/v) or initial glucose concentration is greater than 250 g/L. VHG is a promising technology to reduce energy consumption and labor cost, as well as elevate the efficiency of the fermenter. However, high sugar concentration depresses cell growth and bioconversion. Redox potential control helps cells survive from osmotic pressure and ethanol toxicity by constructing healthier membranes or other potential mechanisms. Yeast grown under VHG condition without redox potential control requires much

longer fermentation times in order to completely utilize substrate [45]; therefore, the improvement of ethanol production by redox potential control would be expected.

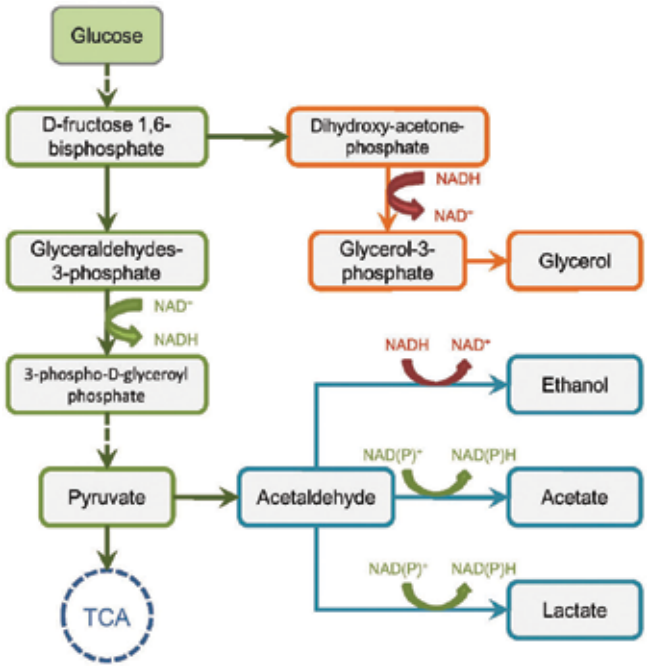

Figure 4. Metabolic pathway of glucose degradation in *Saccharomyces cerevisiae*.

Lin et al. [45] controlled redox potential under −150 mV, −100 mV, and no control conditions and demonstrated that VHG ethanol fermentation under −150 mV resulted in the highest final ethanol concentration and the highest ethanol-to-glucose yield. Compared with the case of 200g glucose/L, the effect of redox potential control becomes significant under VHG conditions [45]. Jeon and Park [46] cultivated *Zymomonas mobilis* and *S. cerevisiae* to produce ethanol in two separate compartments of an electrochemical bioreactor. The results showed that *Z. mobilis* favors the reducing environment, but *S. cerevisiae* produced more ethanol under higher redox potential conditions [46]. Na et al. [47] observed that ethanol production was enhanced in the anode compartment than in the cathode one, although the reduced environment would be better for fermentation process.

7.2. Correlation between cell growth and redox potential

During ethanol fermentation, changes of redox potential are caused by two major substances, electron donor NAD(P)H resulting from dissimilatory processes (e.g. glycolysis) and assimilatory processes (e.g. biomass formation), and electron acceptor oxygen dissolved from sparging and/or agitation. The redox potential profiles are thus correlated to cellular activities and oxygen tension.

A typical redox potential profile resembles a bathtub curve. In the beginning, yeast was inoculated into the autoclaved medium where redox potential is as high as normal oxygen tension. Yeast consumes oxygen as the final electron acceptor during respiration process for rapid propagation, causing a steep fall of redox potential (Stage I, **Figure 5**). When dissolved oxygen is nearly depleted, yeast modulates the respiratory requirement from aerobic to anaerobic stages where a short transition is seen in order to alter relevant gene expression and pathways (between Stage I and II, **Figure 5**). After adjustment, yeast cells accelerate their growth rate in the exponential phase with rapid glucose utilization. Although ethanol production is a redox neutral process in theory, the use of reducing substrate like sugar tends to lower fermentation redox potential. The trend of decline in redox potential continues as fermentation proceeds and could drop as low as to −300 mV if there is no other oxidizing reagent present in the fermentation broth (Stage II–III, **Figure 5**). Due to the substrate depletion and the decline of cell viability attributed to ethanol toxicity, the lowest trough in redox potential level is observed (Stage III, **Figure 5**). Near the end of fermentation, an abrupt increase in redox potential is attributed to constant aeration or well agitation. Technically, an uprising curve appearing reveals that the fermentation is about to finish (Stage IV, **Figure 5**).

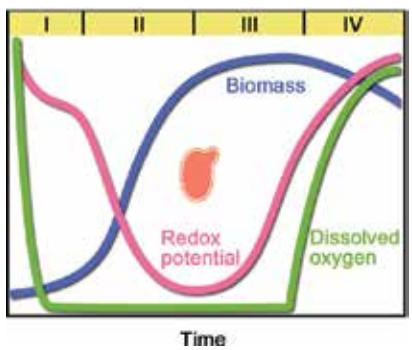

Figure 5. Profiles of redox potential, biomass, and dissolved oxygen.

7.3. Process design using redox potential

The performance of VHG ethanol fermentation can be further improved by (1) searching for the optimal redox potential setting and (2) extending redox potential control period to prolong the exponential growth phase. Three redox potential control schemes are collected [48]. The simple aeration-controlled scheme (ACS) has a short redox potential–controlled period. For glucose-controlled feeding scheme (GCFS), glucose was supplemented along with dissolved oxygen presented in the feed stream. For combined chemostat and aeration-controlled scheme (CCACS), a constant glucose was fed along with air supply determined by redox potential–controlled device. The GCFS extends the redox potential–controlled period by offering enough glucose for yeast propagation and maintaining the low residual glucose. As a result, the ethanol yield is increased noticeably. The operation of GCFS as a fed batch, as such the buildup of ethanol causes yeast cessation, resulting in incomplete fermentation. The CCACS is a set of

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continuous equipment that feeds the fresh medium into a fermenter and discharge spent broth into aging vessels at a constant dilution rate. Sterilized air was used to adjust the fermentation redox potential at a predetermined level. In the chemostat fermenter, both intracellular and extracellular factors should reach their respective steady states. Thus, constant growth rate and yeast viability are sustained under a preset redox potential level, which is helpful to prolong the redox potential–controlled duration and to maximize the benefits from redox potential control. The CCACS achieved the longest controlled period and the highest ethanol yield among all three schemes. However, a chemostat device alone could not result in zero glucose discharge. The incorporation of aging vessel design into fermentation operation thus was developed [49].

8. Future work of redox potential and fermentation

Although many fermentation processes have been well developed with long-term operability, cost saving is an endless effort, particularly for the production of biofuels and bio-based chemicals at bulk quantity. Every penny in cost savings is destined to bring huge economic returns. Since redox reactions and homeostasis are the basis for intracellular metabolism, monitoring and controlling redox potential status inside a cell could potentially re-route metabolic material and energy flow. Numerous works have been done and confirmed that proper redox potential control could alter cellular metabolism, thereby enhancing the conversion of targeted metabolites.

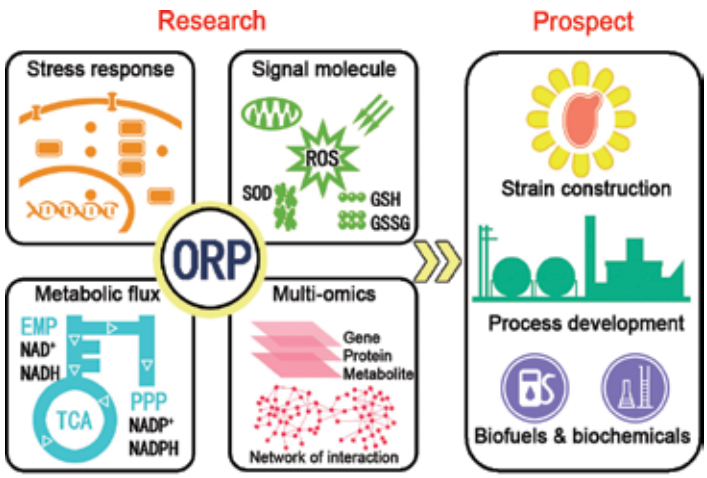

Figure 6. Research and prospect in redox potential–controlled fermentation.

With the availability of technologies that can detect intracellular redox potential levels, an integrated approach, including gene expression, protein biosynthesis, and biomolecular interacting network, should be employed to identify effects of redox potential control on the multiple hierarchy (**Figure 6**). The underlying mechanism of this phenomenon can then be

elucidated at molecular and bioprocess engineering levels. The more details obtained, the better applications of redox potential control can be exploited. Consequently, robust strains and optimized processes can be developed toward high-yield production.

Future perspective of redox potential control is attractive. Fermentation will be carried out using gene-modified strains featuring tailor-made redox potential balance. The strain will be subjected to tight regulation through precise redox potential level. Metabolic flux profiles obtained at different redox potential levels will be quantified to achieve the maximum production of various desired metabolites or used to locate potential bottleneck for strain improvement. Benefits from the development of new redox potential–controlled fermentation technology are thus anticipated.

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Chapter 3

Solid-State Fermentation in a Bag Bioreactor: Effect of Corn Cob Mixed with Phytopathogen Biomass on Spore and Cellulase Production by *Trichoderma asperellum*

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Additional information is available at the end of the chapter

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Abstract

The solid-state fermentation (SSF) is the best option to produce spores of biological control agents (BCA), because the spores have a long shelf life, compared with the obtained in liquid cultures. The spore production under SSF conditions using polyethylene bioreactors (bag-type) is a new topic. Only little information mainly about bioreactors design and adequate conditions to spore production is available. The main aim of this study was to use the corn cob as substrate in SSF and produce spores of the fungi BCA *Trichoderma asperellum* in a polyethylene bioreactor. In the process was added biomass of the phytopathogenic fungi *Colletotrichum gloeosporioides* and *Phytophthora capsici* as inducers of hydrolase enzymes (endoglucanases, exoglucanases and chitinases). It is possible to obtain high levels of spores, cellulases and chitinases using a polyethylene bioreactor under SSF conditions by *T. asperellum* and corn cob as substrate. Under the SSF conditions evaluated, the biomass of *C. gloeosporioides* has an inducer effect just on the spore production. However, *P. capsici* have effect on all response variables evaluated. The spore production was twice when used *P. capsici* as inducer. The most influential factor under SSF was the moisture. Levels of 66 and 50% of this factor increase the yield in all response variables evaluated (sporulation, cellulases and chitinases), *C. gloeosporioides* and *P. capsici*, respectively.

Keywords: spores, cellulase, *Trichoderma asperellum*, solid-state fermentation, bag bioreactor

1. Introduction

The diseases induced by phytopathogens are the leading cause of losses in the most crops worldwide. It is well known that the control of such diseases through the use of chemical pesticides is not effective, they generate resistant strains phytopathogenic, the wastes are toxic and they have carcinogenic effects [1, 2]. In the last years, the alternative proposed is the use of antagonist microorganism of phytopathogens, which results in an adequate biological control, which is highly effective and environmental friendly [2]. For this, the production of high concentrations of the spores of biological control agents (BCA) is necessary, and so currently there are several production processes of different microorganisms. The solid-state fermentation (SSF) is the best to this aim, because the spores have a long shelf life, compared with the obtained in liquid cultures [3, 4]. In other way, the spore production in SSF is relatively easiest, so it can be realized by personal with no experience, and therefore make possible the technology transference to farmers [3]. The spore production under SSF conditions using polyethylene bioreactors (bag-type) is a new topic. Only little information mainly about bioreactors design and adequate conditions to spore production [1, 5, 6] is available. In SSF, most of the time wastes from other manufacturing process are used; therefore, this potential is commonly investigated in developing countries [1]. Wastes of rice, maize meal, corn cob, rice husk, banana husk, wheat bran and tea leaves, among others have been used as substrate to spore production by SSF [3, 5, 6]. There are some compounds that can be added to the substrate of SSF in little proportions to induce some interest metabolite. For example, there are reports of the addition of casein and gluten to produce proteases, waste shrimp silage to chitinase production, among others [7, 8]. The main aim of this study was to use the corn cob as substrate in SSF and produce spores of the fungi BCA *Trichoderma asperellum* in a polyethylene bioreactor. In the process was added biomass of the phytopathogenic fungi *Colletotrichum gloeosporioides* and *Phytophthora capsici* as inducers of hydrolase enzymes (endoglucanases, exoglucanases and chitinases).

2. Materials and methods

2.1. Microorganism and culture conditions

The *T. asperellum* (T2-10) and *P. capsici* were kindly proportioned by the Agricultural Parasitology Department of the UAAAN (Universidad Autónoma Agraria Antonio Narro, Saltillo, México). *C. gloeosporioides* was proportioned by the Food Research Department of the UAdeC (Universidad Autónoma de Coahuila). The fungi were cultivated and conserved in a milk-glycerol 8.5% solution. Potato dextrose agar (PDA) was used to reactivate the fungi. In HACH® tubes, 5 mL of PDA was taken, then they were closed and sterilized at 121°C for 15 min. The tubes in slant were inoculated with the fungal strains and incubated at 30°C for 5 days. The conservation was at ± 4°C.

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2.2. Phytopathogen biomass production

A cornmeal medium (17 g/L) was used to produce phytopathogen biomass. This medium was maintained under shaking for 1 h at 58°C. Then, it was filtrated and sterilized (15 at 115°C). The inoculation of phytopathogens was as follows: *C. gloeosporioides* (1×10^6 spores/mL) and *P. capsici* (10 PDA plugs from a culture of 7days old), and incubated at 28°C for 7 days under shaking (200 rpm).

2.3. Substrates

In this work, we evaluate as substrate corn cob (CC) proportioned by the Mexican Institute of Maize, UAAAN Coahuila, México. The material was dried, ground, fractioned (300–1680 μm) and stored under low moisture conditions for further evaluation. This material was used as a substrate on SSF without pretreatment.

2.4. Solid-state fermentation

Polyethylene bags were used as bioreactor in all experiments. Sporulation and cellulase production were evaluated. Plackett-Burman design (PBD) was used in this experiment to determine the most influential factors on spore and enzyme production by *T. asperellum* under SSF conditions on a bag bioreactor. The factors such as temperature (°C), pH, substrate (g), inoculum (spores/g), moisture (%), phytopathogen biomass (%) and incubation time (days) were evaluated, one maximum (+1) and one minimum (−1) (**Table 1**). Spore counting was done at the end of SSF process using a hemocytometer. The fermented material was placed in a Falcon® tube with 10 mL of distilled water. The enzymatic extract was homogenized in a vortex (1 min) for further determination of enzyme activity.

2.5. Enzyme activity determination

After SSF each sample was analyzed to determine cellulase activity [9], chitinase activity [10] and reducing sugars [11]. The carboxymethylcellulose activity (CMCA) was carried out at 50°C for 30 min. Sample (1 mL) and substrate (1 mL of carboxymethylcellulose 1%) were the mix reaction. Citrate buffer (1 mL at 50 mM, pH 4.8) and substrate (1 mL) were the substrate control. The enzyme control was the mix of sample (1 mL) and citrate buffer (1 mL).

The filter paper activity (FPA) was carried out at 50°C for 1 h. Sample (1 mL) and substrate (filter paper Whatman No.1 (1 cm×5 cm) in 1 mL of citrate buffer at 50 mM, pH 4.8) were the reaction mix. The control substrate was the mix of citrate buffer (2 mL) and filter paper. Sample (1 mL) and citrate buffer (1 mL) were the enzyme control.

Chitinase activity was carried out at 37°C for 1 h. The reaction mix, enzyme and substrate control were done similar to carboxymethylcellulose activity. In this case, substrate (chitin oligosaccharides) and buffer solution (acetate 50 mM, pH 4.0) were replaced.

Sugar concentration was determined after each enzyme reaction. An enzyme activity (U) was defined as the amount of enzyme that catalyze the release of 1 μmol of glucose per minute.

Run	A	B	C	D	E	F	G
1	−1	−1	−1	1	1	1	−1
2	1	−1	−1	−1	−1	1	1
3	−1	1	−1	−1	1	−1	1
4	1	1	−1	1	−1	−1	−1
5	−1	−1	1	1	−1	−1	1
6	1	−1	1	−1	1	−1	−1
7	−1	1	1	−1	−1	1	−1
8	1	1	1	1	1	1	1

Code	Factors	High value	Low value
A	Substrate (g)	30	15
B	pH	8.0	6
C	Inoculum (spores/g)	1×10^{7}	1×10^{5}
D	Temperature (°C)	30	24
E	Moisture (%)	66	50
F	Inducer (%)	3	1
G	Time (days)	7	5

Table 1. PBD matrix used to determine the influence of different variables (A, B, C, D, E, F and G) on spore and enzyme activity in SSF by *T. asperellum*.

2.6. Design and statistical analysis

A PBD was used to SSF. Spore and enzyme production were the response variables. Data were analyzed by ANOVA using STATISTICA 7.0 software; when needed mean treatments were compared using Tukey's multiple range procedure. A *p*-value of less than 0.05 was regarded as significantly different.

3. Results

3.1. Screening of significant factors using Plackett-Burman design

Studies were performed in eight runs each one to identify the combination of factors which allow us to obtain a significant level of spore, cellulase and chitinase production by *T. asperellum* on corn cob under SSF conditions using phytopathogen biomass (*C. gloeosporioides* and *P. capsici*).

3.2. Solid-state fermentation with C. gloeosporioides biomass

Table 2 summarizes the results obtained in SSF using the biomass of *C. gloeosporioides* blended with corn cob. The sporulation index is favored by the treatments F and G (7.3×10^{8} and 6.2×10^{8} Spores/g CS, respectively), with no significant difference among the values. The conditions of treatment C allow the best production to CMCA, FPA and CA (2.582, 1.549 y 5.118 U/g), respectively).

Treatment	Sporulation index (Spores/g)	Enzyme activities		
		CMCA	FPA	CA
A	4.2E+08 ± 6.25E+07^{cd}	1.847 ± 0.02^{b}	1.183 ± 0.17^{b}	3.671 ± 0.46^{b}
B	4.0E+08 ± 4.30E+07^{d}	0.132 ± 0.02^{d}	0.165 ± 0.06^{cd}	1.795 ± 0.05^{f}
C	4.8E+08 ± 5.10E+07^{cd}	2.582 ± 0.08^{a}	1.549 ± 0.05^{a}	5.118 ± 0.28^{a}
D	2.3E+08 ± 6.65E+07^{e}	0.204 ± 0.10^{d}	0.006 ± 0.00^{d}	2.149 ± 0.06^{ef}
E	2.3E+08 ± 7.35E+07^{e}	0.529 ± 0.10^{d}	0.443 ± 0.05^{c}	2.996 ± 0.08^{cd}
F	7.3E+08 ± 9.55E+07^{a}	1.958 ± 0.17^{b}	1.381 ± 0.23^{ab}	3.692 ± 0.13^{b}
G	6.2E+08 ± 8.15E+07^{ab}	1.243 ± 0.02^{c}	0.390 ± 0.01^{c}	2.538 ± 0.01^{de}
H	5.2E+08 ± 3.50E+06^{bc}	2.474 ± 0.47^{a}	1.193 ± 0.25^{b}	3.418 ± 0.29^{bc}

Numbers in each column followed by a common letter are not significantly different (P<0.05).

Table 2. Enzyme production and sporulation index of *T. asperellum* on a mixture of corn cob and *C. gloeosporioides* biomass under SSF conditions.

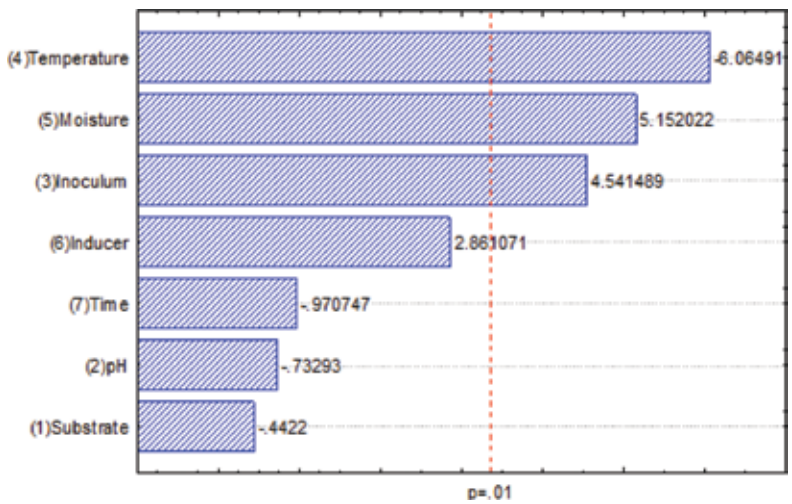

Figure 1. Pareto plot of the standardized effects on the spore production of *T. asperellum* using corn cob in SSF with *C. gloeosporioides* as inducer.

In the first case, the spore production was influenced by the temperature in a negative way. Between the range of the values (24 and 30°C), the study shows that 24°C is the best to produce a better sporulation index and possibly if we reduce the value, the sporulation can be major. The moisture, inoculum and inducer are the other factors that also have influence on spore production, just in a positive way. It means, it is necessary to increase the value of each factor (**Figure 1**). The moisture, pH and inoculum were the factors more determining endoglucanase production (CMCA). These factors had positive values, which mean that high values allow high enzyme activity. A significant effect was observed with the substrate concentration, but this effect was negative, so low amount of substrate is needed to obtain high enzyme yields (**Figure 2**).

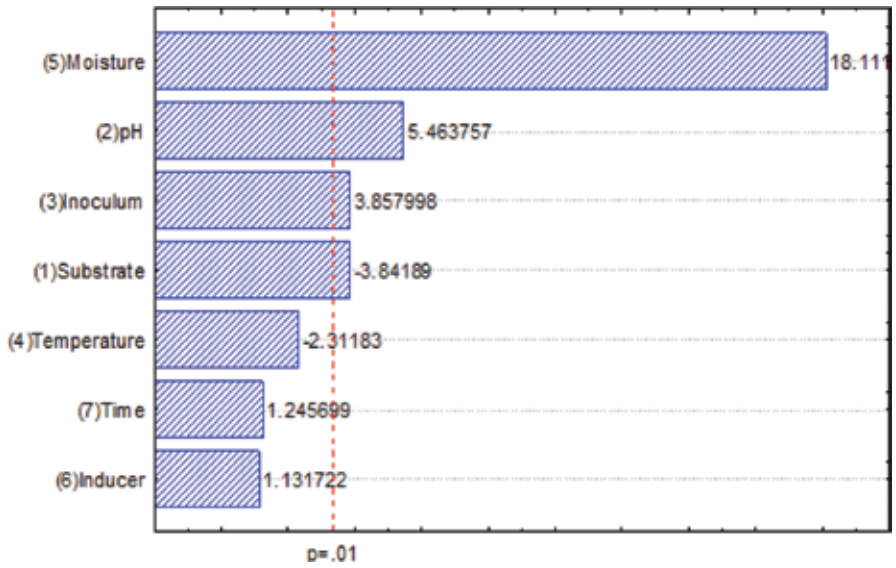

Figure 2. Pareto plot of the standardized effects on CMCA from an extract of *T. asperellum* using corn cob in SSF with *C. gloeosporioides* as inducer.

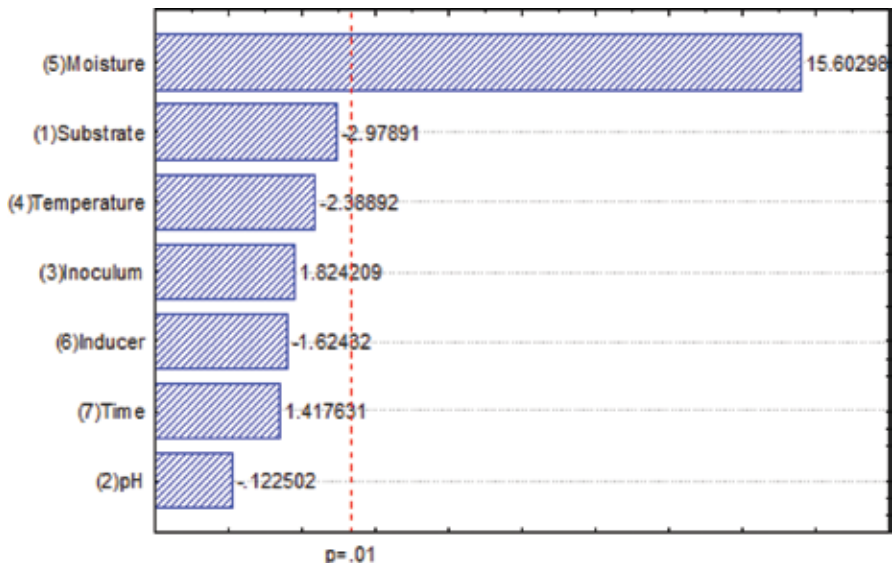

Figure 3. Pareto plot of the standardized effects on FPA from an extract of *T. asperellum* using corn cob in SSF with *C. gloeosporioides* as inducer.

The exoglucanase (FPS) in the same way to CMCA was influenced by the moisture (Positive). Low levels in the substrate and temperature show the best enzymatic yields (**Figure 3**). The moisture was the factor with major influence on the chitinase production. The substrate and

inducer were also significant, but in negative way, it is necessary to use low values to increase the yield. The time and pH were important, so these factors must be in high levels (**Figure 4**).

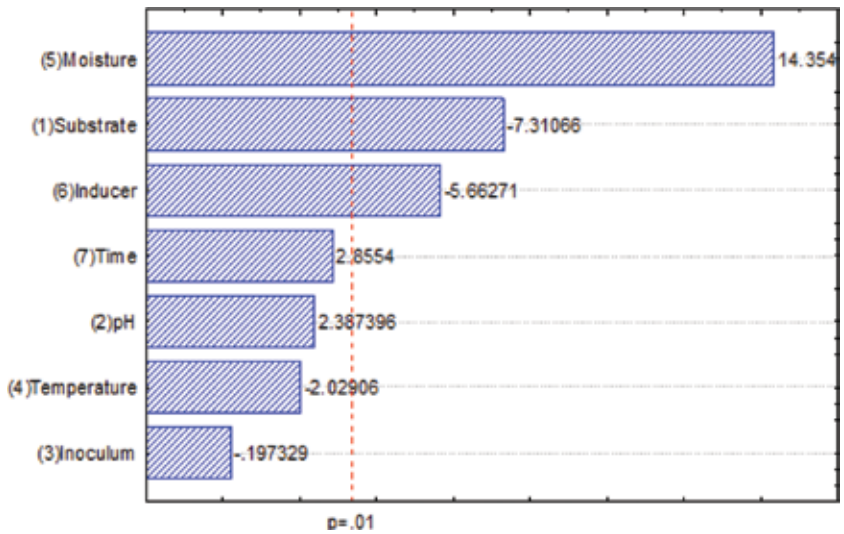

Figure 4. Pareto plot of the standardized effects on chitinase from an extract of *T. asperellum* using corn cob in SSF with *C. gloeosporioides* as inducer.

3.3. Solid-state fermentation with P. capsici as inducer

Now, **Table 3** shows the results obtained in SSF using the biomass of *P. capsici* as inducer. The conditions of treatment G allow the best production to all dependent variables evaluated. The values obtained were sporulation index (1.2×10^9 Spores/g CS), CMCA (7.825 U/g), FPA (2.764 U/g) and CA (3.609 U/g).

Treatment	Sporulation index (Spores/g)	Enzyme activities		
		CMCA	FPA	CA
A	3.1E+08 ± 2.0E+06^{d}	4.238 ± 0.32^{c}	1.660 ± 0.02^{d}	2.848 ± 0.22^{b}
B	1.4E+09 ± 1.5E+08^{a}	4.861 ± 0.13^{bc}	2.466 ± 0.14^{b}	3.216 ± 0.15^{ab}
C	2.6E+07 ± 1.0E+06^{f}	0.062 ± 0.02^{e}	0.121 ± 0.00^{g}	1.527 ± 0.25^{d}
D	8.1E+08 ± 9.0E+06^{b}	5.012 ± 0.40^{b}	2.272 ± 0.01^{c}	2.978 ± 0.10^{b}
E	4.4E+08 ± 6.0E+07^{c}	4.814 ± 0.29^{bc}	2.200 ± 0.02^{c}	3.082 ± 0.04^{b}
F	3.7E+08 ± 3.0E+07^{d}	1.538 ± 0.56^{d}	0.696 ± 0.01^{e}	2.144 ± 0.24^{c}
G	1.2E+09 ± 1.1E+08^{a}	7.825 ± 0.21^{a}	2.764 ± 0.01^{a}	3.609 ± 0.18^{a}
H	1.1E+08 ± 3.0E+06^{e}	1.168 ± 0.21^{d}	0.310 ± 0.13^{f}	1.956 ± 0.22^{cd}

Numbers in each column followed by a common letter are not significantly different ($P<0.05$).

Table 3. Enzyme production and sporulation index of *T. asperellum* grown on a mixture of corn cob with *P. capsici biomass under SSF conditions*.

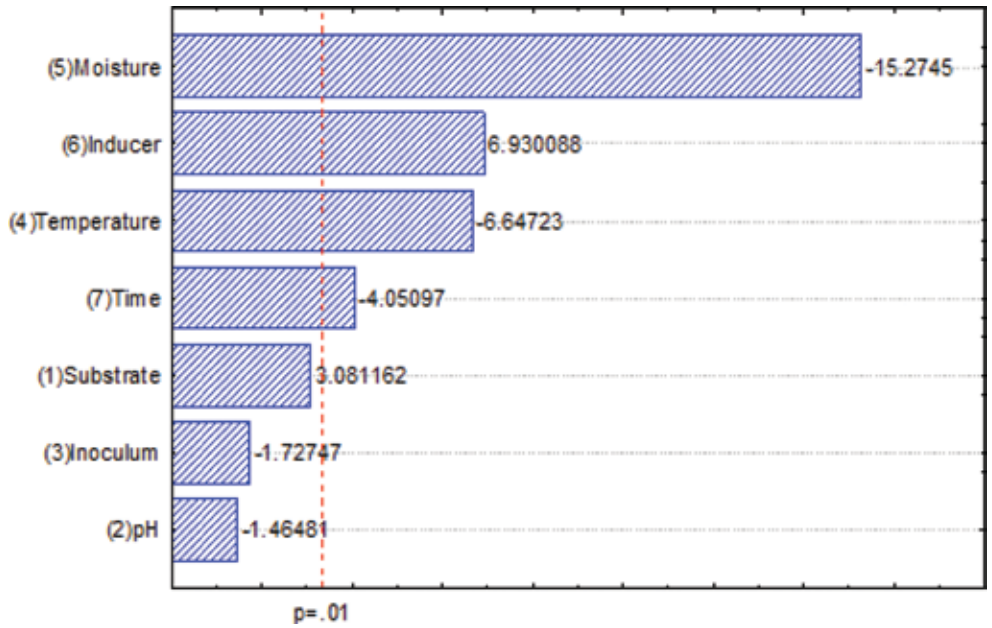

Figure 5. Pareto plot of the standardized effects on the spore production of *T. asperellum* using corn cob in SSF with *P. capsici* as inducer.

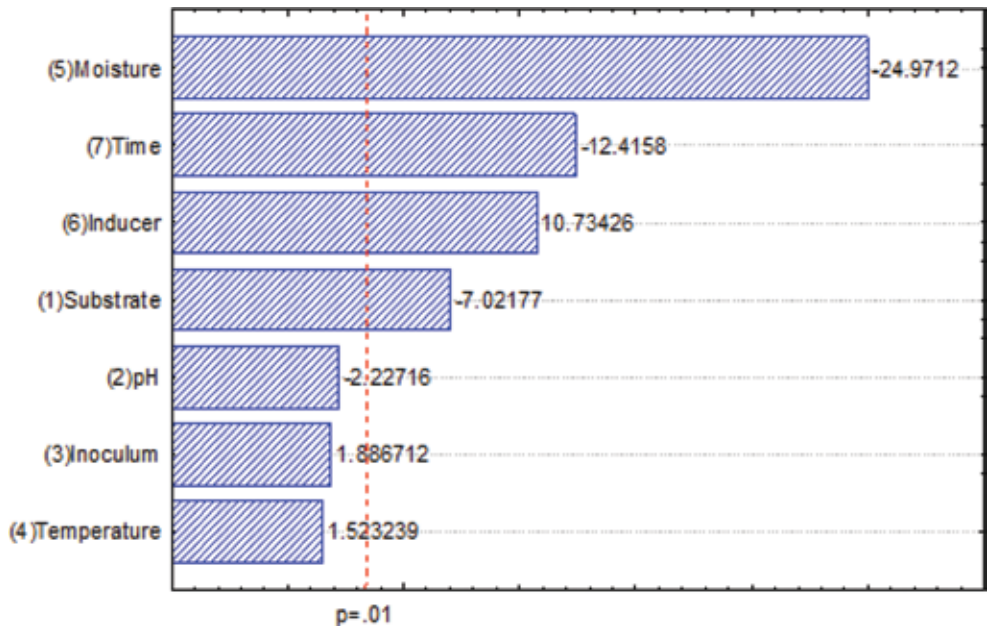

Figure 6. Pareto plot of the standardized effects on CMCA from an extract of *T. asperellum* using corn cob in SSF with *P. capsici* as inducer.

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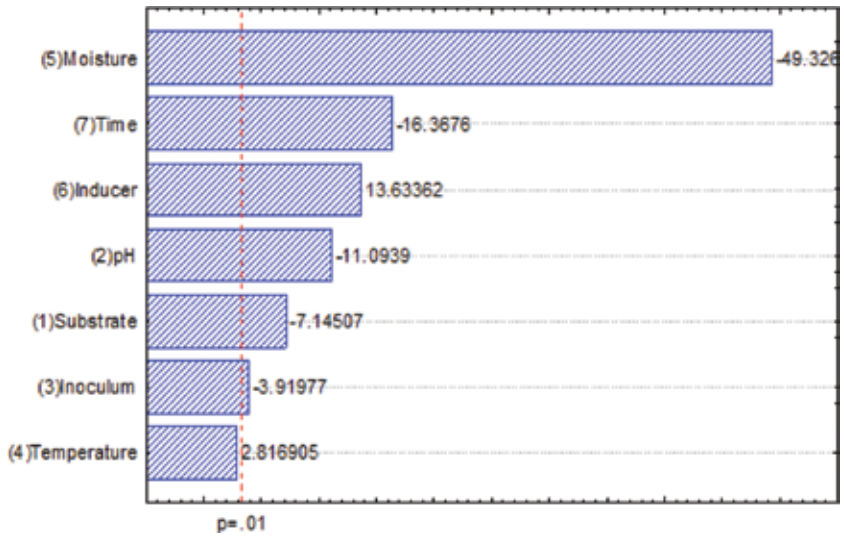

Figure 7. Pareto plot of the standardized effects on FPA from an extract of *T. asperellum* using corn cob in SSF with *P. capsici* as inducer.

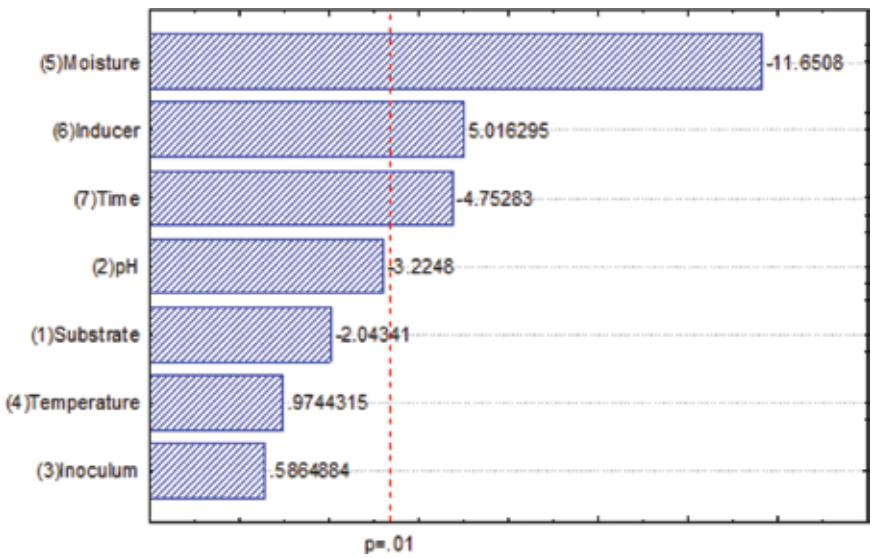

Figure 8. Pareto plot of the standardized effects on chitinase from an extract of *T. asperellum* using corn cob in SSF with *P. capsici* as inducer.

The sporulation of *T. asperellum* was not influenced by the pH and inoculum. But low levels (moisture, temperature and time) and high levels (inducer and substrate) show high spore production (**Figure 5**). In endoglucanase production, four factors are important under the SSF conditions evaluated. Low levels of moisture, time and substrate and high levels of the inducer

show the major levels of enzymatic activity (**Figure 6**). All factors evaluated were significant to the exoglucanase production. Low levels of moisture, time, pH, substrate ad inoculum and high levels of inducer and temperature show the best enzyme yields (**Figure 7**). Finally, low levels of moisture, time and pH and high levels of inducer shows the major chitinolytic activity (**Figure 8**).

4. Discussion

There are several studies that report the production of different enzymes under SSF [12, 13]. Currently, the SSF is a commonly used system because the raw materials such as sugarcane bagasse, wheat bran, among others [14] are cheaper. The control of temperature, pH, moisture, purity of the culture and process time are some factors that difficult the rigorous control of the fermentation process [8].

Sometimes, it is hard to find one combination of the SSF conditions in which we can obtain high yield in all response variables evaluated (sporulation, cellulases and chitinases). In the case of SSF with biomass of *C. gloeosporioides*, the best results were observed in the treatment F to spore production and the treatment C to enzyme activities. However, the treatment F also shows great enzyme yields. So the treatment F allow obtain high values of spores, cellulases and chitinases. Substrate (30 g), pH (6), inoculum (1×10^7 Spores/g), temperature (24°C), moisture (66%), inducer (1%) and time (5 days) were the treatment F conditions.

Now, in the SSF with *P. capsici* biomass, the best results were in the treatment G. It means that the spore, cellulase and chitinase production were high when the conditions are substrate (15 g), pH (8), inoculum (1×10^7 Spores/g), temperature (24°C), moisture (50%), inducer (3%) and 5 days of incubation.

In the start of the study, we think that the addition of certain concentration of phytopathogen biomass could generate an induction effect of some hydrolase enzymes. The production of chitinases when were used *C. gloeosporioides* and cellulases when were used *P. capsici*. This effect is influenced by the phytopathogen composition (chitin and cellulose, respectively).

In the SSF with *C. gloeosporioides*, the induction of enzymes did not happen maybe because the chitinase is a constitutive enzyme result of the natural metabolism of the microorganism. Previously, the same effect in the chitinase production with *Meyerozyma caribbica* in liquid culture using *C. gloeosporioides* as inducer [15] was observed. A similar study was also reported evaluating the β-*N*-acetylhexosaminidase (a chitinase) by *Verticillium lecanii* using shrimp waste silage as inducer and sugarcane bagasse as support [8].

In the case of SSF with *P. capsici*, the inductor was effective and shows an important effect in all enzyme activities evaluated. We did not find reports of the use of biomass to induce some types of cellulase.

In this study, the moisture and temperature are the two important factors. Among the values evaluated, a level of 66% of moisture and 24°C of temperature shows the best yields in spore

and enzymes production on SSF with *C. gloeosporioides* as inducer. In the SSF with *P. capsici* as inducer, the level of moisture was 50%. The two values of moisture used in this study are low which is reported in the literature [8]. They mentioned that the inducer is very important, but also the moisture because they observed that above 75% it can affect the porosity, oxygen diffusion and favor the bacterial contamination. In other hand, low moisture percentage reduces the microbial growth.

This study demonstrated that the biomass addition of any one phytopathogen shows an increment in the spore production by *T. asperellum*. The fungi sporulation starts when the environmental and nutritional conditions become hard to life support. The chemical composition of the inducer possibly causes some stress on *T. asperellum* which accelerate the sporulation process. The experimental stage suggests that high levels of biomass of the inducer increase the sporulation.

Currently, there researches are aimed at the high biomass production of the biological control agents using several systems to produce it. Kancelista *et al*. [16] reported the use of corn cob under SSF by *T. asperellum* obtaining a yield of 3.13×10^9 spores/g. Motta and Santana [17] who working the SSF with empty fruit bunch and a *Trichoderma* spp. The sporulation index was 4.4×10^9 spores/g in a Raimbault columns.

There are few works that report the use of polyethylene bioreactors to produce spores in SSF using some biological control. The use of this kind of bioreactor needs to utilize special plastic bags which allow the gas exchange and microorganism respiration [18]. In some cases, we can use a cotton tap on the bag to allow gas exchange. In this study, the maximal spore production obtained was 7.3×10^8 and 1.4×10^9 spores/g CS to the SSF with *C. gloeosporioides* and *P. capsici*, respectively. Singh *et al*. [6] used a *Trichoderma harzianum* strain and a similar bioreactor, obtaining a production of 8×10^8 y de 4.4×10^6 spores/g CS using tea leaves and sawdust, respectively. Viccini *et al*. [3] did a study of the spore production of *Clonostachys rosea* under SSF conditions using a polyethylene bioreactor and rice grains as substrate. The yield obtained was 1.8×10^8 spores/g CS.

5. Conclusion

It is possible to obtain high levels of spores, cellulases and chitinases using a polyethylene bioreactor under SSF conditions by *T. asperellum* and corn cob as substrate. Under the SSF conditions evaluated, the biomass of *C. gloeosporioides* has an inducer effect just on the spore production. However, *P. capsici* have effect on all response variables evaluated. The spore production was twice when used *P. capsici* as inducer. The most influential factor under SSF was the moisture. Levels of 66 and 50% of this factor increase the yield in all response variables evaluated (sporulation, cellulases and chitinases), *C. gloeosporioides* and *P. capsici*, respectively. When the biomass of *C. gloeosporioides* was used as a inducer, the best SSF conditions with corn cob and *T. asperellum* are as follows: substrate (30 g), pH (6), inoculum (1×10^7 Spores/g), temperature (24°C), moisture (66%), inducer (1%) and time (5 days). In the case of *P. capsici*, the conditions are: substrate (15 g), pH (8), inoculum (1×10^7 Spores/g), temperature (24°C),

moisture (50%), inducer (3%) and time (5 days). Further research on SSF with agroindustrial wastes using polyethylene bioreactors, mainly to the reduction of cost in the process, is necessary. Also, it must be make more analyses to determine the optimal production conditions, as well as, the use of inducers.

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Chapter 4

Characterization of the Solid-State and Liquid Fermentation for the Production of Laccases of *Pleurotus ostreatus*

Gerardo Díaz-Godínez, Maura Téllez-Téllez, Carmen Sánchez and Rubén Díaz

Additional information is available at the end of the chapter

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Abstract

In this chapter, the activity and isoenzymes number of laccases of *Pleurotus ostreatus* grown in solid-state and liquid fermentations are reported. An atypical behavior of this fungus with relation on enzyme production was observed, since the major laccase activity levels were observed in liquid fermentation, whereas the solid-state fermentation has been recognized as better system for enzyme production.

Keywords: laccases, *Pleurotus ostreatus*, solid-state fermentation, submerged fermentation, ligninolytic enzymes

1. Introduction

Laccases are enzymes oxygen oxidoreductases produced by plants, insects, bacteria, and fungi. The most studied laccases are fungal origin, mainly of white rot fungi using different culture systems, mainly in solid-state fermentation (SSF) and liquid fermentation (SmF). In general, it has been suggested that the solid-state fermentation is better for the production of metabolites and enzymes compared with SmF [1]; however, in recent studies has been observed that the basidiomycete *Pleurotus ostreatus* grown in SmF reported higher laccases values compared to those when the fungus grown in solid-state fermentation. *P. ostreatus* strain ATCC 32783 has been studied for the production of intracellular laccases of peripheral and central vegetative mycelium [2], have also been evaluated the solid-state fermentation and SmF systems for laccases

production, observing different levels of activity and number of isoenzymes depending on the culture system [3, 4]. When compared some strains of *P. ostreatus* with ATCC 32783, the latter being better than others [5]. In other study, the effect of pH of the culture medium on the laccases activity of *P. ostreatus* developed in SmF was evaluated, where the activity was seven times higher when the pH of the medium did not change [6]. It has been reported the description of a novel gene encoding a laccase of *P. ostreatus* ATCC 32783 called lacP83, which is preferentially expressed in liquid culture conditions [7]. Recently, the effect of initial pH of development from 3.5 to 8.5 on the laccase activity was evaluated; the pattern of production and the expression profile of five laccase genes of *P. ostreatus* ATCC 32783 grown in SmF, showing that the pH of 8.5 favors biomass production but not enzymatic activity and shows repression of gene expression, however, the pH 4.5 showed higher laccase enzyme activity, reaching up to 78,500 U/L [8].

2. *Pleurotus ostreatus*

2.1. *Pleurotus ostreatus*: phases of growth and composition

The genus *Pleurotus* (Jacq.: Fr.) Kummer (Pleurotaceae, higher Basidiomycetes) comprises a group of edible ligninolytic mushrooms, which have two phases of growth: one is called vegetative or mycelial and is seen as strands of hyphae, which colonize the substrate and the other, the reproductive or fruit body, is represented by the mushroom itself that in basidiomycetes, is called the basidiome. *P. ostreatus* is characterized by a white spore print, with an eccentric stipe and a fan or oyster-shared pileus or cap (5–25 cm). The Latin *Pleurotus* means "beside the ear" and *ostreatus* means "oyster shaped." This fungus is commonly named "oyster mushroom" for the resemblance of its fruiting body a white shell [9]. In this case, the spores are located in a special structure called basidium. In the *P. ostreatus* growth, after spore germination (or inoculation of *in vitro*-grown mycelia), the substrate is invaded by microscopic filaments called hyphae. Hyphae continually grow and branch to form a network of hyphae. Mycelial growth is generally coupled with increased enzyme production and respiration. Hyphae absorb digestive products, penetrating the substrate until its complete hyphal invasion. The vegetative growth is in direct contact with the support (substrate), providing the nutritive materials required for mushroom growth (**Figure 1**)[10].

The growth of all parts of a fungus occurs at the hyphal tips. The mycelial structure grows by synthesizing new wall at the hyphal apex, as they increase in length, additional sites for wall synthesis are formed in the subapical region, originating lateral branches that synthetize wall again confined to the hyphal tip [11]. The formation of a new branch requires the production of a new apex from the existing mature hyphal cell. It has been reported that some enzymes such as proteinases could create weakened zones in the cell wall, which could be pushed out by cytoplasmic flow to initiate branching. So in the mycelial growth, in addition to activation of the cell wall synthesis, enzymes such as chitin synthetase and proteinases, which might weaken the lateral cell walls, are also important. During growth of vegetative hyphae, a large amount of nutrient absorbed from the substrate is stored. Polysaccharide may be stored in the form of glucans in secondary wall layers and/or as glycogen granules in the cytoplasm in the cell. The cells in a hypha are separated by a cross-wall called septum. Septa placement has been

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reported to depend on the position of nuclear division. The septum is formed by chitin deposition on a preformed ring of actin microfilament. Dolipore septa divide hyphae into compartments or cells where movement of cytoplasmic material between them is carefully regulated. It gives rigidity to the hyphae and it can help avoid further injury if damage occurs at the hyphal tip [12]. In a developed colony, the hyphae tip growth or peripheral growth zone forms continuity between hyphae because of the presence of dolipore septa. The growing tips have a constant forward advance, so the mycelium that is left behind seems no longer involved in the growth. At the beginning of the fructification, the characteristic invasive growth of the vegetative mycelium in the substrate is modified. The fruit body initial is formed by increase in mycelial mass, with the formation of additional hyphal branching between the hyphae. The fruit body formation begins with the aggregation of hyphae to form a "knot" that will develop into a primordium and then a mature fruit body with differentiated stem and cap. It has been reported that the most important quantitative change in the cell wall during fruiting is the almost total loss of water-soluble glucan, instead, chitin has been reported important in fruit body development, which is essential for elongation of the stem hyphal walls. The precursor of chitin is N-acetylglucosamine and it is incorporated in the elongation of the hyphae of fruit bodies during expansion. Glycogen is accumulated in the base of fruit bodies at very earlier stages of growth and then disappears from the base as it is accumulated in the cap. In fruit body development, carbohydrates from the culture medium are temporarily store in R-glucan (alkali insoluble glucan) in the wall of mycelia and fruit body primordium hyphae, which is utilized for pileus development in the growing fruit bodies. *Pleurotus* species are cosmopolitan reported mainly as subtropical mushrooms. The optimal temperatures for growth of the mycelium are around 25–28°C and the range of pH is about 6.0–7.0. For fruit body formation, optimal temperature, relative humidity, CO_2, and light are 10–21°C, 85–90%, <1000 ppm, and 1000–1500 lx, respectively [13].

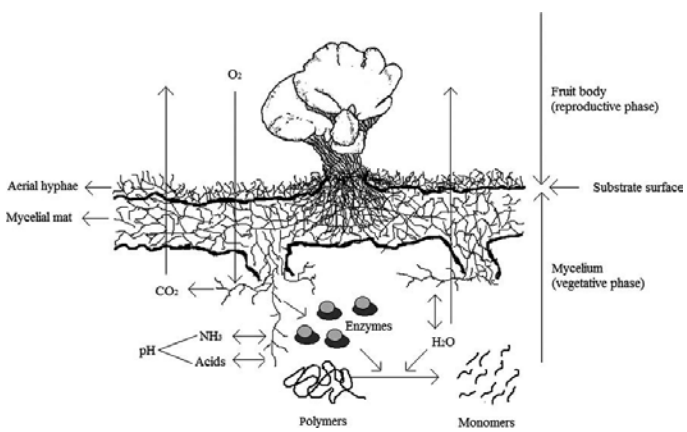

Figure 1. Schematic representation of the growth of *Pleurotus ostreatus* [10].

P. ostreatus can be considered as functional food with nutritional and health benefits in addition to nutritional value [14]. This mushroom contains vitamins as well as an abundance of essential

amino acids. It also has proteins, lipids, ash, glycosides, tocopherols, phenolic compounds, flavonoids, carotenoids, folates, organic acids, etc. [15, 16]. In general, mushrooms contain 90% water and 10% dry matter, and their nutritional value can be compared to those of eggs, milk, and meat [17]. The total energetic value of cultivated species of *P. ostreatus* is 151 J in 100 g of fresh mushrooms [18].

P. ostreatus is the second most cultivated edible mushroom worldwide after *Agaricus bisporus* [13]. Technological improvements have made possible this mushroom cultivation worldwide. It has ability to degrade several lignocellulosic substrates due to its ability to secrete a wide range of hydrolyzing and oxidizing enzymes [19] and can be produced on natural materials from agriculture, woodland, animal husbandry, and manufacturing industries [13].

2.2. Ligninolytic enzymes of *Pleurotus* spp.

From an ecophysiological point of view, white-rot basidiomycetes are microorganisms able to degrade lignin efficiently. However, the degree of lignin degradation with respect to other wood components depends on the environmental conditions as well as the fungal species involved. *Pleurotus* species cause white rot of wood and other lignocellulosic materials, due to their oxidative and extracellular ligninolytic system. The fungal degradation occurs exocellularly, either in association with the outer cell envelope layer or extracellularly, because of the insolubility of lignin, cellulose, and hemicellulose. Three ligninolytic enzyme families have been reported as the enzymatic complex from *Pleurotus* species; manganese peroxidase (EC 1.11.1.13), versatile peroxidase (EC 1.11.1.16) and laccase (EC 1.10.3.2) but lack lignin peroxidase. Recently, was reported that in *Pleurotus ostreatus*, the role that generally played the lignin peroxidase, has been assumed by versatile peroxidase. [20]. Studies on the enzymes secreted by *P. ostreatus* have shown that the concerted action of laccase and aryl-alcohol oxidase produce significant reduction in the molecular mass of soluble lignosulphonates [21].

Additional peroxidases, such as dye decolorizing peroxidases have also been detected in *P. ostreatus* [22, 23]. Lignin biodegradation is an oxidative process, as a consequence *Pleurotus* enzymes can be involved in such processes. The manganese peroxidase gene family (mnps) of *P. ostreatus* is composed of five Mn^{2+}-dependent peroxidases (mnp3, 6, 7, 8, and 9) and four versatile peroxidases (mnp1, 2, 4, and 5), all having related gene and protein structure [24]. Mn^{2+}-dependent peroxidases (MnP) catalyze the H_2O_2-dependent oxidation of lignin and its derivatives [25]. Mn is an obligatory cosubstrate for these enzymes, as it is required to complete the catalytic cycle. In fact, the oxidation of lignin and other phenols by MnP is dependent on free Mn^{2+} ions. This peroxidase does not oxidize nonphenolic lignin structures. It lacks sufficient oxidative potential to cleave the major nonphenolic units of lignin. MnP contains Mn^{2+}-binding catalytic site that is formed by three acidic residues (two Glu and one Asp) and generates Mn^{3+}, which acts as a diffusible oxidizer on phenolic or nonphenolic lignin units through lipid peroxidation reactions [26, 27]. In many fungi, MnP thought to play a crucial role in the primary attack of lignin because it generates a diffusible and strong oxidant (Mn^{3+}). Organic acids such as oxalate and malonate are secreted by white-rot fungi, stimulating the MnP reaction throughout the stabilizing of Mn^{3+} [28, 29]. Versatile peroxidases feature Mn-binding residues as well as conserved Trp involved in the electron transfer that enables

oxidation of nonphenolic compounds. Versatile peroxidases possess two catalytic sites, one for the direct oxidation of low- and high-redox potential compounds, and the other for oxidation of Mn in a preferred manner [23, 30–33]. This dual activity mode of action enables versatile peroxidases to modify a wide range of substrates. It has been suggested a role for versatile peroxidases of *P. ostreatus* in the transformation of azo dyes [23, 30, 34] and carbamazepine [35].

Laccases are blue copper oxidases that catalyze the one-electron oxidation of *ortho*- and *para*-diphenols, aromatic amines by removing an electron and a proton from a hydroxyl group to form a free radical. Their oxidation of the phenolic units in lignin generates phenoxy radicals. Laccases also catalyze the demethoxylation of several lignin model compounds [36–38], such oxidation activity is accompanied by the reduction of molecular oxygen to water. In laccase, histidine and aspartic residues are involved in binding the phenolic compounds, and histidine residue itself is involved in the binding of nonphenolic substrates [39]. Laccase activity in fungal cultures can be increased by the addition of different aromatic compounds to the media, producing different forms of laccase due to the supplementation of aromatic compounds [40, 41]. The ligninolytic system of *P. ostreatus* makes this organism useful in several practical applications of cell-free or purified forms of peroxidases in bioremediation and biotransformation of persistent organic pollutant.

2.3. Laccases

Laccases (benzenediol: oxygen oxidoreductases, EC 1.10.3.2) are enzymes classified as multicopper oxidases. These glycoproteins have the redox ability of copper ions to catalyze the oxidation of a wide range of aromatic substrates where water is obtained as by-product from the reduction of molecular oxygen [42, 43]. Laccases were first time reported in the Japanese lacquer tree (*Rhus vernicifera*) [44]. Laccases has been observed in plants, insects, bacteria, but the most studied are from the fungi classified as of rot-white, where are considered as ligninolytic enzymes because lignin sources are the best substrate for the growth of these fungi. These enzymes occur mainly in basidiomycetes, deuteromycetes, and ascomycetes, but their production in lower fungi has never been observed [45]. There exists a wide diversity of laccases including isoenzymes produced by fungi that have very different physicochemical properties. Numbers of isoenzymes depend on the fungal species [2, 7, 46–48]. In general, laccases show molecular weight between 40 and 100 kDa with 10–50% of their total weight of glycosylation and with isoelectric point (p*I*) around pH 4.0. It has been reported that the glycosylation in fungal laccase plays a role in secretion, copper retention, susceptibility to proteolytic degradation, and thermal stability [49, 50]. The growth conditions of fungi and their physiological states are responsible for the expression of different laccase isoenzymes, which are coded by gene families and differentially regulated [4, 7, 41, 51, 52]. In *P. ostreatus*, 12 possible genes encoding laccases have been reported and only described and characterized 7 isoenzymes laccase: lacc2 [47], lacc4 [53], lacc6 [54], lacc9 [55], lacc10 [56, 57], lacc12 [58] and lacP83 [7]. The characteristics of some purified enzymes from *P. ostreatus* using 2,6-dimethoxyphenol (DMP), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), syringaldazine (SYR) and guaiacol (GUA) as substrate are reported in **Table 1**[**59–65**].

Fungal species	Optimum pH of activity using different substrates				pI	MW (kDa)	Optimum temperature (°C)	Reference
	DMP	ABTS	SYR	GUA				
P. ostreatus			5.8		3.6	67	50	[59]
POXA1b *P. ostreatus*	4.5	3.0	6.0		6.9	62		[54]
POXA1w *P. ostreatus*	3.0–5.0	3.0	6.0		6.7	61	45–65	[60]
POXA2 *P. ostreatus*	6.5	3.0	6.0	6.0	4.0	67	25–35	[61]
POXA3a *P. ostreatus*	5.5	3.6		6.2	4.1	83–85	35	[47]
POXA3b *P. ostreatus*	5.5	3.6		6.2	4.3	83–85	35	[47]
POXC *P. ostreatus*	3.0–5.0	3.0	6.0	6.0	2.9	59	50–60	[56–61]
Lcc2 *P. pulmonarius*		4.0–5.5	6.2–6.5	6.0–8.0		46	50	[62]
P. florida					4.1	77		[63]
P. sajor-caju IV		2.1			3.6	55		[64]
P. eryngii I		4.5			4.1	65	55	[65]
P. eryngii II		4.5			4.2	61	55	[65]

Table 1. Characteristics of laccases from *Pleurotus* spp.

Laccases have a high capacity and nonspecific oxidation which allow their use in many biotechnology applications, such as detoxification of wastewater produced in pulp bleaching process [66] and from industrial plants [67], treatment of elimination of phenolic compounds in beer and processed fruit juices [68], in effluent discoloration and modification of textile fibers [69], as biosensors [70], as drug testing (to distinguish morphine from codeine) [71]. Another important application is in environmental remediation; laccases have shown ability of degrading hazardous compounds that have carcinogenic and/or mutagenic effects, including polycyclic aromatic hydrocarbons (PAH), polychlorinated biphenyls (PCB), 1,1,1-trichloro-2,2-bis (4-chlorophenyl) ethane (DDT), pentachlorophenols (PCP), toluene, benzene, xylene (BTEX), ethylbenzene, and trinitrotoluene (TNT) [72].

2.4. Production of laccases of *Pleurotus* in solid-state and submerged fermentation

SSF has been defined as the bioprocess carried out in the absence or near absence of free water by the use a solid matrix with high water adsorption; the solid matrix could be biodegradable

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or inert, but in both cases must possess enough moisture to support growth and metabolism of the microorganism. Biodegradable solid matrix acts as support and source of nutrients and in the inert matrix, it is only the support and the culture liquid medium must be added [73, 74]. SSF has been a very efficient process for the production of enzymes by filamentous fungi [75, 76], possibly, they reproduce the natural living conditions [77].

SSF is the best culture system to study the morphological and metabolic differences between aerial hyphae and those that penetrate in the solid matrix [78]. It has been reported that SSF is better system than SmF for production of fungal enzymes, because it provides higher volumetric productivities, is less sensible to catabolite repression and yields enzymes with a higher stability at temperature and/or pH [1]. The fermentation could be carried out in less time and the productions of undesirable proteases that degrade enzymes of interest are minimized [78, 79]. Several studies in this field have determined the physiological differences during the growth of microbial cells in the two types of processes. The use of an adequate support for performing SSF is essential, since the success of the fermentation depends on it [80]. Castanera et al. [81] reported that laccase gene transcription is upregulated in an induced SmF but downregulated in the SSF, when were determined the laccases expression profiles in different fungal strains under SmF and SSF using wheat straw extract as inducer.

In a study was found that *P. ostreatus* grown in SSF on wheat bran and vinasse produced twice laccase activity (20 U/ml and three isoforms) than those reported in SmF (10 U/ml and two isoforms) [82]. It has been reported the intracellular activity and isoenzymes number of laccase of 10 strains of *Pleurotus* spp. grown on agar without addition of inducers. Differences in the *in vitro* activities using different substrates (2,6-dimethoxyphenol, *p*-anisidine, or *o*-tolidine) were observed between all the strains; zymogram patterns were similar for strains within same species, independently of any of the three substrate used [2]. Similar results were found in the extracellular extracts obtained of the same strains grown as above was mentioned [7]. In other study was used the same composition of the culture medium to grow *P. ostreatus* (ATCC 32783) in both SmF and SSF using polyurethane foam (PUF) as inert support. Atypical behavior was observed, since in SmF the fungus reported a laccase activity of 13,000 U/L with a biomass production of 5.6 g/L and four laccase isoforms, while SSF had a much lower laccase activity (2430 U/L), with biomass production of 4.5 g/L and three laccase isoforms. These results show that *P. ostreatus* performs much better in SmF than in SSF [4].

P. ostreatus (ATCC 32783) was grown at different initial pH of the culture medium in SSF using PUF. In general, the fungus showed high values of specific growth rate at all pH tested, the higher were at pH 3.5 and 8.5 (0.078 and 0.082 h^{-1}, respectively), whereas at pHs of 4.5, 6.5, and 7.5 were 0.047, 0.034, and 0.047 h^{-1}. Furthermore, the maximum biomass values were low, about 3.7 g/L in all cases. The maximum values of laccase activity were approximately 40,000 U/L observed in fermentation development at pH 4.5 and 6.5. The largest number of isoenzymes was observed in fermentations carried out at pH 7.5 and 8.5 [83].

Pleurotus pulmonarius (Fr) Quélet was cultivated on SSF using corn cob as substrate to produce laccase. The addition of 25 mM $CuSO_4$ increased from 270 to 1420 U/L the laccase production [84]. In other study, *Pleurotus pulmonarius* was grown on SSF using several natural supports, obtaining high laccase activities in wheat bran (2860 ± 250 U/L), pineapple peel (2450 ± 230 U/

L), and orange bagasse (2100 ± 270 U/L) [85]. Recently, the growth of *Pleurotus eryngii* on SSF using different agricultural wastes was reported and its laccase activity was evaluated in mycelium, primordium, and fruiting body. Laccase activities were comparably low in mycelial and primordium. The highest laccase activity was obtained in fruiting body developed on both wheat straw and cotton stalk. The laccase activities of 125.65 and 205.83 U/L of fruiting body were observed on wheat straw +5% of rice bran and on cotton stalk +5 % of rice bran, respectively [86]. Maximum activity of laccase during vegetative phase of growth of *P. ostreatus* can be directly correlated with degradation of lignin in this stage [87].

Different strains of *Lentinula edodes* and *Pleurotus* species were compared for the first time for their ability to produce lignocellulolytic enzymes in SmF and SSF using various plant raw materials. Two strains of *Lentinula edodes* (IBB 123 and IBB 363) appeared to be better producers of laccase than oyster mushrooms. In SSF, *Lentinula edodes* IBB 123 reached laccase activity of 57 U/flask on day 7 of fermentation. *Pleurotus tuber-regium* IBB 624 showed 20 U/flask of laccase activity after 10 days of fermentation, other fungi of this genus produced only 7–16 U/flask of laccase during 7 or 10 days of SSF [88].

P. ostreatus and *Pleurotus sajor-caju* were grown in SSF and their ability to produce laccase and carboxymethylcellulase (CMCase) on different agricultural wastes was studied. *Pleurotus* was inoculated on viticulture wastes, wheat straw, paddy straw, sesame straw, sawdust, and the mixtures of these wastes with wheat bran. Different mycelial growth times were related with different patterns of enzyme activities. During the incubation period, *P. ostreatus* showed its highest values of laccase activity at 10th day and decreased gradually until the first harvest. The highest laccase activity was observed on mixture of wheat straw:bran (2:1) (5.48 U/mg), followed by on paddy straw:bran (2:1) (4.36 U/mg) and on viticulture wastes:bran (2:1) (3.51 U/mg) at 10th day of mycelial growth. The lowest laccase activity was obtained on viticulture wastes (0.30 U/mg) [89]. It has been reported that laccase activity could be regulated, increasing the activity in morphogenesis during the mycelial growth and then the enzyme level decreases rapidly [90]. The laccase production of an indigenous strain of *P. ostreatus* (HP-1) was studied on SSF. Culture parameters, including type and concentration of substrate, moisture content, inoculum size, temperature, pH, surfactant presence, and nitrogen source, were optimized by conventional one factor at a time methodology. Maximum laccase activity of 3952 U/g of dry substrate was obtained with wheat straw as substrate, incubation temperature 28°C, five agar plugs as inoculum, pH 5.0, 60% moisture content, surfactant concentration 0.015 g/L, and combination of L-asparagine and NH_4NO_3 at 10 mM concentration each as nitrogen source. Laccase activity was increased with the use of various aromatic inducers and $CuSO_4$. Highest laccase activity of 14189 U/g of dry substrate was obtained using 0.28 mM $CuSO_4$ under optimized conditions [91]. *P. ostreatus* was grown in SSF conditions for production of laccase, manganese peroxidase, and lignin peroxidase. Highest enzymes levels (laccase 455.11, manganese peroxidase 210.77, and lignin peroxidase 54.50 U/ml) were observed at 7 days in a medium containing 5 g wheat straw (66% w/w moisture), 4 ml inoculum at pH 4.5 and 30°C, using 1% (v/v) glycerol as carbon source, 0.2% (w/w) urea as nitrogen source, 1% (w/v) 2,2-azinobis 3-ethylbenzthiazoline 6 sulphonate as laccase inducer and 1% (w/v) $MnSO_4$ for

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manganese peroxidase, 1% (w/v) $CuSO_4$ as metal ion for laccase, and Mn^+ for manganese peroxidase [92].

Different concentrations of apple pomace were evaluated on laccase production by *P. ostreatus*. During the first four days of fermentation, there was not laccase production. The maximum laccase activity (114.64 U/ml), was observed at 9 days of fermentation in the medium with 2.5% (w/v) apple pomace. This activity was approximately 2.8 times (30.24 U/ml) and 0.9 times (60.49 U/ml) higher than that of *P. ostreatus* with 5% (w/v) and without apple pomace, respectively. These results suggest that *P. ostreatus* might use the nutrient content of apple pomace (rich in carbohydrates, dietary fiber, and minerals) without laccase activity in the initial stages of cultivation (approximately 4 days) [93].

Recently, optimization of the laccases production of *P. ostreatus* grown on sugarcane bagasse in SSF was worked. Water activity, pH, temperature, and concentrations of $CuSO_4$, $(NH_4)_2SO_4$, KH_2PO_4, asparagine, and yeast extract were variables used in the optimization. The concentrations of $CuSO_4$ and $(NH_4)_2SO_4$ had a significant influence on the production of laccase, but the use of yeast extract and the addition of ferulic acid as inducer provided increases of laccase activity of 5.7 times and 2.0 times, respectively. The highest laccase activity of 151.6 U/g was produced at the 5th day of SSF [94].

Cocultivation of *P. ostreatus* MTCC 1804 and mutant *Penicillium oxalicum* SAUE-3.510 was studied for the production of xylanase-laccase mixture under SSF condition. Growth compatibility between both fungi was analyzed by growing them on potato dextrose agar plate, obtaining 58% and 33% higher levels of xylanase and laccase production, respectively. A mixture of sugarcane bagasse and black gram husk (3:1) was the best solid substrate and support for fungal colonization and enzyme production during co cultivation. Maximum activity values of xylanase (8205.31 IU/g) and laccase (375.53 IU/g) during SSF were observed using 4 g of solid support with 80% of moisture content. The coculture system was efficient in the production of xylanases and laccases that may be employed in agroindustrial waste degradation [95].

P. ostreatus (ATCC 32783) was grew under SmF conditions and found the production profile of laccases as well as the isoenzymes patterns through zymograms. Four laccase isoenzymes were produced, one throughout the fermentation time and three only during growth stationary phase. The maximum laccase activity (12200 U/L) was observed in the growth stationary phase. Kinetic parameters of a purified isoenzyme (enzyme produced throughout the fermentation), such as the apparent molecular weight of 43.7 kDa, K_m 90 μM, V_{max} 1.18 ΔAbs/min, and p*I* of 2.3, were obtained [3].

On the other hand, the growth and activity of laccases from of five different strains of *P. ostreatus* developed under SmF conditions with and without copper added to the culture medium was studied. It was observed that the concentration of $CuSO_4 \cdot 5H_2O$ (0.25 g/L) did not affect the growth of the strain ATCC 32783, however, other strains showed lower growth rates and less biomass, the ATCC 201216 strain almost was inhibited. ATCC 32783 strain showed the highest values of laccase activity in the presence of copper reaching up to 37490 U/L, whereas in the culture without copper was obtained 1086 U/L; ATCC 201216 strain in the presence and absence

of copper produced 1400 and 1000 U/L, respectively. These results suggest that not all strains have the same answer to the presence of Cu in the culture medium, and the sensitivity to Cu be could use to select strains with high laccase production for commercial exploitation [5].

The activity and isoenzymes number of laccase from *P. ostreatus* ATCC 32783 grown in SmF conditions using a buffered and nonbuffered media were studied. For both culture media, the initial pH was 3.5. Laccase activity was around 100–500 U/L during the 100–400 h (approximately) of fermentation in both media. Buffered culture medium showed minimal pH changes, while the pH in nonbuffered medium changed drastically, reached a value of 6.5 after 240 h of fermentation. The highest laccase activity (3200 U/L) at 500 h of fermentation was obtained in the buffered medium and in nonbuffered culture medium was only of 450 U/L. One laccase isoenzyme was observed during the entire fermentation process in both media, but in the nonbuffered medium, an additional isoenzyme was produced when the pH reached a value of 6.5. These results suggest that some laccase isoenzymes are regulated by pH signals and also observed that the fungus produces metabolites to regulate the pH of the medium [6].

A gene called lacP83 that encode a laccase isoenzyme of *P. ostreatus* ATCC 32783 grown in SmF was described. Using the PCR inverse strategy, a 2887 bp sequence was obtained from a genomic library of *P. ostreatus*. The coding sequence was of 1527 bp long with 17 exons and the protein encoded had 509 amino acids, shows a putative signal peptide and conserved Cu binding domains. In the promoter region (466 bp upstream of ATG), putative binding transcription factors such as metal response element, xenobiotic response element, a stress response element, and a defense response element were found. The gene and protein sequences of lacP83 had 85–94% and 90–96%, respectively, of similarity with laccases of *Pleurotus* previously reported. This laccase showed differences in its promoter sequence and apparent molecular weight [7].

Recently, the effect of pH on the expression of five genes of laccases (lacc1, lacc4, lacc6, lacc9 and lacc10) and isoenzymes profiles produced by *P. ostreatus* ATCC 32783 developed under SmF conditions was evaluated. The initial pH of the culture media was adjusted at 3.5, 4.5, 6.5, and 8.5. In this research, it was observed that pH is a very important factor for growth, development and production of enzymes, and metabolites of this fungus. The specific growth rate increased with the increase of initial pH of the culture medium, higher biomass values were obtained at pH 6.5 and 8.5; highest laccases activity was obtained at initial pH of culture media of 4.5 and 6.5 and determined at the same values of pH reaching up to 77,500 U/L. The isoenzyme patterns were different depending on the initial pH of the culture medium, to acidic pH was observed up to three isozymes (29, 47, and 65 kDa), at pH near neutrality were observed four isoenzymes (29, 38, 47, and 65 kDa), and alkaline pH three isozymes (29, 47, and 65 kDa) were observed. Since the expression of four genes of laccases (lacc1, lacc4, lacc6, and lacc10) and four isoenzymes was observed, it was suggested that lacc6, lacc10, lacc4, and Lacc1 correspond to isoenzymes of 65, 47, 38, and 29 kDa, respectively. The authors suggest that the pH has a very important role as a transcriptional factor that determines the expression profile and pattern of production of laccase enzymes under conditions SmF [8].

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Section 2

Fermentation Parameters and Modeling

Chapter 5

Factors Affecting Rumen Fermentation Using Batch Culture Technique

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Additional information is available at the end of the chapter

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Abstract

The method of batch culture has been widely applied to evaluate feed value and screen feed additives. The advantages of using this in vitro technique as compared to in vivo methods are many, including low cost, simplicity, requirement of small quantities of feed or additives and the ability to screen large numbers of samples under similar experimental conditions. However, the number of factors associated with the batch culture could alter fermentation outcomes. This chapter discusses the potential impact of series factors on in vitro fermentation and the considerations on improving application of batch culture in ruminant nutrition. The factors that are discussed include inoculum source, gas-recording methods, substrate particle size, substrate delivery method, ratio of rumen inoculum to buffer in mixture of media and addition of soluble carbohydrate in media. Some recent important results obtained using batch culture technique have been highlighted and discussed. Any particular batch system being accepted as the 'standard' procedure seems difficult. However, before any protocol can be adopted, sufficient data need to be developed to reduce the variation and improve the consistence of the measurements.

Keywords: batch culture, feed evaluation, gas production, inoculums, rumen fermentation

1. Introduction

Rumen fermentation plays a major role in feed digestion and microbial production in ruminants. The rate and extent of feed digestion in the rumen, rumen fermentation pattern and amount of microbial protein production ultimately determine the feed value, nutrient provision and animal productivity. Therefore, determining the feed digestibility in the rumen is necessary to predict

animal production and optimum ration formulation. In addition, substantial feed additives are presently used to improve or modify rumen fermentation and their activities need to be determined. The use of animal to measure either feed digestibility or activity of feed additives is a reliable approach but disadvantages are numerous such as time consuming, expensive, require large quantities of feed (or feed additives), and unsuitable for large-scale feed evaluation. As a result, many biological methods which simulate the rumen fermentation process have been developed.

The method of batch culture has been widely applied to screen and compare various feeds and feed additives (e.g., feed enzymes). The advantages of using in vitro techniques as compared to in vivo methods using animals include low cost, its simplicity, small feedstuff requirement and particularly the ability to screen large numbers of samples under similar experimental conditions [1]. However, a number of factors used in the batch culture method including inoculum source, recording system of gas production, method of substrate dispersal in the bottle, sample size and method of substrate preparation could alter fermentation results [2–4]. For example, venting methods for gas measurement is a noticeable issue. In a closed system, gas accumulates and the rise in pressure in headspace may affect the rate of substrate fermentation [5]. Different venting systems to relieve gas pressure have been compared, but results on feed digestion have been inconclusive [2, 6]. Tagliapietra et al. [3] reported that using manual pressure measurements, headspace volume, venting frequency and amount of fermentable substrate need to be carefully balanced to avoid high headspace pressures that could alter fermentation kinetics. Other researchers have reported placing substrates in porous bags within incubation vials [1, 7] or placing it freely into the inoculum [8, 9]. Greater amounts of methane were observed from samples directly dispersed in vials as compared to that enclosed in bags [4]. It is possible that the bags create a microenvironment that is distinct from that of free inoculum and may vary with changes in the pore size of bags [10]. The substrates that are incubated in batch culture need to be processed to obtain an adequate particle size prior to incubation because of lack of mastication and rumen contraction occurs in animal. The use of a finely ground sample reduces the risk of sampling bias, especially for forage samples, but fine particles may exit the bags prior to true digestion. All these factors related to batch culture have not been standardized across the laboratory, and they could significantly impact the fermentation results, thus increase the variability and reduce the reliability of the method. The objective of this chapter is to discuss several key factors that potentially influence the outcomes of the batch culture and to provide useful information to better use the batch culture technique in the evaluation of feeds or feed additives in ruminant nutrition.

2. What is the batch culture?

Batch culture is a technique for large-scale production of microbes or microbial products in which, at a given time, the fermenter is stopped and the culture is worked up. The 'batch culture' fermentation is also known as 'closed culture' system. In this system, at the beginning, the nutrients and other additives are added in required amounts. There is no refill of nutrients once the fermentation process has started and the product is recovered at the end of the process.

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In the beginning, microorganisms grow at a rapid rate due to availability of excess nutrients. As time passes, they increase in number with rapid use of the nutrients and simultaneously produce toxic metabolites. The batch culture that is currently used to evaluate ruminant feeds or feed additives is primarily based on the in vitro technique developed by Tilley and Terry [11] and modified by Goering and Van Soest [12]. The batch culture consists of collection of rumen fluid as inoculum, inoculation of dried, ground feed samples contained in a flask with a buffering and nutritive in vitro medium. Sample digestion is measured following anaerobic degradation by rumen bacteria. The batch culture can measure the kinetics and volume of gas production (mainly CO_2, CH_4), as well as gas profiles, rate, and extent of substrate digestion, which can then be used to evaluate feed values (ranking feed) and feed additive screening. The kinetics of gas production or feed digestion can be a developed model to predict feed intake, microbial protein synthesis, and metabolizable energy.

During the fermentation of feedstuff, the truly digested substrate is partitioned among volatile fatty acids, gas and microbial biomass. Gas production occurs when substrate carbohydrates are fermented to generate acetate or butyrate but no gas is produced with fermentation of carbohydrate to generate propionate. However, gas is also produced when volatile fatty acid causes gas to be released from the bicarbonate buffer [13]. Although gas production is a reflection of the generation of volatile fatty acids and microbial mass as a result of substrate fermentation, gas measurements only account for substrate that is used for volatile fatty acids and gas production and does not consider substrate utilized for microbial growth. Therefore, the volume of gas produced during fermentation is highly associated with the amount of substrate digested. Currently, the gas production technique is commonly used to evaluate and predict feed value and screening feed additives for ruminants. One major advantage of in vitro gas measurement technique is that it focuses on the appearance of fermentation products and non-fermentable substrates do not contribute to gas production [13]. Since gas production does not consider the amount of substrate converted into microbial biomass, the substrate digestibility that is estimated based on gas measurement is considered as apparent digestibility [14]. Feed protein degradation does not contribute to gas but the high ammonia nitrogen concentration in in vitro systems might prevent the release of gas due to its highly basic nature. As one of major measures of batch culture, gas measurement is widely used to predict rate and extent of feed digestion in the rumen as well as feed intake and microbial protein synthesis [14].

3. Factors affecting batch culture fermentation

3.1. Effect of inoculum source

The inoculum is often the major source of variation on the variable measurements in the use of batch culture technique to study fermentation kinetics of ruminant feeds. The effect of inoculum source on in vitro gas production was considerably discussed in a review by Rymer et al. [2]. Considerable animal variation in the quality of rumen fluid inoculum, prepared identically, is known to exist both within and among donor animals [15, 16]. The variation of batch fermentation due to inoculum source is ultimately attributed to the variation of microbial

population profiles and microbial activities in the rumen. Therefore, all the factors that potentially affect the ruminal microbial activity would affect inoculum quality, thus varying the batch fermentation. In this section, the effects on batch fermentation of the inoculum from sampling schedule, different species, rumen versus faeces as well as inoculum preparation are discussed.

3.1.1. Effect of donor animals, diet and collection time

The donor animals, type of diet and the inoculum collection time may all have an effect on consistency of fermentation results between cultures. It is well known that there is considerable individual animal variation on rumen pH and rumen fermentation pattern under the same feeding and management conditions. Therefore, it is often recommended to collect rumen inoculum from several animals and then combined to reduce the variation. Recently, we have conducted a batch culture to compare rumen inoculum of cattle with low- and high-feed digestion. It was observed the differences in gas production and dry matter digestibility of barley straw when the low- and high-feed-digesting rumen inocula were used. However, the use of such inoculum did not result overall in the differences in gas production kinetics. The effect of the inoculum sources on the in vitro effective dry matter digestibility agrees with previous reports that a difference in the activity of the inoculum exist among individual donor animals [16]. In another batch culture using always the same inoculum (low- versus high-feed-digesting cattle), we observed that the gas production and dry matter digestibility of barley straw werenot affected by inoculum source. The results suggest that inoculum from high-feed-digesting cattle did not necessary improve in vitro digestion of straw.

The rate and extent of feed degradability in the rumen vary with the type of feeds and feed processing. Therefore, diet is considered as a significant factor influencing the inoculum activity. Cone et al. [17] reported that the degradability of starch from different feed sources was greater for the donor cow fed a diet containing equal concentrate and hay compared with a hay-based diet. However, the composition of the concentrate mixture had only a minor effect on degradability values. It is clear that the ruminal microbial activity was different between cows fed hay versus hay and concentrate mixed diet. However, manipulating concentrate composition would not dramatically change ruminal microbial profiles. Mertens et al. [18] reported that the higher-fibre diets tended to produce more gas than the lower-fibre diets, which may explain by more acetate production with high-fibre diet, since fermentation of substrate fibre generates primarily acetate and gas is produced when substrate is fermented to generate acetate or butyrate rather than propionate. Huntington et al. [19] showed a similar response when dry cows were fed a diet of either straw or grass silage with rolled barley, and no differences in the gas production with a diet of a dried grass. Menke and Steingass [20] indicated that there was little difference in gas production of treated straw when hay in the diet of donor animals was replaced with treated straw. The inconsistent effect of donor animal diet suggests that it is more important to ensure the minimum microbial activity in the rumen fluid, rather than ensuring that donor animals are fed the substrate incubated.

Rumen microbial activity is increasing following feed ingestion, thus different sampling times have been applied to collect inoculum in literature either for obtaining high activity (i.e., 2 h

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after feeding) or for reducing variation (i.e., before feeding). Cone et al. [21] reported the increased rate of fermentation with rumen fluid that was collected after the morning although the total gas production was not affected. Menke and Steingass [20] stated that sampling rumen contents just before feeding reduced variation in activity of the inoculum. Although differences in microbial activity of inoculum occur at different sampling times, it appears that the most important factor is whether the sampling schedule will allow collection of inoculum with sufficient microbial activity. Payne et al. [22] observed less variation between replicates when rumen fluid was collected either 4 or 8 h after feeding, compared with before or 2 h after feeding.

The rumen fluid preparation procedure had relatively little effect on gas production [23]. However, Bueno et al. [24] reported an increase of in vitro organic matter digestibility by increasing the proportion of the solid phase relative to liquid phase in inoculum preparation and concluded that the contribution of microorganisms from the solid phase of rumen inoculum is important, especially in studies to evaluate high-fibre feeds. Recently, comparing the rumen inocula from low- and high-feed-digesting cattle, we did not find the differences in fibre digestibility of barley straw between the two inoculum sources, which may be explained by the method of inoculum preparation. Although whole ruminal contents were collected, rumen inoculum was obtained by squeezing manually, and it would represent primarily the bacteria associated with liquid or loosely associated with feed particles but not with bacteria tightly associated with particles. The proportion of bacteria associated with rumen feed particulate has been found to range from 50 to 70% and mainly characterized as fibrolytic bacteria [25].

3.1.2. Inoculum from different species

Rumen fluid from sheep is often used as inoculum on the batch culture because housing sheep is easier and less expensive than the cattle, whereas the results obtained with batch culture technique are mainly used to evaluate feeds for beef or dairy cattle. As a result, numbers of studies were conducted in comparison of rumen fluid between cattle and sheep on in vitro gas production and rumen fermentation [24, 26]. Cone et al. [26] compared rumen fluid from cows and sheep fed a similar diet, and they found that the gas production was lower with sheep rumen fluid, but there was a good relationship between volumes of gas produced by the two inocula. They concluded that sheep rumen fluid could replace cow rumen fluid for accurate determination of 24 and 48 h gas production and the gas production profile. However, rumen fluid of cows could not be replaced by that of sheep for the rate of gas production determination. Similarly, Bueno et al. [24] observed the similar gas production and degradability between sheep and cattle under the same feeding and management conditions. However, kinetics of gas production differed between species and so dynamic determinations, such as rate of gas production data, using sheep inoculum cannot be extrapolated to cattle. Bueno et al. [27] found the similar gas production and organic matter degradability of tropic forage between cow and sheep rumen fluid, whereas rumen fluid from sheep resulted in gas production with a longer lag time (6.1 h versus 4.2 h). Differences in microbial composition of rumen fluid from these sheep and cattle appeared to especially affect kinetics of fermentation, but not the end point

measures. Few studies were compared between cattle and buffalo on the effects of rumen fluid on rumen fermentation. Calabrò et al. [28] found higher gas volume and earlier maximum rate of substrate degradation with cow than buffalo inoculum. All of these data indicate that species of donor animal will affect rumen fermentation.

3.1.3. Rumen versus faecal inocula

Use of faecal inoculum in batch culture has been paid great attention in scientific community during last two decades as it would overcome the need for surgically modified animals. The comparison between rumen fluid and faecal inoculum on in vitro gas production and extent of feed fermentation were well documented in several review articles [2, 15]. In general, the use of faecal inoculum give lower cumulative gas production and feed digestibility than use of rumen fluid although a good correlation is often determined. It suggests that the microbial activity in faecal inoculum is lower than in rumen inoculum. The difference between rumen and faecal inoculum may vary with feed degradability in the rumen. When the diet of the donor animal is highly fibrous, such that the microbial activity of the rumen is low, then differences between rumen fluid and faecal inoculum would be smaller, but when high-productive animals are used, the faecal inoculum are of limited value. Mauricio et al. [29] stated that the faecal inoculum could replace rumen fluid where incubations were over extended periods and cumulative gas volumes were examined since the gas release kinetics differed up to 48 h of incubation between the two inocula. Cone et al. [26] concluded that cow rumen fluid cannot be replaced by cow faeces for determination of 24 h gas production, but to be a good alternative for cow rumen fluid to accurately determine 48 h gas production. Mould et al. [15] suggested that faeces may replace rumen fluid as an inoculum for end-point measures (i.e., degradability or cumulative gas volume at the end of extended incubation periods); faecal material is likely an unsuitable inoculum for estimating rate of fermentation.

3.2. Manual versus automated methods

The gas generated from batch fermentation is generally measured either manually using the manual pressure transducer developed by Theodorou et al. [5] or automatically with the automated systems as described by Pell and Schofield [30], Cone et al. [21] and Davies et al. [31]. It has been reported that the headspace gas production associated with feed fermentation can be manually measured by inserting a needle attached to a pressure transducer into the vials at fixed time points [1, 8], or measured automatically using a transducer recording system [32]. Theoretically, the automated recording system, which vents gas at regular intervals may be more accurate than the manual system as where headspace gas can reach higher pressures. Accumulation of gas (i.e., the rise of gas pressure) may influence the release of gas from buffered ruminal fluid [3] and reduce the fermentation rate of substrate [5]. In closed systems, where gas is not released and accumulates, the rise of pressure in the headspace may cause a staircase effect in the recorded data. Especially with fast-fermenting substrates, some of the headspace gas may be forced into the liquid phase, and this dissolved gas may not be released instantly in the following reading, thus affecting successive measurements. Several studies were carried out to compare the gas

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produced using manual pressure transducers and automated pressure systems. The studies by Rymer et al. [2] and Gierus et al. [6] have observed greater gas production with the manual procedure than automated system. Similarly, we previously used two gas production systems, which were differed in gas pressure recording (automated versus manual), headspace and sample size of the bottle. Serum bottles (100 mL) sealed with a rubber stopper were used for manual gas pressure recording and a 500-mL Ankom gas production module (a computerized system with automated pressure transducers, Ankom Technology, Macedon, NY, USA) equipped with an Ankom pressure sensor module including a microchip and a radio transponder was used for automated gas pressure recording. The result also showed that the gas production was different when gas pressure was recorded using the two systems but it was interacted with the type of substrate incubated. The gas production was higher using manual system when the substrates had higher digestibility such as alfalfa hay and wheat distiller grains, whereas no difference in gas production was observed with the incubation of barley straw which had lower digestibility. The similar gas production of barley straw between the two systems may reflect the slower digestion rate of straw generating less gas. In addition, the gas production values from manual and automated recording systems in our study were calculated from different formulas, this may have biased gas production estimates. For the manual system, the gas volume was calculated using the equation described by Mauricio et al. [33]: gas volume, mL = 0.18 + (3.697 × gas pressure) + (0.0824 × gas pressure^2), whereas for the automated system, the gas volume was estimated according to Avogadro's law (gas volume, mL = gas pressure × [V/RT] × 22.4 × 1000, where V is headspace volume in the bottle in litres, R is the gas constant 8.314472 L kPa/K/mol, and T is the temperature in Kelvin). Rymer et al. (2005) reported the stronger relationships between laboratories with manual system than with automated system and suggested that the increased complexity and cost of automated system may not be repaid by increased value of the results. However, the automated system produced good reproducibility among laboratories [21].

3.3. Effect of material delivery

The feed substrates can be incubated directly by dispersing in the medium or incubated in a filter bag. Incubating feeds in filter bags has been widely applied in batch culture [1, 8] because of its practical convenience. In comparison with dispersing the substrate into the medium, enclosing feed in bags has the advantage of being able to simultaneously determine in vitro digestibility of dry matter and fibre without the need to capture residues after incubation. However, incubating feeds in bags can have concerns on restricting microbial access to the substrates, particle loss from the bags during incubation, and the accumulation of the fermentation products which may inhibit microbial activity [34]. The lower in vitro dry matter and fibre digestibility was reported when feeds were incubated in filter bags as compared to when feeds were dispersed in the medium [35]. Krizsan et al. [36] suggested that this lower feed digestibility may arise from the inability of microbes to readily gain access to substrates within the bags, thus lowering the digestion. Additionally, the possible poor fluid exchange within the bags may result in an accumulation of the fermentation products which could further inhibit the fermentation. Ramin et al. [4] reported lower methane production for feed incubated

in filter bags than dispersed in medium because of reduced feed digestion in vitro; however, the proportion of methane to total gas production was greater for feeds incubated in bags than for feeds dispersed in the medium. It was suggested an alteration of microbial population or fermentation pattern of the feeds incubated in bags versus feeds dispersed in the medium. There was also interaction between feed and method (i.e., bag versus dispersing) on ranking of methane output. It was concluded that the bag method should not be used when measuring methane emission during 48 h of incubation. In contrast, our recent study [9] showed that the incubation of feeds in filter bags consistently increased the digestibility of dry matter and fibre as compared to when the feeds were dispersed in the medium. The discrepancy with other studies may be resulted from the low densities of feed substrates being incubated. In this study, Barley straw, alfalfa hay and wheat distiller grain were ground through either 1- or 2-mm sieve and were incubated. It was observed that some feed particles were floating on the top of media and adhered to the sides of the bottles as a result of agitation during incubation. Obviously, this portion of the substrate would not come in direct contact with microbial populations and thus feed digestion would be compromised. Additionally, incubating substrate in bags to measure dry matter digestibility may have potentially resulted in overestimation of digestibility due to possible washout of feed particles from bags. The washout fraction varied with substrate and ranked as wheat distiller grain (18.8%) > alfalfa hay (12.1%) > barley straw (5.9%). However, because the washout fraction could primarily depend on the soluble fraction, and the soluble fraction is considered to be highly fermentable in the rumen, the impact of washout fraction would be minimal for the dry matter digestibility at longer incubation hour, for example, 24 h, whereas it would have a significant impact on the gas production kinetic measurement. He et al. [9] suggested that the method of substrate delivery could be a primary factor to be considered if the dry matter digestibility is a key variable measured. Therefore, lower microbial activity within the bags, altering microbial population or fermentation pattern of the samples incubated in bags compared with those directly dispersed in the medium show negative aspects of the bag method, but such disadvantages may have limited impact when using the batch culture for screening feed additives or ranking feedstuff. The practical convenience of using bags is highly attractive, thus commonly used currently.

3.4. Effect of substrate particle size

Feed digestion in the rumen requires that microorganisms colonize and produce enzymes that hydrolyse feed particles. Increasing the feed surface area increases the accessibility of microbes to substrate, thus potentially increasing feed digestibility. Anele et al. [37] reported that more processed barley grain (i.e., lower processing index at 0.75 which is calculated as ratio of density after rolling to the density before rolling) produced more cumulative gas volume than less processed barley samples with processing index of 0.85 due to less fermentable nutrients. Yang et al. [38] also reported higher in vitro gas production and dry matter digestibility of ground barley (1-mm sieve) as compared to dry-rolled barley (processing index at 0.80), suggesting that grinding increased the surface area available for microbial attachment. However, Rymer et al. [2] suggested that with highly soluble feeds such as some cereal grain, as long as the feed has undergone some abrasion, its particle size does not affect estimates of gas production rate. Lowman et al. [39] reported the similar gas production profiles of

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incubated naked oats that were cut at half, quarter, coarse and finely ground except the whole naked oats. Similarly, McAllister et al. [40] found the similar in situ dry matter digestibility of halved and quartered grains but significant lower dry matter digestibility of whole grain. Seed grains are generally protected by the pericarp and a processing by rolling or grinding is necessary to make the nutrient-rich endosperm available to the microbes, and to increase the rate and extent of digestion. However, there are many evidences that particle size of processed grain has significant impact on in vitro digestibility. Rymer et al. [2] indicated that maize grain that was steam-flaked, rolled or left intact had the same rate and extent of gas production when it was ground through a 1-mm screen but not when it had been ground through a 4-mm screen. With fibrous and more slowly degraded feeds, gas production rate increases as particle size decreases [39] and it appears that the increased gas production has resulted from an increased surface area as a result of grinding, thereby allowing better microbial access. It seems that there is interaction between feed particle size and type of feeds on in vitro gas production and dry matter digestibility. In the study of He et al. [9], a greater digestibility of dry matter and fibre of alfalfa hay ground 1 mm over 2 mm was observed; however, the digestibility of dry matter of barley straw did not respond to the particle size, and even less gas production and fibre digestibility with barley straw ground through a 1-mm screen as compared to a 2-mm screen was noticed. There was no clear explanation on this unexpected finding and authors speculated that finer straw adhered to the bottle more readily due to its greater buoyancy resulting in a lower digestibility.

The feeds that are incubated in vitro are often finely ground (e.g., ground through 1-mm sieve) since the particle size reduction during in vitro incubation is minimal due to absence of mastication and ruminal contractions. The use of finely ground sample also reduces the risk of sampling bias considering usually only less 1.0 g of sample is included in incubations. Yang et al. [38] concluded, based on a comparison of 60 barley samples either ground or dry rolled, that grinding is likely an appropriate processing method to evaluate digestion characteristics of barley using batch culture technique. In fact, the starch digestibility of ground barley after 24 h of incubation was similar to in vivo values observed in the rumen [41]. There was also less variability in digestibility and better correlation between chemical composition of barley and in vitro digestibility for the ground than the rolled barley. The advantage of using finely ground sample has no concern on processing quality associated with kernel uniformity. However, one can make the argument that barley that is tested in vitro should be processed in a manner similar to the form that it is fed to the animal. Although this approach does not consider the impact of mastication on digestion, it is equally clear that fine grinding also eliminates any sample-mediated differences in the particle sizes that may be generated after dry rolling of barley. Nocek [42] stated that reducing the variability of particle size by grinding through 1-mm sieve may not mimic the in vivo conditions ideally but it does tend to improve the precision of both in vitro and in situ measures. Yang et al. [38] reported the low correlation for digestibility of dry matter between ground and rolled barley (R^2 = 0.12), and suggested that the processing associated with kernel uniformity affected at least partly the digestibility of rolled barley. It can be concluded that the impact of particle size on in vitro feed digestibility can be significant but vary with the type of feed incubated. If the gas production technique is to be used as a means of feed evaluation, it may be necessary to require a standardized particle size

and sample preparation procedure in order to reduce variation among experiments and laboratories. Adoption of a standardized approach to sample preparation may be possible to enable comparison between independently produced gas production and digestion data of different feeds. Additionally, as substrate particles are continually changing shape, size and composition in the gut, it seems unlikely that gas production or dry matter digestion data will represent kinetics of plant biomass as it is digested in the rumen.

3.5. Ratio of rumen inoculum to buffer

The ratio of rumen inoculum to buffer varies considerably in the various batch culture techniques from 1:9 to 1:4 Cabral Filho et al. [43]. Increasing the proportion of rumen inoculum in the incubation medium reduced lag time of gas production, but increased the volume or the rate of gas production [23, 30]. Navarro-Villa et al. [44] incubated with three different ratios of rumen fluid to buffer (i.e., 1:2, 1:4, and 1:6), and observed the increased gas production per unit of dry matter input, CH_4 to gas production and CH_4 to total volatile fatty acid ratio in all feeds incubated with increasing the proportion of rumen fluid in the mixture. The increase in CH_4 output due to change of rumen inoculum to buffer ratio can be resulted from different fermentation pattern, such as for barley grain appeared to be associated with higher acetate to propionate ratios and for barley straw was due to higher volatile fatty acid production. There was also a quadratic response of dry matter digestibility to increased ratios of rumen fluid to buffer with feed dependent, wherein decreasing the ratio resulted in a decline in digestibility with barley grain, an increase with grass silage and an increase (between 1:2 and 1:4) followed by a larger decrease (between 1:4 and 1:6) with barley straw. The decrease in ratio of rumen fluid to buffer would decrease microbial activity of the mixture media, thereby reduced feed digestibility. Pell and Schofield [30] included rumen fluid at the proportions of 5, 10, 20 and 40% in the total medium mixture, and observed the increase of alfalfa hay digestibility with increasing the proportion of ruminal fluid. It suggested that a 20% inoculum is sufficient to ensure the maximum rate of fibre digestion but lower percentages of inoculum are not sufficient. The increased lag time without altering maximum gas productions by lowering the ratio of rumen fluid to buffer appeared to reflect the time required for the microbial numbers to increase to levels comparable with those in the higher inocula. The microbial activity in rumen fluid can be determined by measuring absorbance of the inoculum following a 50-fold dilution at 600 nm and it is recommended a minimum microbial activity of 94 mg bacterial DM/ml [45].

3.6. Effect of concentrate addition on roughage fermentation

The inclusion of readily digestible carbohydrates in forage-based diets for ruminants can restrict microbial digestion of structural polysaccharides because rumen pH can be below the optimum [46]. The rumen pH below the optimum level is especially unfavourable for microbial fibrolysis. However, when poor quality of roughage such as straw is incubated in batch culture, there may be nutrient deficiency to support microbial growth or lack of fermentable carbohydrate to attract microbes to adhesion on the substrate, consequently reducing digestibility of substrate. Barrios-Urdaneta et al. [47] reported that the low available energy content of the

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straw cell wall that was incubated in vitro resulted in low fibre digestion even after long hours of incubation (i.e., 72 h). In addition, the low energy was also responsible for low numbers of bacteria associated with the substrate and a low level of polysaccharidase activity, both of which were corrected by the inclusion of energy supplements. Several studies indicated that the source of carbohydrate inclusion could also influence in vitro cell wall fermentation of crop straw. The higher in vitro straw cell wall digestion was observed with addition of pectin versus soluble sugars or starch [47] or when supplemented with sugar beet pulp, a source of highly digestible structural carbohydrates, compared with barley grain as a source of starch [46]. Barrios-Urdaneta et al. [47] suggested that the effect on increased cell wall digestion of straw was mainly attributed to higher bacterial adhesion to cell wall particles at early incubation time. We conducted a batch culture to incubate barley straw alone or barley straw plus a concentrate mix. For the treatment of straw + concentrate, 30% of barley straw was replaced by the equal amount of concentrate mix which consisted of 60% corn distillers grain, 22% canola meal, and 18% mineral and vitamin supplement in dry matter basis. The concentrate was incubated in a second bag within serum bottle. We observed greater rate of gas production and a shorter lag time with adding concentrate than the incubation of barley straw alone. An increased soluble fraction and dry matter degradability as well as increased fibre digestibility of straw by adding concentrate were noticed. The concentrate used in our study consisted of primarily corn distillers' grain which contained very low starch, but high protein and fibre. The fibre in corn distillers' grain has twice hemicellulose compared to original corn and it is highly fermentable in the rumen. Additionally, the protein from concentrate would favour microbial growth compared with straw alone by providing necessary nutrients. It is suggested that adding concentrate would increase microbial colonization on straw and consequently improved dry matter and fibre degradation of poor quality substrate in the rumen. In our study, although rate of gas production was higher, the volume of gas production was lower by adding concentrate, and along with higher digestibility of dry matter, it is suggested that the fermentation efficiency would be improved by adding the concentrate. Doane et al. [48] also noted that gas production of the in vitro fermentation was negatively related to fibre degradation. The lower fibre content of the substrate and the increased fibre degradation by adding concentrate may explain the lower volume of gas production in our study. The positive response of in vitro digestion of poor-quality feed substrates to high fermentable carbohydrate addition suggests necessary consideration when needing to determine the potential digestibility of poor-quality roughage.

4. Conclusions

Several factors including inoculum source, gas venting system, substrate particle size and delivery, ratios of inoculum to buffer, and concentrate addition to media can influence the outcomes of fermentation in batch culture. The rumen inoculum plays a major role in the fermentation in batch culture. The purpose of the inoculum is to provide a suitable microflora to degrade a feed over time and to use the outcome to provide an estimate of rate or extent of feed digestion. The microbial activity of the inoculum can be considerably varied with animal

species (e.g., cattle versus sheep), diets, sampling schedule following feeding time, but the most important consideration is to ensure sufficient microbial activity in the inoculum and to reduce the variation of microbial activity among inocula. A means of reducing the variation, perhaps by increasing the number of donor animals and standardizing the inoculum collection time, is likely required. Many researches have been conducted to compare rumen fluid and faeces and aimed to develop an alternative to rumen fluid. The advantage of using faecal inoculum is primarily to reduce the requirement to rumen cannulated animals. However, it should be recognized that faecal and rumen inocula are slightly different. It appears that faeces have the potential to replace rumen fluid if long term in vitro end-point measurements are considered, whereas rumen fluid should be used if short-term or kinetic data are needed. Gas production that is main measurement in batch culture is highly adaptable and powerful research tools at present ruminant nutrition research. The discussion of different venting systems and substrate delivery methods is inconclusive. It suggests that other factors such as bottle size, headspace and type of feeds incubated could be interacted with these systems. The particle size of substrate incubated has consistent influence on rate and extent of feed digestion. The recommendation on the particle size of feed may be not easily provided and may depend on type of feed (e.g., concentrate versus roughage) and the objective of the study. Varying ratios of rumen fluid to buffer volume changes microbial activity in fermentation media, thus potentially alter rate of fermentation and lag time. The recommendation is to ensure sufficient microbial activity in the mixture of fermentation media without too much rumen fluid which may increase proportion of gas from inoculum over substrate. Finally, adding highly fermentable carbohydrate is helpful to maximize the fermentation of poor-quality feeds.

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Chapter 6

Kinetic Modeling of 1-G Ethanol Fermentations

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Additional information is available at the end of the chapter

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Abstract

The most recent rise in demand for bioethanol, due mainly to economic and environmental issues, has required highly productive and efficient processes. In this sense, mathematical models play an important role in the design, optimization, and control of bioreactors for ethanol production. Such bioreactors are generally modeled by a set of first-order ordinary differential equations, which are derived from mass and energy balances over bioreactors. Complementary equations have also been included to describe fermentation kinetics, based on Monod equation with additional terms accounting for inhibition effects linked to the substrate, products, and biomass. In this chapter, a reasonable number of unstructured kinetic models of 1-G ethanol fermentations have been compiled and reviewed. Segregated models, as regards the physiological state of the biomass (cell viability), have also been reviewed, and it was found that some of the analyzed kinetic models are also applied to the modeling of second-generation ethanol production processes.

Keywords: ethanol fermentation, kinetic modeling, unstructured and unsegregated models, inhibition phenomena, bioreactors

1. Introduction

The interest in producing industrial bioethanol essentially comes from economic and environmental issues. Bioethanol can be produced from batch, fed-batch, and continuous processes, as well as in some cases using flocculating yeasts [1–6].

The development of efficient control strategies for the main operating variables in ethanol fermentations, such as pH, temperature, residual sugars concentration, agitation speed, foam level, among others, requires accurate dynamic models. In addition, mathematical models are important tools for the design, optimization, and control of bioreactors. Bioreactor models seek to describe the overall performance of the bioreactor and consist of two submodels: a balance/

transport submodel that describes mass and heat transfer within and between the various phases of the bioreactor and a kinetic submodel that describes how the rates of the microorganism's growth, substrate consumption, and product formation depend on the key local environmental variables [7].

In ethanol fermentation, the main bioreactions can be summarized by the reductive pathway S $\rightarrow$ X + P + CO_2. According to this reaction, substrates S (glucose and fructose, which result from hydrolysis of sucrose as the limiting substrate), in anaerobic conditions, are metabolized to produce a yeast population X, ethanol P (mainly produced by yeast through the Embden-Meyerhof-Parnas metabolic pathway), and carbon dioxide (CO_2). The hydrolysis of sucrose promoted by the invertase present in the yeast is not the limiting step of ethanol production in industrial processes. The stoichiometry of ethanol-formation reaction from glucose is given by the classical Gay-Lussac equation: $C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2$

According to Doran [8], both *Saccharomyces cerevisiae* yeast and *Zymomonas mobilis* bacteria produce ethanol from glucose under anaerobic conditions without external electron acceptors. The biomass yield from glucose is 0.11 g/g for yeast and 0.05 g/g for *Z. mobilis*. In both cases, the nitrogen source is NH_3, and the cell compositions are represented by the formula $C_{1.8}O_{0.5}N_{0.2}$. From these data, Doran [9] proposed the following stoichiometric equation for ethanol fermentation (the values of stoichiometric coefficients *a*, *b*, c, *d*, *e*, and *f* are presented in **Table 1**):

$$aC_6H_{12}O_6 + bNH_3 \rightarrow cCH_{1.8}O_{0.5}N_{0.2} + dCO_2 + eH_2O + fC_2H_6O(\text{molar basis})$$

Kinetic modeling of growth, ethanol production, and substrate consumption by yeasts has been traditionally conducted using an unsegregated and unstructured approach for the biomass. This approach ignores the presence of individual cells and structural, functional, and compositional aspects of the cell, describing the complex processes of growth, ethanol production, and substrate consumption through simple kinetic equations [10–15]. **Figure 1** shows a simplified scheme of this approach for the ethanol fermentation process by yeasts and bacteria.

Microorganism	Stoichiometric coefficients					
	a	*b*	*c*	*d*	*e*	*f*
Yeast	1	0.16	0.81	1.75	0.35	1.72
Bacteria	1	0.074	0.37	1.89	0.17	1.87

Table 1. Stoichiometric coefficients for ethanol fermentation.

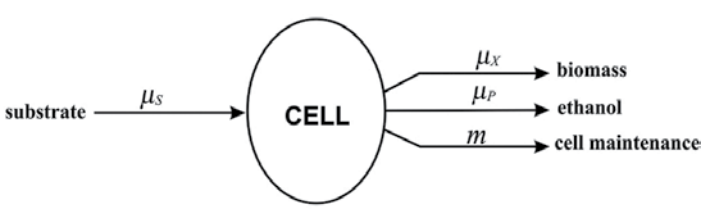

Figure 1. Kinetic modeling of ethanol fermentation based on an unsegregated and unstructured approach for cells (yeasts or bacteria).

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2. Kinetics of cell growth and ethanol formation

In ethanol fermentation, the kinetics of growth and ethanol production are generally the following:

$$\mu_X = f_1(S)g_1(P) \tag{1}$$

$$\mu_p = f_2(S)g_2(P) \tag{2}$$

where μ_X and μ_p, are, respectively, the specific rate of yeast growth and ethanol production, whereas S and P represent the limiting substrate and ethanol concentrations.

2.1. Effect of substrate concentration

The functions $f_1(S)$ *and* $f_2(S)$ are generally of the Monod type [11], except when an inhibition caused by high concentrations of substrate or diffusional limitations occurs due to high cell concentrations.

$$f(S) = \frac{\mu_{\max} S}{K_S + S} \text{(Monod equation)} \tag{3}$$

The inhibition caused by the excess of substrate has generally been modeled by applying the Andrews equation [16–19], though there are other types of equations that are less commonly used [20].

$$f(S) = \frac{\mu_{\max} S}{K_S + S + S^2/K_I} \text{(Andrews equation)} \tag{4}$$

In the case of continuous processes operated near to the steady state, the inhibition concentrations of the substrate are rarely identified. However, inhibitory concentrations can occur during the start-up of these processes or in situations resulting from changes in the substrate feed load.

Atala et al. [21], modeling the effect of temperature upon the kinetics of ethanol fermentation with a high concentration of biomass in a continuous system with total cell retention, used an inhibitory factor (IF) of the exponential type to describe the inhibitory effect of the substrate upon the kinetics of cell growth, which was inserted in the expression of $f(S)$, being $f(S)$, in this case, given by the Monod equation.

$$IF = (e^{-K_I S}) \tag{5}$$

Tsuji et al. [22] evaluating the performance of different ethanol fermentation systems (conventional chemostat, multiple bioreactors, cell recycle bioreactor, extractive bioreactor, and immobilized cell bioreactor) expressed the specific growth rate by an equation analogous to Eq. (1):

$$\mu_X = \mu_1(S)\mu_2(P) \tag{6}$$

One of the analyzed cases considered growth inhibition by substrate, represented by a hyperbolic equation:

$$\mu_1(S) = \frac{\mu_{\max} S}{K_S + S}\left(\frac{K_i}{K_i + S}\right) \tag{7}$$

Sousa and Teixeira [23] reported that one of the main disadvantages of the systems that use flocculating cells (bacteria or yeast) is the reduced reaction rates caused by diffusional limitations of the substrate within the flocs and that, in most cases, the diffusion rate is lower than the reaction rate, which means that the process is controlled by diffusion. Sousa and Teixeira [23] reported that it is generally accepted that yeast flocs are formed by a mediator cation (usually Ca^{2+}) from the interaction between protein and mannans on adjacent cell walls. According to Sousa and Teixeira [23], one means through which to avoid diffusional limitations within the flocs is by using polymeric additives, which act by widening the bridges formed between adjacent cells.

Fontana et al. [24] reported that when the yeast flocs are suspended in a sucrose solution, various phenomena occur simultaneously: the sugar penetrates by diffusion in the aggregates and is hydrolyzed into glucose and fructose by an invertase that is primarily located on the yeast's cell wall. These two sugars diffuse inside and outside of the particle and are fermented in ethanol and CO_2, which in turn diffuse back in the liquid medium.

Fontana et al. [24] assumed that the Fick's law was valid for the aggregate and that the temporal variation of the concentration of each component involved in the transformation is represented by the following equation:

$$\frac{\partial C_i}{\partial t} = D_{ef,i}\frac{\partial^2 C_i}{\partial x^2} + \sum r_i \tag{8}$$

where C_i is the concentration of the component i in the aggregate in time t and distance x as of the floc surface; $D_{\text{ef},i}$ is the effective diffusion coefficient, while $\sum r_i$ is the sum of the consumption or production rates of component i, which are given by Michaelis-Menten-type equations, such as the following:

$$\sum r_i = -r_{S_{\max}}\frac{S}{K_S + S}\ \text{(Sucrose)} \tag{9}$$

$$\sum r_i = +Y_{G/S} r_{S_{\max}}\frac{S}{K_S + S} - r_{G_{\max}}\frac{G}{K_G + G}\text{(Glucose)} \tag{10}$$

where S and G represent, respectively, the concentration of sucrose and glucose, while $Y_{G/S}$ is the conversion factor in glucose based on the hydrolyzed sucrose ($Y_{G/S}$ = 0.505g-glucose/g-sucrose). One relation identical to Eq. (10) can be obtained for fructose.

However, the theoretical descriptions of the diffusional resistances in systems that make use of flocculating microorganisms are generally conducted by introducing a factor into the Monod

http://dx.doi.org/10.5772/65460

equation that takes into account the reduction in growth rate due to mass transfer limitations. One equation of this type is that proposed by Contois [25].

$$f(S) = \frac{\mu_{\max} S}{K_S X + S} \text{(Contois equation)} \tag{11}$$

According to Menezes et al. [26], the Monod model is appropriate at low cell concentrations, while the Contois model is more appropriate at high concentrations, given that the variable saturation term, $K_S X$, can describe the diffusional limitations present in high cell concentrations. Oliveira et al. [27], modeling a continuous process of ethanol fermentation in a tower bioreactor with recycling of flocculating yeasts, obtained a high value for K_S, which was attributed to the diffusional limitations caused by the high cell concentrations reached in the bioreactor.

2.2. Effect of ethanol concentration

The dependence of μ_X and μ_P on the ethanol concentration is due to the fact that this product has been reported in the literature to act as a noncompetitive inhibitor both for growth and its own production [10, 28–32].

Noncompetitive inhibition is characterized by the fact that in the graph of $1/\mu_X$ or $1/\mu_p$ versus $1/S$ (**Figure 2**), for each ethanol concentration (P), straight lines with different slopes

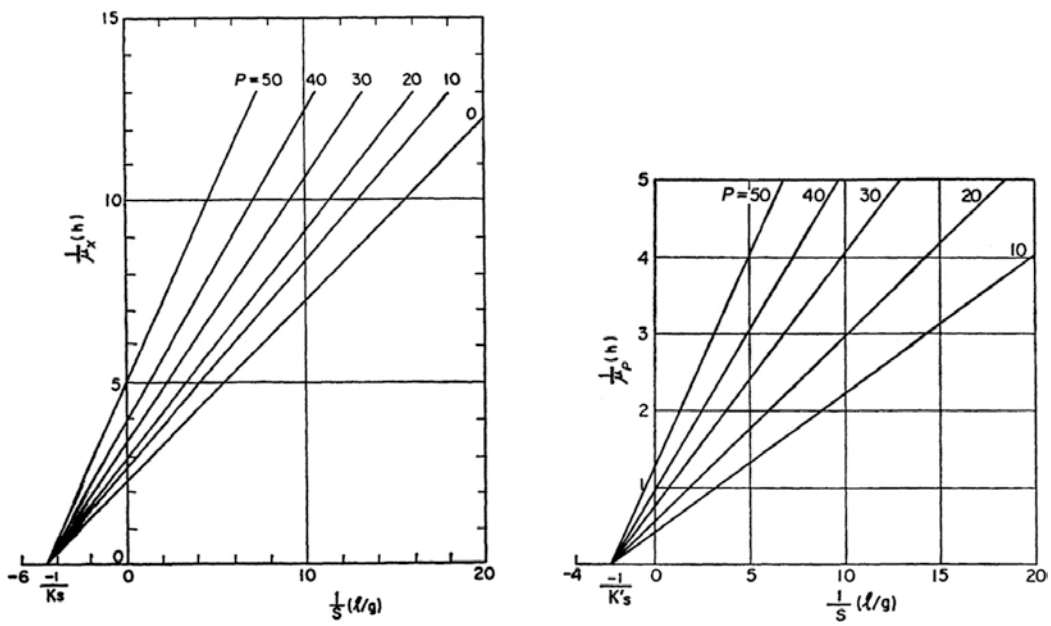

Figure 2. Lineweaver-Burk graph for the specific rates of cell growth (μ_X) and ethanol production (μ_P) (adapted from Aiba et al. [31]).

$\left(\frac{K_{S,i}}{\mu_{max,i}g_i(P)}, \mathrm{i} = 1,2\right)$ and different intercepts $\left(\frac{1}{\mu_{max,i}g_i(P)}, \mathrm{i} = 1,2\right)$ are obtained, but the same intersections with the abscissa are maintained ($-1/K_{S,i}$; $i = 1, 2$).

The molecular base of the mechanism through which the ethanol exerts an inhibitory effect upon fermentation is complex so long as this component, which acts as a denaturing agent, not only acts directly upon the proteins and causes an inactivation or inhibition of the enzymes from the glycolytic pathway but can also act upon the integrity of the lipid membranes, affecting the essential factors, including membrane components, such as transport proteins and the enzymes linked to it [33].

Table 2 presents the main equations proposed for $g_1(P)$ and $g_2(P)$, which are first approximations of much more complicated effects [10, 28–32, 34–42].

Linear (L)	$g(P) = \left(1 - \frac{P}{P_m}\right)$	(12)
Generalized nonlinear (GN)	$g(P) = \left(1 - \frac{P}{P_m}\right)^n$	(13)
Hyperbolic (H)	$g(P) = \left(\frac{K_P}{K_P + P}\right)$	(14)
Parabolic (P)	$g(P) = \left(1 - \frac{P}{P_m}\right)^{0.5}$	(15)
Exponential (E)	$g(P) = (e^{-K_P P})$	(16)

Table 2. Types of commonly proposed equations to describe the inhibitory effect of ethanol upon μ_X and μ_P.

The type of inhibition that affects cell growth is not mandatorily the same as that which affects ethanol production, as it is necessary to determine separately each effect, as proposed by Oliveira et al. [27]. According to Bonomi et al. [17], one of the major difficulties during the development of a mathematical model that fits experimental data of ethanol fermentation is the definition of the type of product inhibition exhibited by the yeast's metabolism. Bonomi et al. [17] reported that this inhibition is characterized by the behavior of the specific growth and production rates with an increase in ethanol concentration, while holding constant the substrate concentration. When developing a mathematical model for a batch system of ethanol production, Bonomi et al. [17] set three values for substrate concentration and determined the corresponding pairs of values (μ_X, P) and (μ_P, P) in each fermentation test. These points—(μ_X, P) and (μ_P, P)—were then plotted to define the types of existing relations between the specific rates and ethanol concentration which, in this case, were both exponential. The values of the specific growth and ethanol production rates were calculated based on experimental data, using the geometric approach proposed by Le Duy and Zajic [43].

The conceptual limitation of the hyperbolic and exponential inhibition is that they predict cell growth and production for all of the ethanol concentrations, even though many experimental tests have shown that cell growth and production cease upon reaching a given high concentration of ethanol [44]. The models of linear, generalized nonlinear, and parabolic inhibition consider that there is a determined concentration of ethanol above which growth and production do not occur. In these models, the P_m parameters represent the ethanol concentrations for which the growth and production processes are completely interrupted.

http://dx.doi.org/10.5772/65460

In the linear, generalized nonlinear, and parabolic models, the exponents of the term ($1–P/P_m$) are called by Levenspiel [40] as "toxic powers." The values of toxic powers are indicative of how the term of inhibition ($1–P/P_m$) strongly affects the specific growth and ethanol production rates. With the rise in toxic power, the intensity of inhibition increases for a determined ethanol concentration.

In the hyperbolic and exponential inhibition models, the K_P parameters do not admit a physical meaning and can be considered simple empirical constants that apparently depend on the cultivation mode: batch or continuous [31, 32, 37]. Aiba and Shoda [32] argued that the fact that the hyperbolic inhibition constant of the specific growth rate (K_P) has been lower in a batch culture (K_P = 16.0 g/L) than in a continuous culture (K_P =5 5.0 g/L) suggests the possibility that a chemical affinity of ethanol for a key participating enzyme in cell growth appeared in batch experiments. By contrast, the fact that the hyperbolic inhibition constant of the specific ethanol production rate (K'_P) has been lower in continuous cultures (K'_P = 12.5 g/L) than in batch cultures (K'_P = 71.5 g/L) suggests that the ethanol inhibition upon another key enzyme responsible for the fermentation activity was more expressive in continuous experiments.

Another inhibition model commonly used in the literature is that proposed by Luong [37]:

$$g(P) = 1–\left(\frac{P}{P_m}\right)^{\beta} \tag{17}$$

where P_m continues to be the ethanol concentration above which no growth or production can occur, and β is an empirical constant.

One different proposal to describe the inhibitory effects of ethanol upon μ_X and μ_P was presented by Wang and Sheu [45] when they applied multiobjective optimization methods to estimate the parameters of kinetic models of batch and fed-batch processes for ethanol production, using one yeast that is highly tolerant to ethanol (*Saccharomyces diastaticus*). In their study, the kinetic models for the specific rate of cell growth and product formation were represented as follows:

$$\mu_X = \left(\frac{\mu_m S}{K_S + S + S^2/K_{IS}}\right)\left(\frac{K_P}{K_P + P + P^2/K_{IP}}\right) \tag{18}$$

$$\mu_P = \left(\frac{\nu_m S}{K'_S + S + S^2/K'_{IS}}\right)\left(\frac{K'_P}{K'_P + P + P^2/K'_{IP}}\right) \tag{19}$$

Olaoye and Kolawole [46], modeling the kinetics of ethanol fermentation in batch culture of *Kluyveromyces marxianus*, used a semiempirical approach to describe the fermentation process. To model the temporal profile of the biomass concentration, the authors inserted Eq. (20) in the cell mass balance ($dX/dt = \mu_X X$) and analytically integrated the resulting equation to obtain the so-called logistic growth curve (Eq. 21). The ethanol concentration was described, directly applying the modified Gompertz equation (Eq. 22), which represented the empirical part of the proposed mathematical model. The authors did not report the values of the model's parameters.

$$\mu_X = \mu_m\left(1-\frac{X}{X_m}\right) \tag{20}$$

$$X = \frac{X_m}{1+\left(\frac{X_m-X_0}{X_0}\right)e^{-\mu_m t}}; X_0 = X(0) \tag{21}$$

$$P = P_m \exp\left\{-\exp\left[\frac{\mathrm{Pr}_m \exp(1)}{P_m}(\lambda - t)+1\right]\right\} \tag{22}$$

where P_m and Pr_m are, respectively, the maximum concentration and maximum productivity of ethanol; λ is the time of duration of the lag phase, anterior to the exponential phase of ethanol production.

The logistic equation has been used to model fermentation kinetics due to its mathematical simplicity. According to Mitchell [7], the logistic equation can, many times in a single equation, offer an adequate approximation of the entire growth curve, including the lag phase and the cessation of growth in the latter stages of fermentation.

2.3. Effect of cell concentration

The inhibition models presented thus far have been sufficient to satisfactorily describe a large number of fermentations. However, in continuous processes with cell recycling, high cell densities are obtained in the fermenter, and the consideration of other factors, such as the inhibition caused by the excess of biomass, may well be necessary for a better description of the bioprocess kinetic behavior.

The inhibition of cell growth by cell concentrations has been modeled using the following generalized equation [44]:

$$h(X) = \left(1-\frac{X}{X_{\max}}\right)^{\delta} \tag{23}$$

where $X_{\max}$ is the maximum cell concentration that would be reached if ideal conditions for growth were observed, that is, an adequate supply of nutrients and the absence of inhibitory effects [44]. Analogous to the term of inhibition caused by the product, δ indicates the intensity of the inhibition due to the high cell concentrations.

Jarzebski et al. [47], modeling a continuous ethanol fermentation process with high yeast concentrations in a membrane filtration module system, used the following expressions for μ_X and μ_P, in which other formats can be observed for the terms that describe the inhibitory effects of the biomass itself:

$$\mu_X = \left(\frac{\mu_0 S}{K_S+S}\right)\left[1-\left(\frac{P}{P_m}\right)^{A_1}\right]\left[1-\left(\frac{X}{X_m}\right)^{A_2}\right] \tag{24}$$

$$\mu_P = a\exp(-bX) \tag{25}$$

http://dx.doi.org/10.5772/65460

In cases occurring inhibition of cell growth and product formation by the biomass itself, the expressions of the specific growth and ethanol production rates must be augmented to incorporate these inhibitory effects, that is,

$$\mu_X = f_1(S)g_1(P)h_1(X) \tag{26}$$

$$\mu_P = f_2(S)g_2(P)h_2(X) \tag{27}$$

As regards the procedures of many authors using a kinetic expression for μ_P detached from μ_X, Bu'Lock et al. [10] reported that this does not mean that there is no association between these rates, so long as the ethanol production has been commonly reported in the literature as a process associated with growth. Bu'Lock et al. [10] justified the adoption of such a procedure due to the simplicity and to the better adaptation of such equations to experimental data.

In relating the kinetics of ethanol production to the kinetics of cell growth, the procedure has been to apply the Luedeking-Piret model:

$$\mu_P = \alpha\mu_X + \beta \tag{28}$$

The Luedeking-Piret model is based on the classification of products of the fermentation process as associated ($\alpha > 0$, $\beta = 0$), nonassociated ($\alpha = 0$, $\beta > 0$), and partially associated with cell growth ($\alpha > 0$, $\beta > 0$) [12]. Since ethanol is a product of the primary metabolism of the yeasts, the majority of described cases assumes $\alpha > 0$ and $\beta = 0$, as reported by Oliveira et al. [19] when they modeled the batch ethanol production, using the expression $\mu_P = \alpha\mu_X$, with α = 4.17 g/g. However, it is possible to find descriptions in the literature of ethanol production, parts associated and not associated with cell growth, that is, $\alpha > 0$ and $\beta > 0$, as is the case with that reported by Guidini et al. [18] that used the following equation to describe μ_P in a fed-batch ethanol fermentation process with flocculating yeasts (*S. cerevisiae*):

$$\mu_P = \underbrace{\left(\frac{Y_{P/S}}{Y_{X/S}}\right)}_{\alpha}\mu_X + b \tag{29}$$

Rivera et al. [48], modeling a fed-batch ethanol fermentation process with a strain of industrial yeast (*S. cerevisiae*), used a modified version of the Luedeking and Piret model in which the β coefficient is given as a function of the substrate concentration (S):

$$\mu_P = Y_{P/X}\mu_X + \underbrace{\left(\frac{\beta_m S}{K_{\beta S} + S}\right)}_{\beta} \tag{30}$$

Ghosh and Ramachandran [49], analyzing the effect of *in situ* product removal on the stability and performance of a continuous bioreactor with cell separator for ethanol production, emphasized the use of the Luedeking-Piret model to represent the kinetics of product formation.

In **Table 3**, typical kinetic parameter values for ethanol fermentation are presented [10, 19, 28–32, 34–41].

Table 3 shows large variations in the values of kinetic parameters, demonstrating that these parameters are strongly dependent upon the operational conditions for which they were adjusted, from the culture medium and from the microorganisms used in the fermentation.

Oliveira et al. [50] analyzed the scale-up effects on kinetic parameters and predictions of a mathematical model developed for a continuous process, in small scale, of ethanol fermentation in a tower bioreactor with flocculating yeast recycling, and concluded that the scale-up did not affect the parameter values and that the model continued to be valid to describe the process in the newly investigated scale.

Although the great majority of mathematical models reviewed thus far have been developed for free cell systems, these are equally valid for naturally or artificially immobilized cell systems. However, physiological changes in microbial cells caused by immobilization can significantly affect the values of the kinetic parameters of such models. Moreover, internal and external diffusion effects in microbial particles and flocs can affect the fermentation kinetics. Admassu et al. [38], modeling the hydrodynamics and the profile of product concentration in a tower fermenter for the continuous production of ethanol with flocculating yeasts, reported that the growth and reaction rates for these flocculating microorganisms are frequently limited by mass transfer.

Vicente et al. [51], developing a new technique to measure kinetics and mass transfer parameters in flocs of *S. cerevisiae*, modeled the kinetics of oxygen consumption using the following equation:

	Parameters of $f_1(S)$, $f_2(S)$ $h(X)$	**Parameters of $g_1(P)$ $g_2(P)$**				
		L	**GN**	**P**	**H**	**E**
$\mu_{max,1}$ (/h)	0.11–0.56					
$\mu_{max,2}$ (g/g/h)	0.21–1.90					
$K_{S,1}$ (g/L)	0.07–0.57					
$K_{S,2}$ (g/L)	0.33–60.0					
$P_{m,1}$ (g/L)		87.0–95.0	73.0–87.5	93.6		
$P_{m,2}$ (g/L)		114.0–135.0	87.5	99.0		
n_1 (–)			0.41–4.0			
n_2 (–)			0.41			
$K_{p,1}$ (L/g or g/L)					16.0–105.2	0.016–0.029
$K_{p,2}$ (L/g or g/L)					12.5–71.5	0.015–0.094
X_{max} (g/L)	100.0–330.0					

Table 3. Typical values of kinetics parameters in ethanol fermentation.

http://dx.doi.org/10.5772/65460

$$r_C = -\left(\frac{p_1 C}{p_2 + C}\right) X \tag{31}$$

where r_C is the oxygen consumption rate (mg O_2/(L h)) from which the specific rate of respiration q_O can be calculated, C is the dissolved oxygen concentration (mg O_2/L), X is the active biomass concentration (g/L), p_1 corresponds to $q_{O,m}$ (mg O_2/(g h)), $q_{O,m}$ and p_2 corresponds to K_m (mg O_2/L).

According to Vicente et al. [51], although Eq. (31) represents a Monod-type kinetic model, the calculated values of p_1 and p_2 are only apparent and have no direct relationship with the usual kinetic parameters. Vicente et al. [51] argue that the designations $q_{O,m}$ and K_m were not used because they are generally applied to suspended free cell cultures and that, in this case, cell aggregates were studied, which significantly change the overall behavior of the system and, therefore, the meaning of such parameters.

3. Kinetics of substrate consumption

The kinetics of substrate consumption can generally be described by the Herbert-Pirt model, according to which the substrate is consumed for cell growth and maintenance and the production of a specific product [26]:

$$\mu_S = \frac{\mu_X}{Y^*_{X/S}} + \frac{\mu_P}{Y^*_{P/S}} + m \tag{32}$$

where $Y^*_{X/S}$ and $Y^*_{P/S}$ are, respectively, the stoichiometric coefficients of substrate conversion in cells and product based on the substrate consumed exclusively for each process.

Substrate consumption for cell maintenance refers to the substrate used in the generation of energy for distinct growth functions, such as the maintenance of the concentration gradients between the interior and exterior environment of the cell (osmotic work), synthesis of the cell components that are being continuously degraded, among others [12].

Equation (32) considers that the specific rate of cell maintenance m is a constant, hypothesis, which Ramkrishna et al. [52] do not adopt. According to these authors, the cells suffer a process of degradation in stages in which, at the first stage, the cells would lose their cell viability and, at a second stage, they would die if their maintenance requirements were not attended. To recover the viability, the nonviable cells would need a substrate that would be the same used for growth (exogenous substrate) or an internally stored substrate (endogenous substrate). From these considerations, the following modification in the mathematical representation of the metabolism of maintenance can be introduced, in turn substituting the constant term m in Eq. (32) by a Monod-type expression [26, 53]:

$$\zeta = \frac{\zeta_{\max} S}{K_{S,m} + S} \tag{33}$$

Equation (33) shows that, at high concentrations of substrate, there is a predominance of exogenous metabolism with $\zeta_{max} \rightarrow m$ when $S >> K_{S,m}$, whereas at low concentrations, the endogenous metabolism predominates with $\zeta \rightarrow 0$ when $S \rightarrow 0$.

Generally, the kinetics of substrate consumption is not described using the Herbert-Pirt model to its full extent. The more common approach is the use of apparent coefficients of substrate conversion in cells ($Y_{X/S}$) and ethanol ($Y_{P/S}$), relating μ_S to μ_X or to μ_p by means of these coefficients (Eqs. (34) and (35)). Another approach to describe μ_S is that represented by Eq. (36).

$$\mu_S = \frac{\mu_X}{Y_{X/S}} \tag{34}$$

$$\mu_S = \frac{\mu_P}{Y_{P/S}} \tag{35}$$

$$\mu_S = \frac{\mu_X}{Y_{X/S}} + m \tag{36}$$

Applications of these approaches can be found in the studies listed in **Table 4**.

Sinclair and Kristiansen [12] emphasize the importance of not confusing the stoichiometric coefficients with apparent coefficients as is normally reported in the literature. The stoichiometric coefficient is a constant that depends on the chemical equation, relating the substrates and the products ($Y^*_{P/S}$ = 0.511g-ethanol/g-glucose in the fermentation of glucose to ethanol). The apparent coefficient is the ratio of the mass of a product formed by the total mass of a consumed substrate, which could be participating in multiple reactions, forming a variety of products, including new cells. In this sense, the following definitions for the stoichiometric and apparent coefficients are convenient:

Study	μ_S	Reference
Optimization of an industrial bioprocess of ethanol fermentation with multiple stages and cell recycle, using techniques of factorial design and response surface analysis in combination with phenomenological modeling and simulation	Eq. (34)	[54]
Ethanol fermentation modeling in a tower bioreactor with flocculating yeasts	Eq. (35)	[38]
Analysis of the steady-state stability and modeling of the dynamic behavior of a continuous ethanol fermentation process in a gas-lift tower bioreactor with high cell densities	Eq. (35)	[36]
Bifurcation analysis of two continuous membrane fermentor configurations for ethanol production	Eq. (36)	[55]
Modeling, simulation, and analysis of an ethanol fermentation process with control structure in industrial scale	Eq. (36)	[56]
Modeling of a fed-batch ethanol fermentation process with a strain of industrial yeast (*Saccharomyces cerevisiae*)	Eq. (36)	[48]
Modeling of a fed-batch ethanol fermentation process with flocculating yeasts (*S. cerevisiae*)	Eq. (36)	[18]

Table 4. Mathematical models used for modeling of substrate-consumption kinetics in 1-G ethanol fermentation processes.

http://dx.doi.org/10.5772/65460

$$\bullet Y^{*}_{X/S} = \frac{\text{Mass of new cells formed}}{\text{Substrate mass consumed only for the formation of new cells}} \tag{37}$$

$$\bullet Y_{X/S} = \frac{\text{Mass of new cells formed}}{\text{Total mass of substrate consumed}} \tag{38}$$

$$\bullet Y^{*}_{P/S} = \frac{\text{Mass of product formed}}{\text{Substrate mass consumed only for the formation of product}} \tag{39}$$

$$\bullet Y_{P/S} = \frac{\text{Mass of product formed}}{\text{Total mass of substrate consumed}} \tag{40}$$

Oliveira et al. [57], modeling a continuous ethanol fermentation process in a two-stage tower bioreactor cascade with flocculating yeast recycle, used simplified ($\mu_S = \mu_P/Y_{P/S}$) and generalized ($\mu_S = \mu_X/Y^{*}_{X/S} + \mu_P/Y^{*}_{P/S} + m$) kinetic expressions to describe μ_S and obtained similar predictions of the state variables by both employed approaches.

Bonomi et al. [17], modeling the ethanol production using cassava hydrolyzate in a batch bioreactor, defined the following equation for the mass balance of substrate:

$$\frac{dS}{dt} = -\frac{1}{2}\left(\frac{1}{Y_{X/S}}\mu_X X + \frac{1}{Y_{P/S}}\mu_P X\right) \tag{41}$$

According to these authors, the definition of the apparent coefficients $Y_{X/S}$ and $Y_{P/S}$ guarantee that the terms $\mu_X X/Y_{X/S}$ and $\mu_P X/Y_{P/S}$ are equal; this equality was also reported by Aiba et al. [31] and Ghose and Tyagi [28]. Bonomi et al. [17] argue that the two terms are not exactly equal due to the fact that the calculated values of $Y_{X/S}$ and $Y_{P/S}$ are affected by different experimental errors and the values of μ_X and μ_P are calculated using estimates of other parameters of the model. These authors justify the introduction of the average among the aforementioned terms in Eq. (41), as a means through which to minimize the propagation of errors discussed above.

By contrast, Jin et al. [42], modeling the kinetics of batch fermentation for ethanol production with *S. cerevisiae* immobilized in calcium alginate gel, presented the following mass balance equation for the substrate, without the introduction of the 1/2 factor in the equation:

$$\frac{dS}{dt} = -\left(\frac{1}{Y_{X/S}}\mu_X X + \frac{1}{Y_{P/S}}\mu_P X\right) \tag{42}$$

One equation like $\mu_S = \mu_X/Y_{X/S} + \mu_P/Y_{P/S}$ was also employed by Marginean et al. [58] to model, simulate, and develop proportional integral derivative (PID) control strategies for temperature and the pH of an ethanol production process in a continuous stirred tank reactor (CSTR).

A different proposal was presented by Limtong et al. [35] to model a continuous process of ethanol fermentation in a tower bioreactor with recycle of flocculating yeasts. The authors determined linear relations between the product concentration (P (g/L)) and the specific rates of glucose consumption (μ_S) and ethanol production (μ_P). The ratios between the corresponding angular and linear coefficients of the straight lines (1.63/3.74 and 0.020/0.046)

provide a reasonable estimate of the value of $Y_{P/S}$ (=0.43 g/g), which demonstrates the consistency of such relations (Eqs. (43) and (44)).

$$\mu_S = -0.046P + 3.74(\text{g/g/h}) \tag{43}$$

$$\mu_P = -0.020P + 1.63(\text{g/g/h}) \tag{44}$$

Another situation to be analyzed is when there is more than one fermentable sugar present in the medium, as is the case in the production of beer. Ramirez [59], modeling the dynamic of batch beer fermentation, considered the glucose (G), maltose (M), and maltotriose (N) to be the three majority sugars contained in the fermentative medium. The specific consumption rates of these sugars were described by equations that exhibit a kinetic pattern of preferential use of these substrates, that is, the preferred sugar (G) is first used until its complete exhaustion; next, the second sugar (M), of intermediate preference, is consumed; and lastly, the third sugar (N), the least preferred, is consumed. According to Ramirez [59], this pattern of sequential use is modeled by inserting terms of inhibition of the consumption of a less preferential sugar by one or more preferential sugars in such a way that the specific consumption rates of these sugars μ_i are given by

$$\mu_G = \frac{V_G G}{K_G + G} \tag{45}$$

$$\mu_M = \frac{V_M M}{K_M + M}\left(\frac{K_G^{'}}{K_G^{'} + G}\right) \tag{46}$$

$$\mu_N = \frac{V_N N}{K_N + N}\left(\frac{K_G^{'}}{K_G^{'} + G}\right)\left(\frac{K_M^{'}}{K_M^{'} + M}\right) \tag{47}$$

where V_i is the maximum specific consumption rate of the sugar i (g/g/h), K_i is the saturation constant for the sugar i (g/L), and $K^{'}_i$ is the constant of inhibition caused by the sugar i (g/L).

Additionally, the specific rates of cell growth (μ_X) and ethanol production (μ_E) were given by the following equations [59]:

$$\mu_X = R_{XG}\mu_G + R_{XM}\mu_M + R_{XN}\mu_N \tag{48}$$

$$\mu_E = R_{EG}\mu_G + R_{EM}\mu_M + R_{EN}\mu_N \tag{49}$$

where R_{Xi} and R_{Ei} are, respectively, the stoichiometric yield of the biomass and ethanol per gram of sugar i consumed (g/g).

A similar approach was employed by Lee et al. [60] when they modeled the batch ethanol production by *S. cerevisiae* from a mixture of glucose and maltose. One term ξ was included in the equation of μ_M to represent the glucose repression effect upon the maltose consumption. The final set of the mathematical model equations is presented as follows, highlighting the prediction of diauxic growth in the expression of μ_X and the production of ethanol from the two sugars in the expression of μ_E.

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$$\frac{dX}{dt} = \mu_X X = \left(\frac{\mu_{G,\max} G}{K_G + G} + \frac{\mu_{M,\max} M\xi}{K_M + M}\right)\eta X \tag{50}$$

$$\frac{dG}{dt} = -\mu_G X = -\left(\frac{\mu_{G,\max} G}{Y_{X/G}(K_G + G)}\eta\right)X \tag{51}$$

$$\frac{dM}{dt} = -\mu_M = -\left(\frac{\mu_{M,\max} M\xi}{Y_{X/M}(K_M + M)}\eta\right)X \tag{52}$$

$$\frac{dE}{dt} = \mu_E X = \left(\frac{Y_{E/G}\mu_{G,\max} G}{Y_{X/G}(K_G + G)} + \frac{Y_{E/M}\mu_{M,\max} M\xi}{Y_{X/M}(K_M + M)}\right)\eta X \tag{53}$$

$$\eta = \left(1 - \frac{X}{X_{\max}}\right)\left(1 - \frac{E}{E_{\max}}\right) \tag{54}$$

$$\xi = \frac{1}{1 + G/k_i} \tag{55}$$

4. Loss of cell viability

The loss of cell viability during continuous ethanol fermentation processes with high cell density has been observed by many authors; however, few studies consider this phenomenon in the kinetic modeling of the process.

Jarzebski et al. [47] studied a continuous system of ethanol fermentation consisting of a perfect mixture reactor and a filter with a membrane for separation and posterior recycling of the cells for the fermenter. These authors compared the predictions of an intrinsic model, in which the loss of cell viability was considered, with the predictions of a modified nonintrinsic model where this phenomenon was not considered. The authors concluded that the predictions provided by the two models were similar, and for proposals of simulation and additional analyses of the process, both models could be used. The intrinsic model was thus called because the substrate and ethanol concentrations in this model are defined as regards a corrected volume that neglects the volume occupied by the cells in systems with high cell densities. Monbouquette [61] presented a detailed mathematical development for the formulation of mass balance equations in terms of these intrinsic concentrations.

Lafforgue-Delorme et al. [62] studying a system similar to that of Jarzebski et al. [47] pointed out the need to consider other factors other than the dilution rate and concentration of yeasts that would be important for the modeling of processes with high cell densities, as is the case of continuous ethanol fermentations with cell recycling. These authors developed a model considering the following aspects: dilution and yeast purge, broth viscosity, filter plugging, limitation by substrate, physiological state of the yeasts (cell viability), and inhibition phenomena linked both to ethanol and biomass. They also introduced the concept of steric "stress," according to which, at high cell densities, there would be a reduction in the specific growth rate due to the lack of space for cell division. The effects of inhibition, owing to high cell

concentrations and steric stress, were described, respectively, by the terms $K_X/(K_X + X_V)$ and $(1-X/X_m)$, where K_X is an empirical constant, X_v is the viable cell concentration, and X is the total concentration of cells (viable + nonviable). The final expressions of μ_X and μ_P in the proposed model are given by Eqs. (56) and (57). The predictions of the model agreed satisfactorily with the experimental data both for the operation of the bioreactor with total recycle as well as for the operation with partial recycle.

$$\mu_X = \left(\frac{\mu_{\max} S}{K_S + S}\right) \left(1-\frac{P}{P_m}\right)\left(\frac{K_X}{K_X + X_V}\right)\left(1-\frac{X}{X_m}\right) \tag{56}$$

$$\mu_p = \mu_{pm} \exp\left(-\frac{K_P X_V}{D}\right) \tag{57}$$

Augusto [63], investigating the influence of the specific rate of oxygen consumption in a continuous ethanol fermentation with high cell density, established the range of 0.1–0.8 mmol O_2/(g-cell h) as being that which the oxygen participates in the metabolism as a micronutrient that is essential to the synthesis of the cell membrane compounds, which would in turn increase the tolerance of the membrane to ethanol and to other inhibitors produced in the fermentation. The greatest tolerance resulted in a lower specific rate of cell death and in a greater efficiency of substrate conversion in ethanol due to the reduction in the value of the maintenance coefficient by the activation of the oxidative catabolic pathway. According to this author, this range of oxygen consumption for which the positive effects of this nutrient are observed in the bioconversion of the substrate would be dependent on the microorganism used, on the fermentation medium, and on the mode in which the process is conducted (batch, fed-batch, and continuous). To calculate the many parameters of fermentation, Augusto [63] segregated the microbial population into viable and nonviable cells, this procedure being possible due to the availability of experimental measures of the concentration of each type of cell separately.

Hojo et al. [41], studying the ethanol production with a strain of flocculating yeast in CSTR with and without cell recycle, concluded that the cell viability was of utmost importance in developing the mathematical model of the process with cell recycle and that cell death is a phenomenon that should be considered in the kinetic modeling of prolonged continuous fermentations in cases in which the hydraulic residence time is high. The kinetic expressions for the specific rates of cell growth (μ_X), substrate consumption (μ_S), ethanol production (μ_P), and cell death (μ_d) were represented by

$$\mu_X = \left(\frac{\mu_{\max} S}{K_S + S}\right) \left(1-\frac{P}{P^*}\right)^n ; \mu_{\max} = 0.6/\text{h}^{-1},\ K_S = 0.57\ \text{g/L}\ ,\ P^* = 80\ \text{g/L},\ n = 1.8 \tag{58}$$

$$\mu_S = \frac{\mu_X}{Y_g}; Y_g = 0.014\ \text{g/g} \tag{59}$$

$$\mu_p = A + B\mu_S; A = 0.065\ \text{g/g/h}; B = 2.24\ \text{g/g} \tag{60}$$

$$\mu_d = k_d; k_d = 0.0054\ \text{h}^{-1} \tag{61}$$

In the aforementioned works, it was considered that the microbial population consisted solely of viable and nonviable cells, with the latter being incapable of growing and producing the

desired product. Although inactive in both processes, it was assumed that the nonviable cells remained intact, which means that cell lysis phenomenon was not considered.

For Borzani [64], when intending to apply such an approach, the segregation of the microbial population must be performed considering the active and inactive cells in the growth process, as well as the active and inactive cells in the production process. According to Borzani [64], this differentiation is justified by the fact that a cell that is considered to be active in a given process may not be active in another, or vice-versa. Though quite realistic, this approach is rarely applied, given the enormous experimental difficulty to quantify the concentration of each group of cells separately.

Using an approach that is quite similar to that suggested by Borzani [64], Ghommidh et al. [65], modeling the oscillatory behavior of *Z. mobilis* in continuous cultures for ethanol production, segregated the microbial population in three distinct groups: viable cells that grow and produce ethanol (X_v), nonviable cells that do not grow but produce ethanol (X_{nv}), and dead cells (X_d). The processes of ethanol production, cell growth, loss of viability, and cell death were represented according to the scheme shown in **Figure 3**.

Starting from the scheme proposed by Ghommidh et al. [65], Jarzebski [66] modeled the oscillatory behavior of the state variables X, S, and P in a continuous ethanol fermentation process with *S. cerevisiae*, introducing the concept of combined effect of inhibition by substrate and ethanol simultaneously, since, according to that author, the inhibition by substrate would depend on the ethanol concentration and vice-versa. Taking into account this combined effect of inhibition, Jarzebski [66] proposed the following equations to describe the specific rates of viable cell growth (μ_v), conversion of viable cells into nonviable cells (μ_{nv}), and cell death (μ_d):

$$\mu_v = \left(\frac{\mu_{\max} S}{K_1 + S}\right)\left(1 - \frac{P}{P_c}\frac{S}{K_2 + S}\right) \text{ for } P < P_c(K_2 + S)/S \tag{62}$$

$$\mu_{nv} = \left(\frac{\mu_{\max} S}{K_1 + S}\right)\left(1 - \frac{P}{P_c'}\frac{S}{K_2 + S}\right) - \mu_v \text{ for } P < P_c'(K_2 + S)/S \tag{63}$$

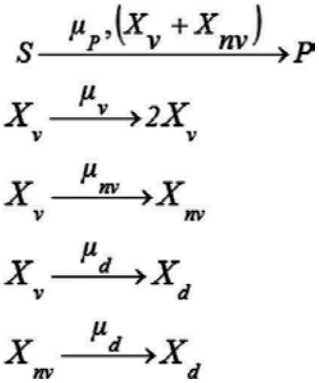

Figure 3. Schematic representation of the cell processes involved in ethanol production by *Zymomonas mobilis* in continuous cultures, according to the model proposed by Ghommidh et al. [65].

$$\mu_d = -\mu_v \text{ for } P < P_c^{'}(K_2 + S)/S \tag{64}$$

Watt et al. [67], using the mathematical model proposed by Jarzebski [66], simulated the continuous ethanol fermentation process for different feed volumetric flow rates and substrate concentrations in the feed stream.

The mathematical modeling of ethanol fermentation processes in which the loss of cell viability occurs is generally conducted by dividing the cell population into two distinct groups: viable cells (X_v) which would be growing and producing ethanol and nonviable or dead cells (X_d), which would be inactive in both processes [68]. The conversion rate from the viable to the nonviable cells is considered to be the first order regarding the concentration of viable cells [12]. The specific rates of cell growth, ethanol production, substrate consumption, and loss of cell viability are defined as regards the viable cell concentration, which refer to the effectively active cells in all of these processes. Mass balance equations for viable and nonviable cells are developed separately. The mass balance equations for ethanol and substrate are similar to those of the conventional model (model that does not incorporate the loss of cell viability) with the previously discussed modifications in the terms involving the specific rates.

Based on these premises, Oliveira et al. [69] developed a mathematical model for a continuous ethanol fermentation process in a tower bioreactor with recycle of flocculating yeasts, in which the loss of cell viability was considered and the predictions of this model were compared with those of the conventional model. Both models provide similar predictions and were equally appropriate for the fermentation process modeling. Later, in another publication, the authors analyzed the scale-up effects on the kinetic parameters and on the predictions of the modified model, and found changes in the values of some of the parameters [70]. In addition, the predictions of the modified model agreed better with the experimental data than did those of the conventional model, especially for the cell concentration variable.

A better description of the fermentation process by the modified model is always the desired result, primarily in those cases in which the levels of cell viability are significantly different than 100%. The cell viability level in ethanol fermentations with high yeast densities has been reported as being strongly dependent on the rate of aeration imposed upon the system [34], varying from 40% to 90% [10, 34, 38, 71]. Under anaerobic conditions, unsaturated fatty acids are not synthesized and the yeasts become more sensitive to ethanol [72]. However, the high levels of cell viability in aerated systems are achieved at the expense of the reduction in ethanol yields [70]. Thus, the rate of aeration is an important variable to be optimized in these systems, seeking to provide an adequate level of oxygen dissolved in the medium [70].

Other aforementioned works in which the segregated approach, regarding cell viability, was applied to describe the microbial population are as follows: Kalil et al. [54], Atala et al. [21], Costa Filho et al. [56], Nelson and Hamzah [53], and Watt et al. [67].

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5. Conclusions

The facility to model the kinetics of ethanol fermentation processes is due to the fact that the governing factors of these processes (limitation by substrate, inhibition, loss of cell viability, death, among others) are well known and that they have a large quantity of mathematical models that have already been developed and made available within the literature.

The present work compiles, in a single publication, a reasonable quantity of kinetic models that are potentially applicable to the adjustment of experimental data of ethanol fermentation processes obtained under the broadest and most varied operating conditions. The models can also be applied to the production processes of another generation, such as is the case of obtaining ethanol from lignocellulosic feedstocks (second-generation bioethanol) for which the literature presents the use of such models as being confirmed by the following recent publications:

- Scott et al. [73]: Attainable region analysis for continuous production of second-generation bioethanol.
- Vásquez et al. [74]: Modeling of a simultaneous saccharification and fermentation process for ethanol production from lignocellulosic wastes by *K. marxianus*.
- Liu et al. [75]: Fermentation Process Modeling with Levenberg-Marquardt Algorithm and Runge-Kutta Method on Ethanol Production by *S. cerevisiae*.

In general, many fermentation studies have confirmed that the unstructured models poorly describe dynamic experiments in which composition and biomass activity change [13, 15]. By contrast, the use of a more detailed approach of cell metabolism, aimed at better describing the dynamic behavior of the process, can lead to the development of structured models containing a large number of variables and parameters. In these cases, the parameter estimation can become a difficult task due to the large experimental effort required and to the need to apply complex numerical methods, which can lead to obtaining parameter values without physical meaning. To illustrate such a scenario, Rivera et al. [76] used a structured model to interpret experimental data of a tower bioreactor for ethanol production by immobilized *S. cerevisiae.* The model contains 34 kinetic parameters and 9 parameters related to the glycolytic and respiratory (tricarboxylic acid [TCA]) pathways. Thus, greater experimental and computational efforts would be required to estimate the parameters associated with this mathematical model.

The class of structured models that are potentially useful is formed by simply applying the structured formulation, through which the description of the quantity and of the biomass properties is performed by using two or three variables, resulting in the so-called two- or three-compartment models. These models combine a better description of the system's behavior with a reasonable mathematical complexity and a smaller number of parameters [77].

Therefore, it is important to balance the complexity of the model with its identification and to seek expressions that are as simple as possible and that are capable of accurately describing the process in both dynamic and steady states [69].

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Chapter 7

Microbial Population Optimization for Control and Improvement of Dark Hydrogen Fermentation

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Additional information is available at the end of the chapter

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Abstract

Dark hydrogen fermentation (DHF) is a process that can achieve two simultaneous objectives: the production of bioenergy and reduction of pollution. Complex microbiological communities containing efficient producers of hydrogen usually carry out the process. Ordinarily, control and operation strategies optimized the process by chemical and physical factors that usually provide only short-term solutions and adverse effects on microbial properties. Microbial population optimization methods are designed to overcome these problems using knowledge on microbiological aspects, especially regarding optimizing microbial community structure and property. Optimizing microbial community structure and property should be an explicit aim for the (i) design and operation of reactors for DHF process, (ii) creating conditions that select for the stable and productive growth of desired microbes, and (iii) preventing or limiting growth of organisms that would be reducing hydrogen yields. Microbial population optimization could be managed by biostimulization by adding nutrient species specific for their community, bioaugmentation by adding dominant species or efficient hydrogen-producing bacteria into the system, and online process control for maintaining their community.

Keywords: dark fermentation processes, biohydrogen production, sludge population optimization, molecular biological techniques, microbial community structure

1. Introduction

In recent years, the worldwide awareness of global climate change, urban air pollution, and security of future supply of energy carriers stimulates the study on alternative fuels. Hydrogen is a clean and promising fuel when it is ultimately derived from renewable energy sources. It is also efficient and environmentally friendly, as it has high energy content and water is the sole

end product [1, 2]. Today, approximately 95% of commercial hydrogen is generated by steam reforming of natural gas and gasification of coal [3]. As these processes use fossil fuels, they are not environmentally friendly. An alternative way to circumvent the dependence of hydrogen production from fossil fuels is to utilize the potential of hydrogen producing microorganisms to drive hydrogen from widely available biomass. Given these perspectives, biological hydrogen production hashigh potential as an alternative energy source. Dark fermentative hydrogen production from wastewater yields relatively higher hydrogen production rates than other biohydrogen production processes [4], with the benefit that the substrate cost (wastewater) is free. For example, a fermentative hydrogen-producing process produces hydrogen at a higher rate (0.5–65.0 l $H_2 l^{-1} d^{-1}$) compared to a light-driven process (0.04–4.3 l $H_2 l^{-1} d^{-1}$) [5]. In addition, the major advantages are low energy demands, resulting in minimal pollution, operation without light sources, no oxygen limitation problems, and low capital costs for at least small-scale production facilities (100–1000 $m^3 H_2 \cdot h^{-1}$) [5, 6–9]. Both mesophilic and thermophilic continuous dark fermentative hydrogen production have been investigated. Thermophilic operation may be particularly appropriate when meeting legislation for the treatment of feedstock containing pathogens or coupled to a process with associated waste heat. Otherwise, because of the energy input needed, thermophilic operation is less likely to be the technically and economically favored option.

An economically feasible biological approach for hydrogen generation is the conversion of (often negatively valued) organic wastes into hydrogen-rich gas using fermentative bacteria [2, 10]. Various organic waste materials and wastewater from corn, palm oil, soybean, and meat processing plants have been studied for hydrogen production [11, 12]. As dark-fermentative hydrogen production processes involve non-sterile feedstock, mixed microflora derived from natural sources has been commonly used. Theoretically, 4 moles of hydrogen are produced from glucose concomitantly with 2 moles of acetate (Eq. 1,3) with only 2 moles of hydrogen produced when butyrate is the main fermentation product (Eq. 2,4). From the above reactions, it can be concluded that the highest theoretical yield of hydrogen is associated with acetic acid as the fermentation end product. In practice, however, when contents of acetic acid and butyrate in mixture are higher than that of propionate, the yield of hydrogen is higher than in other cases [6, 13]. Typically, 60–70% of the aqueous product during sugar fermentation is butyrate and low hydrogen yields (up to 2.5-2.9 mol H_2/mol glucose) compared to the theoretical yield of 4 mol H_2/mol glucose for fermentation with only acetate as liquid end fermentation product [14]. Hydrogen yields can be improved by increasing hydrogen production through reaction (1) and decreasing or preventing reaction (2). This could be accomplished through dark hydrogen fermentation (DHF) with thermophiles or extreme thermophiles, operating at temperatures above 60°C [15, 16].

Mesophilic (35°C)

$$C_6H_{12}O_6 + 2H_2O \rightarrow 4H_2 + 2CO_2 + 2C_2H_4O_2 \rightarrow \Delta G^\circ = -184.2 \text{ kJ mol}^{-1} \quad (1)$$

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$$C_6H_{12}O_6 \rightarrow 2H_2 + 2CO_2 + C_4H_8O_2 \rightarrow \Delta G^\circ = -244.2 \text{ kJ mol}^{-1} \quad (2)$$

Thermophilic (60°C)

$$C_6H_{12}O_6 + 2H_2O \rightarrow 4H_2 + 2CO_2 + 2C_2H_4O_2 \rightarrow \Delta G^\circ = -20.1 \text{ kJ mol}^{-1} \quad (3)$$

$$C_6H_{12}O_6 \rightarrow 2H_2 + 2CO_2 + C_4H_8O_2 \rightarrow \Delta G^\circ = -84.2 \text{ kJ mol}^{-1} \quad (4)$$

Higher temperatures thermodynamically favor hydrogen production. Besides, elevated temperatures contribute to better pathogenic destruction and limit hydrogen consumption by hydrogen consumers (methanogens, homoacetogens, sulfate reducers). Normally 67% of the original organic matter will remain in solution (chemical oxygen demand (COD) basis) under optimal conditions of the DHF process. For achieving a full gain of chemical energy preserved in biomass, a coupled process is required that involves the recovery of the remaining organic matter and production of methane, electricity, bioplastics, and hydrogen by photofermentation process. Two-stage processes are already well developed, and they could conceivably be adapted for both hydrogen and methane production [17], and hydrogen and electricity generated from microbial fuel cells [18]. The efficiency of DHF from food waste in anaerobic mesophilic and thermophilic acidogenesis, followed by a two-phase digestion or photofermentation, has also been assessed [19]. Overall, many technologies for the improvement of biohydrogen production have been increasingly examined to determine their likely successful industrial implementation and sustainability for the generation of alternative renewable bioenergy.

A large number of microbial species, including strict and facultative anaerobic chemoheterotrophs such as Clostridia, Enteric bacteria, *Caldicellulosiruptor* spp., *Thermotoga* spp., and *Thermoanaerobacterium* spp., are efficient producers of hydrogen, while degrading various types of carbohydrates [20]. When using mixed microflora, experimental conditions to suppress methanogenic activity (which consumes hydrogen) and favor hydrogen producing metabolism are necessary. These include inoculum conditioning, optimizing operating conditions such as hydraulic retention time (HRT), pH and substrate concentration, and reducing hydrogen partial pressure [4, 7, 21]**.** Some challenges for optimizing dark hydrogen fermentation processes have been summarized by Hawkes et al. [7] and there has been considerable progress in research in the last few years, although an economically and technically feasible process is not yet established. In general, control and operation strategies are used to optimize the process by chemical, physical, and biological factors independently that usually provide only short-term solutions by adversely affecting the microbial properties of the system. The process is usually carried out by complex microbiological communities containing efficient producers of hydrogen. Recently, many studies [19, 22–28] have demonstrated molecular evidence related to these various effects. Most of *Clostridium* species have been recognized as desirable bacteria for mesophilic, whereas *Thermoanaerobacterium* species,

C. thermocellum, *C. cellulose*, and *C. thermoamyloticum* have been recognized as desirable bacteria for thermophilic conditions. Knowledge and information of microbial community structure and function is the key to improvement of hydrogen productivities through microbial population optimization. Microbial population optimization is a solution based on the existing knowledge of the microbial community data to overcome various technical barriers, such as low hydrogen yields, biomass washout, inhibition by hydrogen, non-stable hydrogen production, and short-time reactor operation. Microbial population optimization requires an integrated knowledge of the microbiology and the physicochemical characteristics of the process. Knowledge on microbiological aspects includes microbial consortia structure and function, the interactions that occur within, and the microbial key players for hydrogen production and their kinetics. The strategies that can be employed following an analysis of the population structure and function include controlling the growth of undesirable microorganisms (i.e., methanogens, propionic acid bacteria, and lactic acid bacteria) that consume hydrogen, while enhancing the numbers and stability of the hydrogen-producing bacteria.

2. The dark hydrogen fermentation process

2.1. Basic principle for dark hydrogen fermentation

Fermentative hydrogen production yields theoretically a maximum of 4 moles (498 ml-H_2/g^{-1} glucose) of hydrogen from glucose concomitantly with 2 moles of acetate, and 2 moles (249 ml-H_2/g^{-1} glucose) of hydrogen are produced from glucose concomitantly with 1 moles of butyrate. A large number of microbial species, including strict and facultative anaerobic chemoheterotrophs, such as *Clostridia*, enteric bacteria, and *Thermoanaerobacterium*, are efficient producers of hydrogen. Fermentation of glucose to hydrogen, pyruvate, and acetyl CoA, which can be converted to acetyl phosphate, subsequently results in the generation of ATP and the excretion of acetate. Pyruvate oxidation to acetyl CoA requires reduction by ferredoxin (Fd). Reduced Fd is oxidized by hydrogenase, which generates oxidized Fd and releases electrons as molecular hydrogen (Eq.5–8). The practical yield is even lower when other metabolic compounds such as propionate, ethanol, and lactate are produced as the fermentation products. These metabolic products bypass the major hydrogen-producing reaction in carbohydrate fermentation as a consequence of thermodynamic limitations [9].

$$C_6H_{12}O_6 + 2H_2O \rightarrow 4H_2 + \rightarrow 2CO_2 + 2C_2H_4O_2 \tag{5}$$

$$C_6H_{12}O_6 \rightarrow 2H_2 + \rightarrow 2CO_2 + C_4H_8O_2 \tag{6}$$

$$\text{Pyruvate} + \text{CoA} + 2\text{Fd}(\text{ox}) \rightarrow \text{Acetyl-CoA} + 2\text{Fd}(\text{red}) + CO_2 \tag{7}$$

http://dx.doi.org/10.5772/64208

$$2\,\mathrm{Fd(red)} \rightarrow 2\,\mathrm{Fd(ox)} + \mathrm{H_2} \quad (8)$$

The proton-reducing ability of Fd_{red} and NADH is thermodynamically limited by the maximum hydrogen partial pressures (P_{H2}) of 0.3 and $6x10^{-4}$ atm (60 Pa), respectively. This confers that as long as the P_{H2} is still less than 0.3 atm, hydrogen production can continue with transferring electrons from Fd_{red} which contains electrons from oxidative decarboxylation of pyruvate by pyruvate:ferredoxin oxidoreductase (PFOR). Meanwhile, the oxidation of NADH by NADH:Fd oxidoreductase (NFOR) can generate Fd_{red} that subsequently generates additional hydrogen when the P_{H2} is maintained less than 60 Pa. However, the P_{H2} limited to hydrogen generation via the oxidation of NADH could be increased to 0.1–0.2 atm at a temperature of 70°C [16]. Therefore, increasing cultivation temperature is necessary to overcome thermodynamic limitation, thereby resulting in a decrease of the Gibbs free energy of conversion according to the second law of thermodynamics ($\Delta G = \Delta H\text{-}T\,\Delta S$) [29]. Thermophilic microorganisms produce generally higher hydrogen yields compared to mesophiles because they are thermodynamically favorable [30]. High hydrogen yields in the range of 314.0–473.0 ml-H_2/g^{-1} sugars have been previously reported using thermophiles such as *C. thermocellum* and *Thermoanaerobacterium thermosaccharolyticum* and extreme thermophiles such as *Thermotoga elfi*, *Caldicellulosiruptor saccharilyticus,* and *Caldanaerobacter subterraneus* [15, 31–34]. In a practical sense, through controlling the fermentative types of microorganisms, it is possible to maximize the amount of hydrogen produced by fermentation.

2.2. Dark hydrogen fermentation by mixed cultures

Dark fermentation process in combination with environmental biotechnology in terms of organic wastes or residue treatment with industrial biotechnology that aims for hydrogen maximization and mixed culture fermentation could thereby become more attractive compared to pure culture fermentation, as mixed cultures are applied originally in the waste treatment fields. Compared to pure culture fermentation, mixed culture fermentation does not require sterilization of the media, offers better adaptation capacity due to its high microbial content and the possibility of mixed substrate co-fermentation, and also allows a continuous fermentation process [35]. Undefined mixed cultures taken from different natural sources need pretreatment or enrichment, by manipulating the operation of the fermentation process and/or by varying the sources of the natural inoculum in order to obtain the required metabolic capacities and the corresponding microbial population for development of the dark fermentation process [36, 37]. To prepare the inoculum for hydrogen production by fermentation of carbohydrates, the original anaerobic sludge is first pretreated to suppress methanogenic archaea, which consume hydrogen generated and subsequently enrich hydrogen-producing bacteria in various reactor configurations [38]. Pretreating anaerobic seed sludge under harsh conditions, spore-forming bacteria involved in anaerobic conversion of carbohydrates to hydrogen could have a better chance to survive compared to the non-spore-forming methanogenic archaea. The spores formed can be activated when the required environmental conditions are provided during subsequent enriching for hydrogen production [39]. Methods,

including heat shock, load shock, acid, base, and chemical pretreatments are usually applied to pretreat anaerobic seed sludge for fermentative hydrogen production

2.2.1. Heat shock

Heat shock has been the most common and effective method for eliminating methanogenic archaea and is achieved by steam heating the seed sludge at 75–121°C with an exposure time between 15 and 120 min, which is relatively easy and inexpensive. The heat shock may also suppress the activity of non-spore-forming propionate producers, but could not effectively deactivate homoacetogens [21, 40]. The existence of homoacetogenic bacteria results in a decrease of hydrogen production because these bacteria further consume hydrogen produced from the fermentation process for the production of acetate [41]. In addition, Duangmanee et al. [42] have previously observed that an inoculum pretreated by heat shock was not stable for hydrogen production in the continuous reactor, and a repeated heat treatment was needed every month to maintain some stability in hydrogen production.

2.2.2. Load shock

During load shock using the pulse load technique in batch and organic fermentation, or hydraulic shock in continuous fermentation, volatile fatty acids (VFAs) tend to accumulate in the fermentative reactor in high concentrations, associated with acidic conditions, and they inhibit methanogens [42, 43]. Applying a load shock with a pulse load of about 40–50 g-sugar/l, the pretreated anaerobic sludge effectively suppressed methanogenic activity [24, 44]. Furthermore, O-Thong et al. [24] have described that load shock-pretreated seed sludge could result in high level of hydrogen production similar to the heat shock-pretreated seed sludge and that load shock would be technically easier to do and more economical than heat shock for implementation on an industrial scale.

2.2.3. Acid and alkali pretreatment

The bioactivity of methanogens during the conventional anaerobic process treatment of organic wastes occurs in neutral to slightly alkaline environments (pH 6.8–8.0) [38]. Limiting methanogenesis can be achieved by adjusting the acidity of the anaerobic sludge substantially away from the preferable range to either pH 3–4 or pH 12. The acid or alkali pretreatment is considered to be technically easier than the heat shock pretreatment for industrial scale implementation [21]; however, the inoculum obtained from an acid or alkali pretreatment requires a much longer acclimatization time of 10 to 30 days to establish hydrogen production [45].

2.2.4. Methanogen inhibitors

2-bromoethanesulfonate acid (BESA), an analog of the coenzyme-M in methanogens, is a chemical that deactivates methanogens. Using BESA at concentrations of 25–100 mM has been found to effectively inhibit the bioactivity of methanogens; however, treating an anaerobic sludge at these levels would not be cost effective for a commercial scale operation [39].

http://dx.doi.org/10.5772/64208

3. Molecular methods for microbial community structure and function studies

Molecular monitoring techniques such as fluorescence in situ hybridization (FISH) [46], a combination of FISH and microautoradiography (FISH–MAR) [47], stable isotope probing (SIP) [48], denaturing gradient gel electrophoresis (DGGE) [49], ribosomal intergenic spacer analysis (RISA) [23], and clone libraries have been developed for studying microbial community and function. These methods are used intensively in natural and engineered systems for wastewater treatment. Principles of and deeper insights on these molecular tools are available elsewhere (e.g. [50]). Among these techniques, cloning and the creation of a gene library, DGGE, TRFLP, RISA, and FISH stand out. DGGE was one of the first techniques used to describe DHF microflora [51, 52]. DGGE is a rapid and simple method that provides characteristic band patterns for different samples, allowing quick sample profiling, while retaining the possibility of a more thorough genetic analysis by sequencing of particular bands. DGGE provides information about the structure of microbial communities and can relatively quantify species abundance through DNA band intensities. Cloning provides very precise taxonomical information, but is time consuming and requires specialized personnel and hence, its introduction in the DHF process has been slow. FISH helps identify microorganisms at any desired taxonomical level, depending on the specificity of the probe used. It is the only quantitative molecular biology technique, although quantification is either complex or tedious and subjective. Combination with a confocal laser scanning microscope allows the visualization of three-dimensional microbial structures (granules and biofilms). Both DGGE and FISH have been extensively employed. Other techniques such as RISA [23] provide information on microbial diversity and species dominance. The advantages and disadvantages of the molecular techniques frequently applied to microbial ecology research in DHF process are shown in **Table 1**.

Molecular methods	Nucleic acid extraction	PCR	Advantages	Disadvantages
Fluorescence *in situ* hybridization (FISH)	No	No	- Direct analysis and quantification - Suitable for targeting specific group/species -Easy and fast if required -Allows direct visualization of non-cultured microorganisms -Differential/preferential detection of active microorganisms	- Require genes/RNA with high number of copies - Limit for total diversity mapping - The design of a specific and unambiguously restrictive probe for a certain group of microorganisms is not always possible - The design and optimization of a new probe is a difficult process - Structural analysis of granular requires a confocal microscope
Denaturing gradient gel	yes	yes	- Permits rapid and simple monitoring of the spatial	- Bias from PCR

Molecular methods	Nucleic acid extraction	PCR	Advantages	Disadvantages
electrophoresis (DGGE)			temporal variability of microbial populations if just band patterns are considered - It is relatively easy to obtain an overview of the dominant species of an ecosystem - It is adequate for analysis of a large number of samples (far more than cloning)	- The number of detected bands is usually small, which implies: the number of identified species is also small; the bands correspond, although not necessarily, to the predominant species in the original sample - The sequences of the bands obtained from a gel correspond to short DNA fragments (200–600 bp), and so phylogenetic relations are less reliably established than with cloning of the whole 16S rRNA gene. In addition, short sequences are less useful for designing new specific primers and probes. - GC content of the amplified DNA can modulate the *Tag* polymerase activity
Cloning and sequencing	Yes	Yes	- Contain larger sequence - Complete 16S rRNA sequencing allows: very precise taxonomic studies and phylogenetic trees of high resolution to be obtained - Identification of microorganisms that have not been yet cultured or identified - Covers most microorganisms, including minority groups, which would be hard to detect with genetic fingerprinting methods	- A large number of clones must be sequenced for positive diversity - Sequences need to be compared with each other and libraries - Very time consuming and laborious, making it unpractical for high sample throughput - It is not quantitative. The PCR step can favor certain species due to differences in DNA target site accessibility - Bias from PCR, total universal primers cannot be totally universal bacteria - Exponential amplification of the DNA mixture may result in ratio discrepancies between the amplified 16S rDNA fragments and the original mixture

Table 1. Brief description of frequently molecular methods that have been used for microbial community analysis in dark fermentative hydrogen production.

For decades, a biological reactor has been considered as a black box. The new insights in microbiology have helped to improve the design and performance of new generation reactors [53, 54]. Probably it is true that it is not essential to know the phylogenetic position of the individual microorganisms that dwell inside a system for the design of a biohydrogen facility. But the knowledge on microbial community structure and function is needed. The more recent reports on microbial community structures of DHF processes still interpret the results with reactor performance and metabolic by-products (indirect function) [55, 56]. However, we are

http://dx.doi.org/10.5772/64208

still uncertain regarding which microorganisms can function effectively in DHF and whether the whole community takes part. Thus, deeper insight into the function is required, not just community structure. The latter is due to a general shortcoming in all these molecular tools. However, some attempts have been made in this direction, as FISH–MAR and FISH combined with biosensors could be applied to reveal the microbial community structure and function in parallel. Furthermore, other techniques such as DNA microarrays are being developed with the goal of being able to infer the in situ physiology of the microorganisms [57], and these should find application in the hydrogen-producing biosystems.

Post-genomic research and systems biology tools such as metaproteomics will greatly contribute to the development by providing functional performance insights of the microorganisms and their metabolism [55]. Recent work on post-genomics involving microbial ecosystems has expanded to both natural microbial biofilms and activated sludge [56, 58]. These cutting-edge technologies are aimed at using new molecular tools to understand the microbial community structures in relation to functions [55] or metabolic transformations [58]. It is commonly known that 16S rRNA genes approaches have copy numbers and PCR bias problems. Housekeeping genes with a single copy are now the focus for population genomic analysis. Multilocus sequence typing (MLST) of housekeeping genes could provide a deeper insight on how microbial populations evolved [59]. These modern molecular monitoring techniques are vital tools and could also be applied for DHF, as they will particularly break new ground for the quantification and dynamics of microorganisms in complex consortia.

A whole variety of analytical methods for both microbial community structure and function are now available. However, microbiologists and engineers should take efforts to apply these tools for quantitative studies of DHF. With a more thorough understanding of the microbial community and its dynamics, an improvement of expectations and optimization of fermentative processes will be possible. The microbial community structure and microbial function may be further optimized by adding species and specific nutrients for the dominant species.

4. Molecular evidence in dark hydrogen fermentation processes

4.1. Effect of inoculum types and conditioning on microbial community structure

It has been previously reported that the methods for seed preparation can affect both start up and overall efficiency of the hydrogen-producing reactors [7]. Quick recovery from process upsets in full-scale applications may also require large quantities of readily available hydrogen-producing seeds. Therefore, induction of hydrogen accumulation in fermentative consortia is related to the inhibition of hydrogen consumers which is essential for its further scale-up and industrial applications. Several types of inocula have been used for anaerobic hydrogen fermentation, such as anaerobic-digested sludge [60], sewage treatment sludge [61], agricultural soil [62], sludge compost [63], and isolated bacteria [64]. In addition, several methods are used for conditioning inocula such as acid conditioning [65], heat conditioning [60, 62], chemicals conditioning such as 2-bromoethanesulfonic acid (BESA) [66], short hydraulic retention time (HRT) without conditioning [67], and overload conditioning [24]. All condi-

tioning methods aid in inhibiting methane formation, as well as accelerating the enrichment of hydrogen producing bacteria, such as spore-forming *Clostridium* species, as these are highly tolerant to extreme environments [68]. The effect of conditioning on hydrogen production rates is inoculum dependent, with appreciable hydrogen production yields being demonstrated with anaerobic-digested sludge and agricultural soil [69]. Several studies (e.g. [23, 51, 70–73] reveal that heat-conditioned anaerobic-digested sludge guarantees the highest hydrogen production yields. Heat shock treatment of sludge gave highest hydrogen yield (2 mol H_2/mol glucose), while base treatment of sludge gave lowest hydrogen yield (0.48 mol H_2/mol glucose) [74]. Sung et al. [71] illustrated that hydrogen production using heat-treated seeds declined after 1-month operation and repeated heat treatment of sludge to recover from reactor every month is not credible. However, others claim that high yields can be achieved without heat treatment [68]. Zhu and Beland [75] have demonstrated that heat shock and acid treatment methods completely repressed methanogenic activity, while base treatment methods did not completely repress methanogenic activity and also significantly affected hydrogen production. Hwang et al. [76] reported that the acidic conditions (pH 4.5–6) can act as a weak inhibitor, but not complete long-term inhibition of methanogenic activity. Elsewhere, it has been shown that acid pretreatment is particularly effective for enhancing the growth of lactic acid bacteria (LAB) [52, 77]. Five methods for preparation of hydrogen-producing seeds (base, acid, 2-bromoethanesulfonic acid (BESA), and load shock and heat shock treatments) as well as an untreated anaerobic digested sludge were evaluated for their hydrogen production performance and responsible microbial community structures under thermophilic conditions (60°C) by O-Thong et al. [24]. The results showed that the load shock treatment method was the best for enriching thermophilic hydrogen-producing seeds from mixed anaerobic cultures as it completely repressed methanogenic activity and gave a maximum hydrogen production yield of 1.96 mol H_2 mol^{-1} hexose with a hydrogen production rate of 11.2 mmol H_2 l^{-1} h^{-1}.

In general, microbial profiles in fermentative production processes occur as a result of a combination of process conditions, such as feedstock characteristics, environmental conditions (pH, temperature, and H_2 partial pressure), and metabolic pathways existing in the microbes involved [51]. Iyer et al. [51] investigated hydrogen-producing bacterial communities from a heat-treated soil inoculum by RISA. They found that species of Clostridiaceae, Bacillaceae, and Enterobacteriaceae responded to hydrogen production at 30°C and a 30-h HRT. The gene pool at 30-h HRT, as determined by 16S rRNA gene sequences, was more diverse than at the 10-h HRT, as only Clostridiaceae were detected at this later point. The application of DGGE indicated *Clostridium tyrobutyricum*, *Lactobacillus ferintoshensis*, *Lactobacillus paracasei*, and *Coprothermobacter* species to be dominant in bacterial communities developed from pH-pretreated inocula [68]. *Lactobacillus* species are common coexisting bacteria in hydrogen fermentation processes. However, they have adverse effects on hydrogen production by competing for sugars and producing acidic products [78, 79]. Interference by lactic acid bacteria is often prevented by feedstock heat treatment at 50°C or by thermophilic fermentation at temperatures beyond 50°C [80]. Load shock and heat shock treatments under thermophilic conditions resulted in a dominance of *T. thermosaccharolyticum* while base- and acid-treated seeds were dominated by *Clostridium* and BESA-treated seeds were dominated by *Bacillus* sp. [24]. The comparative experimental results from hydrogen production performance and

http://dx.doi.org/10.5772/64208

microbial community analysis showed that the load shock treatment method was better than base- and acid-treated, heat shock, BESA-treated methods for enriching thermophilic hydrogen-producing seeds from anaerobic-digested sludge. Load shock-treated sludge was implemented in palm oil mill effluent (POME) fermentation and was found to give maximum hydrogen production rates of 13.34 mmol H_2 $l^{-1}h^{-1}$ and resulted in a dominance of *Thermoanaerobacterium* spp. Load shock treatment is an easy and practical method for enriching thermophilic hydrogen-producing bacteria from anaerobic-digested sludge. The efficiency of preparation methods could be considered based on hydrogen production yield together with microorganisms revealed in the process. Therefore, the microbiological aspects and hydrogen production performance information are needed to identify effective methods for preparation of hydrogen-producing seeds.

4.2. Effect of reactor design and operation on microbial community structure

Various reactor types seeded with the same inoculum and operating under similar process conditions could develop microbial communities with different properties. For instance, in batch mode under mesophilic conditions with glucose as a substrate, microbial communities became dominated by *Clostridium butyricum*-like species [51], *Clostridium* spp. [52] *C. butyricum* [81], and *Clostridium sp*_T5zd [77]. Conversely, a continuous stirred tank reactor (CSTR) was dominated by *Clostridium sporogenes*-like and *Clostridium celerecrescens*-like species [82]. Yet, in an anaerobic membrane reactor (MBR), the main population consisted of Clostridiaceae, Flexibacteraceae, *Clostridium acidisoli, Linmingia china,* and *Cytophaga* [23]. *Clostridium* spp. were also dominant in a CSTR used to produce hydrogen from sucrose at 35°C, pH 5.5, and HRT 12 h, as analyzed by DGGE [51, 71]. Xing et al. [83] followed communities in a CSTR operating on molasses at a low pH with acidophilic bacteria from sewage, which established an ethanol–acetate hydrogen-producing community after 28 days. This was also consistent with other studies, i.e., the hydrogen production rate increased with the increase of *Ethanologenbacterium* sp., *Clostridium* sp., and *Spirochaetes*. Some types of *Clostridium* sp., *Acidovorax* sp., *Kluyvera* sp., and *Bacteriodes* were found throughout all periods of reactor operation [83]. It appeared that hydrogen production depended not only on hydrogen producers but also on cometabolism in the whole community.

In common with many other systems, in batch fermentations without pH control, it has been found that microbial communities change with pH [77], and their biodiversity decreased considerably as the pH decreased from 6.5 to 4.5. Kim et al. [81] reported the effect of substrate concentration on dark hydrogen fermentation using a CSTR. At the peak of hydrogen production yields, all bacterial species detected by DGGE analysis were *Clostridium* spp. and at inlet sucrose concentrations below 20 gCOD l^{-1}, the hydrogen yield per hexose consumed decreased, while *Clostridium scatologenes* (an H_2-consuming acetogen) was found in the sludge. Moreover, it has been shown that short HRT operation without anaerobic sludge preparation allowed for more microbial diversity and increasing the system robustness [22]. Species that differ in optimal growth conditions but are metabolically similar are then present, sharing the same function. Such advantages allow for flexibility in performance when perturbations in process conditions occur. Overall, under mesophilic conditions, hydrogen may be produced

by a large group of bacteria such as the three main groups belonging to the low-GC(guanine-cytosine)gram positive bacteria, i.e., Clostridiaceae, Enterobacteriacea, and Bacillaceae. A number of studies have focused on the analysis of the 16S rRNA gene to understand the species richness of microbial communities in lab-scale reactors under mesophilic conditions, as shown in **Table 2**.

Substrate	Processes and operation condition	Dominating microorganisms	H_2 yield (mol H_2 mol^{-1} hexose)	H_2 rates (l H_2 $l^{-1}d^{-1}$)	References
Glucose	Batch experiment, pH 5.5 and 36°C	Clostridiaceae Enterobacteriaceae *Streptococcus bovis*	0.47	4.6	[52]
Carbohydrate-containing wastewater	Two-step process using CSTR, pH 5.5, HRT 6 h, 36°C and complete-mix cylindrical photoreactor, HRT 25 h, pH 8.0, 32°C	*Clostridia* *Rhodobacter capsulatus*	2.1 and 2.5	4.5 and 0.3	[84]
Glucose	Anaerobic membrane reactor, HRT 3.3 h, pH 5.5, mixed at 200 rpm and 35°C	Clostridiaceae Flexibacteracae *Clostridium acidisoli* *Linmingia china* *Cytophaga*	1.1	15.36	[23]
Food waste	CSTR, HRT 5 d, pH 5.6 and 35°C	*Thermotogales* *Bacillus* spp. *Prevotella species*	0.03–0.1	0.22	[19]
Sucrose	CSTR, HRT 24 h, 37°C and pH 5.5	*Clostridium* sp. *Bacillus* sp.	2.3	0.1	[71]
Glucose	CSTR, pH 5.5 and 30°C at 30 h and 10 h HRT	Bacillaceae Clostridiaceae Enterobacteriaceae Only Clostridiaceae at HRT 10 h	1.61	10.4	[51]
Rice slurry	Batch experiment, pH 4.5 and 45°C	*Clostridium* sp. 44a-T5zd	2.5	2.1	[77]
Sucrose	CSTR, gas sparging at 300 ml/min, pH 5.3 and 35°C	*Clostridium tyrobutyricum* *Clostridium ptoteolyticum* *Clostridium acidisoli*	1.68	6.45	[81]
Sucrose	CSTR, HRT 12 h, pH 6.8 and 35°C	*Clostridium ramosum*	0.9–3.5	9.1	[85]
Glucose	CSTR; glucose to peptone ratio (5:3) 35°C, pH 7 and HRT 12 h	*Clostridium sporogenes* *Clostridium celerecrescens*	0.6	6.8	[82]
Glucose	Batch; glucose to peptone ratio (5:3) 35°C, pH 7 and HRT 12 h	*Clostridium butyricum*	1.11	5.2	[83]

Table 2. Microbial community structure, operational conditions and reactor performance of fermentative hydrogen production process from various organic wastes under mesophilic condition.

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Different microbial community structures develop within different temperature regimes. For instance, a comparative study on hydrogen production from food waste between mesophilic and thermophilic acidogenic conditions revealed that biogas produced in thermophilic conditions was methane free, whereas methane was still detected under mesophilic conditions [19]. Species such as *Thermoanaerobacterium thermosaccharolytium* and *Desulfotomaculum geothermicum* were detected in the thermophilic acidogenic culture, while *Clostridium* and *Bacillus* species were detected in the mesophilic acidogenic culture with DGGE. The composition of microbial communities in thermophilic dark hydrogen fermentation production was investigated in more detail using quantitative FISH and DGGE [8, 22, 86]. This demonstrated that *Thermoanaerobacterium* made up almost half of the total community in thermophilic dark hydrogen fermentation production.

In thermophilic fermentative hydrogen production, a number of microbial species are known, including *C. thermoamylolyticum* [84], *C. cellulose, C. thermocellum, T. thermosaccharolytium* [22, 84], *D. geothermicum* [19], *Saccharococcus* sp. clone ETV-T2 [8], *Mitsuokella jalaludinii* [84]. *Thermoanaerobacterium* sp. and the related genotypes are found to be dominant in many thermophilic fermentations operating at 55°C and neutral pH with feedstocks, including starch, organic waste [22], and cellulose-rich materials [84]. Thus, many studies on microbial consortia of thermophilic fermentations resulted in the detection of the same dominant species. This is in contrast to observations from mesophilic fermentations, and it might therefore indicate that thermophilic conditions lead to a convergence of microbial populations. In this way, thermophilic reactors can provide an additional benefit for the application in sludge population optimization. One of the problems of bioreactor operation is washout of microorganisms. Trickling biofilter reactors (TBR) have been proposed as a solution to this problem, with continuous hydrogen production under thermophilic conditions being successfully demonstrated [22, 23]. In those studies, the TBR was dominated by *T. thermosaccharolytium* and *Clostridia* and *Bacilli* in the phylum *Firmicutes*.

Microbial community structure dynamics in the ASBR for biohydrogen production from palm oil mill effluent during changing of hydraulic retention time (HRT) and organic loading rate (OLR) was studied by denaturing gradient gel electrophoresis (DGGE) aiming at improved insight into the hydrogen fermentation microorganisms. The microbial community structure was strongly dependent on the HRT and OLR. DGGE profiling illustrated that *Thermoanaerobacterium* spp., such as *T. thermosaccharolyticum,* and *T. bryantii*, were dominant and probably played an important role in hydrogen production under thermophilic conditions. The shift in the microbial community from a dominance of *T. thermosaccharolyticum* to a community where *Caloramator proteoclasticus* also constituted a major component occurred at suboptimal HRT (1 d) and OLR (80 gCOD $l^{-1}d^{-1}$) conditions [25]. The information showed that the hydrogen production performance was closely correlated with the bacterial community structure. A number of studies have focused on the analysis of the 16S rRNA gene to understand the species richness of microbial communities in lab-scale reactors under thermophilic conditions, as shown in **Table 3**.

Substrate	Processes and operation condition	Dominating microorganisms	H_2 yield (mol H_2 mol^{-1} hexose)	H_2 rates (l H_2 $l^{-1}d^{-1}$)	References
Glucose	Fed batch experiment, HRT 0.5 d, pH 6.6, and 60°C	*Thermoanaerobacterium thermosaccharolyticum* KU-001	2.4	3.5	[70]
Cellulose	Batch experiment, stirring at 200 rpm, pH 6.4, and 60°C	*Clostridium* and *Bacillus T. thermosaccharolyticum Clostridium thermocellum Clostridium cellulosi*	2.0	1.35	[87]
Starch in wastewater	Batch experiment, pH 6.0 and 55°C	*Thermoanaerobacteriaceae Saccharococcus* sp. clone ETV-T2	0.68	2.8	[8]
Cellulose	Batch experiment, pH 6.5 and 55°C	*Thermoanaerobacterium Clostridium thermoamylolyticum*	0.2	0.82	[84]
Food waste	CSTR, HRT 5 d, pH 5.6, and 55°C	*Thermoanaerobacterium thermosaccharolytium Desulfotomaculum geothermicum*	0.9–1.8	4.56	[19]
Glucose	Trickling biofilter reactor (TBR), HRT 2 h and 55–64°C	*T. thermosaccharolyticum*	1.1	23.25	[22]
Food waste	CSTR; HRT 5 d, pH 5.5, and 55°C	*T. thermosaccharolyticum*	2.2	1.4	[86]
Artificial garbage slurry	Jar fermentor; HRT 1d, pH 6.0, and 60°C	*T. thermosaccharolyticum*	1.99	4.46	[88]

Table 3. Microbial community structure, operational conditions and reactor performance of fermentative hydrogen production process from various organic wastes under thermophilic condition.

In addition to volatile fatty acids (VFAs), anaerobic fermentations may also lead to the production of reduced end products such as ethanol, butanol, and lactate [5], thus reducing H_2 yield potential. Therefore, bacterial metabolism must avoid VFAs by efficient product removal [7, 8] or metabolic engineering. Stripping gas may be used to remove H_2 from the liquid phase to prevent product inhibition. N_2 is used often, but it increases the costs of H_2 purification. For economical reasons, CO_2 might be a better choice, as it is relatively easy to separate from the gas phase. Using CO_2 rather than N_2 for stripping H_2 resulted in a higher production of H_2 and butyrate [79, 89]. High CO_2 partial pressures had little effect on hydrogen-producing bacteria but were inhibitory to other competitive microorganisms such as acetogens and lactic acid bacteria. The microbial community structure under CO_2 sparging conditions was dominated by *C. tyrobutylicum*, *C. proteolyticum*, and *C. acidisoli*. CO_2 sparging has another

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beneficial effect on reactor performance by improving mixing and contact between substrate and microorganisms and also decreased the effects of hydrogen partial pressure [89, 90].

4.3. Microbial key players in dark hydrogen fermentation

Figure 1 summarizes the richness of the microbial key players of mesophiles. Fermentative hydrogen production has been studied for a large group of pure cultures, including species of *Enterobacter*, *Bacillus*, and *Clostridium*. However, hydrogen-producing microflora obtained from natural sources, which are able to survive on non-sterile substrates, contain mostly *Clostridium* spp., such as *C. butyricum*, *C. acidosoli*, *C. tyrobutylicum*, and *C. acetobutylicum*. Although the numbers of case studies are still low to infer solid conclusions, they indicate that the *Clostridium* genus represents the major group in dark mesophilic fermentation under mesophilic conditions. Various *Clostridium* species are found in mesophilic environments, but only four species are highly frequently observed (*C. acetobutylicum* (24%), *C. tyrobutyricum* (9%), *C. acidisoli* (16%), and *C. pasteurianum* (13%)) and related with high hydrogen yield [52,81]. However, *C. saccharolyticum, C. butyricum, C. sporogenes, C. celerecrescens, C. cellulosi*, and *C. beijerinkii* were also found to be strong hydrogen producers [83]. Others species (*Citrobacter sp., Sporolactobacillus racemicus, Streptococcus bovis*, and *B. racemilaticus*) that differ in optimal growth conditions from *Clostridium* but are metabolically similar are allow for flexibility in performance when perturbations in process conditions occur.

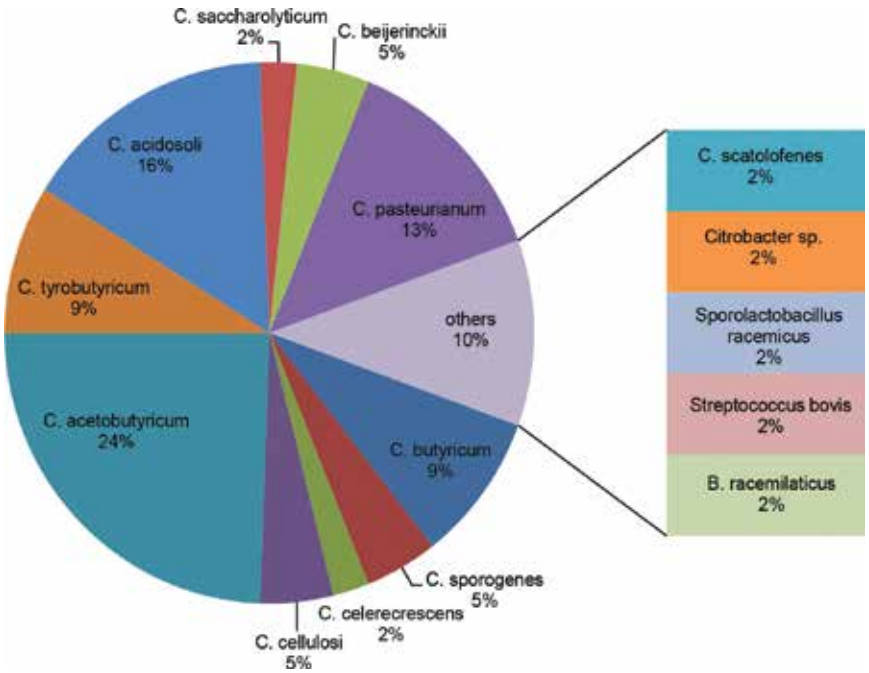

Figure 1. Summary of all fermentative hydrogen-producing bacteria frequently observed based on molecular tools studied under mesophilic conditions.

The *Thermoanaerobacterium* genus represents the major group in dark thermophilic fermentation. **Figure 2** summarizes the richness of the microbial key players of thermophiles. Thermophilic conditions clearly show that *T. thermosaccharolyticum* is a key player in fermentative hydrogen production. *Thermoanaerobacterium* spp. have also been found to dominate in a long-term hydrogen production reactor. Bacteria species are highly frequently observed under thermophilic conditions and they are *Thermoanaerobacterium* sp. (47%) and *T. thermosaccharolyticum* (30%). The microbial community structure of thermophilic mixed culture sludge used for biohydrogen production from palm oil mill effluent was analyzed by fluorescence in situ hybridization (FISH) and 16S rRNA gene clone library techniques. The microbial community was dominated by *Thermoanaerobacterium* species (~66%). The remaining microorganisms belonged to *Clostridium* and *Desulfotomaculum* spp. (~28% and ~6%, respectively). The hydrogen-producing bacteria were isolated and their ability to produce hydrogen was confirmed. Three hydrogen-producing strains, namely HPB-1, HPB-2, and HPB-3, were isolated. The 16S rRNA gene sequence analysis of HPB-1 and HPB-2 revealed a high similarity to *T. thermosaccharolyticum* (98.6% and 99.0%, respectively). The *Thermoanaerobacterium* sp. HPB-2 strain was a promising candidate for thermophilic fermentative hydrogen production with a hydrogen yield of 2.53 mol H_2 mol^{-1} hexose from organic waste and wastewater containing a mixture of hexose and pentose sugars. *Thermoanaerobacterium* species play a major role in thermophilic hydrogen production as confirmed by both molecular and cultivation-based analyses [91]. Various Clostridium species (*C. cellulose, C. thermoamyloticum, and C. thermocellum*) that differ in optimal growth conditions from *Thermoanaerobacterium* but are metabolically similar are allow for flexibility in performance when perturbations in process conditions occur. Other species (*Saccharococcus spp., D. geothermicum*, and *Bacillus* spp.) could allowed for more microbial diversity and increasing the system robustness.

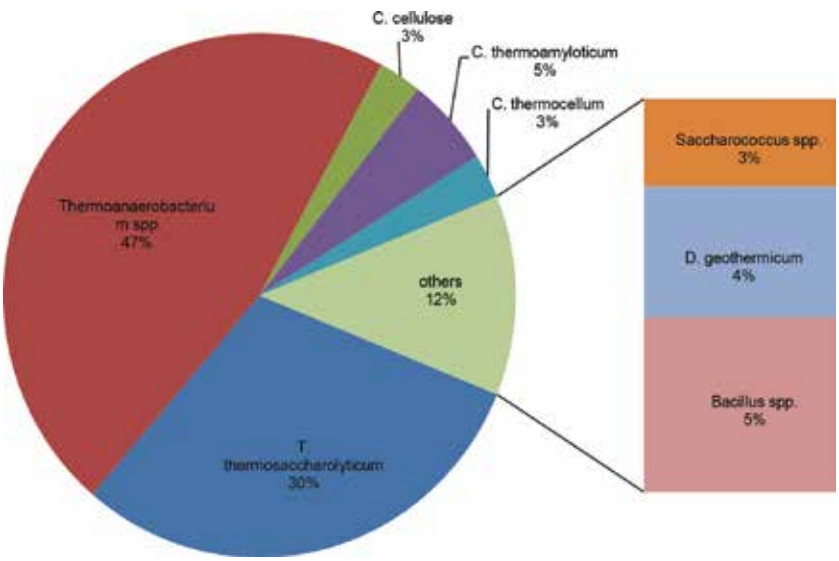

Figure 2. Summary of all fermentative hydrogen producing bacteria frequently observed based on molecular tools studied under thermophilic conditions.

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5. Microbial population optimization for dark hydrogen fermentation

Different species likely possess different growth properties (growth rates, affinity constants with substrates, and yields), and perhaps different capacities in coping with stress arising from variations in growth conditions. Obviously, the species with the most desirable properties would be selected to perform a required function. The possibility of selecting species with better properties has huge potential for improving the performance (efficiency and reliability) of a DHF system. Unfortunately, we still lack knowledge concerning the species to be selected and how they may be selected. Furthermore, 16S rRNA sequence-based identification does not allow inference of functional properties. The correlation between microbial community composition and reactor performance would provide a rationale to further improve the efficiency of fermentative hydrogen production. The characterization of the microbial community as a whole contributes to meaningful data regarding structure and function of such communities and their activities.

The interest in hydrogen as a clean energy carrier has strongly increased recently. Cost-effective generation of hydrogen through fermentation will have an important role in making this idea a reality. Future dark hydrogen fermentation from organic wastes depends on a thorough understanding of the microbiological community structure and function for enhanced or controllable hydrogen production and reactor. Sludge population optimization aims to obtain the best performance of a system through maximizing the properties of the sludge such as kinetics, yields, and robustness to environmental disturbance. A systematic investigation on the effects of a number of operational conditions on fermentative hydrogen production community and their properties is essential for sludge population optimization. The operational parameters to be studied include pH, temperature, hydraulic retention time, sludge retention time, organic loading rate, and nutrient concentration.

Additional improvements of microbial communities should be considered such as creating conditions that select for the stable and productive growth of desired microbes, while preventing or limiting growth of organisms that reduce hydrogen yields. Microbial population optimization could be achieved by biostimulation using the additive of various nutrient species specifically for the community, bioaugmentation using the additive of dominant species or efficient hydrogen-producing bacteria into the system, and online process control for maintaining their community.

A successful selection of such organisms, in particular those responsible for hydrogen production, will be used for recovery from off-set reactors by bioaugmentation strategy. To achieve high and stable hydrogen yield and long-term operation, it is necessary to control the growth of undesirable microorganisms such as hydrogen-consuming bacteria, propionic acid bacteria, and lactic acid bacteria via pH adjustment and reducing of H_2 partial pressure. The absence of hydrogen-consuming bacteria leads to relatively high hydrogen concentrations in the biogas and would significantly reduce costs for gas purification. Enhancement of hydrogen-producing bacteria via specific nutrient supplements will improve the reliability and performance of the process. Sludge population optimization strategies under thermophilic conditions shown in **Figure 3**.

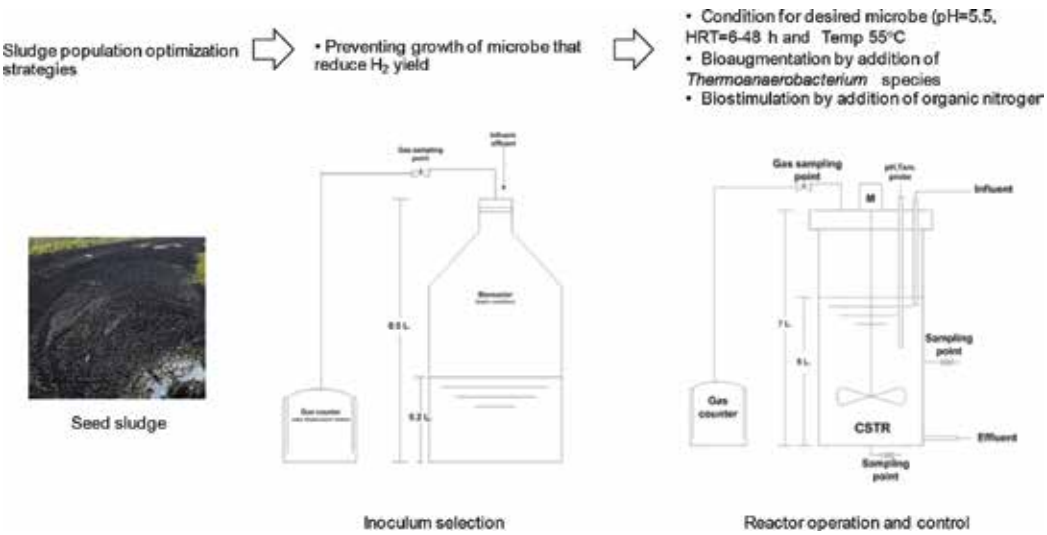

Figure 3. Summary of sludge population optimization strategies under thermophilic conditions.

6. Future directions

The use of hydrogen as a clean energy carrier has recently attracted great interest. The cost-effective generation of hydrogen via fermentation will have an important role in this endeavor. Future DHF from organic wastes depends on microbiological community structure and function for enhanced or controllable hydrogen production and reactor. Sludge population optimization aims to obtain the best performance of a system through maximizing the properties of the sludge such as kinetics, yields, and robustness to environmental disturbance. A systematic investigation on the effects of a number of operational conditions on fermentative hydrogen production community and their properties is essential for sludge population optimization. The operational parameters on the appearance of function of microbial species to be studied include pH, temperature, hydraulic retention time, sludge retention time, organic loading rate, and nutrient concentration. Additional improvements on microbial communities should be considered such as creating conditions that select for the stable and productive growth of desired microbes, while preventing or limiting growth of organisms that would reduce hydrogen yields. Microbial population optimization could be managed by biostimulization with the addition of nutrient species specific for their community, bioaugmentation by addition of dominant species or efficient hydrogen-producing bacteria into the system, and online process control for maintaining their community. A successful selection of such organisms, in particular those responsible for hydrogen production, will be useful for the recovery of off-set reactor by bioaugmentation strategy. To achieve high hydrogen yield and long-term operation, it is necessary to control the growth of undesirable microorganisms such as hydrogen-consuming bacteria, propionic acid bacteria, and lactic acid bacteria via pH adjustment and reduction of pH_2. The absence of hydrogen-consuming bacteria leads to

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relatively high hydrogen concentrations in the biogas and would significantly reduce costs for gas purification. Enhancement of hydrogen-producing bacteria via specific nutrient supplements will improve the reliability and performance of the process.

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Chapter 8

Biosensors in Fermentation Applications

Jianguo Shi, Derong Feng and Yiwei Li

Additional information is available at the end of the chapter

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Abstract

Biosensing technology offers new analytic routes to the use and study of fermentations, taking advantage of the high selectivity and sensitivity of the bioactive elements it exploits. Various biosensors had been commercially available today; they provide fermentation processes with convenient, accurate, and cost-effective ways of monitoring for key biochemical parameters. In this chapter, the basic ideas and principles of biosensors, especially applications of the most popular biosensors related to fermentations were highlighted.

Keywords: biosensor, electrochemical techniques, enzyme electrode, amino acid, sugar, alcohol

1. Introduction

Biosensor is a field of interdisciplinary studies and applications, which is underlain by many theoretical and technical fundaments from life science, physics, analytical chemistry, information technology, and so forth. The study of biosensors is a branch of analytical biology. It is largely aimed to construct rapid, stable, and facile analytical devices and analytical technologies used thereby. As a novel analytical technique, biosensors features small size, high sensitivity, high analytical specificity, and rapid accessibility, ready to realize reagentless analyses. This technology has made its way in great advances and attracted attentions since it was first proposed in 1960s.

The first biosensor was reported to be constructed and succeeded in measuring medical data, a biological electrode by Pro. Clark and co-works in 1962. **Figure 1** is a schematic of it. The electrode is fabricated via fixing a layer of immobilized glucose oxidase (glucose oxidase, EC 1.1.3.4) membrane onto an ion selective electrode that is capable of detecting dissolved oxygen

concentrations. When working, glucose oxidase catalyzes the conversion from the substrates β-D-glucose (the analyte, exists in the solution environment) and molecular oxygen into gluconic acid and hydrogen peroxide (H_2O_2), the products. The electrode could detect oxygen changes in the environment as it is consumed by the enzymatic reaction and transmit the sensing signal into form of voltages, and, in turn, the glucose concentration can be determined, for it is proportional to the dissolved oxygen concentration in certain range. In this method, the high specificity of enzymatic reaction and powerful detecting ability of electrochemical electrode was judiciously integrated so that the biochemical reaction can be monitored through a physicochemical detector. After this exemplification, biosensors had being hot topics among the researchers worldwide. Now, biosensors had found their way in both practical applications and scientific researches in many forms of commercially available products.

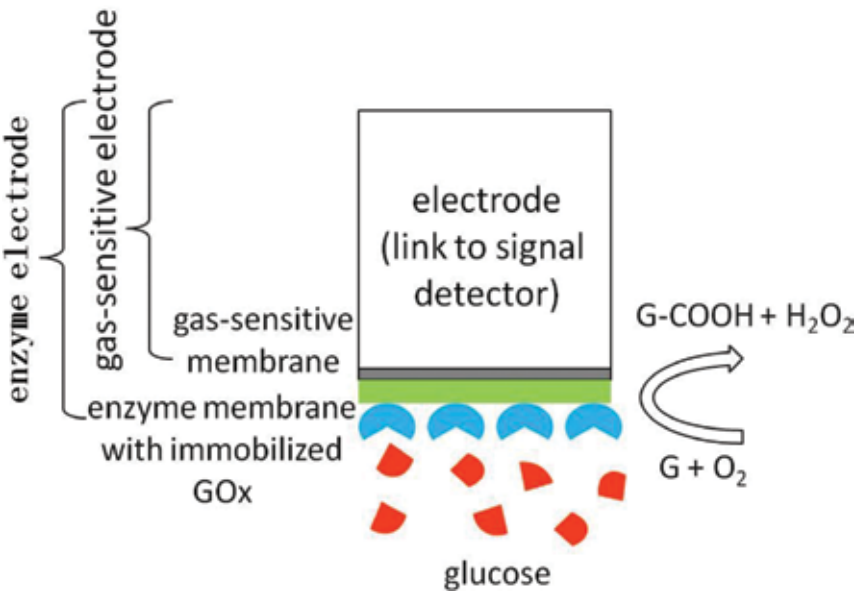

Figure 1. A schematic representation of the classic Clark enzyme electrode with glucose oxidase (Gox) as its biological element.

1.1. Classification of biosensors

As is recommended by IUPAC in 1999, a biosensor is an independently integrated receptor transducer device, which is capable of providing selective quantitative or semiquantitative analytical information using a biological recognition element [1].

A typical biosensor is made up of two main parts: (a) a biological element that can give any form of detectable signals, enzymatically catalyzed reactions, and biomolecular recognitions are the most referred, among others. Enzymes, antibodies, and nucleic acids are often exploited as the biological element; (b) a transducer by which the signal produced by the biological element can be detected and converted into measurable electrical signal.

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To construct a biosensing system, three main elements are often required, they are as follows: a biological element, a transducer, and a signal processing system [2]. A schematic of a typical biosensor system can be seen in **Figure 2**.

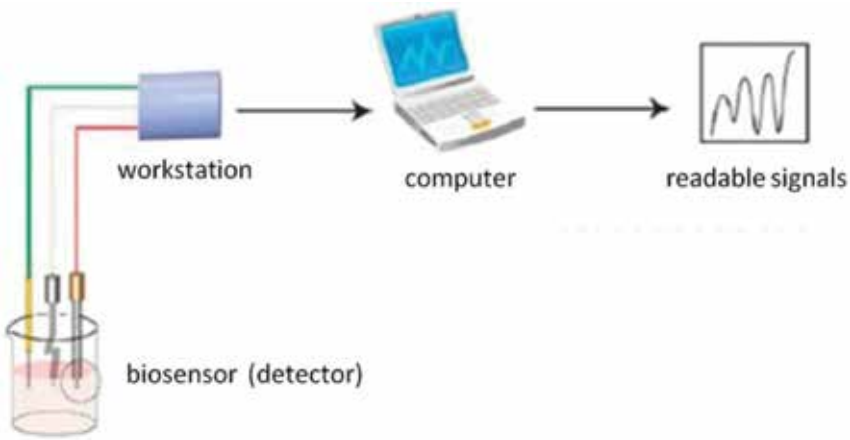

Figure 2. Basic elements to construct a biosensor system.

According to the difference of the biological element and transducer utilized, biosensors can be divided into several categories as what shall we introduced in the following listed in **Figure 3**.

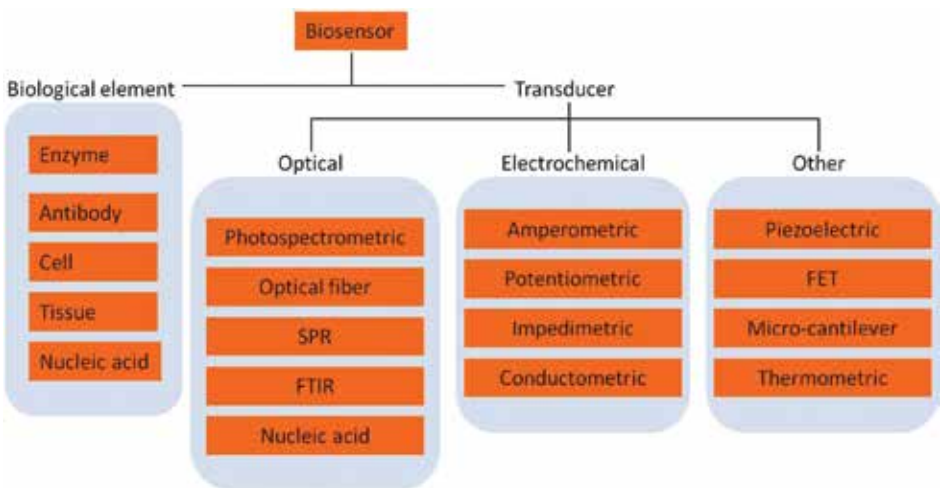

Figure 3. Classifications of common biosensors.

As is shown in the figure, electricity, light, sound, heat, and force, almost all physical dimensions have been available to be used as the target property for constructing a biosensor.

Biosensors based on different physical principles have both their advantages and disadvantages and befit various kinds of analyzing targets. Electrochemical biosensors are inexpensive, easy to be prepared, and available to meet various ranges of analyte concentration [3]; this strategy is most often used in both fermentation and other practical applications. Optical ones can detect biological parameters with UV-visible [4], infrared [5], fluorescent [6], and chemiluminescent [7] lights. Thermal ones detect heat released in physicochemical processes. For fermentation uses, heat is released by both cellular and non-cellular processes to facilitate monitoring the fermentation progress [8]. Other strategies are often seen in comparatively highly specific uses, for example, SPR and microcantilever sensors are good choices to facilitate biomolecular researches [9], although they are often cost non-saving and high in instrumental and operative requirements.

Biosensors, other than those fit into the definition given above, and some sensing technologies that are aimed at detecting biologically related parameters yet contain not any biologically active elements are also counted, and they are usually called "generalized biosensors." Examples of generalized biosensors include for instance: mass spectrometric measurements used in off-gas analyses in fermentation processes [10, 11] and cytometry for fermentation process controls [12].

Out of its prevalence, robust analyzing capability, cost-saving, and facile instrumenting, in this text, we put the most focus on the most studied and relatively mature in practical applications such as electrochemical biosensors.

1.2. Basic principles

Electrochemical biosensors are based on various kinds of electrodes by which electrical signals can be produced and sensed. There are three main techniques widely used for electrochemical biosensors [13]:

1. *Potentiometric*. It includes zero-current potentiometry and techniques of applying amplitude controlled current onto the working electrode. In these methods, electrical potential is detected for measurements.

2. *Amperometric*. Amperometric method is based on detecting current produced by applying a known potential. There are mainly two forms of amperometric methods: constant-potential amperometry and amperometry with applying various potential waveforms. Most of the biosensors used in fermentations are of amperometric type.

3. *Impedimetric*. It is also called impedance spectrometry, which is based on detections of impedance, conductance, and capacitance of a certain electrochemical system. It is more often used in theoretical analyses of the sensing surface.

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2. Applications

2.1. Amino acid detection

Amino acid is one of the most important biomolecules for life, and they act as building blocks of numerous proteins, precursors in the synthesis of many biologically functional molecules and energy resource, in some cases. For human beings, counting in the eight essential and two semi-essential ones, in total about 22 natural amino acids are required to maintain a healthy human body. In food and medicine applications, amino acid is a vital material.

Biosensing tactics for amino acids are mainly realized by using enzyme biosensors. The enzymes are often amino acid oxidases as L-amino acid oxidase [14], glutamate oxidase [15–17], leucine dehydrogenase [18], tyrosinase [19, 20], and L-phenylalanine dehydrogenase [21].

Glutamate is very important in medical uses [22, 23], and sodium salt is a widely used seasoning additive, which is now mainly produced by large-scale cellular fermentations. Amperometric enzyme electrodes that detect glutamate is one of the most widely and maturely used biosensors. In majority of the now commercially available glutamate biosensors, a typical strategy is to integrate a glutamate oxidase onto a platinum electrode. When use, preset potential is applied upon the electrode to electrochemically catalyze the oxidation of the enzymatic reaction's product—hydrogen peroxide. Computer records the electrical current produced in this process and translates it into the corresponding concentration as the readout [24]. In a research, glutamate oxidase (EC 1.4.3.11) and $NADP^+$-dependent glutamate dehydrogenase (EC 1.4.1.3) were co-immobilized onto an oxygen electrode to fabricate an enzymatic MSG detector for food uses. By exploiting those two enzymes, monosodium salt of glutamate and glutamic acid can be distinguished to perform more accurate measurements [25]. Rita et al. [26] constructed a glutamate enzyme sensor by immobilize L-glutamate oxidase (GLOD, EC 1.4.3.11) and Gox on glass carbon electrode. To minimize the interference, the enzyme electrode was then modified with the polymer Nafion, a very widely used material to improve the sensing selectivity of amperometric enzyme sensors. The electrode can perform simultaneous measurement of L-glutamate and glucose without any obvious interference. Tang et al. [27] constructed glutamate enzyme electrode with NAD^+-dependent glutamate dehydrogenase (EC 1.4.1.3). To improve its sensitivity, the electrode was modified with nanocomposite. The electrode has a very rapid response and good stability (remains 85% sensing intensity after 4 weeks). For quantifications of total L-amino acid, enzyme electrode based on immobilized L-amino acid oxidase can be a very good choice [28].

Stasyuk et al. [29] used recombinant yeast cells as the arginine activity source to establish an amperometric biosensor along with immobilized urease. The cell-enzyme coupled sensor reportedly exhibited a linear range cross 3 orders of magnitude up to 0.6 mM and can give the result within no more than 1 min. Another strategy is by coupling arginase (EC 3.5.3.1) and urease (EC 3.5.1.5). An arginine biosensor was constructed on ion-selective field effect transistors (ISFETs) surface via co-immobilizing arginase and urease. When working, arginine catalyzes the conversion of arginine into ornithine with release of urea, which, in turn, is

degraded by urease to produce ammonium ions. Production of ammonium is accompanied by the subtle change of pH and thus can be detected by the transmitter [30].

All amino acids exist in humans are of L-form, for enzyme dealing with D-amino acids are in lack in human body. Therefore, were there D-amino acids in food or medical products, it is of danger to cause problems of safety and monitoring of their presence is often one part of many fermentation products' quality control. To meet this need, an enzyme electrode was developed by co-immobilizing D-amino acid oxidase (DAAO, EC 1.4.3.3) and peroxidase onto polymer electron mediator modified electrode [31]. Zain et al. devised a D-serine-sensitive electrochemical detector via immobilizing DAAO onto polymer functionalized metal electrode. The detector is reported to have ideal interference resistance toward most of the neurochemicals and can be used for *in vivo* D-serine detections [32].

Other than enzyme-biosensing methods, amino acid measurement can also be accomplished by enzyme-free techniques. Dai et al. [33] prepared nanoporous nickel-modified boron-doped diamond electrode with electron-assisted hot filament chemical vapor deposition method, and the electrode can capture redox processes of L-alanine and can perform anti-interference, sensitive detections of it. Seki et al. developed a tryptophan sensitive, potentiometric detector with microbial cells as the biological element. In the scenario, an auxotrophic bacterial strain *Escherichia coli* WP2—a mutant requiring tryptophan for its growth was monitored with a light-addressable potentiometric sensor. When L-tryptophan is in present, the bacterial metabolic result of it will cause pH changes, which can be detected and used for quantification by the sensor [34].

2.2. Sugar detection

In fermentation processes, sugars can either a vital substrate for cellular fermentations or in some cases the target product (e.g. oligosaccharides in isomalto-oligosaccharide [35] and chito-oligosaccharide preparations [36]; glucose from enzymatically degraded starch). The most commercially available and under-research biosensors for sugars are aiming at detections of monosaccharides. For oligo- and polysaccharides, very mature sensing platform is rarely seen mainly because accessible biological elements that exhibit good biorecognitions toward them are difficult to obtain.

Up to now, glucose is the target analyte in most biosensor researches during which its medical uses are most concerned. For fermentation uses, there had been many kinds of commercial biosensors for choice; the majority of them are operated under off-line mode.

A very widely applied glucose biosensor is much the same like the typical glutamate enzyme electrode aforementioned, with the only difference being that the enzyme alters into usually glucose oxidase (Gox). Although the rare metal-based enzyme glucose biosensors are easy to prepare, cost acceptable, and often renewable (in a typical mode of Pt-based Gox electrode, the enzyme is immobilized onto a polymer permeable membrane), biosensor is achieved by covering the enzyme laden side tightly to the Pt electrode surface. When the detected signals are seen obviously declined after a period of use, operators need to only replace the enzyme membrane with a new one from the same manufacturer to refresh the sensor. Pt base is

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electrochemically inert and thus is resistant to repeated use (**Figure 4**). Difficulties in modifying the base electrode to functionalize it, high cost of the electrode hinders it from altering into disposable forms, etc., are often motives for researchers to develop more sophisticated ones.

Commercial blood glucose biosensor had been used to monitor glucose concentration in the fermentation broth. The result shown the method is potent to fill formation needs and is a good alternative for HPLC analysis and reducing sugar assay [37]. White et al. used screen-printed Gox electrode as the glucose detector to perform real-time fermentation control [38]. The electrode is a classical form of electrochemical detector, which is extremely inexpensive, highly reproducible, and repeatable, and very large-scale production is suitable. To overcome the problem of on-line sensing, the researchers introduced flow injection analysis system to rid the gap between sampling and the sensor.

In fermentations, high-temperature processing is often inevitable for preventing the fermentation processes from biological contaminations. However, biosensors are in most cases non–high-temperature tolerant. To tackle the problem, Phelps et al. proposed a glucose-sensing device that is autoclavable and can be repeatedly used [39]. Unlike the conventional immobilized enzyme electrodes, the electrode consists largely of a semipermeable membrane in protecting the sensing surface from fouling by the fermentation broth, a Pt electrode functions as its conventional versions, and a chamber with conduits through which electrolyte and enzyme solutions can be filled or discarded. The design circumvented the contact between the intense autoclaving conditions and the temperature vulnerable enzyme.

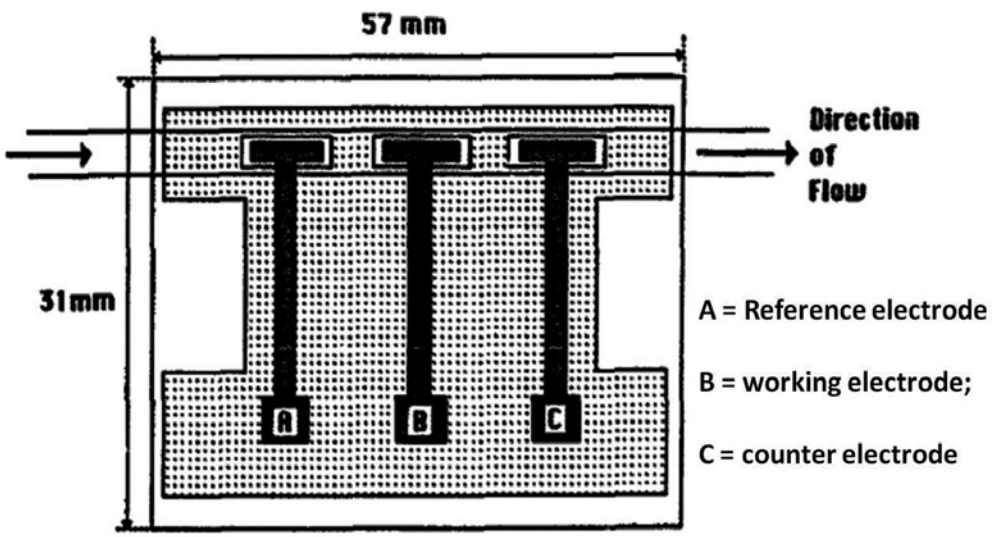

Figure 4. A schematic representation of screen-printed electrode. The base electrode is derived from a printing technology called "screen printing," which uses a mesh with predrawn patterns hollowed out and let the printing ink to through it so that the pattern is printed onto the base material. Enzyme electrodes prepared with this method are near two dimensional so they are potable. The high reproducibility of its preparing progress grants high reproducibility to electrodes made in the same batch.

Another strategy aiming at on-line biosensor design can be seen in a research that uses non-immobilized, liquid Gox as its biological element. The base electrode is not in direct contact with the enzyme, and it only detects the catalytic product in the enzyme solution [40]. Other than the on-line uses, this design open a new way to devise biosensors that are target at measuring high-concentration glucose in consideration that it is especially useful in fermentations. In large number cases of fermentation processes, glucose concentrations at the initial and early period are often too high, which exceed the upper detection limit of most enzyme-immobilized sensors. Therefore, continuous monitoring is hard to be realized without gradient dilutions that are performed, yet the process is often the key source of sampling error. The enzyme-injected mode can allow the electrode to direct detection of high glucose concentration broths.

Development of enzyme electrode has gone though three main stages, in which Gox electrode is a very good example:

Stage I: Gas-sensitive electrodes represented by Clark enzyme electrode. The measurement is performed in potentiometric method by detecting changes of dissolved oxygen or any acid or base produced in the enzymatic reaction. The problem of this strategy is as dissolved oxygen consumed, measuring results are liable to become awry of the electrode's linear range and as a result brings huge errors to the quantification.

Stage II: Electron mediator-functionalized electrodes overcome the shortcut of gas-sensitive electrodes and established new strategy for enzyme electrode designs. Electron mediators were found by researcher that when they were integrated into the biosensing interfaces, electrons produced by the enzymatic reaction can be relied by the mediator, which, in turn, is oxidized by the base electrode. By this way, electrochemical detection can become oxygen consumption independent. Electron mediators can be either natural substances, for example cytochromes and co-enzymes, or artificial ones as some organic dyes, ferrocene and its derivatives, metal complexes, and some conductive polymers. By using electron mediators, detecting potentials can be effectively lowered so that much interference would thus be eliminated.

Stage III: Direct enzyme electrode was proposed after phenomenon that some proteins can make direct electrochemical communications with the electrode, which is represented by the finding of reversible cyclic voltammetric response on the electrode. Principle of the phenomenon is suggested to be when the redox center of the protein molecule is in close adjacent to the electrode surface, electrons can transport between them directly without the aid of any mediators. By using this mechanism, an obviously improved electron transfer efficiency can be obtained and the sensing capability is enhanced. However, it is often difficult to achieve the required conditions for enzymes. Taking Gox for example, its electrochemical active center —FAD^+ cofactor—is wrapped into the space formed by the dimmer subunits and therefore is difficult to build direct electrochemical communication between the electrode and the enzyme. When Gox is tightly adsorbed onto some electrochemical active materials such as carbon nanotubes and graphenes, the direct electron transfer can be observed. Researchers have to pay more attention and efforts to develop direct enzyme electrodes, as it is a promising strategy to construct more rapid, sensitive, and reagentless biosensors.

2.3. Alcohol detection

Alcohol content in fermentation broths can be realized by many conventional methods, for example hydrometry and gas chromatography. Considered the error limit or high expense or time-costing procedures of them, biosensor is a good alternative.

A colorimetric biosensor was proposed by Kuswandi et al. The sensor was constructed by polyaniline film immobilized alcohol oxidase. When ethanol is in presence, a color change from green to blue can be observed due to the oxidation of polyaniline by the enzyme reaction product H_2O_2. Through the computer processing software, the method can determine alcohol quantitatively range between 0.01 and 0.8% [41]. Gotoh et al. devised an amperometric alcohol sensor based on co-immobilized alcohol dehydrogenase and coenzyme NAD^+, the enzyme electrode shown linear response to solution contains ethanol between 0.05 and 10v/v%. As a reagentless enzyme sensor, it can stand at least weeks of continual detections without addition of the coenzyme [42].

3. Future perspectives

In the applications of fermentation processes, although many tangible advances have been achieved and a bunch of biosensors are now commercially accessible, many questions are still in need of further studies.

First, due to the biologically active species that can serve as biological elements are still in a limited range, the parameters detectable for biosensor in fermentation processes are restricted to the several kinds of target constituents. This, in one hand, can be gradually extended by finding and isolating new suitable biological constituents from the natural world. With respect to enzyme biosensors, dehydrogenase is becoming the most widely used. Over 400 dehydrogenases have been discovered or isolated; many biological constituents would be allowed to be detected by dehydrogenase sensors. Dehydrogenases often have their isozymes, and they require NAD^+ or $NADP^+$ or quinones as the cofactors. Comparing to oxidases, the redox center of dehydrogenases is not wrapped tightly by the protein components so more liable to establish direct electronic communications with electrodes. One of the most thorny problems is to establish methods for cofactors' immobilization to realize the reagentless biosensors. On the other hand, new generations may provide opportunities. One technical route is developing molecular imprinting sensors, in which artificial polymers that mimic the structure of natural enzymes, antibodies, or antigens to produce the alike high specificity can be synthesized. Even though, in some examples, the potent polymers are obtained, there still a long way ahead of the application of the technique. Another route is to establish aptamers, usually short nucleic acid chains or peptides judiciously devised and synthesized. Aptamers are alternatives yet can provide the same biorecognition functions to their natural forms. Many usable aptamers have been established, and it is a critical mission to its applications establishing a high-throughput selecting technology to accelerate the discovery of new aptamers.

Second, endeavor to improve the base electrode is never ended, and it is one of the core topics of electrochemistry. By modification, electrodes can be endowed with new functions or get their

sensor capacities enhanced. The most promising materials for electrode functionalization include nanocomposites, conductive polymers, novel electron mediators, liquid ions, and so forth. Among others, nanocomposites such as carbon nanotubes, graphene, and metal nanoparticles are attracting most of researchers' interests as they have both robust physical and chemical properties that are useful for improving sensing capabilities (e.g., limit of detection, sensitivity, selectivity, anti-interference, and electrochemical stability) and huge potential to exploit for conducting immobilization of biological elements. Nanocomposite per se is also a platform for preparing complex composites via combination of the materials mentioned above.

Third, miniaturization, integration, and automation of biosensors in fermentation uses are still at its preliminary stage. Although many commercial biosensors have, to a great extent, facilitated the detection of several kinds of constituent, it is uneasy to realize multiple parameter automatic controls for the whole fermentation process. Aside of developing more and more diverse biosensors fit for different targets, testing conditions, microfabrication technology, and Internet of Things are promising tools for achieving this goal.

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Section 3

Products from Fermentation Process

Chapter 9

Biogas - Turning Waste into Clean Energy

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Additional information is available at the end of the chapter

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Abstract

Expertise in biogas production using anaerobic digestion (AD) can offer many benefits in addition to being an alternative source of energy. This process involves plant digesters and provides an alternative destination for biomass that would eventually go unutilized and deposited in a trash heap. The application of the appropriate plant digester technology can generate energy, and the gas produced can be used for many purposes, such as water and space heating, lighting, and grain drying. In this context, agro residues are one of the most abundant energy sources available world wide. Nevertheless, the bioconversion of organic matter to biogas is a complex process of AD that involves many reactions among several microorganisms living in a stable community. Microorganisms from many diverse genera of obligate anaerobes and facultative anaerobes constitute these steps, and four groups are recognized to be the most frequent in biogas production plants. These groups, in order of substrate hydrolysis, are hydrolytic, acidogenic, and acetogenic bacteria, followed by the core group, the methanogenic archaea. All together, they compose the operation of a systematized activity with synergistic effects that ensure the stability of the process.

Keywords: anaerobic digestion, methanogens, methane, hydrogen, waste utilization

1. Introduction

Increased efforts to reduce the utilization of petroleum have encouraged the development of new technologies for the utilization of alternative energy matrices for the production of different compounds such as novel fuels. Among available biofuels, biogas has been produced for over approximately 2000–3000 years for sanitation purposes [1]; however, the first documented

generation of biogas comes from a carefully designed installation from England in 1895. The interest in its usage grew during World War II when France and Germany started to build biogas facilities and used them to fuel vehicles and tractors. After the war, interest in biogas waned, but recovered during the oil crisis of 1973 with improved technology. Nowadays, Germany is by far the world leader in biogas generation.

Biogas is generated from anaerobic digestion (AD) in a bioreactor (also called a digester unit). Its production can be done through a batch or continuous process, in one-, two-, or multiphased steps, and it utilizes mainly organic matter from waste as the substrate. It is considered a carbon-neutral biofuel since it uses carbon dioxide that was recently taken up by plants from the atmosphere and is able to return it through the fermentation of waste residues [2]. This biofuel also protects the environment from pathogens by reducing the waste that would rot in the open air, which would have increased the possibility of attracting disease-carrying vectors. Moreover, it considerably reduces air and water pollution, helps the conservation of forests, and replaces inorganic fertilizer with its digested residues [3]. According to the European Union, biogas has the potential to produce 25% of all clean energy. It can be used to produce electricity, heat, and vehicle fuel, thus substituting conventional sources of energy that produce greenhouse gases.

In recent years, biogas production has increased greatly. This can be evidenced by the rapid construction of biogas plants, which have been built exclusively in Europe. The world's biogas production in 2012 reached 17.2 ktoe/year (the equivalent of millions of tonnes of oil per year) and Europe alone produced 60% (about 10.5 ktoe/year) of this amount. In 2013, European Union production grew to 13.4 ktoe/year, a 27.6% increase, and it is expected to reach 33.0 ktoe/year by 2022. Several European countries face enormous issues related to the excess of organic waste production from industry, agriculture, and households. AD can also contribute to waste minimization by eliminating the accumulation of harmful and persistent wastes while simultaneously lowering prices for waste disposal.

Taking into account the importance of biogas production, this chapter will discuss, in general, the production of this clean energy source. Therefore, the following topics will be addressed: (1) biogas composition; (2) types of substrate used for their production; (3) overview of biogas production; (4) physical and chemical AD; and (5) anaerobic bioreactors. Specifically, greater emphasis will be given to important aspects of fermentation, such as: (1) the microorganisms and the trophic groups involved in each step (hydrolytic bacteria, acidogenic bacteria, acetogenic bacteria, methanogenic groups); (2) factors affecting biogas production efficiency (temperature, pH and chemical aspects of biomass); (3) the biochemical substrates by the population of microorganisms.

The bioreactor types and their strategies for biogas production will be discussed superficially. However, greater emphasis will be given to important aspects of fermentation, such as: (1) the microorganisms involved, and the trophic groups involved in each step (hydrolytic bacteria, acidogenic bacteria, acetogenic bacteria, and methanogenic groups); (2) factors affecting the efficient production of biogas (temperature, pH and chemical aspects of biomass); (3) the biochemical changes in substrates by the microorganism population.

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2. Biogas composition

The typical composition of biogas is methane (CH_4), carbon dioxide (CO_2), and sulfuric elements (H_2S). The approximate percentage of biogas components is shown in the **Table 1** [4].

Biogas composition	Typical analysis (%/volume)
Methane	55–65
Carbon dioxide (CO_2)	35–45
Nitrogen (N_2)	0–3
Hydrogen (H_2)	0–1
Hydrogen sulfide (H_2S)	0–1
Oxygen (O_2)	0–2
Ammonia (NH_3)	0–1

Table 1. Approximate percentage of biogas components [4].

The main cause of the high variation in percentages of biogas composition (**Table 1**) is due to the substrate utilized. The fact that methane is present at high concentration makes biogas a very attractive source of energy considering that methane has a heating value of 8500 kcal/m^3 and that CO_2 has no energy associated with it. The heating value of biogas is on average 5000–7000 kcal/m^3, approaching nearly 12,000 kcal/m^3 when in a high degree of purity (65% CH_4). Comparatively, a cubic meter of biogas has the same calorific power as 0.613 L of gasoline, 0.579 L of kerosene, 0.553 L of diesel, 0.454 L of cooking gas, 1.536 kg of wood, and 0.790 L of ethanol and produces the equivalent power of 1.4208 kW.

Typically, 0.2–3% of biogas is composed of gases that enter the digester with air included in the substrate (N_2 and O_2). Among these, nitrogen and CO_2 (produced during the digestion process) are included in the inert gases that compose the total biogas mix. On the other hand, the remaining NH_3, O_2, and H_2S gases are unwanted gases due to their toxicity to strict anaerobes that are essential for the process. Both O_2 and H_2S can be removed from biogas through chemical processes such as iron based processes, for example, with the addition of iron chloride, while NH_3 can be degassed through an H_2SO_4 absorber.

Another component, hydrogen sulfide (H_2S), is normally present in biogas as a by-product from anaerobic digestion. It is considered a major cause of corrosion of metal parts and degradation of engine oil, and during the fermentation process, it can precipitate metal elements. This gas is prevenient to the degradation of sulfur-containing proteins (i.e., cysteine and methionine), and besides being prevenient to normal metabolism of fermentation organisms, it has to be removed from the biogas before utilization.

3. Types of substrates

The most utilized residues for biogas production are found in animal manure, agriculture residues, and general organic wastes from food (both vegetable and animal in origin), organic fractions of municipal waste and from catering, sewage sludge and residues from crops dedicated to energy (i.e., biofuels), such as sugar cane and sorghum. These can be classified into various criteria: its origin, organic content, methane yield and dry matter content (**Table 2**). These substrates usually have a high content of sugar, starch, proteins, or fats, which are decomposed through AD. **Table 2** shows several substrates and their classifications according to organic content, carbon:nitrogen ratio, percentage of dry matter, percentage of volatile solids in dry matter, and its biogas yield [5]. It is noticeable how the utilization of different biomasses has a consequence in the biogas yield, for example, it can vary from 0.15 m^3/kg VS (volatile solids) (utilizing straw) to 0.9 m^3/kg VS. When the utilized substrate is concentrated whey, a

Biomass type	Organic content	C:N ratio	DM[a] (%)	VS[b] (% of DM)	Biogas (yield m^3/kg VS)
Pig slurry	Carbohydrates, proteins, lipids	3–10	3–8	70–80	0.25–0.50
Cattle slurry	Carbohydrates, proteins, lipids	6–20	5–12	80	0.20–0.30
Poultry slurry	Carbohydrates, proteins, lipids	3–10	10–30	80	0.35–0.60
Stomach/intestine content	Carbohydrates, proteins, lipids	3–5	15	80	0.40–0.68
Whey	75–80% lactose, 20–25% protein	NR	8–12	90	0.35–0.80
Concentrated whey	75–80% lactose, 20–25% protein	NR	20–25	90	0.80–0.90
Flotation sludge	65–70% proteins, 30–35% lipids	NR	NR	NR	NR
Fermented slops	Carbohydrates	4–10	1–5	80–95	0.35–0.78
Straw	Carbohydrates, lipids	80–100	70–90	80–90	0.15–0.35
Garden wastes	NR	100–150	60–70	90	0.20–0.50
Grass	NR	12–25	20–25	90	0.55
Grass silage	NR	10–25	15–25	90	0.56
Fruit wastes	NR	35	15–20	75	0.25–0.50
Fish oil	30–50% lipids	NR	NR	NR	NR
Soya oil/margarine	90% vegetable oil	NR	NR	NR	NR
Alcohol	40% alcohol	NR	NR	NR	NR
Food remains	NR	NR	10	80	0.50–0.60

[a]Dry matter.[b]Volatile solids. NR, not reported.

Table 2. Substrates commonly utilized for biogas production, its composition, and average biogas yield [5].

500% increase in growth can be observed (**Table 2**). Generally, the C:N ratio also affects the production of biogas. As can be seen in **Table 2**, low C:N ratios (between 3 and 20) produce a yield ranging between 0.25 and 0.78 m^3/kg VS. Higher C:N ratios (above 20, reaching up to 150) do not produce greater yields, since the greater yield obtained is 0.56 m^3/kg VS, approximately 30% lower than that obtained at lower C:N ratios.

In spite of the numerous advantages of utilizing biogas digesters, there are still challenges that need to be overcome in order to maximize fuel production. Methanogenic archaea, microorganisms that produces methane, have specific requirements such as temperature and pH, and they must be maintained within specific ranges for optimal production, which increases the production cost of biogas [6]. Another challenge is hydraulic retention time (HRT), which is the normal time that the input substrate spends in the digester before it is removed. At tropical temperatures, the HRT is 30–50 days, although in colder atmospheres, it may go up to 100 days without heating, which requires a bigger digester volume and raises costs. While digesters can save energy at small-scale production on farms, finding the right economic balance for large-scale production is yet another challenge.

4. Overview of biogas production

Biogas production is an established process in which there is little information available on the microorganisms involved using different wastes. Thus, an understanding of the microorganisms' activity and the factors that can influence biogas composition are crucial in order to maximize fermentation performance and reduce process costs. Therefore, in order to discover which microorganisms are involved in anaerobic digestion, sequencing of 16SrRNA and metagenomics [7] has been performed, as well as the analysis of the methyl-coenzyme M reductase encoding gene, as this is a marker for identification of archaea that are specifically methanogenic [8]. DNA isolated from different bioreactors using different substrates demonstrated a very direct link between reactor type and taxonomic groups. For example, in a stirred digester fed with fodder beet silage, mainly Bacilli, Clostridiales, Deltaproteobacteria, and Bacteroidetes have been found [9], while the microbial population of a thermophilic digester described in another study was particularly rich in Clostridia [10]. Another important relationship is the microorganism present according to the physical location of the digester [11]. The results of several studies inferred that, in the first and second phases of AD, at least 58 species of 18 genera are involved, which categorize biogas production as mixed fermentation.

4.1. Microorganisms and the biochemistry of AD

The production of biogas is performed by a microbial consortium through four main reactions: hydrolysis, acidogenesis, acetogenesis, and methanogenesis, where organisms from the bacteria and archaea domains are involved in consortia that lead to substrate conversion into CH_4 and CO_2 among other gases. The microorganism types involved and an overview of the substrate process are illustrated in **Figure 1**.

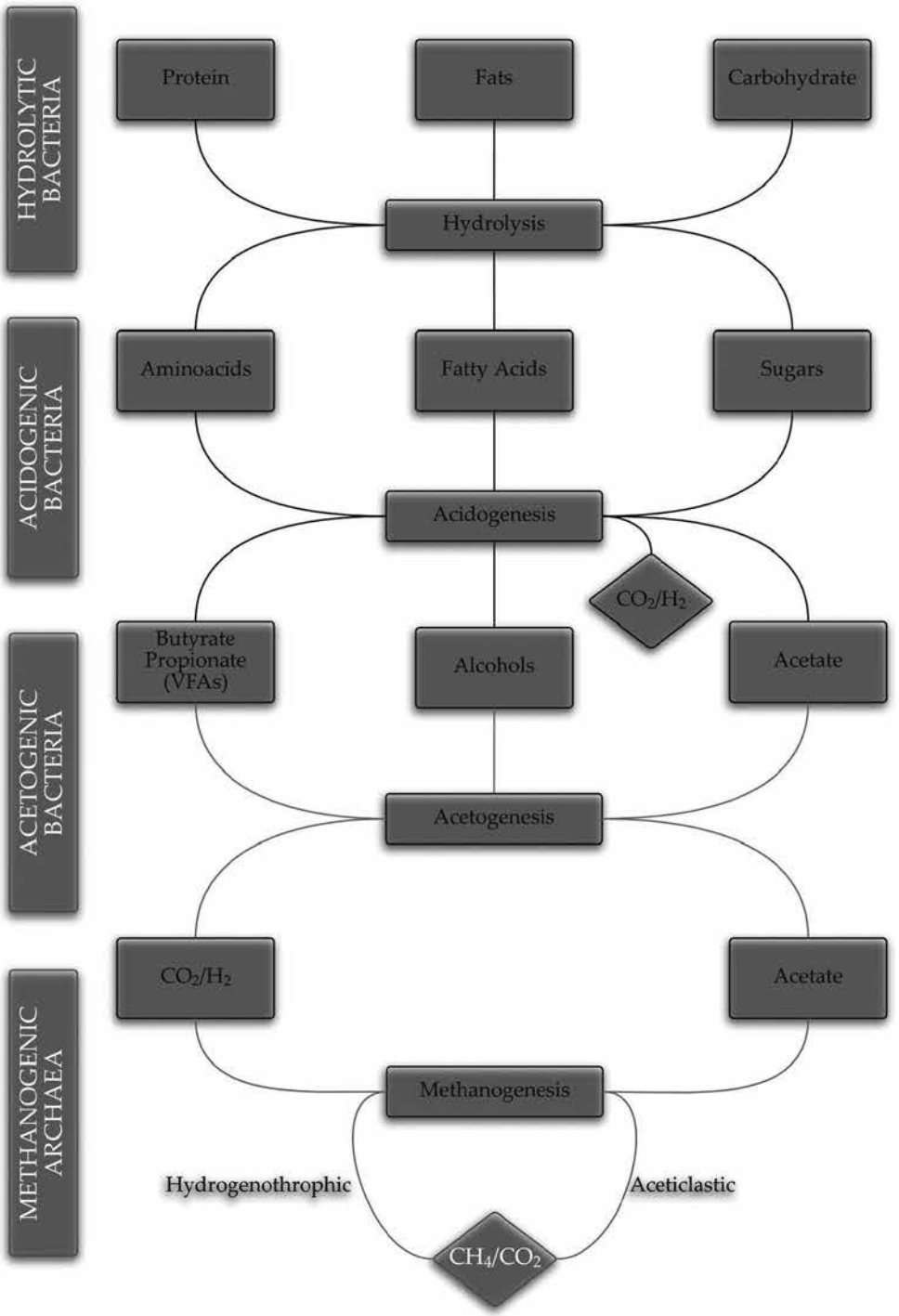

Figure 1. Microorganisms involved in each catabolic step during biogas biosynthesis.

4.1.1. Hydrolytic bacteria

Anaerobic digestion starts with the polymer hydrolysis of fats, proteins, and carbohydrates into monomers that are suitable for further digestion. Hydrolytic bacteria, which can be either facultative or strict anaerobes, are capable of hydrolyzing the bonds of these compounds, converting them into oligomers, monomers, amino acids, and unsaturated fats. For example, cellulose [$(C_6H_{12}O_6)_n$], an insoluble substrate commonly found in sludge, is hydrolyzed by bacteria from the genus *Cellulomonas*, resulting in glucose monomers. The hydrolysis of polymers that are difficult to decompose restrains the rate of waste processing, and just half of these compounds experience hydrolysis in a one-stage digester. In some cases, pretreatment involving an aerobic step can be added. The concept of aerobic treatment consists in the knowledge that some aerobic microorganisms can produce hydrolytic enzymes that are able to generate monomers from the polymers present in the biomass. Moreover, inhibitory macromolecules such as lignin may also be transformed, resulting in a less toxic substrate to the microorganisms that start the AD process [12].

Anaerobic digesters that utilize substrates derived from wastewater treatment from industry, such as dairy and agro industries, are usually composed of soluble organic compounds and therefore do not experience this kind of hydrolysis. However, different sugars such as sucrose and lactose must be hydrolyzed despite being soluble, since they are larger than most cells can absorb [13].

4.1.2. Acidogenic bacteria

In regard to the second reaction stage, acidogenic bacteria will then convert these molecules into volatile fatty acids (VFAs) with high carbon numbers such as butyrate, propionate, and alcohols in addition to CO_2, H_2, and acetate [14]. These biochemical steps depend on various factors, like pH, enzyme production by bacteria, diffusion, and adsorption of enzymes by the biomass undergoing the process of digestion. This is executed by microorganisms from the group of anaerobic bacteria of genera such as *Streptococcus* and *Enterobacteria*.

However, VFAs produced during this stage may negatively affect the AD process depending on its concentration in the bioreactor. When unstable, the AD process accumulates VFAs inside the reactor, which results in a drop of pH-value and consequently a decrease in methane yield. This is explained by the low tolerance of methanogenic archaea in an acidic environment. It is demonstrated that different digesters can react differently in response to the same amount of VFA, where, in one digester, the concentration may be optimal and, in another, it is a considerable inhibitor to methane production. One conceivable explanation is the microorganism population, which varies from digester to digester. It can also be explained by the buffering capacity of the substrate.

4.1.3. Acetogenic bacteria

For the third reaction stage, acetogenic bacteria convert VFAs into acetate. Acetogenic bacteria are obligate proton-reducing bacteria (OPR) and are known for the production of H_2 during acetate production. Some VFA conversions are displayed below in Eq. (1):

$$\begin{aligned}
&\text{propionate} + 3H_2O \rightarrow \text{acetate} + HCO_{3^-} + H^+ + 3H_2 \Delta G = +76.1\,\text{kJ/mole} \\
&\text{butyrate} + 2H_2O \rightarrow 2\,\text{acetate} + H^+ + 2H_2 \Delta G = +48.1\,\text{kJ/mole} \\
&\text{ethanol} + 2H_2O \rightarrow \text{acetate}^- + H^+ + 2H_2 \Delta G = +9.6\ \text{kJ/mole}
\end{aligned} \tag{1}$$

In accordance with the examples above, it is important to note that all of them require energy input. However, in the presence of low hydrogen concentrations provided by the digester, the reaction moves to the product side to maintain equilibrium. To this end, they only live in coexistence with a H_2, utilizing species, which are the methanogenic archaea. A genus such as *Desulfovibrio* oxidizes alcohols and organic acids into acetate and transfers the electrons released to sulfate. Genera such as A*minobacterium* and A*cidaminococcus* ferment amino acids, trans-aconitate and citrate into acetate, CO_2, and H_2. Sulfate-reducer organisms such as the acetogenic *Desulfovibrio*, which oxidizes organic acids and alcohols to acetate and transfers the released electrons to sulfate resulting in a higher energy yield than fermentation, are deeply involved in compound decomposition by AD. These bacteria form cultures from obligated and facultative anaerobes to ferment available substrates such as lactate and alcohol from the acidogenic step.

4.1.4. Methanogenic group

The last phase of anaerobic digestion is catalyzed by a group of microorganisms from the archaea group. This group is subdivided into two groups: a hydrogenotrophic methanogenic group and aceticlastic methanogenic group. The first group utilizes the H_2 produced by the OPR group. Their affinity to uptake hydrogen is on the order of parts per million, making them very efficient in maintaining the substrate with a very low hydrogen partial pressure. The aceticlastic methanogenic group consists of only two genera: *Methanosarcina* and *Methanothrix*. These microorganisms can produce methane from acetic acid, and approximately 70% of all methane produced in biogas reactors originates from this conversion. The reactions of the processes are displayed below (Eqs. (2) and (3)).

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O \tag{2}$$

$$CH_3COOH \rightarrow CH_4 + CO_2 \tag{3}$$

Methanogenic archaea have, in their metabolism, the enzyme methyl-CoM reductase. This hexamer is a large complex composed by two copies of three different subunits (α, β, and γ) containing a unique coenzyme, the nickel phorphinoid factor F_{430} and with activity deep inside the complex for protection from the surrounding water. This complex catalyzes the release of the CH_4 from methyl-CoM [15]. The F_{430} ring needs a nickel atom that is stabilized in the reactive state, which is an important property of this enzyme because the substrate methyl-coenzyme M is rather inert, which makes the reaction easier.

Acetoclastic archaea are well known for their slow doubling time (1–12 days in thermophilic conditions) because of their relative inefficiency in taking up acetate, but on the other hand, hydrogenotrophic methanogenic bacteria are extremely productive and have moderately quick doubling times (0.5–2 days in thermophilic conditions) [16].

5. Physical and chemical AD parameters

The growth and metabolism of anaerobic microorganisms are essentially impacted by physical and chemical conditions such as temperature, pH value, nutrient supply, mixing intensity, and the additional presence of inhibitors.

5.1. Temperature

A large portion of reactor cost comes from the energy spent to maintain its temperature stable. Thus, an optimum temperature setting is the most critical factor in temperate countries since more energy is needed to maintain the temperature of AD and consequently methane production. Temperature parameters for AD can take place at different levels: cryophilic (below 25 °C) mesophilic (25–45 °C), and thermophilic (45–70°C). There is an inverse relationship between the temperature range and the HRT, meaning that thermophilic digesters have a shorter retention time than mesophilic and cryophilic ones.

Many facilities operate their biodigesters at the optimum temperature of thermophilic microorganisms because this reduces number of pathogens, favors methanogenic bacteria growth, improves the separation of liquid and solid fractions, and improves degradation of the substrate since there is more metabolic activity. Moreover, the methane production in thermophilic digesters is 25% greater than in mesophilic digesters. Nevertheless, the utilization of thermophilic temperatures also has disadvantages such as a higher degree of imbalance due to an increased production of volatile fatty acids. When dealing with manure, for example, reactors had optimal production in mesophilic reactors with the temperature between 30 and 35 °C, with only a 3% difference in the methane yield between these two temperatures. The same substrate at 25 °C had a decrease in methane yield of 17.4% [17]. In another study, two reactors, a one-stage reactor operated at mesophilic temperatures and a two-stage reactor operated at thermophilic (first stage) and mesophilic (second stage) temperatures, had their volatile solid consumption compared. The results demonstrated that a thermophilic (60 °C) stage was especially effective in degrading sludge waste substrates, with a 35% reduction in VFAs compared to the one-stage mesophilic digester.

5.2. pH

The pH value of utilized substrates affects AD by influencing the methanogenic-organisms' doubling time. Moreover, pH also influences the dissociation of some important compounds, such as ammonia, sulfide, and some organic acids. Methane generation takes place in the range of 5.5–8.5 pH, with optimal production in the 7.0–8.5 pH range. Most of the problems in AD can be attributed to acid accumulations and a consequent drop in the pH value. Considering

that CO_2 solubility decreases when the temperature increases, the pH of thermophilic reactors is higher than mesophilic ones and therefore has less carbon dioxide dissolved in carbonic acid form, making it more endurable for methanogenic groups. In a two-phase digester, the hydrolytic-acidogenic and acetogenic phases are separated from methanogenesis, and with this, the pH can be controlled to the optimum range for the first phase (4.0–6.0) and second phase (7.0–8.5). In a single-phase reactor, the pH is usually maintained around the tolerance of the methanogenic group (6.6–8.0) since the other population groups of organisms can tolerate these conditions [18].

5.3. Ammonia

Nitrogen in the form of ammonia (NH_4) is present in the environment of the digester as a gas. It originates from protein degradation and from animal slurry, due to its high ammonia concentration. The precise concentration of free ammonia at which it starts to be toxic remains uncertain, but when dealing with a non-adapted digester (i.e., a digester that has not had enough time to acclimate its methanogenic population to a high ammonia concentration), its inhibition starts at 0.08–0.15 gN/L of free ammonia and 2.5 gN/L of total ammonia. In an adapted digester, it is 0.7–1.1 gN/L of free ammonia and 4–6.5 gN/L of total ammonia [19]. Methanogenic bacteria are very sensitive to the presence of ammonia as its presence can disturb the process in two forms, (1) inhibiting methanogenic enzymes in archaea and (2) entering the archaea cell and causing an unbalance in the electrons and disrupting the process [20].

5.4. Micronutrients (trace elements)

The impact of trace elements and changes in its concentration in bioreactors depends on various factors, such as the microbial community structure; population dynamics; individual trophic group metabolism; and meta-community (e.g., the microbial community as a group, incorporating compounds as well as cells). With that in mind, it is hard to fix micronutrient concentrations that are fully satisfactory for the microorganisms' community present in the reactor.

Although nutritional demand for each microorganism species varies, this topic will explore general guidelines of micronutrients, which are limiting for methane-forming archaea. These microorganisms have specific methanogenic enzyme systems with different requirements when compared to other microorganisms. These systems need specific micronutrients that must be incorporated or added to the substrate for its proper degradation and efficiency of CH_4 production.

Cobalt, iron, nickel, and sulfide are obligatory micronutrients, because they are cofactors of the methane pathway enzymes that convert acetate into methane. In some cases, molybdenum, tungsten, and selenium can be obligatory micronutrients as well as barium, calcium, magnesium, and sodium [21].

These micronutrients are usually present in municipal wastewater, although the digester effluent, in some cases, must be analyzed to ensure their presence in enough quantities and

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guarantee that these nutrients are in a soluble form since micronutrient deficiency can be mistaken with toxicity from the accumulation of volatile fatty acids.

Simple variations in the amounts of elements can disturb the environment inside the digester by unbalancing the substrate process and then causing inhibition of the whole process. For example, under co-limiting conditions, methanogenic activity was lost within ten days by acidification of a methylotrophic digester. In other study, Zn deprivation affected methane production significantly, which could not be later restored by a continuous supply of Zn [22].

6. Anaerobic bioreactors

The biodigester (or anaerobic bioreactors) must guarantee optimal conditions for feedstock transformation to occur, such as the retention of the active biomass and favorable environmental conditions for biomass degradation of organic matter [23]. A report, dating from the 1880s, presents a biodigester, named by its inventor, Donald Cameron (Exeter, England), as a "Septic Tank," which was much more efficient than previous, and more rudimentary tanks since its design promoted microbial growth by adopting an organic material entry and exit system below water level in order to minimize the entry of air and turning of the upper part of the tank [24]. The precursor tank, called the "automatic scavenger," was built by Jean Louis M. Mouras, author of the first reference to the liquefaction of organic matter of wastewater under anaerobic conditions (patented in 1881) [24]. However, it is worth noting that this is not the first AD bioreactor, but one of the first reports in the literature.

The increase in demand of organic matter degradation has allowed for further development of these bioreactors, such as the addition of a heating system [25] and mechanical agitation– – Patent US2605220 [26]. Additionally, there are many studies regarding bioreactor design and the way the digestion is conducted, as described in the next Section (6.1).

6.1. Bioreactors types

The digestion unit is the most important part of a biogas plant; after all, it is where organic matter is reduced into biogas by microorganisms. An anaerobic digester design should allow for a continuously high load rate of organic matter, short hydraulic retention time (to reduce bioreactor volume), and a maximization of methane production. The shape of the bioreactor should take important considerations into account, such as the exchange of heat and the mixture, which is not observed in underground reactors (**Figure 2**). In general, these bioreactors are built from concrete blocks in a rectangular or square shape format that does not benefit the mixture. Furthermore, they have accumulated points (edges) of raw materials that lead to reduction in plant efficiency and require more frequent maintenance and thus idle time [27].

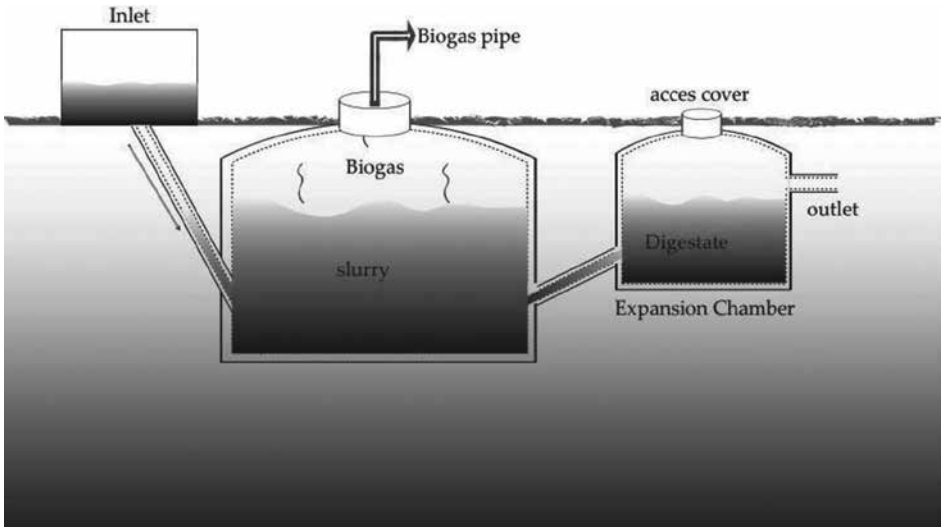

Figure 2. Underground reactor.

The choice of bioreactor for biogas production will depend directly on the characteristics of the raw materials utilized such as dry matter content, rate of degradation, and risk of inhibition. Among the main processing technology options available, there are feeding systems, reactor type, temperature reactor, number of phases, and agitation system (**Figure 3**) [28]. Nevertheless, only the most frequently used options of reactor type and number of phases will be described in more detail in this chapter.

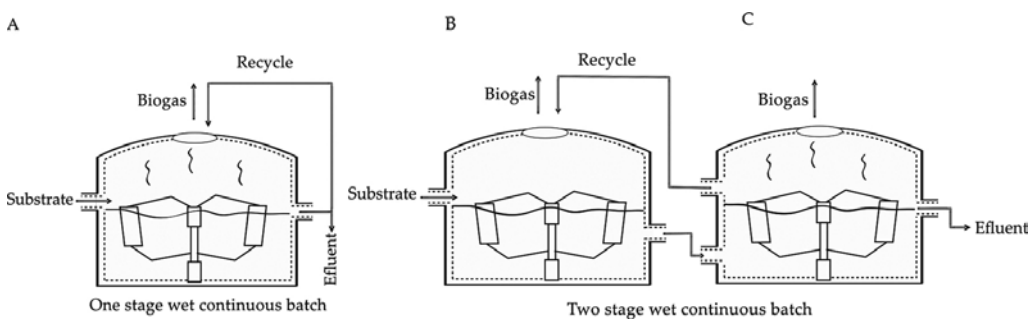

Figure 3. Fermentation modes utilized for biogas production batch digester—one-stage continuously fed system (A); two- or multistage continuously fed system: first stage (B) and second stage (C).

They may be dry or wet, batch or continuous, one step or multistep, and one phase or multiphase and may operate under different temperature conditions (mesophilic or thermophilic). However, the main bioreactor groups commonly employed are as follows: (1) batch bioreactors (**Figure 3A**); (2) continuous fed system: (a) one stage (**Figure 3B**); and (b) two stage or multistage [29] (**Figure 3C**).

6.1.1. Batch bioreactors

In this type of system (**Figure 3A**), a digestion vessel is loaded once with the feedstock then sealed off and left to ferment until gas production decreases. Then, the bioreactor is emptied and filled again with a new batch of feedstock. It is worth noting that part of the digestate should be left in the vessel, which will serve as inoculum for the next batch [30]. This type of bioreactor is generally utilized for feedstock that has a high solid content (between 30 and 40%) and with a high fiber content [31], and it requires little daily attention and it is notable for its simplicity. Moreover, batch reactors may be more suitable when using small amounts of substrate [32].

However, batch bioreactors have some limitations, for example (1) high variation in gas quality and production; thus, a series of batch digesters are employed, which are fed sequentially to generate a reasonably homogenous production of biogas; (2) a considerable time requirement to empty and load the batch digesters; (3) biogas losses during discharging the bioreactors; and (4) limited bioreactor heights [29]. The production of methane may vary from 44.6 to 290 mL/g VS for yard trimmings and rice straw as substrate, respectively [2].

6.1.2. Continuously fed system

For continuous digesters, unlike the batch bioreactors, the feedstock is constantly fed mechanically or by flow force by the newly entered feedstock, enabling uninterrupted production of biogas [33]. Among the types of continuous digesters, the multiple tank system (or multistage system) stands out, which will now be described.

6.1.2.1. One-stage, two-stage, or multistage continuous fed system

As previously discussed in this chapter (Section 4.1), there are four biochemical reactions in anaerobic digestion: hydrolysis, acidogenesis, acetogenesis, and methanogenesis. When all of these biochemical reactions take place in one reactor, it is called a one-stage continuously fed system (**Figure 3B**), in contrast, when the biochemical reactions occurs separately in two reactors, it is called a two-stage (or multistage) continuously fed system (**Figure 3C**) [27].

Organic waste treatment systems that use the two-stage system present advantages over one-stage systems, such as high biogas production rates and yields. One study demonstrated a 13% increase in methane production from cellulosic material in a process that used a two-stage process compared to a single phase [34]. A similar increase was obtained using olive mill solid residues as the substrate [35]. Another study [36] compared one- and two-stage digestions for the treatment of thin stillage. It obtained approximately 57% total volatile fatty acids to the total chemical oxygen demand ratio, while the digestion obtained from one stage is only 10%. Additionally, the use of two-stage digestion also increased the production of methane, from 0.26 L CH^4/g of the chemical oxygen demand added (one stage) to 0.33 L CH_4/g of the chemical oxygen demand added [36]. This is because the system that performs the separation stages of the biochemical anaerobic digestion benefits the selection and development of different microorganisms for each stage. In addition, the conditions in each respective phase are controlled to generate an optimal environment for the action of each microorganism [37].

Acidogenic bacteria are the prevailing microorganisms in the first stage while the methanogenic group is dominant in the second one. In addition, as previously discussed, the intense production of acids inhibits methane formation in a one-phase system. Hence, the second stage favors bacteria that perform the production of methane gas [28]. The multiple-step system allows a faster, higher performance, and less expensive process than those that use single-stage digester, even though multistage digesters were more expensive to build and maintain [38]. The methane yield from municipal solid waste using a two-stage reactor can be 21% greater than the methane yield obtained from a single-stage process [39].

6.2. Microorganisms retention

In general, the generation time of hydrolytic and acidogenic bacteria ranges from approximately 1–3 days, whereas methanogenic and acetogenic bacteria range from about 1–4 and 5–12 days, respectively [13]. Due to the slow growth of microorganisms during the process of digestion, a reactor operated in a continuous mode can result in washout. Therefore, the rate of loading and unloading cannot exceed the maximum growth rate of microorganisms. In addition, the calculation of this rate is one of goals of process optimization. Additionally, one other way to prevent this type of accident is to use immobilized cells [19]. The use of microbial consortium retention contributes to increased performance of the anaerobic phase [40]. The use of support material such as toasted coconut shells and wood chips produced 720 and 144 L/kg VS of biogas, respectively, while the use of expanded clay showed nearly no production [40].

Anaerobic filters use inert supporting materials such as clay fibers, polyvinyl-chloride sheets, polyurethane foam, polypropylene membranes, carbon fiber textiles, tire rubber, zeolite filters, glass, and polyethylene fibers [40]. It is practical at this point to highlight that not only is the type of support material directly related to the performance of the anaerobic reactor, but so are other factors such as specific surface area, porosity, surface roughness, pore size, and orientation of the packaging material [40].

Microbial immobilization on the surface and in the pores of the inert material allows a reduction in the hydraulic residence time, which can decrease from 30 days to under a week, and it consequently reduces reactor volume and initial cost and increases the yield [32]. Among the used systems are (1) fixed- or packed-bed reactors and (2) fluidized-bed reactors (**Figure 4**).

6.2.1. Fixed-bed reactors

In its initial application, the fixed-bed system was used as biological filters for sewage treatment, so it is also known as an anaerobic filter (similarly called a biofilm reactor or packed bed). In this system, the particles containing the immobilized cells are fixed or packed into the reactor and the liquid flows through the bed. The fixed-bed reactor (**Figure 4A**) allows the application of greater organic loads than those applied in the complete mixture of anaerobic digesters. This system uses one kind of reactor that maintains a high biomass density within the reactor through microorganism retention from biofilms that have developed on the support material [32].

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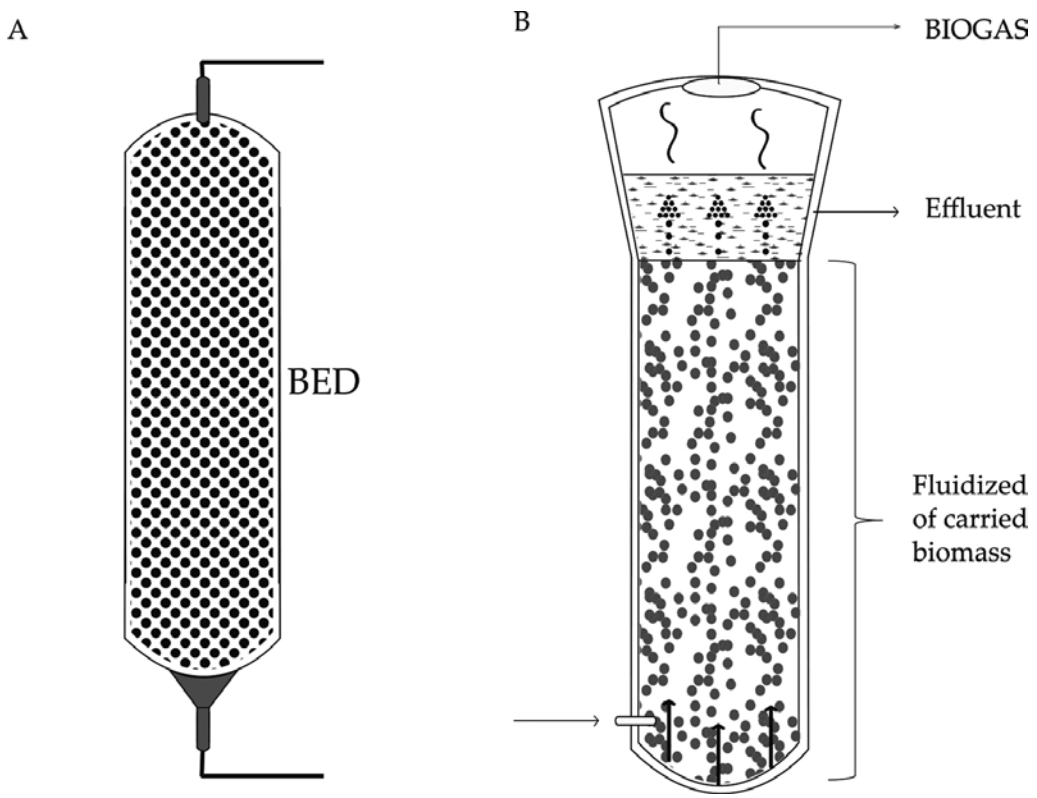

Figure 4. Microorganisms' retention reactors: (A) fixed-bed reactor and (B) fluidized-bed reactor.

6.2.2. Fluidized-bed reactors

In fluidized-bed systems (**Figure 4B**), the supporting material particles are maintained in suspension within the reactor due to substrate flow. This allows the particles to become unrestricted, and therefore, its entire external surface is available for interaction with the feedstock. This type of system has an advantage over packed-bed because we could substrate particulate packed beds. Furthermore, control of the temperature and the pH is more effective than the packed beds [32].

The performance of both reactors (fixed bed and fluidized bed) was compared with that of a fixed-bed reactor under similar conditions (feed gas to steam ratios of 1.5 and 0.75 at a reactor temperature of 750 °C, GHSV (gas hourly space velocity) of 300 L/min) [41]. This study showed a conversion of 75% CH_4 in a fixed-bed reactor. On the other hand, when using the fluidized-bed reactor, the production was much greater, reaching up to 90% conversion. The authors of this study reported the low yield of the fixed-bed reactor creates points of temperatures below the optimum process temperatures.

7. Conclusions and perspectives

Currently, numerous efforts are being made to reduce energy dependence on oil. This requirement has led to the development of new technologies for the use of other energy sources, such as the production of biogas. This biofuel is an important alternative to ensure the supply of clean and affordable energy and to contribute toward reducing the accumulation of waste, as biomass can be used as raw materials for biogas production. However, obtaining high yields is still a major challenge. One solution is to optimize the process, adjusting some of the physical and chemical parameters, such as temperature and pH. This is because this fermentation process involves several microbial groups and therefore needs to be adjusted to the environment of each of these groups. One way to do this is to include fermentation stages, in which more than one reactor is used, allowing the maintenance of optimum conditions for each microbial group involved in each step. Another challenge is the hydraulic retention time, which is the normal time that the input substrate spends in the digester before it is removed. A solution for this is microorganism retention, where they are imprisoned within inert materials, allowing the microorganisms to remain longer inside the reactor. It is worth noting that a deeper understanding of the physiology of each microbial gender participating in the process should be performed in order to be able to more precisely optimize the process parameters. Finally, despite biogas production being an age-old process, little is known about this process. Therefore, further studies on this process are necessary to achieve greater production and thus more amplified outcomes of this process.

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Chapter 10

Production Processes for Monoclonal Antibodies

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Additional information is available at the end of the chapter

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Abstract

Antibodies are glycoprotein structures with immune activity. They are able to identify or induce a neutralizing immune response when they identify foreign bodies such as bacteria, viruses, or tumor cells. Immunoglobulins are produced and secreted by B lymphocytes in response to the presence of antigens. The first monoclonal antibodies (mAbs) have emerged from a survey of hybridomas, and nowadays mAbs are produced mostly from cultivations of these cells. Additionally, there are studies and patents using a range of cells and microorganisms engineered for the production of mAbs at commercial scale. For some years, new methodologies have advanced with new production processes, allowing scale-up production and market introduction. Large-scale production has revolutionized the market for monoclonal antibodies by boosting its production and becoming a more practical method of production. Production techniques have only had a sizable breakthrough due to molecular techniques. Various systems of production are used, including animal cells, microorganisms, plants, and mammary glands. All of these require the technological development of production process such as a stirrer, a wave bioreactor, and roller bottles.

Keywords: monoclonal antibodies, bioprocess, bioreactors, antibodies, mAbs

1. Introduction

Monoclonal antibodies (mAbs) have been widely used as a way to successfully achieve a broad range of extracellular targets with high specificity [1]. mAbs have various applications in

diagnosis and therapy for several diseases such as cancers, autoimmune diseases, sexually transmitted infections (STIs), and others [2, 3]. In recent years, the use of mAbs has been expanded due to significant advances in design. The effect of decreasing immunogenicity in humans, improvement in their bioavailability, optimizing the affinity and antigen-binding specificity, and other advances in protein engineering are improving therapeutic mAb profiles (**Figure 1**) [2].

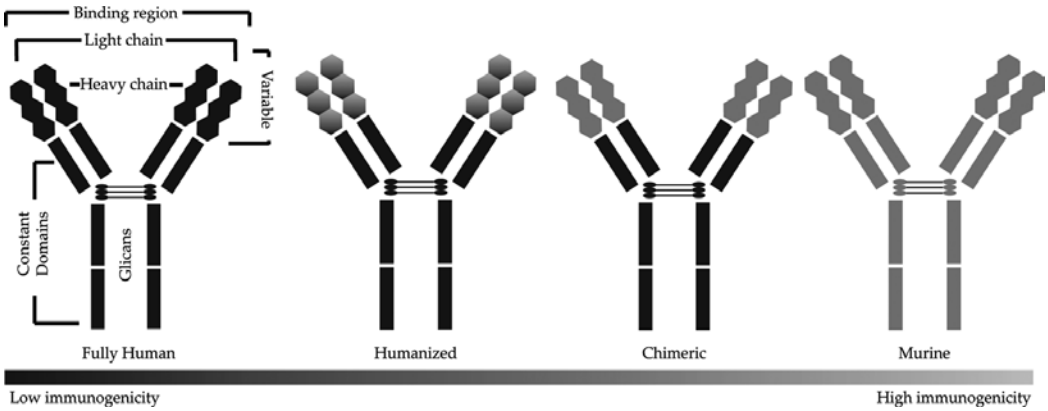

Figure 1. Schematic overview of a monoclonal antibody, showing their heavy and variable chain.

With the advent of genetic engineering, it has been possible to develop new methods to obtain monoclonal antibodies, both for improvement with regard to these humanized antibodies and for production models [4–6]. Advances in molecular and cell biology for the development of more efficient antibodies have allowed advances in diagnostic and therapeutic areas. Such advances have triggered improvements in production processes, allowing for the reduction of production costs and thus leading to an increase in the popularization of treatments with mAbs. All process improvements provide a consistent and reproducible production of large quantities of mAbs at a moderate cost [4–6].

Large-scale production has revolutionized the market for monoclonal antibodies by boosting its production, making this a more practical method of production. Production techniques have only had a sizable breakthrough due to molecular techniques [1, 7].

In general, a process of commercial production of mAb begins with the generation of an mAb by immunizing an animal or by molecular biology methods involving the identification and optimization of the coding DNA sequence and the construction and identification of a stable high-producing clone. Improvements in cultivation are similar to those applied in other bioproducts that rely on culturing microorganisms or cells, requiring the development of a well-designed culturing process comprising the full range of control and associated operations that will support technical evaluations [1, 8].

mAbs production processes in wave or single-use bioreactor (SUBs) are characterized by flexibility and low operating costs when compared to the production processes in fixed

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stainless steel vats. The development of bioprocesses involving these production platforms can reap greater acceptance by the industry [9–11].

Drugs based on mAbs have been controlled by regulatory agencies around the world. Therefore, it is necessary to elaborate regulatory protocols accompanying the increase in production and the nuances of the characteristics of this class of drugs [10, 11].

The proposed chapter covers the fundamental aspects of monoclonal antibody production methods, with emphasis on methodologies using immobilized cells, wave bioreactor systems, SUBs, and finally the roller bottles technique. Such techniques have been described in the most recent literature, both for murine monoclonal antibody production and for production of antibodies from modified microorganisms.

2. mAbs production techniques

2.1. Hybridoma and phage display

Milstein and Köhler described the first technique developed for stable monoclonal antibody production in 1975. This technique consists of creating a hybridoma, a stable hybrid cell capable of producing a single type of antibody against a specific epitope present in an antigen. Hybridoma construction was initially produced from murine models. The technique consists of removing a pool of activated B lymphocytes from an immunized animal spleen and combining them with immortalized myeloma cells unable to produce the enzyme hypoxanthine-guanine-phosphoribosyltransferase (HGPRT), an important enzyme present in the *salvage* pathway, one of the pathways responsible for nucleotide production [1]. To select hybridoma cells, the pool of cells resulting from the fusion (a mix of hybridoma cells and non-fused B lymphocytes and myeloma cells) are cultivated in a selective medium containing aminopterin, which inhibits the nucleotide de novo synthesis. Myeloma cells lack the *salvage* pathway for nucleotide production. When they are exposed to aminopterin present in selective medium, the de novo synthesis is also blocked, and as a result, myeloma cells are no longer viable since all major pathways for nucleotide production are blocked. In contrast, non-fused, activated B lymphocytes can survive as their *salvage* pathway works perfectly and they can continue nucleotide production even if the de novo pathway is blocked by aminopterin. However, these cells are not immortalized and can replicate only a limited number of times after which they eventually die. With this in mind, only cells capable of replicating indefinitely and synthesizing nucleotides through the *salvage* pathway can survive through selection conditions, and these cells are the hybridomas.

In spite of the fact that the primary recombinant mAbs were delivered utilizing this innovation —including the first medication approved by the Food and Drug Administration (FDA) for therapeutic proposes (**Table 1**)—the great contribution of this technology was mostly to elucidate immune response mechanisms and control in vitro antibody production. Therefore, mAb hybridoma production from murine sources exhibits a genuine downside in human therapeutics (**Figure 1**).

Drug name	Active ingredient	Description	Target	Therapeutic category	approval (FDA)
ACTEMRA®	Tocilizumab	Humanized IgG1κ	IL-6 receptor	Immunological	2010
ADCETRIS®	Brentuximab vedotin	Chimeric IgG1	CD30	Cancer	2011
ARZERRA®	Ofatumumab	Human IgG1κ	CD20	Cancer	2009
AVASTIN®	Bevacizumab	Humanized IgG1	VEGF	Cancer	2004
BENLYSTA®	Belimumab	Human IgG1λ	BLyS	Immunological	2011
BEXXAR	Tositumomab; iodine I 131 tositumomab	IgG2αλ, I131	CD20	Cancer	2003
BLINCYTO	Blinatumomab	BiTE antibody-scFvs	CD19/CD3	Cancer	2014
CAMPATH (LEMTRADA™)	Alemtuzumab	Humanized IgG1κ	CD52	Immunological	2001
CEA-SCAN	Arcitumomab	Murine IgG1 Fab′	CEA	Diagnosys	1996
CIMZIA®	Certolizumab pegol	Humanized Fab′, PEG	TNFα	Immunological	2008
COSENTYX®	Secukinumab	Human IgG1κ	IL-17A	Immunological	2015
CYRAMZA	Ramucirumab	Human IgG1	VEGRF-2	Cancer	2014
DARZALEX	Daratumumab	Human IgG1κ	CD38	Cancer	2015
HERCEPTIN®	Trastuzumab	humanized IgG1κ	HER2	Cancer	1998
EMPLICITI™	Elotuzumab	Humanized IgG1	SLAMF7	Cancer	2015
ENTYVIO	Vedolizumab	Humanized IgG1	α4β7 Integrin	Immunological	2014
ERBITUX®	Cetuximab	Chimeric IgG1	EGFR	Cancer	2004
GAZYVA®	Obinutuzumab	Humanized IgG1	CD20	Cancer	2013
HUMIRA	Adalimumab	Human IgG1	TNF	Immunological	2002
ILARIS	Canakinumab	Human IgG1κ	uman-IL-1β	Immunological/ anti-inflammatory	2009
KADCYLA®	Ado-trastuzumab emtansine	Humanized IgG1; DM1	HER2	Cancer	2013
KEYTRUDA®	Pembrolizumab	Humanized IgG4κ	PD-1	Cancer	2014
LEMTRADA™	Alemtuzumab	Humanized IgG1κ	CD52	Immunological	2001
LUCENTIS	Ranibizumab	Humanized IgG1κ	VEGF-A	Ophthalmic	2006
Muromomab	Orthoclone	Murine IgG2α	CD3	Immunological	1992
Mylotarg®	Gemtuzumab ozogamicin	Humanized IgG4κ, calicheamicin	CD33	Cancer	2000

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Drug name	Active ingredient	Description	Target	Therapeutic category	approval (FDA)
MYOSCINT®	Imciromab Penlelale	Murine IgG2/4κ Fab′;DTPA	Heavy chain of human myosin	Detection of myocardial injury	1996
NUCALA®	Mepolizumab	Humanized IgG1κ	IL-5	Immunological	2015
OPDIVO	Nivolumab	Human IgG4κ	PD-1	Cancer	2014
PERJETA®	Pertuzumab	Humanized IgG	HER2/neu receptor	Cancer	2012
PORTRAZZA	Necitumumab	Human IgGκ	EGFR	Cancer	2015
PRALUENT™	Alirocumab	Human IgG1	PCSK9	Lipid-lowering	2015
PRAXBIND®	Idarucizumab	Humanized IgG1 Fab	Dabigatran (anticoagulant)	Hemostasis	2015
XGEVA®	Denosumab	Human IgG2	RANKL	Bone disorders	2010
ProstaScint®	Capromab pendetide	Murine IgG1κ, GYK-DTPA-HCl	PSMA	Cancer	1996
RAPTIVA®	Efalizumab	Humanized IgG1κ	CD11a	Immunological	2003
RAXIBACUMAB	Raxibacumab	Human IgG1λ	PA of B. Anthracis toxin	Anti-toxin	2012
REMICADE®	Infliximab	Chimeric IgG1κ	TNFα	Immunological	1998
ReoPro®	Abciximab	Chimeric IgG1κ Fab	GPIIb/IIIa	Hemostasis	1993
REPATHA	Evolocumab	Human IgG2	PCSK9	Lipid-lowering	2015
RITUXAN®	Rituximab	Chimeric IgG1κ	CD20	Cancer	1997
SIMPONI	Golimumab	Human IgG1κ	TNFα	Immunological	2009
SIMULECT®	Basiliximab	Chimeric IgG1κ	IL-2 receptor	Immunological	1998
SOLIRIS®	Eculizumab	Humanized IgG2/4κ	C5	Hemostasis	2007
STELARA®	Ustekinumab	Human IgG1κ	IL-12 and IL 23	Immunological	2009
SYLVANT	Siltuximab	Chimeric IgG	IL-6	Immunological	2014
SYNAGIS®	Palivizumab	Humanized IgG1κ	RSV F	Antiviral	1998
NeutroSpec™	FanolesomaB; technetium Tc 99m	Murine IgM	3-fucosyl-N-acetyllactosamine	Diagnosys	2004
TYSABRI	Natalizumab	Humanized IgG4κ	α4β1/α4β7 integrins	Immunological	2004
UNITUXIN™	Dinutuximab	Chimeric IgG1κ	Glycolipid GD2	Cancer	2015
VECTIBIX®	Panitumumab	Human IgG2κ	EGFR	Cancer	2006
VERLUMA™	Nofetumomab	Murine IgG2b Fab	Glycoprotein antigen expressed in a variety of cancers	Diagnosys	1996

Drug name	Active ingredient	Description	Target	Therapeutic category	approval (FDA)
XGEVA	Denosumab	Human IgG2	RANKL	Cancer	2010
XOLAIR®	Omalizumab	Humanized IgG1κ	Human IgE	Immunological	2003
YERVOY®	Ipilimumab	Human IgG1κ	CTLA-4	Cancer	2011
ZENAPAX®	Daclizumab	Humanized IgG1	IL-2 receptor	Immunological	1997
ZEVALIN®	Ibritumomab tiuxetan	murine IgG1κ, Yttrium-90	CD20	Cancer	2002

Table 1. Monoclonal antibody-based therapeutic drugs approved by FDA (Food and Drug Administration) until 2015.

After a few infusions, murine antibody molecules trigger the human anti-mouse antibody (HAMA) response of the human immune system [1, 12]. To work around this issue, new methodologies have been developed to deliver antibodies similar to human molecules, so the technology evolved to less immunogenic chimeric antibodies (constant regions of human antibodies linked to the variable region of the murine source), creating a new set of therapeutic possibilities (**Figure 1**). Subsequently, the need for an even less immunogenic alternative boosted the production of humanized antibodies (only the region that interacts with the antigen epitope is from mouse origin) (**Figure 1**). Even fully human antibodies (**Figure 1**) can be produced from genetically modified mice [13].

A great improvement in mAb production has come with the development of phage display libraries. This methodology helps to investigate interactions between molecules (protein-protein, protein-peptide, and protein-DNA) and consists, basically, in cloning Fab-region-coding genes amplified from B lymphocytes into bacteriophage plasmid vectors. Then the bacterium can be transformed with these vectors, going on to express the heterologous genes from a viral capsid. This capsid contains viral proteins and proteins encoded by the Fab sequence received by that specific cell. Once the library is complete, the affinity between proteins produced from different Fab regions can be tested against the antigen of interest and the cell transformed with the plasmid that contains those genes can be readily sequenced. The advantages of this methodology are the following: the same library has the potential to generate a great number of new antibodies, it is an in vitro process, so it does not require animal immunizations steps, and because of that, toxic antigens can be tested. Also, a greater variety of antigens can be tested, and antibody molecules can be rapidly obtained [13].

2.2. Culture production factors

2.2.1. Cell lines

One of the most critical steps in developing an mAb production system is to choose the cell line. The cells must be stable and secrete the desired protein with the correct conformation at high levels. Based on these requirements, the mammalian cell is the most commonly chosen expression system for mAb production. The main advantage of a mammalian expression

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system is that the cellular machinery is adapted for the production, processing, and secretion of highly complex molecules. The great majority of commercial mAbs are produced in Chinese hamster ovary (CHO) and NS0 cells, originating from plasmacytoma cells that were modified until IgG generation in nonsecreting B cells. Genetic modifications in CHO cells have generated cell lines capable of producing a high quantity of humanized mAbs. These cell lines were able to secrete up to 100 pg/cell/day [14]. Other modifications led to a high production of a chimeric mAb, ranging from 80 to 110 pg/cell/day [15]. NS0 modifications also have been made, leading to higher mAb production rates, ranging from 20 to 50 pg/cell/day [16]. In smaller quantities, hybridoma cell lines are also used in industrial mAb production. Some hybridoma strains are reported to have a production rate up to 80 pg/cell/day [16]. In spite of this, different mammalian cell lines and even more peculiar expression systems such as genetically modified plant cells, genetically modified insect cells, and genetically modified microorganism cells have also been used in mAb production and have gained space in the biopharmaceutical industry [1, 8]

Microorganisms modified by genetic engineering techniques have attracted much focus in industry, because these cells are simpler to handle and to modify when compared to animal cells. Other advantages of production methods using genetically modified microorganisms are that these cells have well-defined expression systems, and the production methodology is reproducible and easy to validate. Modified yeast cells, such as *Pichia pastoris* have a great potential for usage since these cells are known to achieve high secretion levels of heterologous proteins. Yeast cultivation systems for mAb production are easier scale-up and are cheaper when compared to mammalian cell cultivation systems. They can be cultivated in regular stirred tank bioreactors, in batch, or in feed-batch modes of operation. Generally, microorganisms do not have physicochemical and biological characteristics for the appropriate expression and posttranslational processing of mAbs [4].

Modified plants have also gained attention since plants are easy to cultivate and propagate. Other cultivation advantages such as cheap medium, low maintenance cost, and high production yields make plant production a cheaper alternative when compared to mammalian cell cultures [17]. However, there are some limitations—different glycosylation patterns and post-translational processing can also make plant cell utilization difficult [17].

2.2.2. Culture medium

Cultivation media for mammalian cells must have a complex content of ingredients ranging from amino acids to trace elements. To supply the cellular demand of these nutrients, the culture medium uses serum in its composition, however, due to the emergence of diseases caused by defective prions, such as bovine spongiform encephalitis (BSE), there is a great incentive to remove any animal component of culture media composition, especially if the medium is used for industrial production of biopharmaceuticals products. This has led to the emergence of media free from any animal components, including well-defined media for CHO and NS0, the two most utilized cell types in mAb production. The development of a proper medium can be time consuming and very expensive. However, many companies prefer to develop their own production media to maintain the composition between production lots as well as develop an appropriate medium composition for the specific cell type that will be used

and to achieve greater control over production. Added to this, the development of downstream processes that meet the requirement for high-purity products and tests to validate the final product quality raises the overall production cost of a drug based on monoclonal antibodies [1].

Despite the complexity of developing a culture medium, much progress has been made in this area, allowing for greater cell growth and increasing cell conservation time in suitable conditions for the growth and production of molecules of interest [8].

2.2.3. Culture conditions

Growing conditions can directly influence the cell growth and production levels of molecules of interest. Usually, mammalian cell culture conditions for mAb production are very well defined: 37 °C, pH 7.15, and dissolved O_2 (OD) levels at 30–60%. CO_2 level is monitored to mimic the physiological standard between 31 and 54 mmHg. However, changes in cellular conditions have shown great potential to change cellular metabolism toward cellular growth or molecule production and this can be used to increase mAb production. Bioprocesses can be designed to occur in two phases. First, cell growth is optimized to reach a certain cell density. Once this density is reached, the second phase begins and the bioreactor conditions are shifted so the cells continue to grow just at a maintenance rate and directing the metabolism toward monoclonal antibody production. Some CHO cell strains and hybridoma cells are sensitive to changes in temperature and pH. When subjected to temperature and pH values lower than those normally used, values between 30 and 35 °C and 6.7–7.0, respectively, cell growth metabolism is reduced and specific production increases. The growth metabolism reduction also contributes to lower production of some metabolic compounds which are toxic for cell cultures, allowing increased cell viability, which spend more time producing molecules of interest. A good way to monitor the growth stage of a cell culture for controlling changes in cultivation is watching the DO and pCO_2 levels, which can also be adjusted to maximize the production of proteins such as mAbs [1].

2.2.4. Production platforms

The cell culture for mAb production can follow three different types of processes. The simplest of them is batch production, which consists of a closed system where a bioreactor is sterilized and prepared with a medium containing all the nutrients needed for cellular growth and product manufacturing and then, cells are inoculated. There is no feeding system with fresh medium or withdrawal of spent medium. As the process runs, nutrient concentration decreases and waste metabolites are produced, lowering cell viability. In spite of being a simple process, batch is not the most suitable type of production platform for mammalian cell cultures, as the environment inside the reactor quickly becomes unfavorable for cell growth and, at the same time, waste product concentration increases. Cultivation factors such as initial nutrient concentration and waste metabolite production directly determine the maximum concentration that cells can reach in a bath culture. Generally, this type of cultivation reaches a maximum density of 1–2 × 10^6 cells/mL, and then the cell viability drops rapidly [1]. The production process lasts for 4–7 days, when productivity reaches certain concentration of interest [1]. Supernatant is collected and the product is recovered by downstream processes. The time that

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each batch takes to finish also depends on the production kinetics. If the production is growth dependent (production occurs concomitantly with cellular growth), batch processes can be stopped as soon as cells reach the stationary phase. But if the product is not associated with growth (production only starts when the growth rate decreases), the culture needs to be carried for a longer period of time since production only starts at stationary phase.

In contrast to batch, a second type of production process utilized is continuous fermentation. There are two types of continuous production: chemostat cultures and perfusion cultures. Concerning chemostat cultures, fresh medium is added to the bioreactor and fermented medium is removed along with cells at a constant flow rate so that the culture volume remains unchanged. The flow rate (dilution rate) controls cellular growth and when these two variables are equal, the bioreactor reaches equilibrium—cell concentration, nutrient concentration, and product concentration are held constant. In this context, the culture can be kept in equilibrium for several months reaching a cell density of 10–30 × 10^6 cells/mL [1]. To avoid viable cell loss along with the constant outflow of the by-products of cell metabolism, many manufacturing plants have developed a cell-recycling system and thus, the perfusion culture method was developed where cells are kept inside the bioreactor. The disadvantages of continuous fermentation are the use of a large amount of expensive culture media and the difficulty in recovering the product, which comes out fairly diluted. These two disadvantages are consequences of the constant medium flow rate. To work around the product dilution problem, some production manufacturing plants have ultrafiltration systems which retain the product inside the bioreactor [18]. Another obstacle of this type of process is that the establishment of culture conditions for a stable industrial production plant can take months. For this to occur, the strain used must be very stable and have its physiological aspects clearly elucidated, such as growth rate, productivity, and response to certain stress conditions. It is not uncommon to hear that numerous attempts are made before the settlement of a stable production plant is achieved, but, once settled, this production process can bring many advantages, since it can be operated in smaller-volume bioreactors, and therefore have greater production flexibility.

The third type of process for producing monoclonal antibodies is by far the most utilized at industrial scale, which is fed-batch process. In this process, the cell density reaches 8–12 × 10^6 cells/mL, and cell viability in the bioreactor is enhanced by controlled nutrient addition at specified intervals [1]. The production process can take 12–20 days [1]. Usually, the same medium used in the initial culture is also used for feeding, but in a more concentrated version. The feeding solution composition can be designed to supply the cells based on their metabolic state at different culture phases by analyzing and identifying the spent medium nutrients that are being more consumed. Furthermore, the medium used in feeding can be modified to promote cell growth or to stimulate molecule production, since different components may modify the behavior of cells, changing the metabolism for different purposes. The feed solution can also be designed to minimize the production of waste metabolites that cause cell stress when in excess. However, their production is not completely avoidable as they eventually reach harmful concentrations. It is relatively easy to scale up and operate this system. More summarized data about the advantages and disadvantages of each process for mAb production can be seen in **Table 2**.

Production platform	Batch	Feed-batch	Perfusion culture
Advantages	• Simple to scale-up • Control by production lot	• Simple to scale-up • Control by production lot • Production facility is simple • Process is easy to perform and to validate • Higher volumetric production	• Cells are maintained in a relatively optimal biochemical environment • Culture reaches high cell density • Higher volumetric production
Disadvantages	• Difficult to define initial concentration of nutrients • Accumulation of waste metabolites • Degradation of more sensitive products	• Accumulation of waste metabolites • Degradation of more sensitive products	• Lack of homogeneity in the continuous reactor vessel • Challenges regarding long-term operability and maintenance • High cost and long times required for process development experiments • Genetic instability of cells

Adapted from [1, 21] (colocar referencias).

Table 2. Comparison between different operation modes that can be used for mAbs production.

A lot of effort has been made to increase cell longevity in batch and feed-batch modes of operation. It is expected that the longer the cells are maintained viable, the greater the antibodies' production will be. So, in order to maintain cell viability, some culture parameters can be optimized, such as culture media, feed solution, and mAb secretion rates and by-product production. To improve mAb titers in the batch platform, the start medium can be supplemented with glucose and amino acids, increasing mAb production up to eightfold when compared with regular media [9, 26]. Improvements for the fed-batch platform can be achieved by adjustments in feed solution, as mentioned before. Feed solutions containing glucose and aminoacids/glutamine have been reported to increase mAb titers from two to fourfold, reaching production of up to 2 g/L, when compared with the batch production platform [19].

The optimization of the antibody secretion rate can be achieved by high-density cell cultivation. On a fed-batch platform, a high cell cultivation culture can reach an mAb productivity rate of 0.94 g/L/day and a final titration of 17 g/L, while a continuous culture performed at high density conditions can reach final titration and productivity rates of 0.8 and 1.6 g/L/day, respectively [20]. Optimizing mAb secretion highly depends on the cell line chosen for production. Each cell strain can be influenced by the manufacturing conditions and respond differently to increasing or decreasing mAb production and secretion [19]. The accumulation of toxic by-products is a great bottleneck in manufacturing processes since they can inhibit cell growth

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and then directly affect mAb production. Although a few strategies to minimize this by-product accumulation have shown to be promising, some are not applicable for a large-scale production. Optimizing medium composition and feed solutions with substrates that reduce toxic compound production is the most common strategy used at industrial scales of production [19].

Although most mAbs are produced by fed-batch process, there are tendencies indicating that in the future many bioprocesses will be operated in continuous platforms, especially for the production of biopharmaceuticals. On these platforms, the production system will be coupled to upstream and downstream processes [21]. However, for this to actually happen, a great improvement in technological development still needs to be achieved.

2.3. Production systems

The use of monoclonal antibodies as therapeutic drugs requires a large-scale production that far exceeds that of laboratory production (**Figure 2**). Various production systems have been developed and have evolved, while new alternatives are emerging. The production of mAbs at commercial scale can be performed with adherent cells or suspension cells, although the latter is by far the most used and is better established with more efficient production methods available for cells cultivation. Thus, scale-up using suspension cells is easier. Another advantage of the suspension production system is that a bioreactor with a large area for cell adhesion is not necessary since the cultivation of adherent cell productivity is directly linked to the bioreactor's area [22].

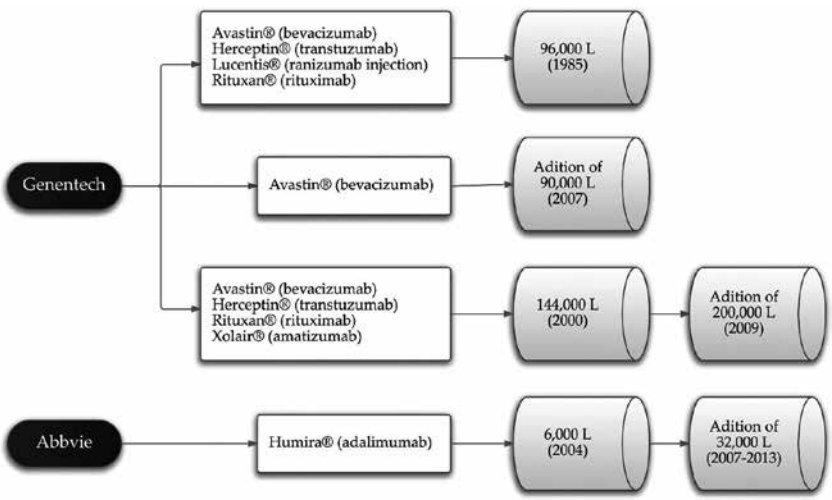

Figure 2. Work volumes used for industrial production of some commercial monoclonal antibodies [27, 28].

Some cultivation issues and worries have arisen regarding the production scale increase, maintenance of product quality, contamination control, demand for oxygen supply, and control over DO and CO_2 removal, among others. Regarding suspension cell cultures, aeration

is in part dependent on the agitation of the culture inside the bioreactor, which can lead to cell shear stress. To work around cultivation problems, major advances have been made in the process itself by developing better culture control and conditions, as well as the improvement and development of new bioreactors [7, 23].

2.3.1. Production systems for cells in suspension cultures

The different types of bioreactors commonly used for mAb production in submerged mammalian cells are stainless steel stirred tank bioreactors (STR), air-lift reactors, and disposable bioreactors. More details on each of these bioreactors are discussed below.

2.3.2. Stainless steel stirred tank bioreactors

Stainless steel stirred tank bioreactors are the most consolidated type of bioreactor used for industrial mAb production and consist of baffle-stirred tanks linked to rotor systems (**Figure 3**). It is a consolidated system, and there is a lot of knowledge and experience surrounding this technology, acquired by its vast industrial use beyond production using mammalian cells.

The cultivation in this bioreactor allows for wide flexibility of working volumes, ranging from 1.0 to 25.0 L [1], since this system is easily scalable to larger volumes due to its high control over production conditions and extensive handling knowledge. The mechanisms and cleaning and sterilization protocols are well defined. Additionally, cultivation parameters for this system, such as gas transfer coefficient, agitation, aeration, temperature maintenance, pH, and

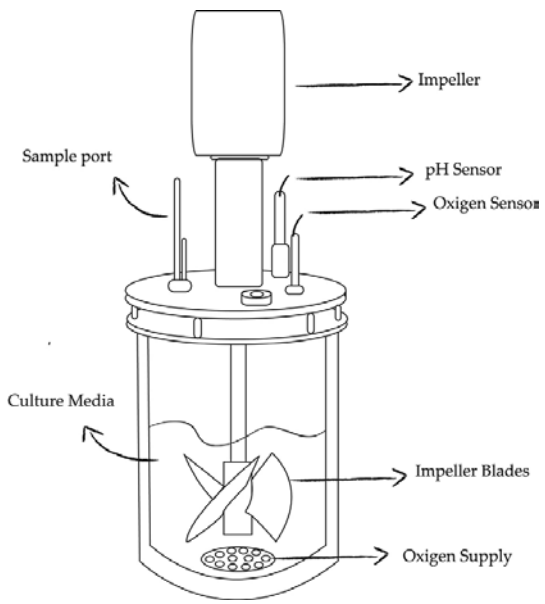

Figure 3. Schematic representation of a stainless steel stirred tank bioreactor. Showing the main components in a cell cultivation.

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others are well controlled and regulated when compared to other production systems. Another advantage of the STR is that it can be used for cultivation of various cell types and in addition, the products obtained from the cultivation in this type of bioreactor are easily approved for therapeutic use, as regulatory terms are well defined for this type of production [11].

However, the biggest disadvantage for the use of STR is the stress caused by shear. It can cause cell lysis and lead to loss in mAb productivity.

2.3.3. Air-lift reactors

Air-lift reactors are also broadly used for the industrial production of mAbs. The reactor consists of tanks with a bubble column inside, and air is injected into the column base (**Figure 4**). The air flows through the column's length to the top of the bioreactor as degassed culture medium flows in the opposite direction to the reactor bottom. This creates a constant gentle mixing of the medium as well as proper culture aeration, annulling part of the shear stress caused by other stirring systems. Other advantages of this operation system are that it is easier to scale-up, contamination problems are more unlikely to occur, and the equipment is simpler. In spite of these advantages, this system is less utilized than STR reactors because

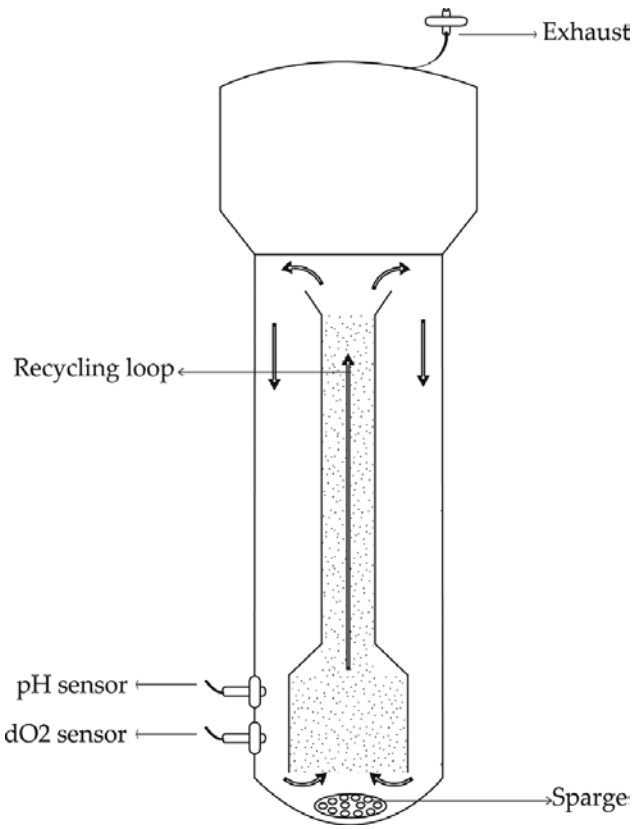

Figure 4. Schematic representation of an air-lift bioreactor. Showing the main components in a cultivation process.

the working volume ranges only from 2.0 to 5.0 L [1] and the air-lift reactor handling is not so well elucidated [11].

2.3.4. Disposable bioreactors

The first single-use bioreactors emerged in the late 1990s with the launch of a wave reactor system. After that, disposable stirred tank bioreactors were developed [11].

This method brought many advantages for mAb manufacturing. At the end of the process, the bioreactor is discarded and replaced by a new clean and sterile one. This eradicates cross contamination between batches and decreases the time consumed with the equipment preparation between batches. When all the advantages of this process are taken in account, the savings made regarding production and investment capital are highly significant when compared with other process methods. The great disadvantage of this production system is the small work volume supported, ranging from 50 to 2000 L [1].

The wave system consists of a sterile plastic bag (CellBag™) lying on a rocking platform (**Figure 5**). The bag is half filled with cultivation medium and half filled with a gas mix of interest. The platform motion creates an undulation movement in the culture, ensuring efficient aeration and culture mixing without causing shear damage [10, 11, 13]. The other available systems combine the convenience of a disposable system with the well-known stirred tank system and they are HyClone S.U.B®, Millipore® (CellReady™), or Xcellerex® (XDR™).

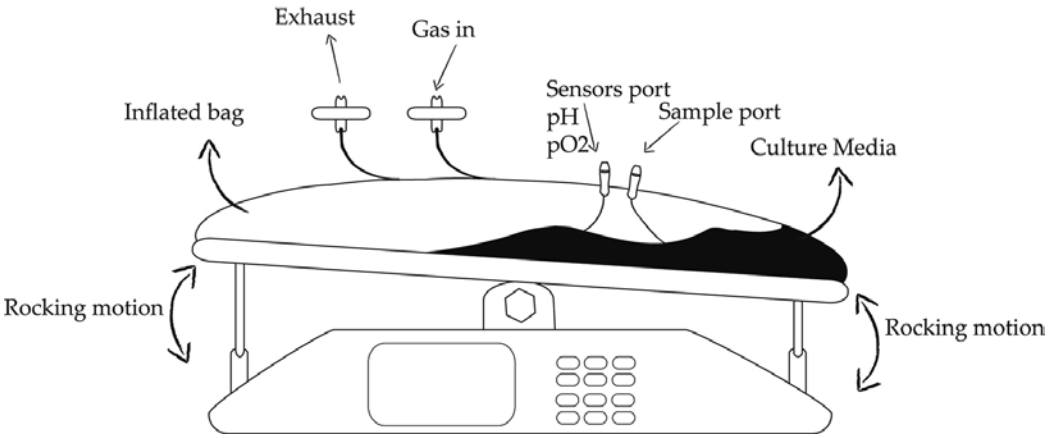

Figure 5. Schematic representation of a disposable wave bioreactor. Showing the main components in a cell cultivation process.

The main features of SUBs are related to their technical characteristics similar to those of stainless steel bioreactors, that is, aeration rate, agitation, reactor geometry, and ease of monitoring internal conditions, a process similar to stainless steel bioreactors [9].

SUBs are being widely used to replace many processes for the production of bioproducts. SUBs may be a cheaper and more efficient alternative from an industrial point of view, and its

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principle can easily replace any bioprocess to adapt the method to the platform of interest to be replaced, such as large tanks and stainless steel or the motion rocking platforms [9, 24].

SUBs have been used in bioprocesses for monoclonal antibody production involving several expression systems, including mammalian cells, microorganisms, plants, mammary glands, etc. Animal cell culture technology is one of the oldest techniques for the production of mAbs.

There is also the production of bottles known as roller bottles, consisting of mammalian cells growing in nutritional and physical conditions controlled in bottles which remain in rotational movement.

2.3.5. Roller bottles

Roller bottles are a rotary motion system for growing cells and for the production of some bioproducts. It has been an alternative to other monoclonal antibody production systems (**Figure 6**). Roller bottles provide conditions that favor the transfer of oxygen and temperature control without aeration, agitation propellers, or circulation pumps. The bottle is mounted on a turntable which gives homogeneity of growth and aeration of the culture medium [11, 25, 28].

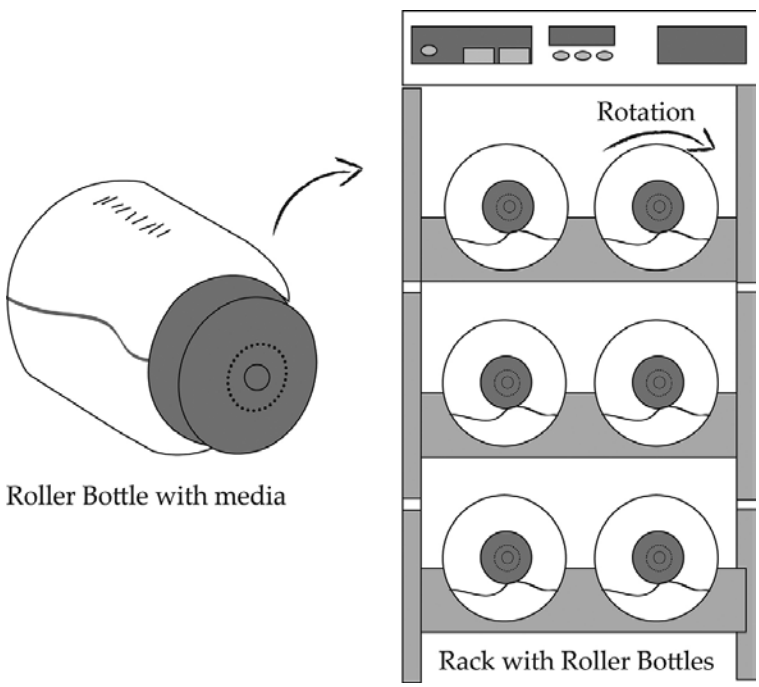

Figure 6. Schematic representation of roller bottles bioreactor and a rack with the rotational motion system in a cultivation for mAb production.

For the production of monoclonal antibodies at commercial scale, the roller bottle technique can be adapted to racks containing tens of bottle in a production line. The advantages of this

technique is the high growth potential linked to ease of handling and monitoring of certain conditions such as temperature and rotation. However, the scale of view requires a large physical footprint, which can make the process less economical [11, 25].

3. Conclusions and perspectives

Actually, the trade of monoclonal antibodies makes up half of marketed biopharmaceuticals, reaching $ 75 billion. For some years, new development methodologies of antibodies have advanced with new production processes, allowing scale-up production and market introduction, and demands for high-quality biologics will continue to increase in the coming decades. Generally, processes are similar to those applied in the scheduling for other bioproducts/biosimilars that rely on culturing microorganisms or cells, requiring the development of a well-designed culturing process comprising the full range of control and associated operations that will support technical evaluations.

In combination with increasing pressure from regulatory agencies for enhanced quality and lower process costs from the health care systems, we are facing an important challenge. It will be necessary to make changes in plant design aiming for highly flexible multi-purpose facilities for small production volumes.

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Chapter 11

Production of Lipopeptides by Fermentation Processes: Endophytic Bacteria, Fermentation Strategies and Easy Methods for Bacterial Selection

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Abstract

Lipopeptides constitute an important class of microbial secondary metabolites. Some lipopeptides have potent therapeutic activities such as antibacterial, antiviral, antifungal, antitumor and immunomodulator. Surfactin, iturin, fengycin, lichenysin and bacillomycin D from *Bacillus* species, daptomycin from *Streptomyces roseosporus* and rhamnolipids from *Pseudomonas aeruginosa* are among the most studied lipopeptides. These molecules are good candidates to replace those antibiotics and antifungals with no effect on pathogenic microorganisms. Microbial lipopeptides are produced via fermentation processes by bacteria, yeast and actinomycetes either on water miscible and immiscible substrates. However, the major bottlenecks in lipopeptide production are yield increase and cost reduction. Improving the bioindustrial production processes relies on many issues such as selecting hyperproducing strains and the appropriate extraction techniques; purification and identification by Polymerase Chain Reaction(PCR), High Performance Liquid Chromatography-Mass Spectrometry(HPLC-MS), Matrix Assisted Laser Desorption Ionization-Time of Flight-Mass Spectrometry(MALDI-TOF-MS); the use of cheap raw materials and the optimization of medium-culture conditions. The purpose of this chapter is to orient the reader on the key elements in this field, including the selection of analytical strategies to get a good microbial strain as well as to show some examples of liquid and solid-state low-cost fermentation processes. Last, we introduce endophytic bacteria as lipopeptide-producer candidates.

Keywords: endophytic bacteria, fermentation, lipopeptide-producers, microbial lipopeptides, quorum sensing

1. Introduction

In recent years, the production of microbial lipopeptides (LPs) has been widely studied for their biotechnological application in several areas including pharmaceutical industry, food preservation and agriculture. Lipopeptides are characterized for their highly structured diversity and their ability to decrease the surface and interfacial tension. Structurally, they consist of a hydrophilic peptide and a hydrophobic fatty acid acyl chain. The number of amino acids generally varies from 7 to 25, whereas the length of the fatty acid chains varies from 13 to 17 carbons. One strain is able to produce several isoforms of the same polypeptide. *Bacillus*- and *Paenibacillus*-related lipopeptides (firmicutes) and *Pseudomonas*-related lipopeptides (Proteobacteria) are the most studied [1,2]. Besides, LPs can also be produced by *Streptomyces* [3,4] and fungal strains [5]. The LPs are highly variable and their structural analogues results from frequent amino acid substitutions. Among the most documented LPs produced by *Bacillus* strains are surfactin, iturin, fengycin and lichenysin. On the other hand, those produced by *Pseudomonas* strains are viscosin, tensin, arthrofactin, massetolid, pseudodesmin, xantholysin, pseudofactin and syringomicin. These lipopeptides as many others, are good candidates to replace those antibiotics and antifungals with no effect on the control of pathogenic microorganisms.

Lipopeptide surfactants are naturally produced as mixtures of several macromolecules belonging to the same family or class. The nutritional parameters can influence the nature of the produced LPs [6]. However, the major limitations on their production are the production costs and yields. A wide range of carbon sources and culture conditions have been reported in order to increase the production of iturins, surfactins and fengycins. Nowadays, a variety of cheap raw materials have been used in their production: rice bran, soybean, potato peels, molasses, etc. In addition, it has been demonstrated that divalent cations have an important influence on LP production, mainly Mn^{2+} and Fe^{2+}. The manganese addition to the medium culture increased yield rates from 0.33 to 2.6 g/L [7]. Furthermore, the presence of $ZnSO_4$, $FeCl_3$ and $MnSO_4$ increased surfactin production in *Bacillus subtilis* [8]. In this context, we will highlight the key parameters for the maximization of LPs production and to development future strategies for optimizing liquid and solid-state fermentations (SSFs). Both fermentation types are important on industrial scale production processes.

Another important issue to address here relates to the microbial-producing strains. The genetic load of the microorganisms is a determining factor on LPs production yields, since the capacity to generate a metabolite is controlled by genes. There is a need for hyperproducer strains. But how can we recognize these over producing microbes? Lipopeptides production can be detected by: (i) culture dependent methods; (ii) methods relying on surface analysis and emulsifying activity; (iii) cell surface hydrophobicity and (iv) chemical identification [9]. An optimal and widely accepted study must combine the genetic identification and the structural

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and genetic analysis of the produced LPs by the particular isolate. This methodology assures the phenotypic and genotypic features of the microbial isolates for LPs production. Currently, the PCR and gene sequencing are quick tools for screening and identification of microbial-producers, as well as to identify the genes involved in the LPs synthesis. Also, the isolation and identification techniques such as liquid chromatography coupled to mass spectrometry (LC-MS) and more recently the MALDI-TOF mass spectrometry, have been considered as the fastest and most efficient tools for identification of LPs in mixtures and peptide sequencing, respectively. These techniques are also useful to identify the most novel LPs and even the small compositional changes in the sequence of amino acids that will determine its properties. There is no doubt, that these tools will increase and incentive the development of this field in fermentation processes.

In our lab, we are particularly interested in bacterial endophytes. By definition, an endophyte is a bacteria or fungus that lives in the internal tissues of plants without disease manifestation. Endophytes are ubiquitous to virtually all-terrestrial plants. With the increasing appreciation of studies that unravel the mutualistic interactions between plant and microbes, functions of endophytes are gaining value, so these microorganisms have become the target of biotechnological developments for biological control of plant pathogens (fungi and bacteria). We have evidence that a large group of endophytic bacteria have the ability to eradicate their competitors (pathogens) from the niche using LPs. In this chapter, we are interested in discussing endophytic microorganisms known as lipopeptide-producers beyond the genus *Bacillus*. We believe this information will be of value for alternative research in agricultural microbiology as well as for the production of antimicrobial molecules. However, given that some endophytic bacteria have a closer relationship with human pathogens, the application on commercial crops as biopesticides is strictly regulated. This last point impacts negatively the use endophytic microbes as tools for disease control and the development of new bioinoculants for agriculture; therefore, such LPs must be produced by fermentation.

Finally, it must be considered that the production of biosurfactants is associated with the physiological status of the bacteria, where quorum sensing (QS) is probably a condition. Quorum sensing is perhaps an overlooked variable in fermentation processes. In this chapter, we try to explain how this phenomenon and other conditions can alter the performance of LP production.

2. Lipopeptides: classes, microbial producers, fermentation processes and downstream processes

2.1. Classes of lipopeptides and their applications

The production of LPs in their active form requires transcriptional induction, translation and post-translational modifications. The main machinery for their synthesis is multi-modular and consists of non-ribosomal peptide synthetases (NRPSs) [10]. Synthetases are organized on modules; each module permits the incorporation of a specific amino acid, subsequent condensation, termination and cyclization of the peptide chain. The synthesized peptides contain

D-amino acids, β-amino acids and hydroxyl- or *N*-methylated amino acids. The integrated system introduces heterogeneity among LPs. The peptide moiety is inactive until it is coupled to a fatty acyl chain. The lipid aliphatic chain, of variable length, fuses with the N-terminal residue of the peptide chain, and then the bioactive LP is generated. After the biosynthesis of the LPs is finished, the molecule is modified by glycosylation or halogenation by specific enzymes associated to the synthetases [11].

	Lipopeptides	***Microbial-producers***	**Activity roles**
Surfactin family	Surfactin linchenysin pumilacidin WH1 fungin	*B. subtilis* *B. polyfermenticus* *B. megaterium* *B. licheniformis* *B. pumilus* *B. amyloliquefaciens*	-Enhanced oil recovery Antibacterial Antiviral Antimycoplasma Antitumoral Anticoagulant Enzyme inhibition
Iturin family	Iturin Bacillomycins Mycosubtilin Subtulene (contains a unique Iso C15-long chain β -amino acid)	*B. subtilis* *B. megaterium*	-Antifungal -Biopesticides
Fengycin family	Fengycin Plipastatin Agrastatin1	*B. subtilis* *B. thuringiensis* *B. circulans* *B. megaterium*	-Strong fungitoxic agent against filamentous fungi -Immunomodulating activities
Pseudomonas sp. Lipopeptides	Viscosin Massetolide Entolysin	*Pseudomonas sp*	-Antibacterial and Antifungal activity against; *Mycobacterium tuberculosis*, Gram positive bacteria, *G. candidum* and *R. pilimanae*
Streptomyces sp. Lipopeptides	Daptomycin	*Streptomyces roseosporus*	-Broad spectrum activity against *staphylococci* (MRSA), beta-hemolytic *Streptococcus spp., Pneumococci, Clostridium spp., and Enterococci sp.* –MRSA *Staphylococcus aureus* -Antiparasitic -Immunosuppressor

Table 1. Important and the most studied lipopeptides from *Bacillus*, *Pseudomonas* and *Streptomyces* and their activity roles.

Many bacteria and some fungi produce LPs, which have several roles including activity against bacteria, fungi, virus and more recently, it has been discovered their antitumor activity. Lipopeptides are also are involved in bacterial motility, in the swarming behavior and in the attachment to surfaces [1]. On the other hand, the extensive use of chemicals to control pathogens (bacteria and fungi) has modified the behavior of these microorganisms in humans and plants. The growing drug resistance, in these pathogens, urges for alternative antimicrobial molecules for clinical and crop protection, as well as for food preservation. As we mentioned above, LPs can be cyclic or linear based on the topology of the peptide chain. Here, we

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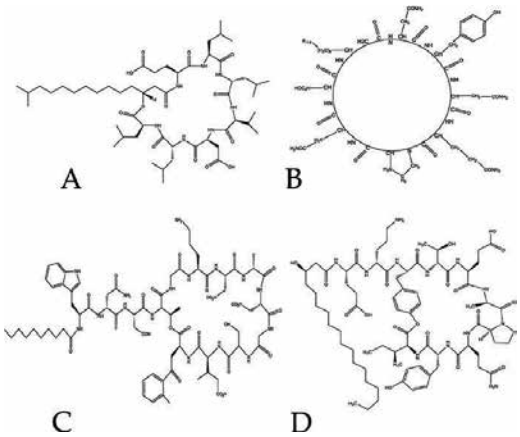

Figure 1. Pharmaceutically and economically important lipopeptides. Structures of representative member of lipopeptide synthesized by *Bacillus* and *Streptomyces*. (A) Daptomycin, (B) surfactin, (C) iturin and (D) fengycin.

present an updated overview on the bioactive LPs and their uses, being the cyclic lipopeptides the most biologically relevant with proved activity and more market applications in several fields. The characteristics of the LPs are discussed below, and their properties, structures and uses have been summarized in **Table 1** and **Figure 1**.

Daptomycin. This structure is a cyclic decanoyl lipid chain attached to 13 amino acids (a 10-member macrolactone and three exocyclic residues) peptide. It is produced by *Streptomyces roseosporus*, a Gram-positive bacterium. It has potent antimicrobial properties and it has been clinically approved for its use as antibiotic since 2003. It is marketed under the tradename Cubicin. The mode of action of daptomycin is still unclear, but two hypotheses have been proposed, the first one states the inhibition of lipoteichoic acid synthesis (proteoglycan component of the cell wall of Gram-positive bacteria); the second states the disruption of bacterial membrane potential (depolarization) via pore formation and its calcium ion dependence. Concomitantly, the bacterial cell loses the ability to accumulate amino acid substrates while leaving glucose uptake intact [12,13]. It has been successfully used to control skin infections, endocarditis, osteomyelitis and soft-tissue infections [14]. The cost of daptomycin in the market is approximately €125/day at dose of 6 mg/kg/day.

Surfactin. Surfactins constitute a major class of antibiotic LPs produced by *Bacillus* spp. They are highly active biosurfactants able to reduce the surface tension of water to 27 mN m^{-1} at 20 μmol. This group consist of a heptapeptide bonded to a C_{13}–C_{15} fatty acyl chain [15]. Surfactins are able to permeate the lipid membranes as dimer and form ion channels in planar lipid bilayer membranes. These compounds are effective against Gram-positive and Gram-negative bacteria and also have antimycoplasma, antiviral and antitumor activity and suppress inflammatory responses through inhibition of phospholipase A2 [16,17]. Surfactin also inhibits phytopathogens such as *Pseudomonas syringae*, *Xanthomonas axonopodis*, *Sclerotinia sclerotium*, *Botrytis cinerea*, *Colletotrichum gloeosporioides* and stimulates plant defense [18,19]. Since surfactins have hemolytic activity their medical applications are limited.

Iturins. Iturin is an antifungal cyclic lipopeptide produced by *Bacillus* spp. These amphiphilic compounds are characterized by a peptide ring of seven amino acid residues including an invariable D-Tyr2, with the constant chiral sequence LDDLLDL closed by a C14–C17 aliphatic β-amino acid. Iturins have a high polymorphism due to amino acid variations. These variants including iturin A, iturin C, iturin D, iturin E, Bacillomycin D, Bacillomycin F, Bacillomycin Lc, Mojavensin A and mycosubtilin [20]. Iturin A has been shown to form potassium ion-conducting channels in lipid bilayers. Iturins can act as biocontrol agents of plant pathogens [14]. They exert their fungicidal action by interacting with sterol components in the fungal membrane. Mojavensin A, a new member of the family, is cytotoxic [21]. From a clinical perspective, a disadvantage associated with iturins is their haemolytic activity.

Fengycins. This class of cyclic lipopeptides includes fengycins and plispastatins produced by some *Bacillus* and *Paenibacillus* strains. Fengicins are decapetides acylated with a β-hydroxylipid tail (C14–C18) and cyclized between the phenol side chain of Tyr 3 and the C-terminus. They act on plasma membrane of fungal cells and have been suggested for agriculture. They have antitumor activity because of the production of reactive oxygen species and mitochondria-dependent apoptosis [22]. They are good candidates for medical applications due their milder haemolytic activity.

Pseudofactin I and II. These compounds are cyclic octapeptides bonded to palmitic acid produced by *Pseudomonas fluorescens* BD5. The C-terminal of the carboxylic group forms a lactone with the –OH of threonine. Their emulsification activity and stability are greater in comparison to other synthetic surfactants, thus have a great potential for bioremediation or biomedicine. For example, Pseudofactin II exerts cytotoxicity in human melanoma [23].

Viscosin is obtained from *Pseudomonas fluorescens*. It has antibiotic activity, is highly surface active and is able to inhibit the migration of cancer cells. In *Pseudomonas*, this LP protects it from protozoan predation. Viscosin increases the efficiency of surface spreading over plant roots and protects germinating seedlings in soil infected with plant pathogen [24].

Linear cationic lipopeptides. Limited research has been performed on this small group. This is surprising as they have the potential to be more accessible than cyclic lipopeptides. This group includes saltavalin (which was named because contains serine, alanine, leucine, threonine, valine and 2,4-diaminobutyric acid, isolated from *P. polymyxa*), jolipeptin (*B. polymyxa*), cerexins (*B. cereus*, *B. mycoides*), tridecaptins (*P. terrae*). All exhibit antibacterial activity against Gram-negative and Gram-positive bacteria.

2.2. Endophytic bacteria as lipopeptide-producers

Due to the nature of the endophytic life style, endophytic microbes establish a long-lasting stable relationship with the plant. In this symbiotic association, the plant provides nutrients and shelter for the microbes and, in turn, the endophyte benefits plants by imparting biotic and abiotic stress tolerance and promoting its growth. Some endophytes are known to produce anti-pest compounds. These bioactive secondary metabolites can be either directly involved in antibiosis and/or triggering induced systemic resistance (ISR). There have been published some reports of the production of LPs by endophytic bacteria that may explain the antifungal

http://dx.doi.org/10.5772/64236

or antibacterial activity on plant pathogens. Recently, Gond et al. reported an endophytic *Bacillus* that produces antifungal LPs and host defense gene expression in maize [25]. Understanding the mechanism of biological control helps to manipulate the environment to create conditions for better biocontrol. Nowadays, in Mexico, some groups are developing research focused in field applications of LPs rather than bacteria, since many of the bacteria belong to genera related to human pathogens. In unpublished studies from our group, we have found strains of bacteria with the ability to produce LPs isolated from plants, including agave, banana and maize (Beltran-Gracia, manuscript in preparation). In **Table 2**, we list some of endophytic strains reported to be lipopeptide-producers.

Endophytic lipopeptide-producers	**Secreted lipopeptides**		
B. subtilis	Surfactin	Iturin	Linchenysin
	Pumilacidin	Bacillomycins	Mycosubtilin
	Fengycin	Plipastatin	
B. amyloliquefaciens	Surfactin	Linchenysin	Pumilacidin
	Fengycin		
B. megaterium	Surfactin	Linchenysin	Pumilacidin
	Iturin	Bacillomycins	Mycosubtilin
	Subtulene	Fengycin	Plipastatin
B. circulans	Fengycin	Plipastatin	Iturin
B. tequilensis	Surfactin	Iturin	Fengycin
B. polymyxa	Polymyxin		
B. macerans	Surfactin	Iturin	Fengycin
Streptomyces sp.	Daptomycin		
Pseudomonas sp.	Viscosin	Massetolide	Entolysin

Table 2. Endophytic bacteria reported as lipopeptide-producers.

2.3. Lipopeptide production by fermentation process: culture conditions and operational conditions

In order to incorporate LPs into industrial processes and for medical, pharmaceutical and agricultural uses, it is required their production by fermentation and their posterior downstream. It is clear that one of the main limitations for commercial applications of LPs are the high production costs and the low yield. To overcome these barriers, many efforts have been focused in improving the fermentation process, which represents a fundamental stage in the global production. Lipopeptides have been reported as growth-associated metabolites. In contrast to other bacterial secondary metabolites, production of LPs is induced when the cells have exhausted one or more essential nutrients, in example, surfactin production is induced

in actively growing cells during the transition from exponential to stationary phase (SP); fengycin synthesis is related to the early SP, and iturins only accumulate in the later SP [1].

The production of LPs can be achieved by liquid fermentation (LF) or solid-state fermentation (SSF) and now, both methods have been proposed for scale up their industrial production. The LF is an advantageous and typical process used for LPs production in controlled bioreactors, while SSF is still in evolution but has gained attention owing its priority to LF, including lower investment for production, less time and higher secondary metabolite yields.

A critical factor into industrial LP production is media optimization. In fact, the nature of the carbon substrate, N, P, Na, Mg, Fe, Zn and Mn ions concentration in the medium, have been shown to influence enormously the nature and quantity of the LP produced by several bacterial strains. An orderly and planned statistical procedure to screen the effect of each component of the media is very useful. For example, a Plackett-Burman procedure was applied to find that glucose, K_2HPO_4, and urea concentrations had the most influence into LPs production by *Bacillus subtilis* of 11 tested variables (glucose, urea, ammonium sulfate, NaCl, $MgSO_4$, KH_2PO_4, K_2HPO_4, $MnSO_4$, $FeSO_4$, $ZnSO_4$). After, a Central Composite Design was conducted to optimize the three selected factors, finding a maximum biosurfactant concentration of 3.1 g/L when using 15 g/L glucose, 6 g/L urea and 1 g/L K_2HPO_4, keeping the other parameters at their minimum values [26]. A similar statistical procedure was also applied to determine the effect of sucrose, ammonium nitrate, NaH_2PO_4, K_2HPO_4, $MgSO_4$, $MnCl_2$, extract yeast as components of culture media to growth *Bacillus amyloliquefaciens*, using the Plackett-Burman design in the production of C15-surfactin, indicated a significant effect of sucrose, ammonium nitrate and NaH_2PO_4. The optimum values of the tested variables were 21.17 g/L sucrose, 2.50 g/L ammonium nitrate and 11.56 g/L NaH_2PO_4 with a production of 134.2 mg/L LP [27]. In a third case, a five-level four-factor Central Composite Design was employed to determine the maximum LP yield by *Bacillus subtilis* testing sucrose, ammonium chloride, ferrous sulphate and zinc sulphate. Optimum fermentation components were 22.431 g/L of sucrose, 2.781 g/L of ammonium chloride, 6.7879 mM of $FeSO_4$ and 0.0377 mM of $ZnSO_4$ to produce 1.712 g/L of LP. Only the ammonium chloride had no significant effect [28].

2.3.1. Carbon sources to optimize lipopeptide production

It is clear the importance of carbon source in any fermentation process due to its impact in the bacterial metabolism as well as in production costs. The nature and quantity of the carbon source are the most important factors that would affect LPs production. Structural and compositional diversity of LPs is substrate dependent. For example, *Bacillus amyloliquefaciens* was grown in a minimal salt medium with different carbon sources (sucrose, dextrose, maltose, lactose, glycerol and sorbitol) where a C:N ratio remained constant at 15.55. The surfactin, iturin and fengycin were detected when dextrose, sucrose and glycerol were used as carbon source. However, in the presence of maltose, lactose and sorbitol only iturin was produced. Also, these carbon sources significantly influenced the antifungal activity of the molecules. Those bacteria grown in media supplemented with dextrose or sucrose produced LPs with the higher antifungal activity. The maximum biosurfactant activity was observed when the growing minimal salt medium was supplemented with sucrose [29]. In a similar study, it was

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reported that among several carbon sources: glucose, sucrose, galactose, maltose, sucrose, glycerol, mannitol, soluble starch and dextrin, evaluated for C15-surfactin production, sucrose was the best carbon source [27].

2.3.2. Nitrogen sources

Several inorganic nitrogen compounds have been tested in LP production trials, i.e. ammonium nitrate, ammonium sulphate, sodium nitrate, urea and glutamic sodium. And looking for cheaper raw materials, complex compounds such as soybean flour, peptone and casein acid hydrolysate have been assayed too [27]. When using organic nitrogen sources it was observed that tryptone enhances the lipopeptide production because it contains several homologous L-amino acids to those found in LPs [30]. A similar behavior was reported in the modified Landy medium, where L-glutamic acid was replaced with various L-α-amino acids at the same concentration (5 g/L). Cottonseed-derived medium (Pharmamedia) proved to be a suitable substrate for the production of 220 mg/L of surfactin from *Bacillus subtilis*; this medium is suitable to achieve high production yields at low cost, which in turn makes it profitable for large scale usage. Moreover, supplementing Pharmamedia with Fe^{2+} (4.0 mM) and sucrose (2 g/L) leads to a maximum production of about (300 mg/L) [31]. Other interesting nitrogen source is rapeseed meal, a low-cost material that was used to synthetize iturin A by *Bacillus subtilis*. The maximum iturin A concentration was 0.60 g/L after 70 h of incubation, which was 20% and 8.0 times higher than that achieved with peptone and ammonium nitrate media, respectively [32].

2.3.3. Divalent ions

To optimize the trace element composition of culture media the use of statistical experimental designs is preferred. Such is the case, where was doubled surfactin concentration when was applied the statistical method Taguchi to determine cation effect of culture medium [33]. The role of Fe^{2+} in the synthesis of LPs is crucial; there are some reports where the supplementation of this cation enhanced the yield of biosurfactants [31,33]. The addition of Fe^{2+} into fermentation medium was utilized to optimize surfactin production from *Bacillus subtilis*, reaching yields up to 3 g/L into minimal salt medium; the optimal Fe^{2+} dosage (4.0 mM) leading to 8-fold and 10-fold increments in cell concentration and surfactin yield, respectively, as compared to those media without Fe^{2+} [34]. *Bacillus amyloliquefaciens* in a culture medium supplemented with 0.2 mM of iron was able to produce 92.78 mg/L of iturin after 5 days with no pH control in the culture. Moreover, if the starting pH at 6.64 and 0.2 mM of ferrous sulphate, an iturin A production of 121.28 mg/L was obtained [35].

2.3.4. Operational conditions

The pH, temperature, dissolved oxygen concentration and degree of aeration affect cellular growth, and consequently, biosurfactant production. Optimal operational conditions vary from strain to strain, and the better growing condition for each particular strain must be determined experimentally. For example, surfactin synthesis by *Bacillus subtilis* can be achieved at temperatures ranging from 25 to 37°C; the optimal temperature for the surfactin produc-

tion by *Bacillus subtilis* DSM 3256 was 37.4°C. In contrast, for thermophilic *Bacillus* spp. surfactins were produced at temperatures above 40°C without detriment on their activity [36]. Regarding pH, a greater LP activity was observed when the pH was adjusted to between 3.0 and 8.0.

Statistical tools had been used to optimize LP biosynthesis considering operational conditions. The response surface methodology has been used to determine the maximum LP production varying the temperature, initial pH and culture cycle. Another important condition to be controlled is the dissolved oxygen. The oxygen acted at different levels, suggesting a complex system for regulating the synthesis of LP in B. *subtilis* ATCC6633. So, the oxygen transfer is one of the critical parameters for process optimization and scaling-up of production of surfactin [36]. Varying the oxygen transfer conditions, the synthesis could be oriented to mixed production or to surfactin monoproduction. The fraction of surfactin towards total LPs produced and the maximal surfactin production both increased with $k_L a$ increase (surfactin concentration about 2 g/L at $k_L a$ = 0.04–0.08 s^{-1}), while the maximal fengycin production (fengycin concentration about 0.3 g/L) was obtained at moderate oxygen supply ($k_L a = 0.01\ s^{-1}$). The production of LP represents a challenge due to its surface properties. Foam causes a severe decrease of oxygen transfer. A significant decrease of $k_L a$ (up to 27%) was measured during fermentation process reduces LP biosynthesis [37].

Classical bioreactors aerated by gas bubbling are not suitable for production of LP biosurfactants due to excessive foaming. The use of antifoam agents is not appropriate because it can affect the bacterial physiology and downstream processing. The alternatives are bioreactor with foam collector, rotating discs biofilm reactor [38], bubbleless membrane aerated bioreactor [39] and three-phase inverse fluidized bed bioreactor [40]. Another alternative to foam control is the use of a strictly anaerobic bioreactor cultivation to produce surfactin. Most interesting, the product yields exceeded classical aerobic fermentations, in which foam fractionation was applied. Additionally, values for specific production rate surfactin (0.005 g/(g h)) and product yield per consumed substrate ($Y_{P/S}$ = 0.033 g/g) surpass results of comparable foam-free processes [41]. The bioreactor design is still a challenge to get better productivities in industrial processes; the LPs synthesis is not an exception.

2.4. Solid-state fermentation (SSF)

Solid-state fermentation (SSF) is an alternative technology for the production of high-value molecules. The SSF could be an alternative method for the LPs production. The SSF uses agro-industrial wastes as substrates, which contributes to reduce the production costs. The productivity of LPs synthetized by SSF depends on initial moisture content, incubation temperature, fermentation time, substrates used and supplementary nutrients such as mineral salts. The temperature is an important parameter for both bacterial growth and LP production. Within a range from 25 to 40°C, the optimal temperature for growth was found to be 30°C, but the biosynthesis of LPs is favored at 37°C. At the beginning, in the firsts 24 h, temperature should be maintained at 30°C, then shifted up to 37°C to enhance LP production [42].

The selection of raw materials to formulate a culture medium, as the previously described, requires experimental tests. Savings in time and resources can be achieved using statistical

http://dx.doi.org/10.5772/64236

methods, which help in optimizing components and concentrations in the formulation of a culture medium. The optimal composition of culture medium in solid-state fermentation to get the highest LP production had been determined using a 'Central Composite Design'. First, a screen to select the major solid substrates was performed, where rapeseed meal, corn flour, soybean flour, bean cake, wheat bran, rice hull and rice straw were considered as candidates to support bacterial growth and biosurfactant production. For the election, a quantity of each solid substrate was supplemented with 1.0 ml of mineral solution with initial pH 7.5 and moisture content 55%. To increase the porosity of the substrates and improve its availability, each of them was mixed with an inert substrate (perlite, vermiculite, beads). After that, both easily digestible carbon sources (glucose, sucrose, starch, L-glutamic, maltose, glycerol, D-galactose) and nitrogen sources (tryptone, peptone, yeast extract, urea, NH_4NO_3, NH_4Cl, $(NH_4)_2SO_4$ at 2% (w/w) were added. The substrates selected to the optimization of the medium composition for LP production by 'Response Surface Methodology' were soybean flour, rice straw, starch and yeast extract. The optimal conditions were 1.79% starch and 1.91% yeast extract by employing 5.58 g soybean flour and 3.67 g rice straw as the solid substrate with initial pH 7.5, moisture content 55% and a 10% inoculum level at 30°C for 2 days. Under these conditions, the experimental yield of LPs reached 50.01 mg/gram of dry substrates [43]. SSF many times is compared with submerged fermentation. By this reason, a comparative study was performed to determine the compositions and properties of LP products purified and the transcription values of LP genes under submerged fermentation and SSF. Results revealed no significant differences in the polarity and structure of the two LP products. But, LP obtained by submerged fermentation possessed higher amino acid proportions, better emulsification activity and antagonistic activity than that from solid-state fermentation. For solid-state fermentation, the transcription accumulation levels of the LP synthetic genes srfA and sfp were higher than for submerged fermentation at the same stage. Transcripts for ituD and lpa-14 remained elevated for a longer period of time under solid-state fermentation conditions, accounting for differences in the production and fermentation periods between both fermentation techniques [44].

2.5. Downstream processing: isolation and purification of lipopeptides from fermentation process

Due to different applications of LPs, we need different levels of purity. Crude LPs can straightaway be used in bioremediation related applications, where the overall economy of the process is the most important concern. On the other hand, partially purified fractions (about 60–80% pure) can suit applications in microemulsion based nanoparticle synthesis, laundry and food industry. However, the requirement for ultrahigh pure product is indispensable, if the LPs are to be considered for pharmaceuticals and human healthcare [45]. As we mentioned before, the intense foaming produced during aerobic liquid fermentation is a big obstacle for the commercialization making their recovery and purification difficult. A great deal of monetary input will be required for purification, this account 60% of the total production cost. Different techniques have been developed to extract and purify LPs. Among the most used techniques to extract are acid precipitation (HCl 6N), solvent extraction (chloroform, ethyl acetate, dichloromethane or mixtures of chloroform-methanol), ammonium sulphate precipitation

with dialysis to remove small molecules and salts, and foam fractionation (utilized for continuous retention process and high purity). For purification normally have been used membrane ultrafiltration techniques, ionic exchange chromatography and adsorption-desorption on resins (XAD-4, XAD-7 HP, HP-2MG, HP-20) or activated carbon. The High Performance Liquid Chromatography(HPLC) (an excellent method for separation of this class of molecules) uses a reverse phase with C18 columns. The LPs separated can be detected using ultraviolet absorbance or diode arrays detectors and each peak separated is collected using a fraction collector for further analysis of their structure.

2.5.1. Example of techniques used for lipopeptide recovery, purification and identification

Membrane ultrafiltration. This technique serves essentially as an intermediate process for the recovery and purification of LPs. The separation of LPs by membrane filtration depends on their molecular aggregation behavior and on their ability to form micelles, since a process become economic feasible when a high MWCO (molecular weight cut-off) membranes were used. In general, the use of low MWCO membranes requires high maintenance due to low permeate fluxes through smaller pores that get easily plugged by monomers and progressive reduction in flux caused by the mechanism of concentration polarization [45].

A two-step ultrafiltration process using large pore size membranes (up to MWCO = 300 kDa) was investigated to separation of LPs aggregated in single and mixed solutions from fermentation culture. In single solutions of LP both surfactin and mycosubtilin formed micelles of different size depending on their concentration. However when the LPs were in the same solution, they formed mixed micelles of different size and probably conformation to that formed by the individual LPs, this prevents their separation according to size. An effective rejection in the first ultrafiltration step was achieved by membranes with MCWO= 10–100 kDa but poor rejection by the 300 KDa membrane [46]. The rejection is a measure of retention capacity of a membrane. However, some properties of LP micelles such as poor stability and non-uniform size distribution limit the use of readily scalable high MWCO membranes for the purification of LP, as smaller sized micelles and monomers can easily pass through the pores of these membranes. An addition of Ca^{2+} ions causes the structural transformation of surfactin monomers to larger micellar aggregates, showing excellent features such as compact structure, narrow size distribution, and improved stability [45].

Chromatographic technique for daptomycin. Daptomycin was purified from clarified fermentation broth using anion exchange chromatography and reverse phase chromatography. The anion resin was a highly cross-linked agarose with dextran surface extender. Daptomycin was eluted from the anion exchange column with a NaCl gradient from 0.2 to 1.0 M in water. The semi-purified daptomycin was then added to a reversed phase column and was washed with water containing 15% of alcohol. The reverse phase resin was a mono-sized, porous resin made of polystyrene and divinyl benzene (source: RPC 30). After, daptomycin was eluted with 40–70% of ethanol. Two reverse phase columns were involved to improve the purity of the final product at different pH. The first column was run at pH 7.5–8.0, while the second one was eluted at pH 3.0–3.1. The purified daptomycin is then filtered and lyophilized under standard conditions with at least 95% of purity [47].

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Resins. Macroporous adsorption resin (MAR) chromatography has been successfully used for separation of bioactive molecules on the basis of hydrophobic/hydrophilic interactions between solute and resin surface. MAR has been resolved problems related with low efficiency to separate LP mixtures into individual families employing a simple stepwise solvent gradient elution under optimal conditions. The adsorption and desorption of solutes on MARs depend upon the properties of the resins such as particle size, pore diameter, surface area and polarity. An example is the performance of a non-polar resin (HP-20) that combine features such as higher surface area, pore size and appropriate polarity, allowing it to have a superior adsorption capacity over other resins. Dual gradient MAR was applied to a cell free broth diluted until its total crude LP concentration was 3 g/L, then it was pumped at a flow rate of 1 ml/min into the column pre-packed with HP-20 resin (15 g) until breakthrough point. After one run of adsorption and desorption, the three LP families were successfully enriched in separate fractions and the recovery yields were 79.5% for iturin, 94.4% for fengycin and 89.4% for surfactin. Their purities in the enriched fractions were found to be 68.3, 77.6 and 91.6%, respectively. This process represents a basis for *in situ* recovery of LPs from the culture broth in continuous mode [48].

2.5.2. HLPC-MALDI-TOF

HPLC is an excellent method for the separation of individual LP separation. The most employed technique is reverse phase chromatography, due to this method can separate this metabolite based on its polarity. The separated products are detected by UV absorbance detection and each individual peak can be collected for further analysis of their structure. Also, use of a diode array detector is recommended. This detector can measure simultaneous wavelengths in a range of 200–600 nm, this means that we can detect LPs as they are eluted from the column in several wavelengths. For mobile phases reported, the methanol:water (80:20) is the most commonly employed, due this phase can elute several LPs as fengicins and iturins. Also, another mobile phase used is acetonitrile:water, as the methanol:water phase, this mobile phase can elute LPs as surfactins. The proportions for a acetonitrile:water can change depending on the LP that you want to separate. The typical column for LP's separation is a C-18 column, the length can vary from 150 to 250 mm, and this depends on the resolution and separation desired. In terms of particle size, 5 μm is the most adequate for the stationary phase. **Table 3A** shows some conditions of HPLC most widely used for separation as well as quantification of LPs derived from a fermentative process [49,50].

MALDI-TOF (matrix-assisted laser desorption/ionization-time of flight) is a mass spectrometry technique that allows the identification of intact compounds. Samples to be analyzed are mixed with a matrix and dried on a stainless steel plate, onto which a laser with various degrees of energy is fired to forming gaseous ions, which can be separated in a time of flight (TOF) analyzer and detected. Now, MALDI-TOF-MS has come to be regarded as a very fast and reliable tool for identification of LPs when compared to the conventional methods like culturing and purifying the LPs. The MALDI-TOF-MS for the identification of LPs has been previously reported [51, 52]. For LP identification, we can use CHCA (α-cyano-4-hydroxycinnamic acid), SA (sinapic acid) and DHB (2,5-dihydroxybenzoic acid). To the best of our

knowledge, DHB matrix is better than CHCA, because we obtain a good quality spectra with intensities above 2E4 and LP isomers can be observed. In **Table 3B** are shown relevant information of the mass range for specific LPs identification derived from MALDI-TOF analysis.

TECHNIQUE	*CONDITIONS*		*LIPOPEPTIDE SEPARATED*
(A) HPLC	COLUMN	C-18, 150mm–250mm length, size particle 5μm	ITURIN, FENGYCIN SURFACTIN AMPHISIN LOKISIN HODERSIN, TENSIN VICOSINAMIDE DAPTOMYCIN VISCONSIN MASSETOLIDE ENTOLYSIN
	MOBILE PHASE	Methanol: water (80:20), Acetonitile:water (80:20 and 40:60), acetonitrile: acetic acid (68:32)	
	RETENTION TIMES	3-10 min iturin 10-16 min fengycin 16-25 min surfactin 28.5 min amphisin 29 min lokisin 29.2 min hodersin 29.9 min tensin 31.6 min vicosinamide	
	DETECTORS	UV-VIS (205, 235, 278, 285nm), diode array.	
(B) MASS SPECTROMETRY	**PEAKS (*m*/*z*)**	**IDENTIFICATED LIPOPEPTIDE**	
	1001.42, 1029.42; 1016.56, 1030.58, 1044.59, 1058.61, 1072.62; 1030.64, 1044.65, **1058.66**, 1072.67	ITURIN A	
	1052.63,1094.45, 1122.47, 1136.55; **1052.64**, 1066.57; **1052.62**, 1066.60, 1080.59	BACILLOMYCIN	
	1435.58, 1449.63, **1463.68** , 1477.66; 1421.61, 1435.66, 1459.69, **1463.71**, 1477.72; 1421.75, 1435.76, 1449.77, **1463.79**, 1477.60,	FENGYCIN	

TECHNIQUE	*CONDITIONS*		*LIPOPEPTIDE SEPARATED*
	1491.81		
	1488.70, 1502.71, 1516.62; **1488.71**, 1502.72; **1488.79**, 1502.79	FENGYCIN (POTASSIUM ADDUCT)	
	1074.58, 1088.57, 1102.55	BACILLOMYCIN (SODIUM ADDUCT)	
	1485.70 , 1499.71, 1513.71; 1471.75, **1485.76**, 1499.77, 1513.79	FENGYCIN (SODIUM ADDUCT)	
	878.47, 892.41, **906.50**	KURSTAKINS	
	1202.75, 1188.73, 1143.70, **1129.68**, 1168.77, 1154.75	POLYMYXINS	
	1421.75, 1407.73, 1393.72, **1379.70**	BACITRACINS	

Table 3. HPLC strategies and conditions (A) used for lipopeptides separation from extracts of culture filtrate and (B) ranges of m/z of typical peaks obtained by MALDI-TOF analysis. In bold, the reference peaks for lipopeptide identification.

2.6. Discovery of endophyte-producers of lipopeptides: combining molecular biology and chromatographic- mass spectrometry methods

To search for endophytic bacteria in nature that can produce LPs, researchers must optimize their screening and identification times. Actually, the strategies for the selection of endophytic microorganisms as candidates for biocontrol or lipopeptide-producers combines antifungal-antibacterial screenings, a molecular analysis of the genes involved in the LP synthesis and then an analysis of the extracts obtained from the culture medium by HPLC and mass spectrometry.

In practice, after endophyte isolation, we identify the purified strain by MALDI-TOF and confirm its identity by 16s rDNA sequencing. Once identified, we select the strain as a function of its antagonistic ability against plant pathogenic strains. For screening antifungal activity of the isolates, we use a dual culture test against fungal strains collected from plants and previously identified. Normally, the plates are incubated for 3–5 days to observe inhibition of the fungal mycelium (**Figure 2**). For screening the presence of LP product biosynthetic gene clusters present in the antifungal isolate, PCR-based screening methods are used for different genes involved. In **Table 4**, we show the summarized sequences and methods for PCR analysis most used for some lipopeptide-genes, then each amplification product is analyzed by agarose gel electrophoresis to compare the predicted base-pair number (**Figure 3**). The sequence data

obtained must be analyzed by BLAST and detailed *in silico* phylogenetic analysis to confirm the PCR product.

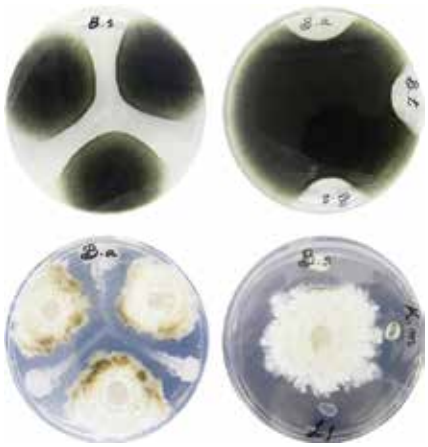

Figure 2. Antagonism of endophytic *Bacillus subtilis (Bs), B. amyloliquefaciens (Ba) and B. tequilensis (Bt), Kocuria marina (Km)* and *Lysinibacillus fusiformis (Lf)* isolated from agave, banana and maize against *Rhizotocnia* sp. and Colletotrichum sp. incubated by 5 days. It is noted that *Bacillus* strains used are the only ones that have antifungal activity, so the other strains should be discarded for identification analysis.

LIPOPEPTIDES	GENES	PRIMERS	PRIMERS SECUENCES (5′–3′)	AT*	PCR++
Iturin	ituD	ITUD-F1	TTGAAYGTCAGYGCSCCTTT	55	482
	ituC	ITUD-R1	TGCGMAAATAATGGSGTCGT	55	594
	ituA	ITUC-F1	CCCCCTCGGTCAAGTGAATA	55	647
		ITUC-R1	TTGGTTAAGCCCTGATGCTC		
		ITUD1F	GATGCGATCTCCTTGGATGT		
		ITUD1R	ATCGTCATGTGCTGCTTGAG		
Surfactin	srfA	SRFA-F1	AGAGCACATTGAGCGTTACAAA	55	626
	srfP	SRFA-R1	CAGCATCTCGTTCAACTTTCAC	52	675
	srf/Ich	SFP-F1	ATGAAGATTTACGGAATTTA	43	428
		SFP-R1	TTATAAAAGCTCTTCGTACG		
		As1-F	CGCGGMTACCGVATYGAGC		
		Ts2-R	ATBCCTTTBTWDGAATGTCCGCC		
Mycosubtilin	Myc/itu	Am1-F	CAKCARGTSAAAATYCGMG CCDASATCAAARAADTTATC	45	419
		Tm1-R			
Fengycin	fen	Af2-F	GAATAYMTCGGMCGTMTKGA GCTTTWADKGAATSBCCGCC	45	452
		Tf1-R			
Piplastin	pps	Ap1-F	AGMCAGCKSGCMASATCMCC	58	1029
		Tp1-R	GCKATWWTGAARRCCGGCGG		

*AT: ANNEALING TEMPERATURE (°C).
+PCR: PCR PRODUCT SIZE EXPECTED (bp).

Table 4. PCR primers commonly used for amplification of lipopeptide genes.

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The crude extracts obtained from culture media must be subject to HPLC separation and their collection for mass identification. The chromatographic profile of acidic methanol extracts and MALDI-TOF spectra of LPs from endophytic *Bacillus amyloliquefaciens* and *B. tequilensis shown* the presence of LPs groups iturins, fengycins and surfactin (**Figure 3**). Mass ranges (*m*/*z*) are 1001.42–1072 for iturin, 1471–75–1513.79, 1488.70–1516.62 for K and Na adducts of fengycin respectively and 1000–1100 for surfactin.

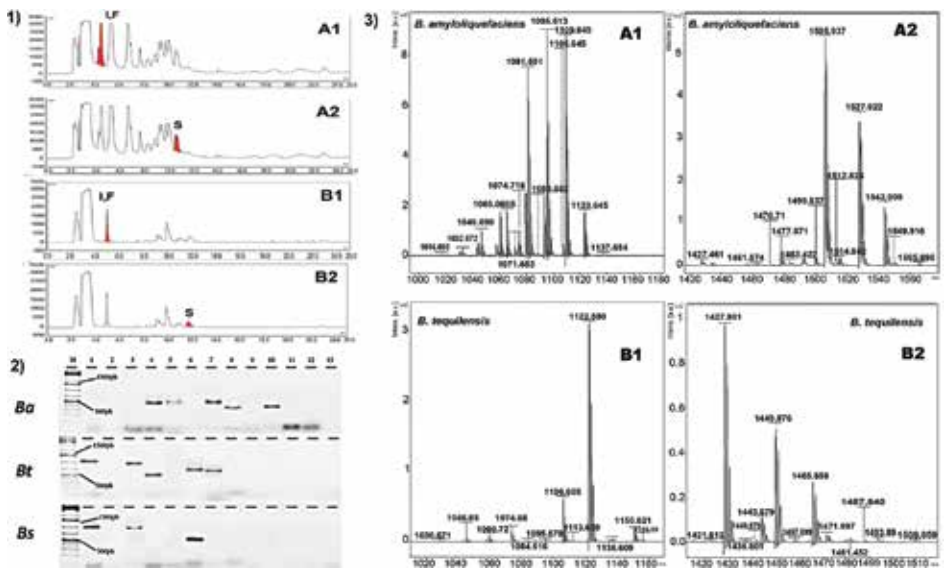

Figure 3. Agarose gel electrophoresis of PCR products of Bacillus endophytes (Below left) and separation and identification of the antifungal lipopeptides from acidic methanol extract by using reversed-phase HPLC(Above left) and MALDI-TOF MS analysis (Right) Section 2: PCR detection of lipopeptide and biosynthesis genes from Bacillus amyloliquefaciens (A), B. tequilensis (B), B. subtilis (C). M DNA ladder, Lane 1 Subtilosin, Lane 2 Sublancin, Lane 3 Plipastatin, Lane 4 Iturin D, Lane 5 Iturin C, Lane 6 Iturin A, lane 7 Surfactin A, Lane 8 Surfactin F, Lane 9 Mycosubtilin, Lane 10 Fengycin, lane 11 Subtilin A Lane 12 Ericin and Lane 13 Surfactin P. Chromatogram profile and MALDI-TOF mass spectrum of B. *amyloliquefaciens* (A) and B. *tequilensis* (B). The chromatograms were obtained under the following conditions: 0-3min: 45%-50% acetonitrile; 3-8min: 50%-80% acetonitrile; 8-25min: 80-100% acetonitrile, temperature of 38°C and a C-18 column (5μm particle size, 250 mm). Mass spectra were obtained with a RP 700-3500 Da, DHB as matrix. In section 1 (**A1** and **B1**), we can observe the mass spectrum of the HPLC fraction collected (within 4 min for A1 and 5 min for **B1**) that represent iturin (Section 3: with mass of 1016.68m/z and surfactin (1065.06 m/z) indicated that both strains produce this lipopeptides. In **A2** and **B2** we observe fengycin, in both cases the mass spectrum contains the representative mass of fengycin (1477 m/z) in section 3.

2.7. Quorum sensing in fermentative processes and lipopeptide production

Quorum sensing (QS) is a form of cell-cell communication by which bacteria communicate by secreting signaling molecules called autoinducers that help regulate gene expression. The QS molecule as N-acylhomoserine lactones in Gram-negatives or AIP in Gram-positives regulates different bacterial functions such as antibiotic biosynthesis, production of virulence factor, bacterial swarming, sporulation, competence and transition to the stationary growth phase.

How are LP production and quorum sensing associated? Surfactin a LP widely mentioned in this chapter book was proposed as a quorum-sensing molecule that activates the process of sporulation and production of biofilm [53]. For regulation of surfactin production by a cell density-responsive mechanism *B. subtilis* utilized a peptide pheromone Com X. Com X accumulates in the growth medium. So, the QS control the *srf* operon expression via Com X. However, few studies have been reported relating QS with fermentative processes. The production of putisolvins, also cyclic lipopeptides, in *Pseudomonas putida* occur at the end of the exponential growth phase, which indicates that the production of putisolvins is mediated through a quorum sensing-mechanism [54,55]. Another example that links QS with LP production is rhamnolipid a glycolipid biosurfactant produced also by *Pseudomonas spp*. The *rhl* quorum sensing system in *P. aeruginosa* regulates the production of rhamnolipid type biosurfactants. RhlA, a rhamnosyltransferase, catalyses the synthesis of fatty acid dimers that subsequently serve as the precursor for RhlB to form monorhamnolipids and dirhamnolipids catalyzed by RhlC. The genes (*rhlA*, *rhlB* and *rhlC*) for these catalyses are under the control of QS. Other studies link rhamnolipids synthesis to nutritional conditions, such as nitrogen exhaustion and the alternative sigma factor σ^{54} for nitrogen limitation. Schmidberger et al. [56], reports an interesting study of *P. aeruginosa* and rhamnolipid synthesis. Using PCRq, gene expression was monitored over entire course of fermentation. They observed until late deceleration phase (or ending log phase), an increase in relative gene expression of the *las*, *rhl* and *pqs* quorum-sensing regulon under nitrogen limitation.

Fermentation processes in terms of batch fermentation constituted a molecular black box in regards to transcriptional activity of the genes of LP synthesis circuitry in both Gram-positive and Gram-negative bacteria. More studies of the molecular biology during fermentation of LPs are needed. Monitoring gene expression of LPs over the entire time course of the fermentation process provides information about regulatory events linked or not to QS. QS is a variable that has largely been ignored in fermentative process studies. It is likely that information on QS in fermentation will help optimize bioreactor conditions, nutrient limitations or perhaps the use of signal molecules of QS to improve production yields of LPs and other microbial products.

3. Conclusions

The examples discussed so briefly in this chapter are by no means exhaustive. Hopefully they serve to illustrate the potential use of bacterial LPs and highlight potential applications in fields of biomedicine and agriculture. We also emphasize the potential of endophytic bacteria as lipopeptide-producers, opening research opportunities to understand some of the mechanisms involved in the biological control that occurs in niches they inhabit as endophytes. Knowledge of this and other topics, will promote the implementation of new molecules that are harmless to humans when we cannot directly apply bacteria in agricultural fields. It is clear that for widespread use of microbial LPs, more research is required focused on production with higher yields and at lower cost, where solid-state fermentation emerges as an important area of study in fermentation processes. This field is very much in its early stages, and progress

will come from a combination of ecological, physiological, structural, genetic and fermentative process approaches.

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Chapter 12

Lactic Acid Bacteria and Fermentation of Cereals and Pseudocereals

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Additional information is available at the end of the chapter

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Abstract

The usage of lactic acid bacteria (LAB) in food as starters in fermentation technologies has a long tradition. Although the theorized idea of host-friendly bacteria found in yoghurt has been formulated only over a century ago, both groups are widely used nowadays. Lactic acid bacteria alone or with special adjunct probiotic strains are inevitable for the preparation of various specific fermented and probiotic foods. Moreover, because of their growth and metabolism, the final products are preserved for a certain time. Growth dynamics of probiotic LAB and Fresco DVS 1010 in milk- and water-based maize mashes with sucrose or flavours (chocolate, caramel and vanilla) were evaluated in this study. Although milk is typical growth medium for the LAB growth, observed strains showed sufficient growth in each of prepared mashes as well as they were able to maintain their content above 10^6 CFU ml^{-1} during storage period (6°C/21 d). Designed flavoured mashes were acceptable from the microbiological point of view, but according to the sensory evaluation they were provided with an attractive overall acceptability and are adequate alternative for celiac patients, people suffering from milk protein allergies or lactose intolerance.

Keywords: lactic acid bacteria, fermentation, biopreservation, probiotics, functional products

1. Introduction

For centuries, human civilization had used different approaches to preserve different types of food products. If we look back in history, we can find the preparation of different types of foods, for example, alcoholic beverages by ancient Egyptians, the preparation of yoghurt and kefir by

the nomadic people from central Asia, fermentation of meat by the Germanic tribes and fish by the Eskimos, preparation of boza by the ancient Persians or fermenting maize by the native tribes in pre-Columbian America [1]. The earliest records about fermentation process were dated back to 6000 BC, and thus fermentation represents one of the oldest food preservation methods [2, 3]. The ancient people probably did not have any knowledge of microbiology, but in the middle of the nineteenth century, Louis Pasteur significantly contributed to the understanding of the fermentation process itself. He established the role of microorganisms and proved that there are many different kinds of fermentation [3]. The original and primary purpose of fermentation was a preservation effect. Subsequently, with the development of many available preservation technologies, plenty of fermented foods were therefore manufactured because of their unique flavours, aromas and textures much appreciated to a consumer [4, 5]. Fermentation process created plenty of traditional food products, such as milk products (cheese, butter and yoghurt), fermented meat, plants and fruits (sausages, silage, sauerkraut, olives and grapes) and finally fermented cereal products such as bread and beer [6]. Fermented food and beverages are defined as those that have been subjected to the effect of microbial enzymes, particularly amylases, proteases and lipases that cause biochemical transformation of polysaccharides, proteins and lipids to non-toxic variety of desirable products with tastes, aromas and textures attractive to a consumer [4, 7].

In food fermentations, conditions of treatment and storage create an environment in which certain types of organism can flourish and these have a benign effect on the food rather than spoiling it. The majority of fermented foods is produced by the activity of lactic acid bacteria (LAB) and fungi, principally yeasts but also, to a lesser extent, moulds. Both groups of organisms share a common ecological niche, are able to grow under conditions of low pH and reduced water activity, although only lactic acid bacteria and facultative yeasts will prosper under anaerobic conditions. They frequently occur together in fermented products, dairy and non-dairy, but in some cases, they play the role of a spoilage agent [8].

Microorganisms responsible for the fermentation process may be presented naturally in the substrate, or may be added as a starter and adjunct cultures [9].

2. Lactic acid bacteria

Lactic acid bacteria (LAB) represent an ubiquitous and heterogeneous species with common feature of lactic acid production as a result of sugar metabolism which leads to an acidification of the environment down to a pH of 3.5 [10]. The monograph by Orla-Jensen (1919) formed the basis of the present classification of LAB that take into account the cellular morphology, mode of glucose fermentation, growth temperature and sugar utilization possibilities [11]. Taxonomically, LAB are divided into two distinct phyla: *Firmicutes* and *Actinobacteria*. Within the *Firmicutes* phylum genera such as *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Enterococcus*, *Tetragenococcus*, *Aerococcus*, *Carnobacterium*, *Weissella*, *Alloiococcus*, *Symbiobacterium* and *Vagococcus* belong. Within the *Actinobacteria* phylum, lactic acid bacteria belong to the *Atopobium* and *Bifidobacterium* genera [12].

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Lactic acid bacteria are Gram-positive, non-sporulating, non-pigmented and non-motile rods and cocci, most of which are non-respiring but aerotolerant anaerobes. They lack cytochromes and porphyrins and are therefore catalase- and oxidase-negative. LAB tend to be nutritionally fastidious, often requiring specific amino acids, B-vitamins and other growth factors. Some do take up oxygen through the mediation of flavoprotein oxidases, thus producing hydrogen peroxide and/or re-oxidizing NADH during dehydrogenation of sugars. The cellular energy is derived from the fermentation of carbohydrates to produce major lactic acid. They use one of two different pathways and this provides a useful diagnostic feature in their classification. Since many species of lactic acid bacteria (LAB) and other food-associated bacteria had a long historical association with human foods, they are recognized as generally regarded as safe (GRAS) bacteria. Infections by LAB are characterized as opportunistic that rely on host factors rather than on intrinsic pathogenicity. Only rare cases of clinical infections have been reported in humans, for example, in patients with endocarditis or with immune deficiency [8, 12–15].

Homofermentative organisms produce only lactic acid from the glucose fermentation during the Embden-Meyerhof-Parnas glycolytic pathway. Heterofermenters produce roughly equimolar concentration of lactate, ethanol/acetate and carbon dioxide from glucose (**Table 1**).

Genera	Morphology	Fermentation	Lactate isomer	DNA (mole % G-C)
Lactococcus	Cocci	Homo	L	33–37
Lactobacillus	Rods	Homo/hetero	D, L, DL	32–53
Leuconostoc	Cocci	Hetero	D	38–41
Streptococcus	Cocci	Homo	L	40
Pediococcus	Cocci	Homo	DL	34–42

Table 1. Principal genera of the lactic acid bacteria [8].

2.1. Starters used in lactic acid fermentation

Genera *Lactobacillus* is recognized as being phylogenetically very heterogeneous and this is evidenced from broad interval of % G-C content. They are in general characterized as Gram-positive, microaerophilic, non-spore-forming and non-flagellated rods or coccobacilli. They are commonly found in a diversity of environments, in dairy and meat-fermented products, in fermented and pickled vegetables, adhered on human-mucosal surfaces (in the gastrointestinal and vaginal tract) as well as in soil and plants [16, 17]. Intestinal lactobacilli (*Lb. rhamnosus*, *Lb. acidophilus*, *Lb. reuteri*, *Lb. plantarum* and *Lb. paracasei*) interact with the host and have been linked with numerous health benefits [18–20]. *Lb. reuteri* is one of the most probiotic bacteria, which are added to infant dried milk formula for babies with lactose intolerance or for realimentation after diarrhoea [21]. Lactobacilli are naturally presented in breast milk, especially species of *Lb. fermentum*, *Lb. rhamnosus*, *Lb. gasseri* and *Lb. salivarius* [18, 22–25]. Liptáková and co-workers [26] identified frequently *Lb. plantarum* from breast milk of healthy mothers.

Lactobacillus species are divided into three groups based on fermentation end products: obligate homofermenters, facultative heterofermenters and obligate heterofermenters [17, 27]. Obligate homofermenters ferment hexoses almost exclusively to lactate but are unable to ferment pentoses or gluconate (*Lb. helveticus*, *Lb. acidophilus*, *Lb. delbrueckii* and others). *Lb. acidophilus* strains are the best known of the health-promoting lactobacilli and it is a part of human gut microflora. As probiotic strain is added to dairy foods for its physiological benefits. The facultative heterofermenters ferment hexoses via the EMP pathway and pentoses due to phosphoketolase activity to lactate, acetate, formic acid and ethanol (*Lb. plantarum* and *Lb. casei*). Obligate heterofermenters such as *Lb. brevis*, *Lb. reuteri*, *Lb. fermentum* or *Lb. kefir* use the phosphoketolase pathway for hexoses and pentoses fermentation and the main products of fermentation are lactic and acetic acid (or ethanol), and carbon dioxide [13, 15, 28].

Genera *Lactococcus* contains the major mesophilic microorganisms used for lactic acid production especially in dairy fermentations (sour milk and cream, lactic butter, fresh, soft and hard cheeses of artisanal and commercial origin). Some of them are suitable for cereal and pseudocereal fermentations [29, 30]. Joseph Lister made the first reported isolation of microorganism responsible for milk fermentation in 1873. He named the culture *Bacterium lactis* that was changed to *S. lactis* later. Orla-Jensen in 1919 differentiated mesophilic lactic streptococci into *S. lactis* and *S. cremoris*, which were included in Group N *Streptococci* [29]. On the present, the genus *Lactococcus* comprises nine species: *L. lactis* (including the subspecies *lactis*, *cremoris* and *hordniae*), *L. garvieae*, *L. piscium*, *L. plantarum*, *L. raffinolactis*, *L. chungangensis*, *L. fujiensis*, *L. formosensis* and *L. taiwanensis* [31, 32]. *L. cremoris* is unable to ferment maltose and ribose, to grow at 4% of salt and to hydrolyse arginine in comparison with *L. lactis*. *L. lactis* subsp. *lactis* var. *diacetylactis* converts citrate to diacetyl, carbon dioxide and acetone responsible for a creamy and buttery aroma in fermented milks, cream and butter and in Camembert, Emmental and Cheddar type of cheeses [13, 15, 33].

Many strains of *L. lactis* produce bacteriocins, which have antimicrobial activity especially against a narrow spectrum of *Lactococci;* however, nisin and lacticin 3147 have much broader activity against a wider range of Gram-positive bacteria. Nisin has been accepted as a food additive to control contaminating microbiota [11, 29]. Sadiq et al. [34] isolated three bacteriocinogenic strains *L. lactis* described as TI-4, CE-2 and PI-2 that were effective against *B. subtilis* and *S. aureus* and the maximum of bacteriocins (nisin A and nisin Z) were produced at 25 and 30°C and at pH 5 and 8, respectively.

Leuconostocs (predominantly *Ln. mesenteroides* subsp. *cremoris*) are the most commonly used heterofermentative dairy lactic acid bacteria that are flavour-producers in a number of fermented dairy products and cheeses. The fermentation of citrate is important in diacetyl and carbon dioxide formation in some types of cheeses. The genus *Leuconostoc* consists of 12 species isolated from plant, fermented foods (meats and vegetables or dairy products), vacuum-packaged, cold-stored meat, honey, Ethiopian coffee fermentation, kimchi, palm wine, cane juice and human clinical sources: *Ln. mesenteroides*, *Ln. pseudomesenteroides*, *Ln. carnosum*, *Ln. citreum*, *Ln. fallax*, *Ln. gasicomitatum*, *Ln. gelidum*, *Ln. holzapfelii*, *Ln. inhae*, *Ln. kimchii*, *Ln. lactis*, *Ln. palmae* [35–38].

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Twelve species of genera *Pediococcus* are currently recognized: *P. acidilactici*, *P. pentosaceus*, *P. argentinicus*, *P. cellicola*, *P. claussenii*, *P. damnosus*, *P. ethanolidurans*, *P. inopinatus*, *P. lolii*, *P. parvulus*, *P. siamensis* and *P. stilesii*. In contrast to other cocci in the LAB, pediococci usually do not form chains of cells [35, 39]. Pediococci are associated with dairy products and dairy environment and have potential impact on texture due to exopolysaccharides production. Garai-Ibabe et al. isolated two strains of *P. parvulus* (CUPV1 and CUPV22) that enable to produce high concentration of 2-substituted (1,3)-β-D-glucan increasing viscosity of the growth media [40]. Pediococci are often found in a large number of several fermented meat and fish products, fermented beans, cereals, olives or sauerkraut. Some strains are proposed to have probiotic activity due to their ability to survive and adhere to the gastrointestinal tract and due to reported immune modulation capability [13, 39, 41].

Streptococcus derives from the Greek 'streptos'—easily twisted like a chain—and 'kokkos'—grain/seed and the term was firstly used in 1874 by Billroth as a descriptor for the chain-forming, coccoid-shaped bacteria. Rosenbach (1884) firstly applied the generic name *Streptococcus* when describing *S. pyogenes*, the chain-forming coccus isolated from suppurative abscess in human. In 1906, Andrewes and Horder examined 1200 streptococci isolated from human, air and milk sources, and on the basis of sugar metabolism, reduction of neutral red and growth characteristics in milk, they distinguished eight groups. Sherman in 1937 produced the first comprehensive systematic classification of streptococcal isolates from environmental, commensal and hospital sources. He excluded from the genus *Streptococcus* all strictly anaerobic cocci and pneumococci because of their extreme sensitivity to bile and introduced four primary divisions: pyogenic, enterococcus, lactic and viridans group [42]. The results of molecular taxonomic studies allowed the major changes in the classification of *Streptococcus* spp.: the 'lactic' streptococci now constitute the genus *Lactococcus* and some members from Sherman's 'enterococcus' division became foundation members of the genus *Enterococcus* [43]. The subdivision of *Streptococci* into seven groups is based on 16S rRNA gene sequence data correlated well with the results of DNA-DNA re-association experiments and numerical taxonomic studies [44–46].

The only *Streptococcus* sp. useful in milk fermentation (production of yoghurt and Swiss- or Italian-type cooked cheeses such as Grana Padano, Gorgonzola, Mozzarella or Fontina) is *S. thermophilus* var. *salivarius*. It has the status of GRAS in the USA and a Qualified Presumption of Safety in European Union due to its long history of safe use in food manufacture. The end products of lactose fermentation are lactate, acetaldehyde and diacetyl. Some strains are able to produce thermophilins, proteinaceous compounds that are inhibitory against listeria and clostridia, especially thermophilin 13 and 1277 have a broad inhibitory spectrum [15, 27, 28, 47–50].

Species from the genus *Bifidobacterium* were originally identified from stool samples of breast-fed infants as bacteria with a strange and characteristic Y shape in 1899 by Tissier and named *B. bifidus*. In 1924, Orla-Jensen recognized the existence of the genus *Bifidobacterium* as a separate taxon but due to the similarities of bifidobacteria with the genus *Lactobacillus* they were included in this genus. In 1957, Dehnart realized the existence of multiple biotypes of

Bifidobacterium and proposed a scheme for the differentiation of these bacteria based on their hexose fermentation pathway [51].

The most frequently found strains in the human gastrointestinal tract include *B. adolescentis*, *B. bifidum*, *B. breve*, *B. catenulatum*, *B. pseudocatenulatum*, *B. longum* subsp. *infantis*, *B. longum* subsp. *longum*, *B. dentium* and *B. angulatum* [52]. Bifidobacteria represent up to 25% of the cultivation faecal microbiota in adults and 80% in infants [53]. According to Matsukiand and co-workers [54], the most often isolated bifidobacteria from adult intestinal tract are *B. catenulatum*, *B. longum* and *B. adolescentis,* whereas *B. breve*, *B. infantis* and *B. longum* predominate in the infant intestine.

The most important species of *Bifidobacterium* for probiotic application are *B. longum*, *B. bifidum* and *B. animalis*. Children receiving *Bifidobacterium*-supplemented milk-based formula (*B. lactis* Bb-12 strain) were protected against symptomatic rotavirus infection. Daily consumption of three cups/day of *B. longum* yoghurts decreased erythromycin-associated gastrointestinal disorders. *B. bifidum* NCFB 1454 was found to be active against certain species of *Listeria*, *Bacillus*, *Enterococcus*, *Pediococcus* and *Leuconostoc* due to bifidocin B production [15, 16, 28, 51, 53, 55, 56].

3. Antimicrobial compounds produced by lactic acid bacteria

Lactic acid bacteria may produce substances and thus create conditions harmful for undesired bacteria, yeasts and moulds which lead to the increase of food shelf life [57]. Temperature and incubation period are the main factors modulating production of antimicrobial substances. Sathe et al. [58] in their study evaluated the impact of the growth phase on antimicrobial activity of *Lb. plantarum* at 30°C. The evaluated strain showed maximal antimicrobial activity at the end of exponential phase of growth, and in stationary phase after 48 h of cultivation, decline in antimicrobial activity was observed. These results are consistent with the study of Batish et al. [59] who observed maximal antimicrobial activity of the same strain after 48 h of incubation at 30°C. The main product of fermentation by lactic acid bacteria is mostly lactic acid. However, under aerobic conditions, carbon dioxide and acetic acid are created as a result of oxidative dissimilation, while hydrogen peroxide as an intermediate product is formed [27]. Most of the isolated and identified antimicrobial substances produced by lactic acid bacteria are with low-molecular weight composed of organic acids, reuterin, hydrogen peroxide, hydroxyl fatty acids, phenolic and proteinaceous compounds [60].

When lactic acid is produced, the pH decreases and consequently the organic acids or small fatty acids (SFAs) become undissociated and represent the main antimicrobial activity of the LAB [61]. It has been shown that organic acids penetrate bacterial membrane of the target microorganism and inhibit transport mechanism in the cell by reducing pH values [62]. The effect of acids depends not only in combination with lowering pH and reduction of redox potential but also on the type and concentration of acid presented in the environment [63]. Acetic acid in comparison to lactic acid was described as being more effective, and is able to inhibit growth of moulds, yeasts and bacteria [5]. Propionic acid inhibits moulds and selected

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Gram-positive microorganism [62]. Phenyllactic acid and pyroglutamic acid are able to inhibit growth of *Aspergillus niger*, *A. flavus* and *Penicillium expansum*, and both were isolated from cell-free extract of *L. plantarum* and *Lb. rhamnosus* GG (LGG) [60, 64]. Liptáková et al. [64] mathematically predicted the inhibitory effect of *Lb. rhamnosus* GG (LGG) on the growth dynamics of *Candida maltosa* YP1 and *Geotrichum candidum* yeasts. At 18°C, growth rates of the yeasts in mixed cultures decreased about 50% compared with rates of its pure cultures. The effectiveness of growth inhibition of *C. maltosa* was dependent on initial LGG concentration; the most antagonistic activity of lactobacilli was determined at log 4 and log 6 initial concentration (**Figure 1**). Greifová et al. [65] described the inhibitory effect of D, L-phenyllactic acid on moulds such as *Alternaria alternata*, *A. flavus*, *Cladosporium herbarum*, *Fusarium nivale*, *Mucor racemosus* and *P. funiculosum*.

Liptáková and co-workers [66] focused on the growth of yoghurt contaminant *C. maltosa* YP1 in milk as influenced with initial different numbers of *Lb. rhamnosus* VT1 (ranged from 1 to 15% v/v) and temperature. The growth parameters of yeast in dependence on the lactobacilli counts at 17°C are summarized in **Table 2**. The antagonistic relationship between *C. maltosa* YP1 and *Lb. rhamnosus* VT1 was based not only on the lactic acid but it was consequence of the other antimicrobial, non-proteinaceous and non-saccharidic substances, identified by Plocková et al. [67] and also pyroglutamic acid, later identified by Liptáková et al. [64].

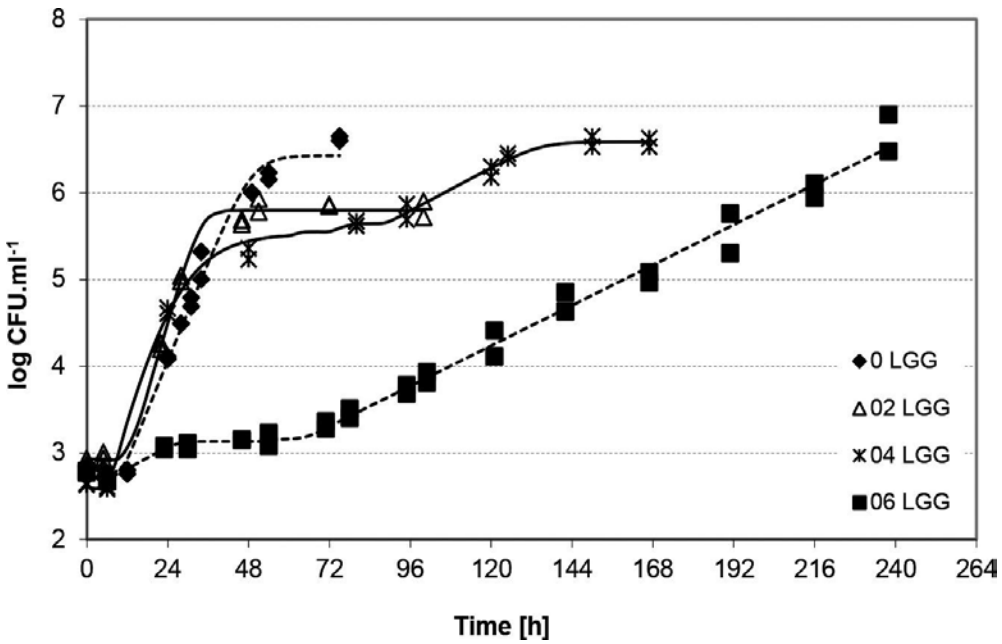

Figure 1. Growth dynamics of *C. maltosa* YP1 in co-culture with *Lb. rhamnosus* GG at 18°C in dependence on various initial lactobacilli concentration (♦ without LGG addition, △ 2 log LGG initial counts, ✳ 4 log LGG initial counts, ■ 6 LGG initial counts).

Initial inoculation of *Lb. rhamnosus* VT1 (% v/v)	Growth rate (log CFU ml^{-1} h^{-1})	Lag-phase duration (h)
1.0	0.062	0.1
	0.064	0.1
5.0	0.055	5.5
	0.052	8.1
10.0	0.046	72.9
	0.046	74.2
15.0	0.041	76.4
	0.043	73.1

Table 2. Values of growth rate (Gr) and lag time (λ) of *C. maltosa* YP1 in milk in dependence of initial numbers of *Lb. rhamnosus* VT1 at 17 ± 0.5°C.

3.1. Hydrogen peroxide

Most lactic acid bacteria produce hydrogen peroxide in the presence of oxygen. After its accumulation, inhibitory effect is mediated through oxidizing effect on membrane lipids and cell proteins of targeted microorganism. The antimicrobial activity of the compound in lower concentrations mostly in food is enhanced by treatment with the formation of hypothiocyanite catalysed by lactoperoxidase system [68]. Fitzsimmons and Berry [69] reported in their study the inhibitory effect of hydrogen peroxide on the growth of *C. albicans*. The minimum inhibitory concentration is less than 0.025% [60].

3.2. Carbon dioxide

Carbon dioxide at low concentrations may stimulate the growth of selected bacteria. Creating an anaerobic environment may be toxic to some aerobic food microorganisms through its action on cell membranes and its ability to reduce internal and external pH values [5].

3.3. Bacteriocins

Bacteriocins are ribosomally synthesized antimicrobial group of heterogeneous peptides with antimicrobial effect that kill or inhibit the growth of other bacterial strains. Typically, LAB bacteriocins have a narrow antibacterial spectrum, but some strains may also produce bacteriocins with a broad antibacterial spectrum. Selected lactic acid bacteria may inhibit the growth of Gram-positive pathogenic and spoilage bacteria, as well as yeasts. It has been reported that bacteriocins also inhibit the growth of some Gram-negative species. Lozo et al. [70] showed the production of bacteriocin 217 (Bac 217) by the strain *Lb. paracasei* subsp. *paracasei* BGBUK2-16 isolated from traditional home cheese that shows inhibitory effect against *Staphylococcus aureus*. Strains of *Lb. fermentum*, *Lb. pentosus*, *Lb. paracasei* and *Lb. rhamnosus* isolated from traditional corn drink made of wheat have produced active bacteriocins against *Escherichia coli*, *P. aeruginosa* and *E. faecalis* [71]. Valdés-Stauber and Scherer [72] isolated and characterized Linocin M18, bacteriocin produced by B. linens M18 in stationary growth phase. This bacteriocin was able to inhibit *Listeria* spp., especially *L. monocytogenes*, *L. innocua*, *L.*

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ivanovii and several coryneforms, Gram-negative bacteria were insensitive. Corsetti et al. [73] in their study described the antimicrobial substances in sourdough and identified them as a bacteriocins-like inhibitory substance. Some leuconostocs, especially *Ln. mesenteroides* subsp. *mesenteroides* Y105 and UL5, are able to produce bacteriocins with antilisterial activity [37, 74]. Some strains of *Pediococcus* spp. may have antimicrobial effect by the production of pediocins against undesirable and pathogenic microorganisms, for example, *Listeria* spp. and *Clostridium perfringens* [75]. Gurira and Buys [76] isolated *P. acidilactici* and *P. pentosaceus* from Bouquet and Gouda cheeses as non-starter lactic acid bacteria which had inhibitory potential against *L. monocytogenes* ATCC 7644 and *B. cereus* ATCC 1178 through the action of pediocins. Altuntas et al. [77] confirmed the antilisterial effect of pediocin producing strain *P. acidilactici* 13 in their study.

3.3.1. Reuterin

Reuterin is a product of glycerol fermentation produced during stationary phase by *Lb. reuteri*, *Lb. brevis*, *Lb. buchneri*, *Lb. collinoides* and *Lb. coryniformis* under anaerobic conditions, which enables to suppress ribonuclease activity [60]. Reuterin has a wide inhibitory spectrum against Gram-negative and Gram-positive bacteria, yeasts, fungi and protozoa: *Salmonella*, *Shigella*, *Clostridium*, *Staphylococcus*, *Listeria*, *Candida* and *Trypanosoma* [78]. An inhibitory effect on the growth of genus *Aspergillus* and *Fusarium* has been reported. The addition of glycerol to the media containing lactic acid bacteria producing reuterin increased its antifungal activity [60].

4. Probiotics and functional foods

4.1. Probiotics

The word probiotic originated from Greek meaning 'for life'. The first definition of probiotics was described by Vergin, 1954, as the opposite to antibiotics, and 1 year later Kolb proposed that the microbial imbalance in the human body as a result of antibiotic therapy could be restored by probiotics. Parker in 1974 defined the probiotics as organisms and substances that contribute to gut-microbial balance. Most frequently cited definition is that of Fuller's (1992), who defined them as 'a live microbial feed supplement, which beneficially affects the host animal by improving its intestinal microbial balance'. According to the recommendations of a Food and Agriculture Organization/World Health Organization (FAO/WHO)-working group on probiotics suggested definition describes probiotics as live microorganisms that when administered in adequate amounts confer health benefit on the host (2002). Health benefits must be scientifically established by clinical studies in humans and published in peer-reviewed journals [79]. A number of genera and strains of bacteria (*Lactobacillus*, *Bifidobacterium*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *S. salivarius* subsp. *thermophilus*, *E. faecium*, *E. faecalis*, *E. coli*, *B. cereus*, *B. subtilis*, *B. clausii*, *B. coagulans*, *B. licheniformis* and *B. polyfermenticus*) and yeast *Saccharomyces boulardii* are used as probiotic mostly in dairy products (milks, yoghurts and probiotic cheeses) but also in non-dairy food and beverages such as dry sausages, soy milk

drink, juices, fermented cereal products Boza, Bushera, Mahewu and Pozol [57, 80–85]. Lee and co-workers [86] investigated the probiotic potential of *B. polyfermenticus* KU3 isolated from kimchi. The spore suspension was resistant to artificial gastric juice and survived for 24 h in artificial bile acid, adhered strongly to HT-29 cell line and anti-carcinogenic activity of *B. polyfermenticus* KU3 was observed. Cell *B. polyfermenticus* strongly inhibited the proliferation of various cancer cell lines such as HeLa, LoVo, HT-29 and MCF-7 (percentage of inhibition between 90.5 and 96.9%). Liptáková et al. [87] observed comparable inhibition effect on the proliferation of HeLa and Caco-2 cells due to the adhesion and metabolism of probiotic *Lb. acidophilus* 145 (95–96%) and *Lb. rhamnosus* GG (68%).

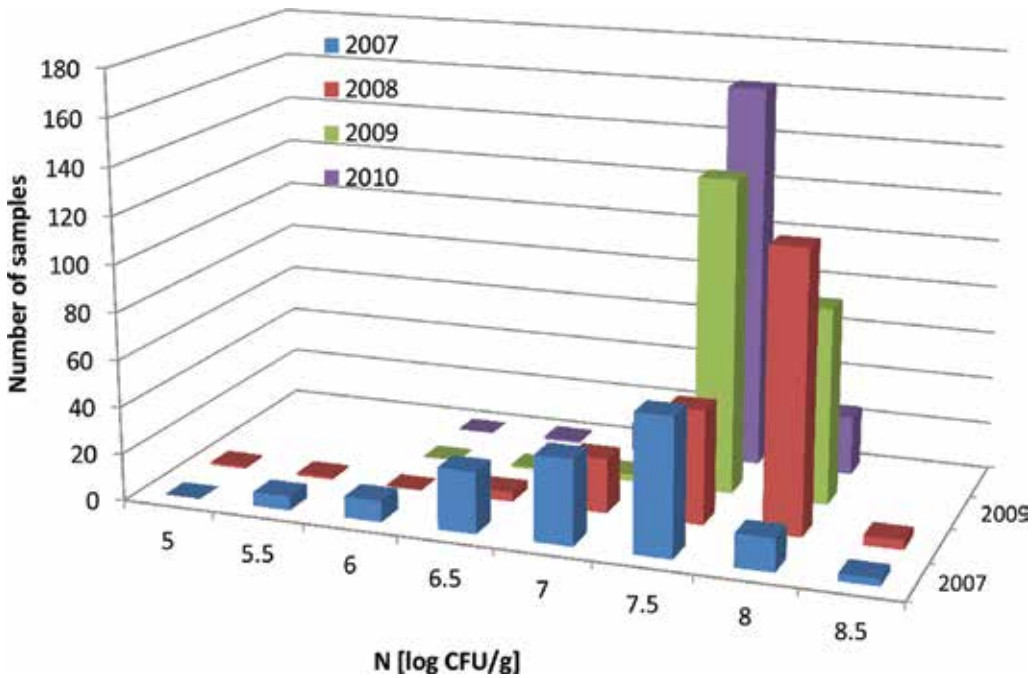

Figure 2. *Lactobacillus acidophilus* contents in the fermented milk at the end of shelf life.

The choice of which microbe to use as a probiotic is determined by many factors: probiotics have to be safe, non-pathogenic and non-toxic species, survive the passage through the intestinal tract and adhere to the intestinal mucosa and organic acid production, lactic and acetic [57, 79]. According to Tripathi and Giri [85], the viability of probiotics in food is affected by many factors such as pH, water activity, redox potential of foods, presence of salt, sugar, hydrogen peroxide, bacteriocins, aroma and colouring compounds, processing, packaging and storage conditions. Probiotic foods should preferably be stored at a temperature between 4 and 5°C. The highest viability of *Lb. acidophilus* LA-5 in yoghurt was observed 20 days at 2°C, but for *B. lactis* BB 12 the optimum storage temperature was 8°C [88, 89]. To realize health benefits on host, probiotic microorganisms must be viable and available in a high concentration of about 10^6 to 10^7 CFU/ml or g at the end of shelf life of product, so minimum therapeutic

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daily dose is usually considered as 10^8 to 10^9 CFU/ml or g [16]. Liptáková et al. [90] determined the concentration of *Lb. acidophilus* 145 in acidophilus milk at the end of shelf life during storage at 6, 8 and 10°C. The number of probiotic *Lb. acidophilus* 145 ranged from 6 to 7 log counts. Over a period of 5 years (2007–2011), Valík et al. [91] monitored the contents of *Lb. acidophilus* in the fermented milk at the end of shelf life. The average values in log CFU/g ranged in interval from 6.85 to 7.47, respectively. In the years 2007 and 2008, 9.87 and 1.01% of samples contained less than 10^6 CFU/g of *Lb. acidophilus* at the end of consumption, while in other years they did not find any sample with number lower than 10^6 CFU/g (**Figure 2**).

The mechanisms of health-improving properties of probiotics are still not completely understood, but their anti-carcinogenic and anti-mutagenic activity, the suppression of allergies, reduction of serum cholesterol level and reduction in blood pressure are known [12, 80, 92, 93]. *Lb. bulgaricus* and *S. thermophilus* are able to ferment lactose, so they have beneficial effects for people suffering from lactose intolerance [94]. *Lb. rhamnosus* GG, *B. lactis* Bb-12, *Lb. acidophilus*, *Lb. casei* Shirota or *Lb. reuteri* have beneficial effects against acute diarrhoea caused by rotavirus, in treatment, shortening or preventing of this disease [95–97]. The administration of *S. boulardii* as non-pathogenic biotherapeutic yeast plays essential role in the treatment or prevention of antibiotic-associated diarrhoea caused by *C. difficile* [83, 98–101]. Probiotics, especially *Lb. acidophilus*, *Lb. plantarum*, *Lb. rhamnosus* and *Bifidobacterium,* are able to reduce faecal enzyme activity which converts procarcinogens into carcinogens (β-glucuronidase, azoreductase, urease, nitroreductase and glycocholic acid reductase) due to short-chain fatty acids production and may thus contribute to a decreasing risk of colorectal carcinoma [79, 102]. Other potential mechanisms for probiotics-induced anti-carcinogenic activity are described in the works of Commune et al. [92] and Faghfoori et al. [103], respectively.

4.2. Fermented cereals and pseudocereals functional products

Recently, there is an explosion of consumer's interest in functional foods; therefore, a key priority for food industry is the development of such products with a high quality and safety [104]. The aim of these products is to have beneficial effect on host health affecting gut microbial composition subsequently with reducing the risk of chronic diseases [105]. Cereals have been investigated in recent years regarding their potential use in the production of functional foods [106].

Possible application of cereals in functional food can be summarized as follows:

- as fermentable substrates for the growth of probiotic bacteria (lactobacilli, bifidobacteria);
- as prebiotics due to their content of non-digestible oligosaccharides (galacto-oligosaccharides and fructo-oligosaccharides);
- as dietary fibre promoting beneficial effects on human host;
- as encapsulation matter for probiotics to enhance their stability [104, 107].

Cereals have been and still are one of the most important sources of human diet [108] and are grown over 73% of total harvest area [109]. A number of cereals are grown in different

countries, including wheat, barley, oat, corn, rye, rice and millet, particularly important from an economical point of view. According to FAO's latest forecast, cereal production in 2015 stands at close to 2525 million tonnes but is still 1.4% below than the record in 2014 [110]. Cereal grains and their derivatives represent an important nutritive component both in developed and in developing countries [111]. They are considered as one of the most important sources of dietary proteins, carbohydrates (starch and fibre), vitamins (B group) and minerals for people all over the world [112].

4.2.1. Nutritional value of cereals

Cereal grains are primarily a source of carbohydrates, and thus a good source of energy [113]. They form about two-thirds up to three-quarters of dry matter [114]. Monosaccharides are the basic components of oligo- and polysaccharides and are most represented in the forms of hexoses (fructose, glucose and galactose) and pentoses, arabinose and xylose [115]. Starch, the major component of cereal grains, occurs in starch granules of different sizes in endosperm.

Within common varieties, 25–27% of starch is presented as amylase and 72–75% represents amylopectin. However, in cereals a portion of the presented starch is not digested and absorbed in the small intestine. This is referred to as resistant starch and it appears to act in a similar way to a dietary fibre [113]. A wide variety of biochemical processes occur in cereals during fermentation as a result of lactic acid bacteria. Fermentation process itself may lead to an increase in the content of reducing sugars, which was confirmed also in a study by Marko et al. [116]. Simple carbohydrates are metabolized directly to organic acids and the glucose as a final product of starch metabolism is utilized immediately [116]. Lambo et al. [117] described the decrease in starch content during fermentation of barley with lactobacilli.

Cereals are in general good sources of proteins. The proportions of essential amino acids and their digestibility mainly determine protein nutritional quality. Because of different production systems, environmental factors, as well as genotype, it is difficult to obtain comparative values of protein contents of different cereals. Thus, ranges of 5.8–7.7% of protein on a dry weight have been measured for rice, 8.0–15.0% for barley and 9.0–11.0% for maize. The amount of lysine, which is the limiting amino acid for all cereals, varies between species with the highest values in oat and rice and lowest in wheat and maize [118]. The most represented is glutamic acid in the form of glutamine [119]. Degradation and depolymerization of proteins during fermentation process depend not only on the metabolic activity of presented bacteria but also on enzymes that naturally occurred in cereals. Peptides are converted to amino acids by the activity of lactic acid bacteria by the specific intracellular peptidases that are subsequently converted to the specific products influencing the aroma and taste of final products [120]. Antony and co-workers [121] in their study pointed out that the fermentation process does not generally significantly change the total protein content of cereals. However, in the case of yeast corn fermentation, Cui et al. [122] found a significant increase ($P < 0.05$) in the total protein content.

Lipids are only a minor component of cereal grains with the amount varying from 1.7 to 7.0% on a dry mass basis, dependent on the type of cereal grain. The germ is the richest source of lipids. In particular, cereals are rich in essential fatty acids and contain only trace amounts of

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saturated fatty acids [123]. Oxidation of lipids during fermentation process creates volatiles that contribute to the flavour of final products. Linoleic, oleic and linolenic acids are oxidized by lipoxygenases by forming hydroperoxides that are formed to aldehydes [124]. Aldehydes are converted to alcohols by alcohol dehydrogenases during fermentation process [125]. Antony et al. [121] in their study did not record any changes in the total lipid content during the millet fermentation with the endogenous microorganisms.

Cereals may contribute to vitamin intake due to the presence of most B-vitamins and appreciable amounts of vitamin E. Wholegrain cereals also contain considerable amount of calcium, magnesium, iron, zinc, as well as lower levels of many trace elements, for example, selenium. The content of minerals ranges from 1.0 to 2.5% [113, 126]. Cereals contain relatively high levels of phytate (0.2–1.4%), concentrated mostly in the aleurone layer, which can bind minerals and there is an evidence of its decreased absorption in the presence of phytate, so minerals are not available to microorganisms. However, at a pH values less than 5.5, phytates are hydrolysed by endogenous phytases, thus minerals are released from the complex [9]. In our investigation, changes in chemical composition of maize flours before and after expiry date were determined (**Table 3**). The percentage of starch and reducing sugars is one of the most important aspects showing the suitability of the tested substrate in fermentation technologies. A decline in the content of reducing sugars (60.1%) and starch (7.9%) was observed. Matejčeková and coworkers [30] recorded a decline of reducing sugars in amaranth flours before and after expiry date of about 31% in their study.

In comparison to milk and dairy products, the nutritional quality of cereals and their products is sometimes inferior, or poor. The reason is the lower protein content in comparison to milk, limitations in the amounts of certain amino acids, notably lysine, and the presence of antinutritive compounds (phytic acid, tannins and polyphenols) and a coarse nature of grains [7, 127]. Cereals typically undergo a range of processes that change the nutritional content. Milling is the main process associated with cereals; also, extrusion is used to produce a variety of different types of products [128].

	Proteins	Lipids	Starch	Reducing sugars
Corn flour 1	3.21 ± 0.00	1.59 ± 0.03	68.71 ± 0.12	4.24 ± 0.01
Corn flour 2	4.46 ± 0.07	2.49 ± 0.00	63.30 ± 0.24	1.69 ± 0.01

Corn flour 1 (before expiry date), corn flour 2 (after expiry date), the results are means ± standard deviation of two determinations.

Table 3. Chemical composition of maize flours before and after expiry date (%).

Helland et al. [106] studied the growth and metabolism of four selected probiotic strains in rice- or maize-based puddings with milk or water. All four tested strains showed good growth and survival in cereal-based puddings.

4.2.2. Fermented cereal and pseudocereal food and beverages

Fermented food and beverages are defined as those products that have been subjected to the effect of microbial enzymes, particularly amylases, proteases and lipases that causes biochemical transformation of polysaccharides, proteins and lipids to non-toxic variety of desirable products with tastes, aromas and textures attractive to a consumer [4, 7]. Microorganisms responsible for the fermentation process may be presented naturally in the substrate, or may be added as a starter culture [9].

Traditional cereal- and pseudocereal-fermented products are made of various kinds of substrates all over the world, mainly widespread in Asia and Africa. Fermentation may have multiple effects on the nutritional value of food [129].

The development of non-dairy-fermented products is a challenge to the food industry by producing high-quality functional products. The main aims of cereal fermentation can be summarized as follows:

- preservation, which relies mainly on acidification (production of lactic, acetic and propionic acid) and/or alcoholic production often in combination with reduction of water activity [130];
- enhances the safety of final products by the inhibition of pathogens [131];
- affecting sensory properties (taste, aroma, colour and texture);
- improves the nutritional value by removing antinutritive compounds (phytic acid, enzyme inhibitors, tannins and polyphenols) and enhances the bioavailability of components;
- reduces the level of carbohydrates as well as non-digestible poly- and oligosaccharides [9].

Cereal fermentations affected by characteristic variables include the following:

- the type of cereal determining the content of fermentable substrates, growth factors, nutrients, minerals, nitrogen sources and buffering capacity;
- duration and temperature of fermentation process;
- water content;
- additional components (sugars, salt and exposure to oxygen);
- sources of amylolytic activity to gain fermentable sugars from starch or other polysaccharides [9, 132].

Fermented cereal-based products are prepared in different parts of the world, mainly in developing countries—Asia and Africa, in combination with legumes to improve overall protein quality of the final fermented products [7]. Petruláková and Valík [133] evaluated the growth and metabolic activity of *Lb. rhamnosus* GG during fermentation of leguminous porridges. Cell density during 21-day cold storage was stable except whole soybean, yellow pea and red bean. Metabolic activity of observed strain caused decrease in pH values to the final 5.6–6.0 and subsequently during cold storage decreased. Fermented products are usually

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prepared in the form of beverages, gruels or breakfast meals. Most of the fermented products are made in Asia (soy sauce), India (idli and dosa) and in the Middle East (kishk). In America, as a basic raw material for the production of cereal-fermented foods, corn is used, in products such as tesgüino (alcoholic beverage of Mexico) or jamin-bang-bread made in Brazil [134]. An overview of traditional fermented food and beverages is summarized in **Table 4**.

Fermented food/country	Raw material/substrate	Microorganism
Idli—South India, Sri Lanka	Rice	*Leuconostoc mesenteroides*, *Enterococcus*, *Torulopsis*
Dosa—India	Rice	*Leuconostoc mesenteroides*, *Streptococcus faecalis*, *Torulopsis candida*
Kishk—Egypt, Syria	Wheat, milk	*Lactobacillus plantarum*, *Lb. casei*, *Lb. Brevis*, yeasts.
Tarhana—Turkey	Wheat, yoghurt	*Lactobacillus bulgaricus*, *Streptococcus thermophilus*, *Saccharomyces cerevisiae*
Ogi—West Africa	Maize, millet, sorghum	*Lactobacillus plantarum*, yeasts, moulds
Pozol—Mexico	Maize	Moulds, yeasts, bacteria
Injera—Ethiopia	Sorghum, maize	*Candida guilliermondii*
Sake—Japan	Rice	*Saccharomyces sake*
Bouza—Egypt	Wheat	LAB
Boza—Albania, Turkey	Wheat, millet	LAB, *Saccharomyces cerevisiae*, *Leuconostoc*
Mahewu—South Africa	Maize	*Lactococcus lactis*
Chicha—Peru	Maize	*Aspergillus*, *Penicillium*, yeasts, bacteria
Uji—Kenya, Uganda	Maize, millet, sorghum	*Lactobacillus plantarum*, *Leuconostoc mesenteroides*

Table 4. Overview of traditional fermented products and beverages [7, 135].

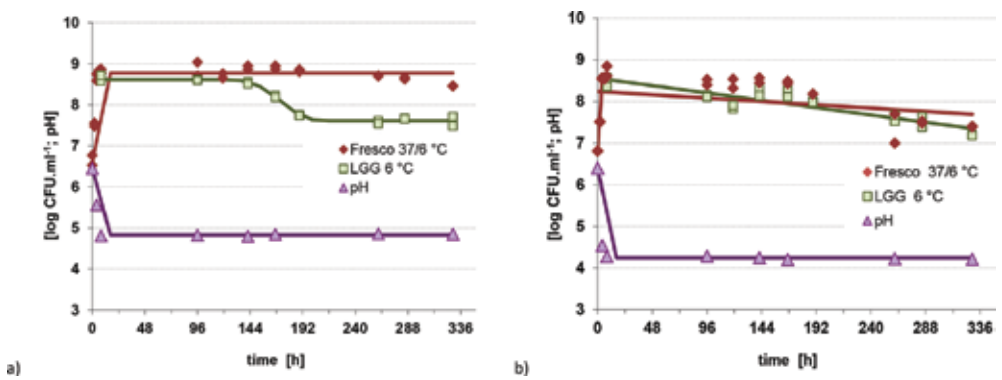

Figure 3. Presumptive counts of the cocci from Fresco DVS 1010 culture and *Lb. rhamnosus* GG (LGG) content in milk-based (a) and water-based (b) maize mashes.

As an example, the growth of Fresco DVS 1010 culture at 37 °C and the survival of probiotic strain *Lb. rhamnosus* GG (6 °C) in milk- and water-based maize mashes with sucrose are demonstrated in **Figure 3** as well as the growth parameters in **Table 5**. In general, the obtained maximal counts of monitored Fresco DVS 1010 culture after 8 h of fermentation process were $N = 10^8$–10^9 CFU ml^{-1} from initial $N_0 = 10^6$–10^7 CFU ml^{-1}, which shows the suitability of tested sweet corn mashes for the growth and survival of lactic acid bacteria. During the refrigerated storage at 6°C (**Table 6**), a decline in the number of probiotic strain *Lb. rhamnosus* GG was observed, but not under the levels of 10^6 CFU ml^{-1} necessary from the legislation point of view.

Microorganism	Substrate corn flour	Gr_f (log CFU ml^{-1} h^{-1})	λ (h)	k_{pH} (h^{-1})
Fresco DVS 1010	Milk	0.522	–	-0.231
	Milk + caramel	0.446	–	-0.345
	Milk + chocolate	0.563	–	-0.172
	Water	0.445	–	-0.481
	Water + caramel	0.508	0.59	-0.298
	Water + chocolate	0.540	–	-0.462

Gr_f, growth rate; λ, lag-phase duration; k_{pH}, rate constant for the decrease of pH. The growth data were fitted using DMFit tool, kindly provided by Dr. J. Baranyi.

Table 5. Growth parameters of Fresco culture, 8-h fermentation at 37°C in maize mashes.

In botanical terms, amaranth, quinoa and buckwheat are not true cereals. They are dicotyledonous plants, and thus not cereals (monocotyledonous). Their seeds are in function and composition similar to true cereals, so they are referred as pseudocereals [136, 137]. Gluten-free pseudocereals increased attention worldwide, because they represent alternative to conventional gluten-containing cereals and industrially are used for the production of gluten-free products, especially for celiac patients. They enrich the nutrition of health people and contribute to their balanced diet. In comparison to cereals, pseudocereals are characterized by the increased availability of proteins, as well as its higher content. Moreover, pseudocereals are the major source of minerals and vitamins, and in comparison to cereals, the content of essential amino acid lysine is higher [138–141].

Due to its chemical composition, amaranth is considered as one of the most nutritious plants that is easy to grow and over 60 species of amaranth are known worldwide [142]. Grains are characterized with balanced composition of essential amino acids, especially lysine and methionine, higher content of proteins (15–17%) and starch (60–65%) [143, 144]. Compared to other cereals, the fat content is higher, ranging from 7 to 8%. Overall, amaranth is a good source of vitamins (riboflavin, niacin and vitamin E) and minerals such as calcium and magnesium [138]. A growing number of studies have investigated the usage of amaranth in cereal technology not only in the production of nutrient-rich gluten-free products but also to enrich diet of health people [145]. Several studies have also reported the possibility to enrich wheat-based products with amaranth to improve the quality and overall nutritional value of final

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products [140]. Matejčeková et al. [146] confirmed in their study the growth of probiotic and potentially probiotic strains (*Lb. acidophilus* 145, *Lb. rhamnosus* GG, *Lb. rhamnosus* VT1 and *Lb. paracasei* subsp. *paracasei*) in water- and milk-based amaranth mashes. The same authors [30] studied the growth and survival of probiotic strain *Lb. rhamnosus* GG in flavoured amaranth mashes, which were acceptable not only from the microbiological point of view but also from the sensory evaluation. Kocková and Valík [147] evaluated the suitability of selected cereals and pseudocereals for the development of new probiotic foods fermented by *Lb. rhamnosus* GG. The highest growth rate was calculated in the case of amaranth flour (0.589 log CFU g^{-1} h^{-1}) and the longest lag phase was observed.

Substrate corn flour	k_d (log CFU ml^{-1} h^{-1})	λ (h)	N_0 (log CFU ml^{-1})	N_{end} (log CFU ml^{-1})
Milk	-0.0212	139.14	8.61	7.61
Milk + caramel	-0.0031	–	8.57	7.91
Milk + chocolate	-0.0200	–	7.91	7.04
Water	-0.0036	–	8.47	7.19
Water + caramel	-0.0033	141.78	8.27	7.65
Water + chocolate	-0.0093	–	7.47	7.03

k_d, rate constant for decrease of the *Lb. rhamnosus* counts; N_0, initial counts; N_{end}, final counts after 14 days of storage period. The growth data were fitted using DMFit tool kindly provided by Dr. J. Baranyi.

Table 6. Parameters evaluating the behaviour of *Lb. rhamnosus* GG in fermented maize mashes during storage at 6°C when added after fermentation.

Substrate buckwheat flour	Gr_f (log CFU ml^{-1} h^{-1})>	λ (h)	N_0 (log CFU ml^{-1})	N_{max} (log CFU ml^{-1})
Milk + vanilla	0.251	2.7	6.80	8.02
Milk + caramel	0.641	1.1	6.25	8.49
Milk + chocolate	0.332	3.0	6.74	8.32
Water + vanilla	0.275	2.4	6.93	8.48
Water + caramel	0.580	–	6.12	8.40
Water + chocolate	0.258	1.3	6.76	8.49

Gr_f, growth rate; λ, lag-phase duration; N_0, initial counts; N_{max}, counts after storage period. The growth data were fitted using DMFit tool kindly provided by Dr. J. Baranyi.

Table 7. Growth parameters of *Lb. rhamnosus* GG in fermented buckwheat-flavoured mashes during fermentation at 37°C.

Together with amaranth, buckwheat and its products are studied in connection with celiac disease. Buckwheat was initially grown mainly in Asia and later has spread to Europe, Australia as well as to USA and Canada. The total carbohydrate content is 67–70%, of which

55% represents starch stored in the endosperm, as in common cereals. Buckwheat has a good content of thiamine, riboflavin and pyridoxine, and also represents a good source of minerals —magnesium, copper and potassium. It is characterized by a unique concentration of phytochemicals, in particular rutin, which has a positive effect on health especially in the prevention of cardiovascular diseases [148, 149]. Pelikánová et al. [109] evaluated the growth dynamics of *Lactobacillus* spp. in sweet buckwheat gruels. The population density of tested lactobacilli reached counts 10^8–10^9 CFU ml^{-1} after 8 (10) h of fermentation, and after a 3-week-refrigerated storage period, the number of lactobacilli slightly increased except *Lb. acidophilus* 145. Liptáková et al. [87] in their study examined the pressed buckwheat products. The most suitable strain for fermentation was *Lb. rhamnosus* GG. Pressed fermented buckwheat water product with vanilla flavour was after 24 h of fermentation and after 5 days of storage evaluated with higher points according to the final evaluation of overall sensory acceptance.

Substrate buckwheat flour	k_d (log CFU ml^{-1} h^{-1})	λ (h)	N_{end} (log CFU ml^{-1})
Milk + vanilla	0.0006	–	8.54
Milk + caramel	-0.0002	–	8.42
Milk + chocolate	0.0009	–	8.89
Water + vanilla	0.0000	–	8.38
Water + caramel	-0.0002	–	8.41
Water + chocolate	0.0000	–	8.49

k_d, rate constant for decrease of the *Lb. rhamnosus* counts; λ, lag-phase duration; N_{end}, final counts after 21 days of storage period. The growth data were fitted using DMFit tool kindly provided by Dr. J. Baranyi.

Table 8. Parameters of *Lb. rhamnosus* GG in fermented buckwheat-flavoured mashes during storage at 6°C.

As for the example, growth and fermentative metabolism of probiotic strain *Lb. rhamnosus* GG in buckwheat mashes with caramel/vanilla/chocolate flavour is summarized in **Tables 7** and **8**. Investigated probiotic strain showed sufficient growth and survival in prepared flavoured mashes with the growth rates ranging from 0.251 to 0.641 log CFU ml^{-1} h^{-1}. At the end of cold storage, densities of *Lb. rhamnosus* GG maintained above the minimum limit of 10^6 CFU ml^{-1}.

The interest of consumers in fermented cereal- or pseudocereal-based products is growing. The development of non-dairy-fermented products including probiotics may lead to enrichment of the diet in patients suffering from celiac disease, people with allergies, or intolerances, but it may contribute to the balanced diet of healthy subjects [149]. If the cereal or pseudocereal products are presented with an attractive sensory taste, it may represent a suitable option for the development of new probiotic foods. Thus, in our study we evaluate the overall sensory acceptability of maize-flavoured (chocolate/caramel) mashes (**Figures 4** and **5**). The overall acceptability was evaluated from 2.80 to 3.30 (four-point scale) that indicated pleasant acceptance except caramel water mash (2.56). Kocková and Valík [135] noted negative effect of a 21-day storage period on overall acceptability buckwheat product with salt fermented by probiotic strain *Lb. rhamnosus* GG from values 3.31 to 2.44. In our study, no decline of overall acceptance during storage period was observed.

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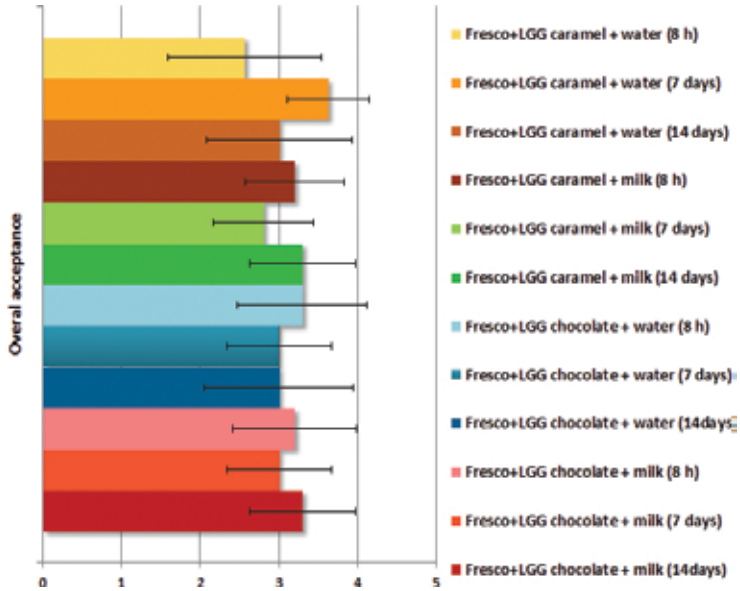

Figure 4. Evaluation of overall acceptability maize caramel/chocolate mashes (LGG—*Lactobacillus rhamnosus* GG).

Figure 5. Photo-documentation of flavoured final maize products.

5. Conclusion

Sustainable diets and cultured consumer interests, for example, in personal health, represent the main driving forces for the development of new functional foods in the world. Throughout the world, many fermented foods that are produced cover a wide range of substances and microorganisms. Ensuring high quality and safety for such a product requires deep under-

standing of fermentation process, types and roles of microorganisms used and specific final product characteristics. Lactic acid bacteria are the alternatives of food biopreservation primarily due to the production of weak organic acids and other inhibitory substances in combination with lowering pH and reduction of redox potential. LAB and their metabolites are able to slow or inhibit the growth of undesirable bacteria, yeasts and toxigenic fungi in food. There is evidence that LAB are also able to reduce the gluten content of cereals that represents increasing problem for 0.5–1% of population worldwide. Many lactic acid bacteria and other microbial strains such as *E. coli* Nissle, *B. cereus*, *B. subtilis* or *S. boulardii* belong to the probiotics with documented positive effects on human health.

The development of fermented cereal- or pseudocereal-based products supplemented with probiotics represents an available alternative to milk products and may lead to enrichment of the diet of people suffering from celiac disease, allergy to milk proteins, lactose intolerance people or otherwise metabolically handicapped consumers, but it may also contribute to a balanced diet of healthy subjects.

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Chapter 13

Solid-State Culture for Lignocellulases Production

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Additional information is available at the end of the chapter

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Abstract

Aspergillus sp. and *Trametes versicolor* solid-state monocultures produced high titers of xylanases and laccases activities (4617 ± 38 and 2759 ± 30 U/$g_{substrate}$, respectively). Fungal biomass was quantified by estimating the ergosterol content of the mycelium, and by a simple material balance the corresponding residual substrate was obtained. Fungal growth and substrate consumption rates showed different behavior for these monocultures ($\mu = 0.03$ and $0.11\ h^{-1}$; $r_s = -0.04$ and -0.0006 $g_{substrate}$/h, respectively). In this case, xylanases production was directly linked to the growth, while laccases were produced during both growth and maintenance phases. Besides xylanases (42% of total *Aspergillus* enzyme), high titers of cellulases (15%), amylases (34%), and invertases (9%), as well as lignin and manganese peroxidases (10 and 24% of the total *Trametes* enzyme), were produced on the corresponding monocultures. When both fungi were used in a coculture mode, xylanases and laccases production decreased (around 85 and 70%), and the proportion of the hydrolases and oxidases changed. This suggested the need for most careful coculture design, in order to produce both enzymatic activities simultaneously even though the enzymatic extracts obtained by mono- or cocultures can be applied in several bioprocesses.

Keywords: *Aspergillus*, coculture, laccases, *Trametes versicolor*, xylanases

1. Introduction

Laccases and xylanases are two of the most important lignocellulases that are employed in several industrial processes. Xylanases (EC 3.2.1.8) are responsible for degrading the xylan, a

major polysaccharide in several cereals cell wall, to its constituent monomers. These enzymes are mostly used in textile, pulp and paper, bread making and feed industries, and the production of juice and fruit extracts [1, 2]. Moreover, laccases (EC 1.10.3.2) are multicopper enzymes that catalyze the oxidation of a wide variety of substrates such as mono-, di- and polyphenols, aminophenols, methoxyphenols, aromatic amines, and ascorbic acid. They have several industrial uses: they degrade toxic fungal metabolites and phenolic compounds and also are used in the design of biosensors, the detection of phenols in wastewaters, in the development of biofuel cells, during bleaching and delignification processes in the pulp and paper industry, and for the production of novel paper products [3]. Together, xylanases and laccases can act for boosting bleaching process of several kinds of pulps, generating a cleaner process in which the use of the hazardous chemicals may be considerably reduced [4, 5]. These enzymes can be produced mainly by fungus, either in submerged or in solid-state cultures. The former is the most widely used as it provides a good control of operational parameters, high productivity and easy downstream processing, homogeneity of the culture and pH, and better oxygen supply and agitation speed management [6]. However, solid-state culture could be better for producing this kind of enzymes as it represents the conditions that fungus finds in nature during the invasion of lignocellulosic material. Regarding this, our research group has advanced in optimizing the components of culture media in order to obtain the highest xylanases and laccases activities and yields by *Aspergillus* sp and *Trametes versicolor*, respectively [7]. Both enzymatic activities have been evaluated for pulp pretreatment, achieving good results [8, 9]. However, the behavior of the corresponding optimized cultures has not been analyzed properly although this information can be used for developing a scalable bioprocess.

On the other hand, the joint use of fungi which produce xylanase or laccase for developing a coculture system may be considered in order to obtain a mixed enzyme preparation, which has both xylanase and laccase activities, for being applied in biopulping and biobleaching processes. This kind of procedure will provide economic advantages because of the reduction in the overall cost of production [10].

Therefore, the objective of this work is to characterize solid-state monocultures with respect to growth, substrate consumption, and xylanases or laccases production and to test a coculture for producing both enzymes at the same solid-state fermentation.

2. Methodology

2.1. Microorganisms

Trametes versicolor CDBB-H-1051 and *Aspergillus* sp were used in these experiments. Stock cultures were maintained on malt-extract agar (malt extract 2%, agar 1.8%) or PDA slants at 4°C with a periodic transfer.

2.2. Culture conditions for solid-state monocultures

For developing the corresponding monoculture, each microorganism was cultivated in solid-state fermentation (SSF) using 4 g of wheat bran and sugar cane bagasse (1:1 w/w) as support

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and substrate. For doing this, the support was moistened with water and autoclaved in 250 mL beakers at 121°C for 20 min.

For laccases production monoculture, an appropriate quantity (around 4 mL) of Kirk medium was added to maintain the desired moisture level of the support (50%) for several experimental units. Each beaker was then inoculated with 4 mycelial plugs (50 mm diameter) taken from the periphery if a *T. versicolor* colony grown on malt extract agar at 30°C for 240 h, withdrawing two experimental units every 24 h for quantifying growth, residual substrate content and oxidases activities, and employing the analytical techniques described in the following sections.

Kirk basal medium composition (in g/L) was as follows: sodium tartrate, 0.275; $MgSO_4 \cdot 7H_2O$, 0.55; K_2HPO_4, 2.2; $CaCl_2 \cdot 2H_2O$, 0.145; $(NH_4)_2SO_4$, 0.44; Glucose, 8.2; $CuSO_4 \cdot 5H_2O$, 0.28; trace elements, 11 mL (in g/L: $MnSO_4 \cdot H_2O$, 0.5; NaCl, 1; $FeSO_4 \cdot 7H_2O$, 0.1; $CoCl_2 \cdot 6H_2O$, 0.185; $ZnSO_4 \cdot 7H_2O$, 0.11; $Na_2MO_4 \cdot 2H_2O$, 0.011; H_3BO_3, 0.011). The pH of the medium was adjusted at 5.0 using 1 M HCl before sterilization.

Inoculum from *Aspergillus* sp was obtained from several 5 day old PDA plates incubated at 37°C. The spores in the agar surface were gently scraped and blended in 10 mL sterile saline and used as spore suspension. The spores were enumerated under microscope using a Neubauer chamber. The experimental units used for developing the *Aspergillus* monocultures for obtaining hydrolytic activities were inoculated using 1×10^8 spores/$g_{substrate}$ and incubated at 37°C for 96 h. Two experimental units were taken from incubation every 24 h, for quantifying growth, residual substrate content and hydrolytic activities, employing the analytical techniques described further.

Basal medium composition employed for this monoculture (in g/L) was as follows: K_2HPO_4, 2; KH_2PO_4, 2; $(NH_4)_2SO_4$, 5.

2.3. Cocultures developing

For developing the cocultures in solid state, the same support employed for monocultures was used. This was moisturized and sterilized as indicated before. In this case, the support was moisturized with Kirk basal medium, and both inoculums (*T. versicolor* and *Aspergillus* sp.) were added, using the same quantities of the corresponding monocultures (see Section 2.2). Several experimental units were subsequently incubated at 30 or 37°C for 240 h. In this case oxidative and hydrolytic activities were quantified for each culture after 240 h.

2.4. Extraction and storage of crude enzymes

After incubation, 80 mL of 100 mM sodium acetate buffer pH 5 was added to each experimental unit, homogenizing with a Multi Braun® mixer and a posterior constant agitation in an ice bath. Afterwards, the enzymatic extracts were recovered by centrifugation at 4000 rpm and stored at 4°C until analyzed. The solids obtained after centrifugation were used for estimate biomass and residual substrate content, as explained below.

2.5. Enzymatic activities quantification

Laccase (Lac) activity was determined by measuring the increase in A_{470} ($\varepsilon_{470\ nm}$ = 26,600 M^{-1} cm^{-1}) due to the oxidation of 10 mM guaiacol in 0.1 M Na-acetate buffer (pH 5.0) after incubation with the crude extract at 25°C for 10 min. One unit of laccase activity (U) was defined as the amount of enzyme required to oxidize 1 μmol of guaiacol per minute of reaction [11].

Xylanolytic (Xyl) activity was assayed by incubating at 45°C for 20 min using the crude enzyme with 1% (w/v) xylan dissolved in 100 mM acetate buffer pH 5.0, and the release of reducing sugars as xylose was monitored at 575 nm after stopping the reaction by the addition of DNS. The optical density obtained was compared with a 1 g/L xylose standard curve. One unit of xylanolytic activity was defined as the enzyme necessary for the release of 1 μmol of xylose under the described conditions [12].

Lignin peroxidase (LiP) activity was estimated by incubating at 25 °C for 20 min the crude enzyme with 4 mM veratryl alcohol diluted in 100 mM tartrate buffer pH 3.5 and 0.4 mM H_2O_2. The formation of veratraldehyde was monitored at 310 nm ($\varepsilon_{310\ nm}$ = 9.3331 mM^{-1} cm^{-1}). One unit of lignin peroxidase activity was defined as the enzyme required for oxidize 1 μmol of veratryl alcohol per minute of reaction [13].

Manganese peroxidase (MnP) activity was assayed by Incubating at 25 °C for 5 min the crude enzyme with 0.1 mM $MnSO_4$, 1 mM H_2O_2, and 1 mM 2,6-dimethoxyphenol (DMP) as substrate diluted in 0.1 M tartrate buffer pH 4.5, measuring the increase in A_{469} nm ($\varepsilon_{469\ nm}$ = 27,500 M^{-1} cm^{-1}). One unit of manganese peroxidase activity (U) was defined as the amount of enzyme required to oxidize one μmol of DMP per minute of reaction [14].

Glucoamylase (Glcamyl) activity was estimated by incubating at 60 °C for 15 min the crude enzyme with 1% (w/v) starch dissolved in 0.15 M sodium chloride buffer pH 5.0. The released reducing sugars as glucose were monitored at 575 nm after stopping the reaction by the addition of DNS. The optical density obtained was compared with a 1 g/L glucose standard curve. One unit of glucoamylase activity was defined as the enzyme necessary for the release of 1 μmol of glucose under the reaction conditions [15].

α-Amylase (α-amyl) activity was determined by incubating at 37 °C for 20 min the crude enzyme with 1% (w/v) starch dissolved in 0.15 M sodium chloride buffer pH 5. The photometric disappearance of starch was quantified after stopping the reaction by the addition of an iodine (I2/IK) mother solution, and the resultant optical density at 580 nm was registered. One unit of α-amylase activity was defined as the enzyme necessary for hydrolyze 0.1 mg of starch [15].

Invertase (Inv) activity was determined by incubating at 30 °C for 30 min the crude enzyme with 0.1 M sucrose dissolved in 0.15 M acetate buffer pH 5.5. The reducing sugars as fructose were quantified after stopping the reaction with DNS. The optical density obtained was compared with a 1 g/L glucose standard curve. One unit of invertase activity was described as the enzyme necessary for the release of 1 μmol of reducing sugars per minute of reaction [16].

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Carboxymethyl cellulase (CMCase) activity was quantified By incubating at 50 °C for 5 min the crude enzyme with (1% w/v) carboxymethyl cellulase low viscosity in 50 mM citrate buffer (pH 5.0). The reducing sugars as glucose were quantified after stopping the reaction with DNS. The optical density obtained was compared with a 1 g/L glucose standard curve. One unit of CMCase activity was described as the enzyme necessary for the release of 1 μmol of reducing sugars per minute of reaction [17].

The total cellulase activity (filter paper activity, FPAse) was assayed by using a 0.5 × 6 cm string of filter paper as the substrate. This was incubated using 100 mM acetate buffer (pH 5.0) and the crude enzyme for 5 min at 45°C, stopping the reaction with DNS. The optical density obtained was compared with a 1 g/L glucose standard curve. One unit of FPAse activity was defined as the amount of enzyme used for the release of 1 μmol of glucose under the assayed conditions [18].

2.6. Biomass quantification

The quantification of fungal biomass was made after quantifying ergosterol content of the biomass in each sample. For doing this, the solid content of each experimental unit was resuspended in 10 mL of water by vigorous agitation. From this homogeneous solid suspension, 1 mL was withdrawn and filtered, to recover the solids. To this, 3 mL of an alcoholic basic solution (25% w/v of KOH dissolved in methanol) was added, boiling the resultant mixture for 5 h. Afterwards, 1 mL of distilled water and 5 mL of *n*-heptane were added, for mixing at the vortex for 3 min. The tubes were let to settle until the phase separation (around 1 h), for recording the corresponding optical density at 230 nm (for detecting the presence of 24(28) DHE, an intermediary of ergosterol pathway) and 280 nm (for detecting the ergosterol presence) of the organic phase. Ergosterol content of the biomass was estimated using the following equation:

$$Ergosterol(\%) = \frac{O.D.at280nm}{290} - \frac{O.D.at230nm}{518}$$

where 290 and 518 correspond to the molar extinction coefficient (M^{-1} cm^{-1}) of crystalline ergosterol and 24(28) DHE, respectively. A 10 g/L mycelium (*Aspergillus* sp or *T. versicolor*) standard curve was developed, for correlate g/L of biomass with ergosterol %. In fact, the calculated ergosterol % contained on each sample was compared with this curve in order to estimate biomass content [19].

2.7. Substrate quantification

The solids obtained from each culture were dried at 60°C for 12 h. The biomass content (estimated as explained before) was subtracted to the corresponding dry weight in order to obtain the real substrate content of each sample.

All the experiments were performed in duplicate, and the results are expressed as the mean of these repetitions and the corresponding standard deviation.

3. Results and discussion

3.1. Behavior of solid-state fermentation monocultures during enzymes production

On previous work, we optimized the culture media components in order to obtain high xylanase and laccase productions on solid-state fermentation, using *Aspergillus* sp or *T. versicolor*, respectively [7]. However, these experiments were made at very low volumes, in which only the information of final enzyme production was obtained at 72 or 240 h, respectively. As these enzymes were used successfully for jonote pulp bleaching [8], we decided to increase the volume of the reaction unit, and analyze the behavior of each culture with respect to fungal growth, substrate consumption and xylanase or laccase production. By doing this, we could obtain the basic information to scale up the process for producing both enzymatic activities in large amounts. Also, it could be possible to design an efficient bioprocess in which both enzymatic units can be produced simultaneously.

In this regard, it is worth mentioning that biomass quantification on solid-state cultures is a complicated work. Fungal growth is not easy to quantify because fungus grows as hyphal filaments that cannot be quantified by the usual enumeration techniques, and specifically on cultures in which an insoluble material is used as the only carbon source, complete recovery of fungal biomass from the substrate is very difficult, because the fungal hyphae tend to penetrate into and binds tightly to the solid substrate particles [20]. It is important therefore to have reliable and convenient methods for measuring fungal growth. For this reason, we used the ergosterol content methodology [19] for quantifying biomass evolution along the culture, and employed a simple mass balance for knowing the corresponding residual substrate. Therefore, this is one of the main contributions of the present work.

With respect to monocultures developed with *Aspergillus* sp., growth seemed to start increasing when the culture was among 20 and 40 h, reaching finally 0.7 g of mycelium/g of substrate at the end of culture. In this case, the fungus showed an approximate growth rate of 0.03 h^{-1}. Substrate consumption showed a constant rate during the first 60 h, stopping after that. Almost

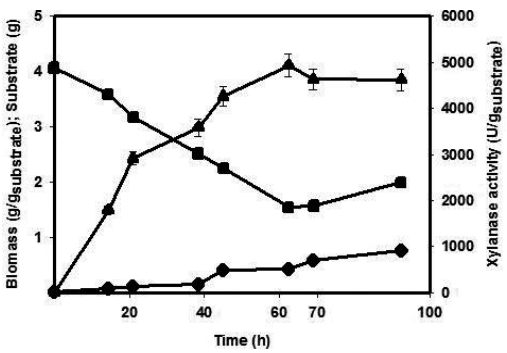

Figure 1. Fungal growth (♦), substrate consumption (▪), and xylanase production (▲) by *Aspergillus* sp along the solid-state monoculture developed on wheat bran:sugar cane bagasse 1:1 (w/w) as substrate for solid-state fermentation, incubated at 37°C for 96 h.

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2.5 g of substrate seems to have been consumed during the culture, at a consumption rate of −0.04 $g_{substrate}$/h. With respect to xylanases production, this started with the same trend as that of fungal growth, and increased constantly, although when the growth stopped xylanolytic activity decreased slightly (**Figure 1**).

With respect to monocultures developed with *T. versicolor* for laccase production (**Figure 2**), it can be seen that in this case there was an adaptation phase of around 50 h, afterwards the fungus started to grow. In this case, it was obtained about 0.6 $g_{mycelium}/g_{substrate}$, with a constant specific growth rate of 0.11 h^{-1}. However, substrate consumption was really slow (r_s = −0.0006 $g_{substrate}$/h), and the fungi only consumed approximately 1.5 g along the culture, which represents 40% of the initial substrate. Laccase production and growth started together, increasing at constant rate, but the enzyme production remained even when biomass stopped growing.

Several studies developed on liquid or submerged fermentation have reported that laccases production has a secondary metabolite behavior; it means the activity is produced mainly during the secondary metabolism [21]. On solid-state culture, this relationship is not well known due to the difficulty of quantifying accurately the total biomass grown in the substrate along the culture. However, other studies in which fungal biomass has been quantified by indirect methods, like that used in the present work, have shown that laccases production is growth related, as happened for laccases produced by *Streptomyces psammoticus* on rice straw [22]. And those results are in accordance with the *T. versicolor* profile obtained in the present work.

The titers obtained on each monoculture for xylanases and laccase activities were high. These results show that solid-state culture is a good alternative for producing high oxidative or hydrolytic activities, in order to employ them for several industrial bioprocesses. At this respect, the results obtained in the present work would represent one basis for developing this process on a full- scale. Thereby, for further characterization of the monocultures, in the next section the production profiles of other oxidative or hydrolytic activities obtained were analyzed.

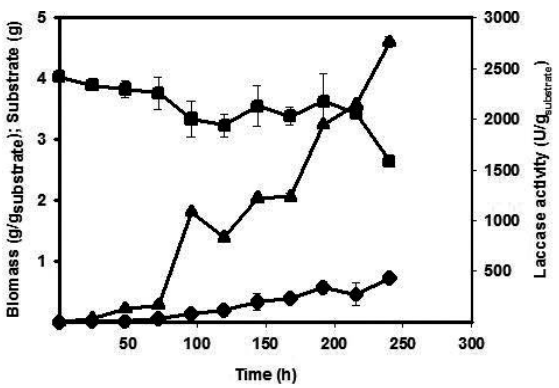

Figure 2. Fungal growth (♦), substrate consumption (▪), and laccase production (▲) by *T. versicolor* in monoculture developed on wheat bran:sugar cane bagasse 1:1 (w/w) as support/substrate for solid-state fermentation, incubated at 37°C for 96 h.

3.2. Enzymatic profiles of crude enzymes

Total enzyme production obtained along *Aspergillus* sp. and *T. versicolor* solid-state monocultures was determined by quantifying different activities: xylanases, oxido-reductases and cellulases, since it is known that these enzymatic families are involved in invasion of lignocellulosic material [23]. Furthermore, due to wheat bran and sugar cane bagasse composition [24], amylase and invertase activities were also measured.

Along *Aspergillus* monoculture, xylanolytic activity was predominant; a peak of this activity was reached after 60 h, followed by a constant level until the end of culture. However, another hydrolases were quantified: cellulases and invertases had maximum titers during the first 20 h, after that their levels remained below 1000 U/g for FPase and 500 U/g for CMCase, while amylases reached their maximum levels after 48 h of cultivation, and these activities were kept until the end of the culture. In this monoculture, no oxidase activities were obtained.

With respect to oxidases, during the first 72 h of monocultures developed with *T. versicolor*, LiP and MnP activities were predominant; from this moment, laccase activity increased continuously to reach a peak around 2700 U/g at the end of process. This activity was almost five times higher than lignin and manganese peroxidases. After 100 h of cultivation, LiP and MnP activities stayed at a level around 250 and 800 U/g, respectively. In this *T. versicolor* monoculture none hydrolytic activity was detected. **Table 1** concentrates final titers of all hydrolase and oxidase activities obtained on each of both monocultures.

Enzymatic activities (U/gs)	***Trametes versicolor***	***Aspergillus* sp**
Laccases	2759 ± 30	ND
LiP	410 ± 10	ND
MnP	996 ± 2	ND
Xylanases	ND	4617 ± 38
FPAses	ND	746 ± 7
Carboximetil-celulases	ND	829 ± 34
Glucoamylases	ND	1762 ± 4
α-amylases	ND	1898 ± 6
Invertases	ND	1023 ± 73

ND indicates the enzyme whose activity was not detected in the corresponding monoculture.

Table 1. Oxidases and hydrolase activities produced by monocultures developed by *T. versicolor* or *Aspergillus* sp on solid-state monocultures.

Taking into account that enzymatic extracts obtained on solid-state cultures by *Aspergillus* sp. and *T. versicolor* had several hydrolytic or oxidative activities, there could be some other uses for them. In this respect, extracts obtained from *Aspergillus* sp. monoculture could be used for saccharification processes of a number of agro-industrial residues [25], while the extract

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obtained from *T. versicolor* monoculture can be proved in color removal of some industrial effluents [26].

3.3. Cocultures for joint production of lignocellulases

Solid-state cultures showed high productions of either hydrolase or oxidase activities in the corresponding monocultures. Previous work of this group has shown that bleach boosting of kraft [9] and jonote [8] pulp can be improved with the employment of the combined action of xylanases and laccases, produced by solid-state cultures as those described in the present work. So, a greater enzyme production would be desirable in order to have a more efficient bioprocess. At this respect, it has been suggested that the cocultivation of microbes in fermentation can increase the quantity of the desirable components on a cellulose complex [27]. On the other side, some reports have shown that laccases or xylanases production can increase in a coculture mode, as happens with *P. ostreatus*, which increased fivefold its laccase production in a coculture with *Trichoderma viride* in submerged fermentation [28]. As discussed earlier, it became interesting to probe if a coculture of *Aspergillus* sp and *T. versicolor* was feasible for obtaining higher xylanase and laccase activities at the same time considering that both fungi used wheat bran and sugar cane bagasse as substrate. In addition, this coculture strategy would provide economic advantages because of reduction in overall cost production. This is why both fungi were inoculated at the same time in the support and two incubation temperatures were probed: 30°C (the best for *T. versicolor*) and 37°C (the best for *A. niger*), developing independent experiments. **Figure 3** shows the enzyme proportions obtained in cocultures developed at 30 or 37°C with respect to the control conditions, obtained in the respective monocultures of each fungi.

With respect to hydrolytic activities proportion, it can be seen that while on monocultures xylanase activity was the predominant hydrolytic activity, glucoamylases were in greater proportion for cocultures developed at 30°C, and α-amylases highlighted on cocultures developed at 37°C (**Figure 3 A**).

Comparing both cocultures, one can see that the highest hydrolytic activities were obtained on that developed at 37°C, which means that temperature could be affecting seriously the *Aspergillus* sp. behavior along this culture. On the other hand, invertase and cellulase activity proportions were low on monocultures, but decreased on both cocultures. The diminution of invertase activity production on coculture modes can be due to the presence of glucose in the media, as Kirk medium was used to moisturize the support in these experiments, and this mineral medium contains glucose. A similar diminution on invertase activity production due to glucose presence in solid-state fermentation was reported previously for *A. ochraceus* in similar culture conditions, which employed wheat bran or sugar cane bagasse as substrates [29].

Laccase activities decreased on both cocultures with respect to those values obtained on monocultures. This behavior could be considered unusual, because in general the coculture strategy is used in order to increase these activities. In fact, the addition of soil microorganisms to white rot fungi cultures has increased laccase and other oxidases production [30]. In this case, the diminution on laccase activity can be explained from different points of view. First of

all, in the present study both fungi were inoculated at the same time to the support. The decrease in oxidases and hydrolases production could be provoked by problems in fungal growth, considering the differences in specific growth rate of each fungal species. This is because mineral medium used in these cocultures contained glucose, and it has been reported as laccase inductor for *T. versicolor* [31]. So, even when *Aspergillus* grows slower in its respective monoculture, in this case it could be consuming the available glucose of the medium faster than *T. versicolor* could, reducing the ability of *T. versicolor* for producing laccases and the another oxidases. However, to probe this hypothesis, some additional experiments would be necessary.

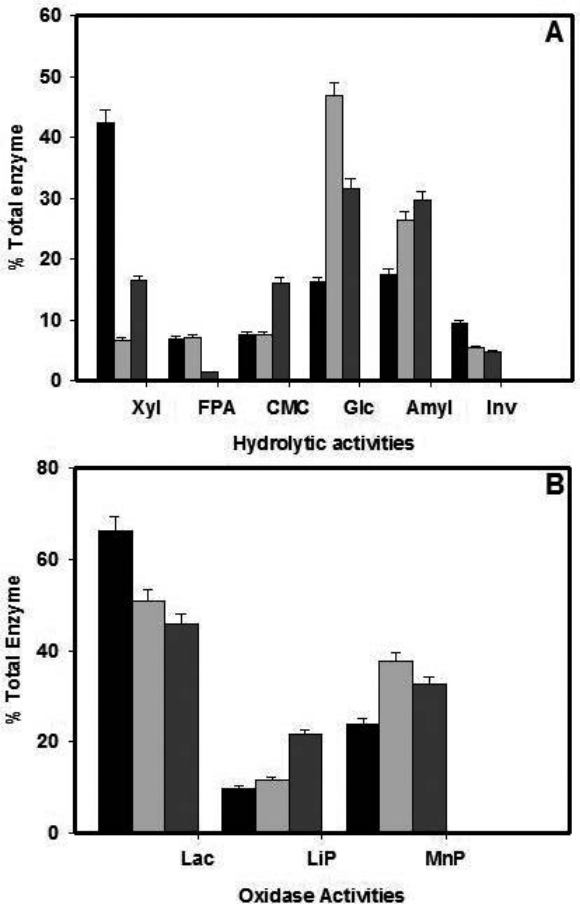

Figure 3. Enzyme proportion of hydrolytic (A) or oxidase (B) activities obtained on monocultures (▪), and cocultures developed at 30°C (■) or 37°C (■).

On the other hand, we must remember that enzymes are produced at different rates along the cultures, as described in Section 3.2. In this case, activities analyzed for this experimental phase are those obtained after 10 days, but the enzyme evolution along both cocultures was not

registered. This is a great limitation for obtaining valid conclusions. It could be possible that one of these enzymes has been increased at any time during the fermentations, but as we did not quantify the enzyme evolution, we could not know if the reported activity was the highest obtained for the corresponding enzyme in this experiment. So, we can only analyze the enzyme activities at the end of the coculture, and these could not be the highest activities obtained in this case.

Finally, even when some hydrolytic or oxidative enzymes decreased on coculture mode, these extracts can be employed on several processes. Agricultural by-products typically vary in their chemical composition and nutritional value, and sometimes are also higher in low-quality fiber, so a specific enzyme complex is required to break it down in order to be used in ruminant feed. Besides, their nutritional value could be increased by biodegradation methods of fiber in the rumen, through efficient delignification [32]. Therefore, the filtrates obtained on both cocultures could be a good alternative for being employed for using agricultural by-products for ruminant feed.

4. Conclusions

The indirect technique used for the quantification of fungal biomass content was useful, meaning a great contribution for analyzing solid-state cultures. From this, it could be seen that both fungi have different behaviors along the culture, in which *T. versicolor* seems to grow faster than *Aspergillus* sp., and consequently its substrate consumption rate is also higher. However, high xylanases and laccases titers were obtained on the corresponding monocultures. In this case, xylanases production seemed to be growth related, while laccases started to produce since growth phase and continued producing even when fungus stopped growing. In addition, the presence of other hydrolases and oxidases activities showed the potential of the enzymatic extracts for being used in several industrial bioprocesses. Coculture mode caused a decrease in xylanase and laccase production. In this respect, it seems that xylanase production is affected by the incubation temperature, and although *Aspergillus* sp. grows slower it could be consuming the glucose contained in Kirk mineral medium, delaying the laccases production by *T. versicolor*. Some modification in the inoculation methodology is needed in order to increase the production of these enzymes by coculture. Based on this, we can conclude that solid-state fermentation, independent or as coculture, could be a promising green biotechnology for producing several lignocellulosic enzymes from agricultural residues that can be used for different industrial applications at a lower cost.

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Chapter 14

Anaerobic Digestion: I. A Common Process Ensuring Energy Flow and the Circulation of Matter in Ecosystems. II. A Tool for the Production of Gaseous Biofuels

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Aleksandra Chojnacka and Mieczysław K. Błaszczyk

Additional information is available at the end of the chapter

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Abstract

Anaerobic digestion, a process that ultimately generates methane and carbon dioxide, is common in natural anoxic ecosystems where concentrations of electron acceptors such as nitrate, the oxidized forms of metals and sulphate are low. It also occurs in landfill sites and wastewater treatment plants. The general scheme of anaerobic digestion is well known and comprises four major steps: (i) hydrolysis of complex organic polymers to monomers; (ii) acidogenesis that results in the formation of hydrogen and carbon dioxide as well as non-gaseous fermentation products that are further oxidized to hydrogen, carbon dioxide and acetate in (iii) acetogenesis based on syntrophic metabolism and (iv) methanogenesis. Approaches to the analysis of methane-yielding microbial communities and data acquisition are described. There is currently great interest in the development of new technologies for the production of biogas (primarily methane) from anaerobic digestion as a source of renewable energy. This includes the modernization of landfill sites and wastewater treatment plants and the construction of biogas plants. Moreover, research effort is being devoted to the idea of separating hydrolysis and acidogenesis from acetogenesis and methanogenesis under controlled conditions to favour biohydrogen and biomethane production, respectively. These two stages occur under different conditions and are carried out in separate bioreactors.

Keywords: anaerobic digestion, microorganisms, hydrogen, methane, syntrophy, renewable energy

1. Introduction

Anaerobic digestion of biomass under mesophilic conditions (anaerobic microbial decomposition/degradation of organic matter), whose final products are methane and carbon dioxide, contributes to the energy flow and circulation of matter in ecosystems. It is a key process in the global carbon cycle that is promoted by the activity of many different groups of microorganisms. Anaerobic digestion commonly occurs in natural anoxic ecosystems such as freshwater sediments, wetlands, marshlands, paddy fields and deeper zones of marine sediments. The digestive tracts of animals, especially ruminants and termites, are also sites of methane production by this process. It is estimated that biological methanogenesis is responsible for more than 70% of total global methane emissions [1, 2].

Anaerobic decomposition of biomass to carbon dioxide and methane only occurs in anoxic environments with a low redox potential, i.e., where concentrations of other electron acceptors including nitrate, oxidized forms of metals such as Mn(IV) and Fe(III) or sulphate are low. The inhibition of anaerobic digestion by nitrate, oxidized metal ions and sulphate is determined by the redox potential. As shown in **Figure 1**, a decrease in redox potential leads to changes in the dominant type of anaerobic respiration towards low energy-yielding processes. The nature of the final electron acceptors present in an environment is a key factor in determining the ecological niches for particular microorganisms.

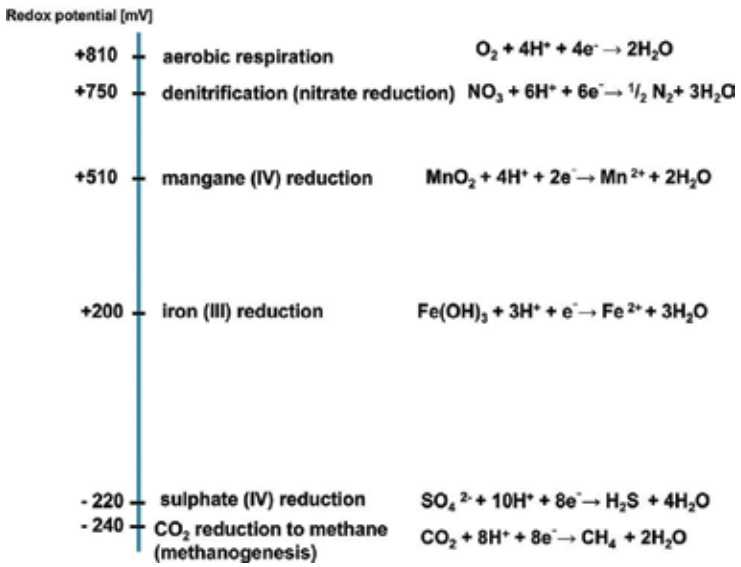

Figure 1. Redox potential for different types of final electron acceptors in anaerobic respiration and energy gain for microbial cells.

The general scheme of anaerobic digestion is well known (**Figure 2**). It is a complex process promoted by the interaction of many groups of microorganisms and has four major steps. The first is hydrolysis of complex organic polymers to monomers. The second step is acidogenesis

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that results in the formation of hydrogen and carbon dioxide as well as non-gaseous fermentation products, i.e., low-molecular weight organic acids (short-chain fatty acids) and alcohols. In the third step, known as acetogenesis, these non-gaseous products are further oxidized to hydrogen, carbon dioxide and acetate, mainly by syntrophic degradation processes. The fourth step is methanogenesis. The final two steps, acetogenesis and methanogenesis, are closely linked and involve syntrophic associations between hydrogen-producing acetogenic bacteria and hydrogenotrophic methanogens. These associations keep the hydrogen partial pressure sufficiently low to allow acetogenesis to become thermodynamically favourable. This process, referred to as interspecies electron transfer, is in fact a hydrogen/formate transfer. Acetate is a direct substrate for methanogenesis and can also be syntrophically oxidized to hydrogen and carbon dioxide [3–8].

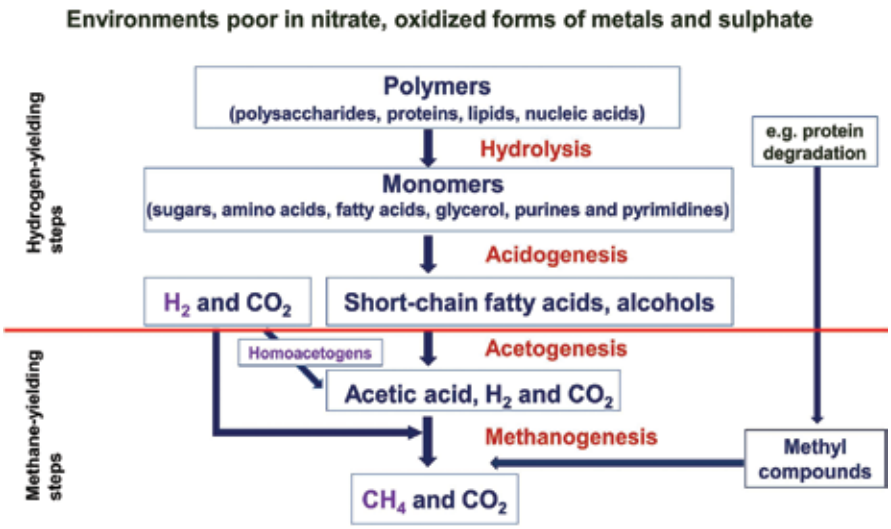

Figure 2. Scheme of anaerobic digestion of polymeric organic matter to methane and carbon dioxide.

Anaerobic digestion is common in landfill sites and anaerobic wastewater treatment plants. The process of anaerobic decomposition of biomass, such as energy crops or organic agro-waste, is commonly used to produce biogas as an alternative energy source. There is currently great interest in the development of new technologies for the modernization of landfills and wastewater treatment plants to control the release of biogas and collect methane to use as fuel. Moreover, for the purpose of innovative technologies based on microbial processes, it is desirable to build modern biogas plants where the hydrogen-yielding (hydrolysis and acidogenesis) and methane-yielding (acetogenesis and methanogenesis) stages of anaerobic digestion are separated to, respectively, favour the production of hydrogen and methane under controlled conditions. Optimization of methane or hydrogen and methane production from organic matter requires a good understanding of anaerobic digestion at the molecular level, namely the structure and diversity of microbial communities and metabolic pathways, leading to transformation of the organic substrate to the desired gaseous products.

2. Meta-omics approaches for exploring microbial communities

Current knowledge of microbial ecology and physiology, derived from culture-dependent techniques, is limited and incomplete because the majority of microorganisms have not been cultivated. It has been predicted that only 1% or less of all microorganisms present in natural ecosystems may be cultivated as a pure culture using standard methods [9]. Moreover, syntrophy is believed to be common in microbial communities, and syntrophic bacteria cannot be grown as a monoculture. However, culture-dependent techniques have permitted the isolation and characterization of some species involved in specific metabolic processes during anaerobic digestion, and numerous genomes have been sequenced. Data from genome sequence analyses supported by the results of physiological and biochemical studies on cultivated bacteria have provided hints as to which physiological groups of microorganisms are responsible for the key steps of anaerobic digestion. Information on methane-yielding microbial communities is now being obtained using culture-independent analytical techniques (**Figure 3**).

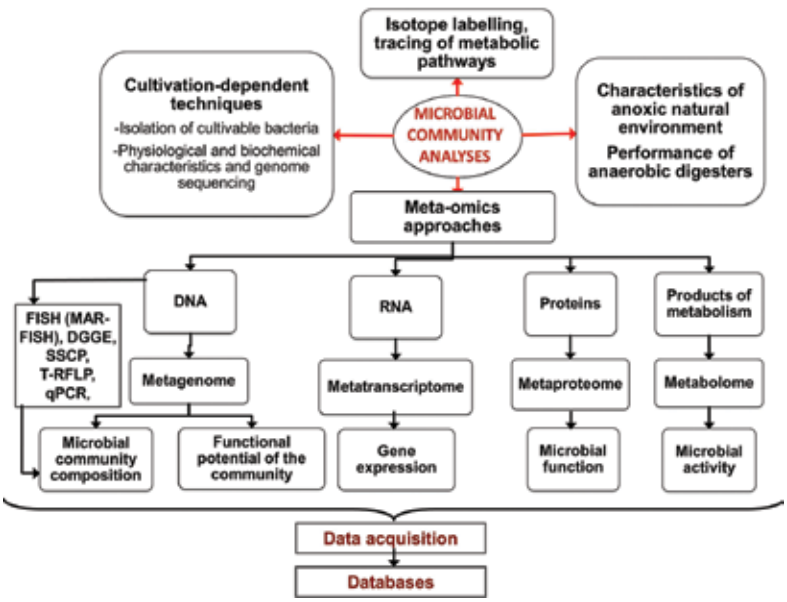

Figure 3. Culture-independent approaches to analyse methane-yielding microbial communities. FISH, fluorescence in-situ hybridization; MAR-FISH, microautoradiography combined FISH; DGGE, denaturing gradient gel electrophoresis; SSCP, single-strand conformation polymorphism; T-RFLP, terminal restriction fragment length polymorphism; qPCR, real-time quantitative PCR.

The recent increase in the number of culture-independent molecular biology techniques and bioinformatic tools for exploring microbial communities has helped to develop the field of meta-omics. Meta-omics encompasses metagenomics, metatranscriptomics, metaproteomics and metabolomics, based on analyses of, respectively, total DNA, mRNA, total proteins and metabolites isolated from the microbial communities [10–14]. Metagenomics shows microbial

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potential by describing the genes present in a microbial community or ecosystem. Metatranscriptomics analyses gene expression and thus represents potential microbial function. Messenger RNA (mRNA) can be sequenced directly or used to generate cDNA (by reverse transcription) that is subsequently sequenced using metagenomics platforms. Metaproteomics is focused on microbial function—it investigates proteins expressed within a microbiome. Metabolomics analyses the intermediates and end-products of metabolism and then shows microbial activity.

The data generated by these novel methodologies have provided significant insights into the structure and function of microbial communities in both natural environments and man-made systems. However, meta-omics-based approaches do suffer from certain limitations: the variable extraction efficiency of DNA/RNA/protein/metabolites may affect the results, and reference databases used for comparative analyses often contain false or missing assignments of DNA and protein sequences or chromatography/mass spectrometry data. For example, metagenomic analyses always generate large numbers of sequences that are of low complexity, unclassified, not assigned or show no hits. Such unidentified reads usually constitute a significant proportion of the total reads, as discussed by Chojnacka et al. [15]. It is noteworthy that the limited number of microorganisms that can be propagated as pure cultures determines the number of sequenced reference genomes available for genomic studies. So far, only five genomes of syntrophic bacteria involved in acetogenesis have been sequenced: *Syntrophus aciditrophicus*, *Syntrophus wolfei*, *Syntrophobacter fumaroxidans*, *Pelotomaculum thermopropionicum* and *Syntrophothermus lipocalidus*, as discussed by Li et al. [16].

Using metagenomic sequence data and genomic assembly procedures, it is possible to reconstruct genomes of bacteria that have not been cultivated. One example is a reconstruction of the genome of *Candidatus* Cloacimonas acidaminovorans [17]. This is a representative of the *Cloacimonetes*, a sub-dominant group of bacteria found in anaerobic mesophilic digesters and gut microflora. They are regarded as syntrophs capable of amino acid fermentation, propionate and butyrate oxidation as well as cellulose degradation and have never been grown in pure culture.

In the case of anaerobic digestion, the combined use of meta-omic approaches and isotope labelling techniques in both natural anoxic environments and bioreactors plus the analysis of reactor performance data will allow us to develop a fundamental understanding of the processes leading to methane production. Meta-omic data can also be used to validate commonly accepted theses.

Other cultivation-independent techniques include isolation of total DNA from microbial communities, amplification, cloning and sequencing of marker genes, most frequently 16S rRNA or others such as *gyrB* or *mcrA* for methanogenic *Archaea*; fluorescence in-situ hybridization (FISH) and its derivatives such as microautoradiography combined FISH (MAR-FISH); community fingerprinting by denaturing gradient gel electrophoresis (DGGE); single-strand conformation polymorphism (SSCP); terminal restriction fragment length polymorphism (T-RFLP) and real-time quantitative PCR (qPCR), as discussed by Dziewit et al. [18].

3. Hydrolysis and acidogenesis: the anaerobic digestion steps yielding short-chain fatty acids and hydrogen

3.1. Hydrolysis

Hydrolysis is the first step in the anaerobic decomposition of organic matter. It involves the conversion of polymeric organic matter (e.g., polysaccharides, lipids, proteins) to monomers (e.g., sugars, fatty acids, amino acids) by hydrolases secreted to the environment by microorganisms. Three key groups of hydrolases are involved in the process of anaerobic digestion: esterases, glycosidases and peptidases, which catalyse the cleavage of ester bonds, glycoside bonds and peptide bonds, respectively [19]. The bacteria most commonly associated with hydrolysis include representatives of the *Firmicutes* (*Clostridia, Bacilli*), *Bacteroidetes* and *Gammaproteobacteria* [20–22]. Usually, the same bacteria are also able to conduct acidogenesis, the second step in the decomposition of organic matter.

Metaproteomic analysis of microbial communities mediating the decomposition of dead plant material in forest leaf litter revealed fungi to be the main producers of extracellular hydrolytic enzymes, the most prominent of which are cellulolytic enzymes: exo- and endo-glucanases as well as β-glucosidases. Other hydrolases involved include phosphatases, pectinases, xylanases, lipases, amylases, chitinases and oxidoreductases. Moreover, the species of fungi – the main cellulase producers – changed depending on the season. In a sample collected in February, *Leotiomycetes* dominated, whereas in samples collected in May, *Eurotiomycetes, Dothideomycetes, Leotiomycetes* and *Sordariomycetes* were the most abundant fungal phyla. Interestingly, no bacterial hydrolases were detected [23].

3.2. Acidogenesis

3.2.1. Fermentation of sugars

During acidogenesis, the products of hydrolysis are converted to non-gaseous short-chain fatty acids, alcohols, aldehydes and the gases, such as carbon dioxide and hydrogen [3]. The dominant end-products of the fermentation process determine the type of fermentation (**Figure 4A**).

The main hydrogen-yielding fermentations under mesophilic conditions are butyric acid fermentation (*Clostridium*-type fermentation) and mixed-acid fermentation (*Enterobacteriaceae*-type fermentation). The common first step is glycolysis (the Embden-Meyerhof-Parnas pathway) when glucose is converted to pyruvate and NADH is formed (Figure 4B). In both types of fermentation, hydrogenases are responsible for hydrogen release. Hydrogenases are metalloenzymes that are divided into two major groups according to the metal in the prosthetic group of the active site: [FeFe] and [FeNi] hydrogenases [24, 25]. In the *Clostridium*-type fermentation, pyruvate is oxidized to acetyl-CoA by pyruvate:ferredoxin oxidoreductase (PFOR) in the presence of ferredoxin (Fd). Reduced ferredoxin is also formed in the reaction with NADH catalysed by NADH:ferredoxin oxidoreductase (NFOR). Hydrogen is released, mainly by [FeFe] hydrogenases that catalyse proton reduction using electrons from ferredoxin.

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Acetyl-CoA is converted to non-gaseous end-products including short-chain fatty acids (acetate, butyrate, lactate, propionate), alcohols (mainly not only ethanol, but also butanol and propanol) and ketones (such as acetone) by a wide range of enzymes. An increased number of non-gaseous products of fermentation decreases the production of hydrogen during acidogenesis. The hydrogen concentration regulates the relative activities of PFOR and NFOR. A hydrogen partial pressure of >60 Pa inhibits NFOR activity and favours the formation of non-gaseous end-products. In contrast, PFOR is still active at hydrogen concentrations of up to 30,000 Pa [3, 26–29].

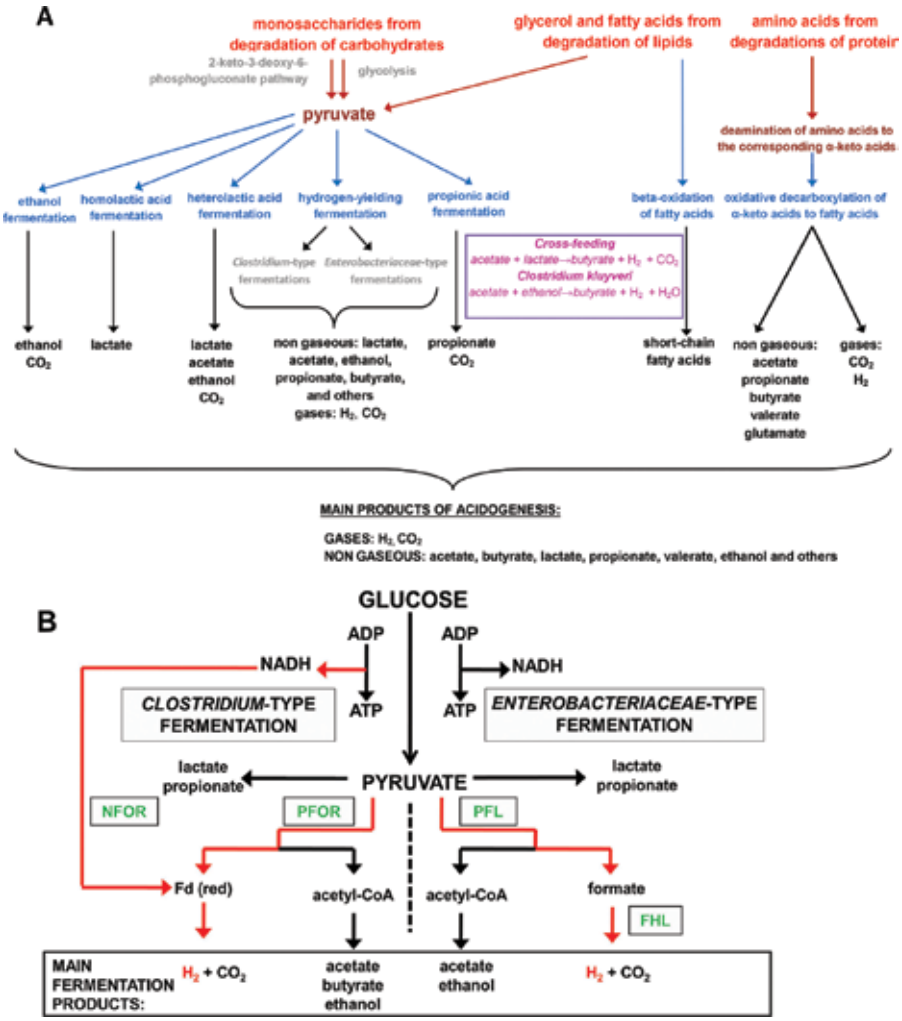

Figure 4. Metabolic pathways of acidogenesis: (A) general overview and (B) glycolytic hydrogen-yielding fermentations. PFOR, pyruvate:ferredoxin oxidoreductase; NFOR, NADH:ferredoxin oxidoreductase; PFL, pyruvate formate-lyase; FHL, formate hydrogen-lyase complex.

In the mixed-acid fermentation (also known as formic acid fermentation), pyruvate is converted to acetyl-CoA and formic acid by pyruvate formate-lyase (PFL). The formic acid can then be degraded into hydrogen and carbon dioxide by formate hydrogen-lyase (FHL) complex. One of the FHL subunits is the [FeNi] hydrogenase Hyd-3. There are two types of mixed-acid fermentation: the *Escherichia coli* type and the *Enterobacter* type. In the *Enterobacter*-type fermentation, hydrogen can also be generated through oxidation of NADH by NFOR in reactions similar to those described for the *Clostridium*-type fermentation. Non-gaseous products of the *Enterobacteriaceae*-type fermentation can include ethanol, short-chain fatty acids (formate, acetate, lactate, succinate) as well as acetoin and 2,3-butanediol [30–32].

Besides glycolysis, other pathways of pyruvate formation exist, e.g., the 2-keto-3-deoxy-6-phosphogluconate (Entner-Doudoroff) pathway. Two intermediates of glycolysis, glyceraldehyde-3-phosphate and fructose-6-phosphate, are also formed in the pentose phosphate pathway. Monosaccharides other than glucose can enter glycolysis or other pathways leading to pyruvate formation. Pyruvate can also be formed from glycerol [33].

In addition to the hydrogen-yielding fermentations, other fermentations occur during acidogenesis, including lactic, propionic and ethanol fermentations. Two types of lactic acid fermentation are distinguished: homolactic and heterolactic, whose products are, respectively, lactate only or lactate, ethanol, acetate and carbon dioxide.

3.2.2. Fermentation of amino acids

Members of the *Clostridiales* (families *Clostridiaceae, Eubacteriaceae, Peptococcaceae, Peptostreptococcaceae*), *Fusobacterales, Synergistetes* (*Aminobacterium colombiense*) and *Cloacimonetes* (*Candidatus* Cloacimonas acidaminovorans) are capable of amino acid fermentation [4, 7]. Amino acids are generally degraded to acetate, propionate, hydrogen and carbon dioxide, with the formation of butyrate and ammonia. This process involves NAD(P)- or FAD-dependent deamination of amino acids to the corresponding α-keto acids with subsequent oxidative decarboxylation of α-keto acids to fatty acids with CoA and phosphate derivatives (Figure 4A). The pathways of fermentation differ depending on the amino acid type [4]. Amino acid mixtures are often degraded by coupled fermentation of pairs of amino acids through the Stickland reaction (e.g., alanine and glycine in *Clostridium sporogenes*). Oxidation of one amino acid is coupled to the reduction in another in a single cell:

$$\text{alanine} + 2H_2O \rightarrow \text{acetate}^- + CO_2 + NH_4^+ + 2H_2$$

$$\text{glycine} + 2H_2O + H^+ \rightarrow 2CO_2 + NH_4^+ + 3H_2$$

$$\text{serine} + H_2O \rightarrow \text{acetate}^- + CO_2 + NH_4^+ + H_2$$

$$\text{threonine} + H_2O \rightarrow \text{propionate}^- + CO_2 + NH_4^+ + H_2$$

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$$\text{histidine} + 4H_2O + H^+ \rightarrow \text{glutamate}^- + CO_2 + 2NH_4^+ + H_2$$

$$\text{proline} + 2H_2O \rightarrow \text{glutamate}^- + H^+ + 2H_2$$

$$\text{glutamate}^- + 2H_2O + H^+ \rightarrow \text{propionate}^- + 2CO_2 + NH_4^+ + 2H_2$$

$$\text{glutamate}^- + 2H_2O \rightarrow 2\ \text{acetate}^- + CO_2 + NH_4^+ + H_2$$

$$\text{aspartate}^- + 2H_2O + H^+ \rightarrow \text{acetate}^- + 2CO_2 + NH_4^+ + 2H_2$$

Notably, glutamate may be fermented through five different pathways by various bacterial species: Pathway 1—through 3-methylaspartate; Pathway 2—through 3-methylaspartate to acetate, propionate, carbon dioxide and ammonium; Pathway 3—through 2-hydroxyglutarate to acetate, butyrate, hydrogen, carbon dioxide and ammonium; Pathway 4—through 4-aminobutyrate to acetate, butyrate and ammonium and Pathway 5—through 5-aminovalerate to acetate, propionate, valeriate and ammonium [34].

3.2.3. Transformation of lipids during acidogenesis

The products of lipid hydrolysis are glycerol and long-chain fatty acids (Figure 4A). Glycerol can enter (i) a reductive pathway and be converted to 1,3-propanediol or (ii) an oxidative pathway and be transformed to phosphoenolopyruvate in a four-step process. Phosphoenolopyruvate can then be converted to succinate and propionate and/or to pyruvate. In the latter case, further transformations of pyruvate occur through glycolytic fermentations as described for sugars [33, 35]. Significant hydrogen production was observed when *Enterobacter aerogenes* [36] and *Klebsiella pneumoniae* [37] were grown on glycerol-rich media.

Long-chain fatty acids are transformed to acetate and hydrogen through the beta-oxidation pathway, requiring syntrophic cooperation between acetogens and methanogens (described in Section 2.3). However, long-chain fatty acids have an inhibitory effect on anaerobic digestion due to their adherence to microbial cell walls, which can block the passage of nutrients through the cell membrane and/or cause flotation of the cells.

3.2.4. Cross-feeding

Symbiotic interactions between lactic acid bacteria and butyrate-producing bacteria involving clostridia, called "cross-feeding", have been detected in the gastrointestinal tract (Figure 4A). Numerous observations in different animal models have described lactate and acetate conversion to butyrate by butyrate-producing intestinal bacteria, stimulated by lactic acid bacteria (for review, see Ref. [38]). The incubation of human microflora in media containing ^{13}C-labelled lactate revealed that butyrate was the major net product of lactate conversion [39]. Other studies performed using ^{2}H-labelled acetate and ^{13}C-labelled lactate showed that acetic and lactic acids are important precursors of butyrate production in human faecal samples [40].

The metabolic pathway of lactate and acetate utilization to produce butyrate proposed for *Eubacterium hallii* and *Anaerostipes caccae* involves the conversion of lactate to pyruvate by lactate dehydrogenase [41, 42]. The next steps are typical of hydrogen-yielding *Clostridium*-type fermentation. Pyruvate is oxidized to acetyl coenzyme A (acetyl-CoA), which is subsequently routed to acetate and butyrate. Additional acetate is converted to acetyl-CoA. Hydrogen can be produced by both PFOR and NFOR complexes and hydrogenases. The conversion of lactate to butyrate is an important factor in maintaining homeostasis in gastrointestinal tracts.

It is commonly accepted that anaerobic digestion requires symbiotic interactions between specific groups of microorganisms. Some studies have indicated that lactic acid bacteria (LAB), often detected within mesophilic hydrogen-producing microbial communities, may support hydrogen production during acidogenesis. Based on our own research and the findings of other groups, we have considered the true role of LAB in bioreactors and their influence on hydrogen producers [38]. Our metagenomic survey of microbial communities in anaerobic bioreactors, performed using 454-pyrosequencing, revealed that *Clostridiaceae, Enterobacteriaceae* and heterolactic fermentation bacteria, mainly *Leuconostocaeae,* were the most dominant bacteria in hydrogen-producing consortia under optimal conditions for gas production. Furthermore, the complete consumption of lactic acid and predominance of butyric acid in the acidic effluent were observed [43].

An analysis of the hydrogen-yielding granular sludge using the FISH technique [44] revealed that *Streptococcus* spp. cells are located inside the granules, surrounded by *Clostridium* cells. This finding indicates the importance of *Streptococcus* spp. in sludge granule formation and the positive role they play within these microbial communities by stimulating hydrogen production.

Others researchers have examined the effects of lactic acid on hydrogen production by communities of fermentative bacteria. In one study, the complete consumption of lactic acid increasing hydrogen production and butyric acid formation was observed [45]. Subsequently, another group demonstrated that lactic acid increased the efficiency of hydrogen production [46]. FISH analysis revealed that *Clostridium* spp. were the dominant hydrogen producers in the examined system.

Many studies have examined the conversion of lactate and acetate to butyrate and hydrogen by clostridial species, and all point to pH as a critical factor for this process. It is noteworthy that the results of studies on gastrointestinal microflora indicate that acidity is a key regulatory factor in lactate metabolism. The pH may influence both bacterial growth and the development of specific groups of bacteria, as well as fermentation processes affecting the relative proportions of short-chain fatty acids (for review, see Ref. [38]).

A phenomenon analogous to cross-feeding observed in the gastrointestinal tract may occur in hydrogen-producing bioreactors and natural environments [38, 43] (Figure 4A).

Clostridium kluyveri ferments ethanol and acetate to butyrate and hydrogen (Figure 4A; for review, see Ref. [47]).

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4. Acetogenesis

4.1. The essence of acetogenesis

The two final steps of anaerobic digestion, acetogenesis (Stage III) and methane formation (Stage IV), are tightly connected. Acetogenesis supplies substrates for methanogens. Three groups of substrates for methane production and three types of methanogenic pathways have been recognized: (i) splitting of acetate (aceticlastic/acetotrophic methanogenesis); (ii) reduction in CO_2 with H_2 or formate and rarely ethanol or secondary alcohols as electron donors (hydrogenotrophic methanogenesis) and (iii) reduction in methyl groups of methylated compounds such as methanol, methylated amines or methylated sulphides (hydrogen-dependent and hydrogen-independent methylotrophic methanogenesis) [2, 48–51].

Due to the limited number of substrates for methanogenesis, methanogens are strictly dependent on partner microbes with which they form syntrophic systems. Syntrophy is a special type of symbiotic cooperation between two metabolically different types of microorganisms, which depend on one another for the degradation of a certain substrate, typically through the transfer of one or more metabolic intermediate. In this case, the partner microbes oxidize non-gaseous products of acidogenesis to acetate, carbon dioxide, hydrogen and formate that are directly utilized by the methanogens, making the entire syntrophic metabolism efficient and thermodynamically favourable. This is the essence of acetogenesis. The process of hydrogen or formate transfer (interspecies hydrogen/formate transfer) between acetogenic bacteria and methanogenic *Archaea* is an excellent example of syntrophy [4, 6, 7].

Under standard conditions, the oxidation of butyrate, propionate, acetate, ethanol and other non-gaseous products of acidogenesis, coupled to hydrogen or formate production, is endergonic, as demonstrated by the positive change in Gibbs free energy. However, when the oxidation processes are coupled to methane production, the conversion is energetically feasible (exergonic) due to the very low hydrogen partial pressure ensured by hydrogen-consuming methanogens (**Figure 5**). Oxidation of non-gaseous products of acidogenesis during acetogenesis is based on reverse electron transfer: the energetically unfavourable movement of electrons that requires the input of energy to drive the oxidation/reduction reaction (**Figure 5**). This involves multiple systems, most of which are membrane-located, comprising formate dehydrogenases (FDHs), ferredoxin:NAD^+ oxidoreductase, hydrogenases, *c*-type cytochromes, quinone reactive complexes, flavoprotein:quinone oxidoreductases and confurcating hydrogenases. Electron confurcation is a key process in reverse electron transfer. It involves a combined biochemical reaction using two dissimilar electron donors to generate a single product. Confurcating hydrogenases couple hydrogen production from reduced ferredoxin with hydrogen production from NADH [7]. The process responsible for energy conservation in syntrophically growing acetogens is called flavin-based electron bifurcation. Electron bifurcation is the reverse process whereby two products are formed, e.g., NADH and reduced ferredoxin from butyryl-CoA (see Section 4.2) [47, 52].

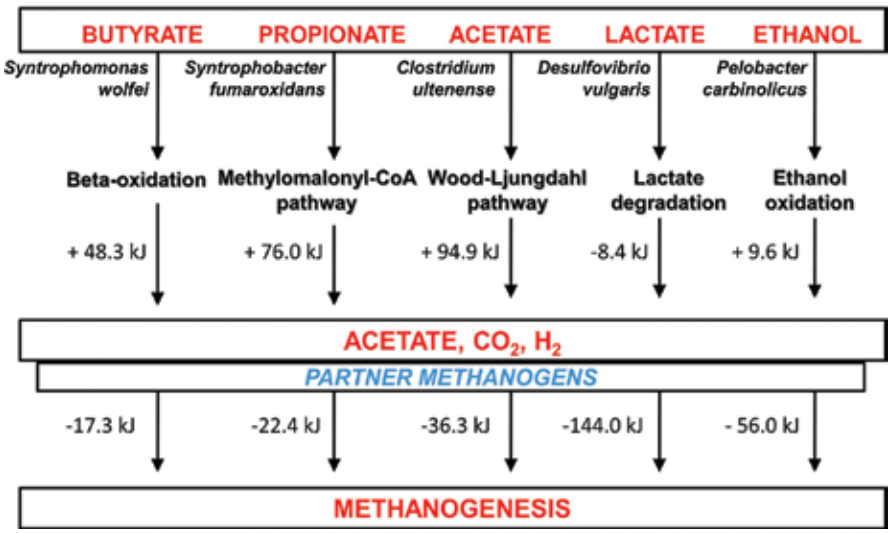

Figure 5. Syntrophic metabolism during acetogenesis—oxidation of non-gaseous products of acidogenesis based on reverse electron transfer in syntrophy with hydrogen-consuming methanogens. The $\Delta G^{0\prime}$ values for acetate, butyrate, propionate and ethanol come from the study of Kamagata [53] and those for lactate oxidation, coupled or uncoupled with the methanogen partner, from the study of McInerney and Bryant [54].

The second known mechanism of interspecies electron transfer in methanogen-yielding communities is direct transfer. This was described between *Geobacter* and *Shewanella* species as the electron donor and methanogen (the electron acceptor), respectively, in environments lacking Mn(IV) and Fe(III) compounds. In this case, pili and outer membrane *c*-type cytochromes are involved in the cell-to-cell electron transfer. Interspecies electron transfer in syntrophic methanogenic microbial communities has been recently reviewed [55].

Our current understanding of the microbial ecology and physiology associated with anaerobic digestion is restricted to culture-dependent techniques and thus is incomplete. The majority of microorganisms involved in the process of anaerobic digestion have yet to be cultivated. It is noteworthy that acetogenic bacteria are unable to grow without their syntrophic partners and cannot be cultivated as a monoculture. Thus, the mechanisms of acetogenesis are poorly characterized at the molecular level. Data derived using recently developed meta-omics approaches are likely to give a deeper insight into syntrophic metabolic pathways of anaerobic digestion.

4.2. Biochemistry of syntrophic oxidation of non-gaseous products of acidogenesis

The metabolic pathways utilized for syntrophic oxidation of common non-gaseous products of acidogenesis include beta-oxidation for butyrate, the methylmalonyl-CoA pathway for propionate, the Wood-Ljungdahl pathway for acetate, the pathway of lactate oxidation recognized in *Desulfovibrio* in the absence of sulphate and the pathway of ethanol oxidation recognized in the genera *Pelobacter* and *Desulfovibrio* in the absence of other electron acceptors.

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In the first reaction of butyrate oxidation, butyrate is activated with acetyl-CoA to butyryl-CoA by butyrate-CoA transferase. This is followed by the conversion of butyryl-CoA to crotonyl-CoA catalysed by butyryl-CoA dehydrogenase, to release electrons as hydrogen or formate, which requires ATP. This process is only possible by a reverse electron transport through electron transfer flavoprotein EtfAB and a membrane-anchored DUF224 protein to the menaquinone pool in the membrane, cytochromes and other electron transfer complexes, terminating at the formate dehydrogenase and hydrogenase/formate dehydrogenase complexes. Crotonyl-CoA is transformed to 3-hydroxy-butyryl-CoA by crotonase and then to aceto-acetyl-CoA by 3-hydroxybutyryl-CoA dehydrogenase. The latter reaction also yields electrons as hydrogen or formate due to reverse electron transfer and the activity of the NADH:hydrogenase/formate dehydrogenase complex. Aceto-acetyl-CoA is split into two moieties of acetyl-CoA by acetyl-CoA acetyltransferase: one is used for butyrate activation and the second is transformed to acetate by phosphotransacetylase and acetate kinase activity, accompanied by the release of ATP [52, 56, 57].

In the first reaction of propionate oxidation, propionate is activated with acetyl-CoA to propionyl-CoA by propionate-CoA transferase. This is then transformed to (S) methylmalonyl-CoA, (M) methylmalonyl-CoA, succinyl-CoA and succinate by, respectively, methylmalonyl-CoA decarboxylase, methylmalonyl-CoA epimerase, methylmalonyl-CoA mutase and succinyl-CoA synthetase. The final step generates ATP. The next reaction is the conversion of succinate to fumarate by fumarate reductase, which releases electrons. This is the first key reaction that requires reverse electron transport. Fumarate is transformed to malate by fumarate hydratase. Malate is then converted to oxaloacetate by malate dehydrogenase in the second key reaction coupled to reverse electron transport. Pyruvate formed from oxaloacetate by pyruvate carboxylase is then transformed to acetyl-CoA by pyruvate:ferredoxin oxidoreductase. Finally, acetyl-CoA is converted to acetate in the third step generating electrons during propionate oxidation. The oxidation of oxaloacetate to fumarate involves coupling menaquinone reduction, proteins encoded by cytochrome c gene homologues, cytochrome b:quinone oxidoreductases, formate dehydrogenases, and hydrogenases including confurcating [FeFe]-hydrogenases [7, 52, 55, 57].

Acetogens that synthesize acetate from hydrogen and carbon dioxide use the reductive carbon monoxide dehydrogenase/acetyl-CoA synthase pathway (reductive CODH/ACS) known as the Wood-Ljungdahl pathway. Acetate-oxidizing syntrophs use the same pathway in reverse (oxidative CODH/ACS). Electrons as hydrogen or formate are released in the reactions catalysed by the carbon monoxide dehydrogenase/acetyl-CoA synthase, methylene-tetrahydrofolate (methylene-THF) reductase and methylene-THF dehydrogenase formate dehydrogenase. Reverse electron transfer during acetate oxidation has yet to be confirmed. It is likely that the same electron transfer mechanism is used in both pathways (reductive and oxidative) [52, 58].

It is believed that ethanol is oxidized to acetaldehyde coupled to NADH formation. Subsequently, acetaldehyde is oxidized to acetate and reduced ferredoxin is formed. Ethanol-oxidizing *Pelobacter carbinolicus* possesses genes encoding membrane-bound ion-translocating

ferredoxin:NAD^+ oxidoreductase and a confurcating hydrogenase that could directly catalyse the oxidation of NADH and reduced ferredoxin to form hydrogen [7, 59].

The key reaction of syntrophic lactate oxidation in *Desulfovibrio* spp. is the conversion of lactate to acetyl-CoA (via pyruvate) by lactate dehydrogenase followed by pyruvate:ferredoxin oxidoreductase, in a reaction that requires reverse electron transfer. The membrane-bound Qmo (quinone-interacting membrane-bound oxidoreductase) complex, cytochromes (involving Hmc, high-molecular-weight cytochrome c complex), menaquinone, hydrogenases (Hyn, Hyd, Hys) and formate dehydrogenases are responsible for reverse electron transport and final hydrogen and formate release. Acetyl-CoA is further processed to acetate by phosphate acetyltransferase and acetate kinase or to ethanol by alcohol dehydrogenase [60].

Worm and co-workers analysed the genomes of the butyrate- or propionate-oxidizing syntrophs *Syntrophus aciditrophicus, Syntrophus wolfei*, *Syntrophobacter fumaroxidans*, *Pelotomaculum thermopropionicum* and *Syntrophothermus lipocalidus,* and identified six syntrophy-specific functional domains [52]. These include the extra-cytoplasmic formate dehydrogenase (FDH) alpha subunit, as well as an FdhE-like protein and FDH accessory protein. The functions of the latter two proteins are tightly connected with FDH. This finding points to the important role of formate in interspecies electron transfer. The fourth domain was detected in CapA, a protein involved in capsule or biofilm formation that may facilitate syntrophic growth. The fifth domain is characteristic of FtsW, RodA and SpoVE proteins involved in membrane integration, cell division, sporulation and shape determination. The final domain was detected in a conserved site of ribonuclease P involved in tRNA maturation.

In the same study, functional domains involved in electron transfer were also identified [52]. These were found in the following proteins: cytoplasmic FDH, extra-cytoplasmic FDH, formate transporter, Fe-Fe hydrogenase, NiFe hydrogenase, Rnf complex, Ech complex, Etf alpha, Etf beta, Bcd, cytochromes c, cIII, b561 and b5 and the DUF224 protein complex.

Notably, the genomes of sulphate-reducing non-syntrophs were found to lack the syntrophy-specific domains. However, these domains are present in other sulphate reducers that have never been tested for syntrophy: *Desulfobacterium autotrophicum*, *Desulfomonile tiedjei* and *Desulfosporosinus meridiei* [52].

4.3. A model of methane-yielding granules

According to the model of methane-yielding granules proposed more than 25 years ago acetotrophic methanogens constitute a central core of the granule surrounded by acetogenic bacteria and hydrogenotrophic methanogens, and the external layer is composed of microorganisms responsible for acidogenesis. The physical distances (proximities) necessary for energetically favourable hydrogen transfer between acetogenic bacteria and hydrogenotrophic methanogens have been estimated from studies on the propionate-, propanol-, ethanol-oxidizing syntroph *Pelotomaculum thermopropionicum* and hydrogenotrophic methanogen *Methanothermobacter thermoautotrophicus*. The proximity needed for efficient interspecies hydrogen transfer depended on the substrate and was estimated at 2, 16 and 32 μm for propionate, ethanol and propanol oxidation, respectively. It is noteworthy that for the less

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energetically favourable syntrophic process ($\Delta G^{0'}$ = +76.0 kJ, +9.6 kJ and +3.0 kJ, respectively, for propionate, ethanol and propanol oxidation), a closer relationship, i.e., a shorter distance between syntrophic partners, is required (for review, see Refs. [53, 55]).

4.4. Syntrophic relationships between acetogenic bacteria and methanogens during anaerobic digestion

The most well-studied examples of syntrophic metabolism in methanogenic communities are described below. The *Syntrophomonadaceae*, a family from the order *Clostridiales*, are highly specialized syntrophic microbes found in methanogenic consortia that can oxidize butyric, propionic and long-chain fatty acids to acetic and formic acids with the production of hydrogen and carbon dioxide—the basic substrates for their partner methanogens [61–63].

The most frequently recognized butyrate oxidizers are representatives of the *Syntrophomonadaceae—Syntrophomonas wolfei, S. bryantii, S. curvata, S. sapovorans, S. palmitatica, S. cellicola, S. saponavida, S. erecta, S. zehnderi; Syntrophothermus lipocalidus, Thermosyntropha lipolytica* and representatives of the *Syntrophobacterales (Deltaproteobacteria)* and *Syntrophus acidotrophicus*. Proteins expressed specifically during syntrophic growth of *S. wolfei* with butyrate have been investigated by proteomic analysis [4, 6, 7, 56].

The propionate-oxidizing bacteria are members of the *Syntrophomonadaceae*—genus *Syntrophobacter* (*S. fumaroxidans, S. wolinii*) and *Smithella propionica* and of the *Peptococcaceae—Desulfotomaculum* (*D. thermocisternum, D. thermobenzoicum* subs. *thermosyntrophicum*) and *Pelotomaculum* (*P. thermopropionicum*) genera [4, 6, 7].

Li and co-workers [16] developed specific PCR assays for propionate-CoA transferase genes (*pct*) to identify and analyse propionate oxidizers in the methane-yielding microbial communities in anaerobic digesters treating various food industry wastes. In addition to *Syntrophobacter fumaroxidans*, six other distinct clusters of putative *pct* genes were detected. The diversity and abundance of the *pct* genes were determined by the nature of the feedstocks of the anaerobic digesters. There was little difference between the *pct* gene profiles of the granular sludge and the liquid phase in the same digester. These authors postulated that the feedstock is a critical factor influencing propionate metabolism in anaerobic digesters. It is noteworthy that such PCR assays may also be used to examine anaerobic decomposition of organic matter in natural environments.

Acetate is the major intermediate product during anaerobic digestion of organic matter to methane and carbon dioxide. It can be directly transformed to methane and carbon dioxide by acetoclastic methanogens (Section 2.4) or syntrophically oxidized to hydrogen and carbon dioxide. The latter reaction requires the participation of two microbial partners: an acetate-oxidizing bacterium and a hydrogenotrophic methanogen. Recognized acetate-oxidizing bacteria include members of the *Clostridia—Thermoacetogenium phaeum*, *Clostridium ultunense, Clostridium sporomusa, Syntrophaceticus schinkii, Tepidanaerobacter syntrophicus, Tepidanaerobacter acetatoxydans, Candidatus* Syntrophonatronum acetioxidans and *Moorella* sp., as well as *Deltaproteobacteria—Geobacter* spp. and *Thermotogae—Thermotogae lettingae* [4, 52]. Analyses using culture-independent techniques have revealed many other uncultured acetate oxidizers.

Ito and co-workers [64] used MAR-FISH combined with phylogenetic analysis of ^{13}C-labelled bacterial 16S rRNA and tracing of [2-^{14}C]-labelled acetate degradation to study metabolic pathways of acetate transformation in methanogenic sludge from an anaerobic digester fed with mineral medium containing powdered whole milk. These analyses identified *Synergistes* Group 4, belonging to the phylum *Synergistetes*, as the major acetate-utilizing group of bacteria. Moreover, acetate oxidizers were shown to win the competition with acetoclastic methanogens from the genus *Methanosaeta* for the utilization of acetate. At high acetate concentrations, the *Synergistetes* showed a lower affinity for acetate and higher utilization rate in comparison with *Methanosaeta*.

Lee and co-workers [8] presented evidence that in anaerobic digesters fed with a medium containing acetate as the sole carbon source, *Spirochaetes* syntrophically oxidize this substrate with hydrogenotrophic *Methanomicrobiales*. Quantitative PCR (qPCR) and reverse transcription quantitative PCR (RT-qPCR) targeting the 16S rRNA genes of cluster II *Spirochaetes* and methanogens (*Methanosaetaceae*, *Methanosarcinaceae*, *Methanomicrobiales* and *Methanobacteriales*) revealed that an increase in the former was correlated with higher numbers of *Methanomicrobiales*. High concentrations of hydrogen inhibited the activity of the *Spirochaetes*.

Synergistetes and *Spirochaetes* are frequently found in anaerobic digesters and natural environments, but little is known about their role in anaerobic digestion besides the fact that the latter are thought to be capable of glucose fermentation.

Interestingly, current knowledge concerning the oxidation of lactate in methanogenic consortia is limited to members of the *Desulfovibrio* genus. These species are capable of syntrophic growth on lactate and ethanol with hydrogenotrophic methane-producing partners in the absence of sulphate. As methanogenesis is thermodynamically unfavourable, such syntrophic metabolism is possible only when other electron acceptors such as sulphate are absent. Otherwise, sulphate reduction occurs. Lactate can also act as a substrate for the non-methanogen *Archaeoglobus*, a known sulphate reducer capable of oxidizing lactate to carbon dioxide [7, 60].

Recent studies on anaerobic digestion of molasses wastewater in an upflow anaerobic sludge blanket (UASB) reactor revealed the significant contribution of *Lactococcus* and *Methanosaeta* and their close interaction in methane production [65]. These authors analysed cDNA obtained by reverse transcription of RNA isolated from methane-yielding sludge samples. They proposed lactate as the major fermentation product, which is subsequently oxidized to acetate, a substrate for *Methanosaeta*.

Chojnacka and co-workers [15] hypothesized that a symbiotic interaction between lactic acid bacteria and clostridia, known as lactate cross-feeding (described in Section 2.2.3.), may also occur in methanogenic communities. Butyrate and hydrogen are the products of lactate transformation. The hydrogen and the products of further syntrophic butyrate oxidation constitute substrates for methanogenesis.

Ethanol is also effectively utilized by the methane-yielding microbial communities [15, 66]. Apart from *Desulfovibrio* species, other well-recognized syntrophic ethanol oxidizers are representatives of the *Deltaproteobacteria*, *Geobacter* and *Pelobacter*—well-known Fe(III) reduc-

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ers. To be an energetically effective reaction, the oxidation of ethanol to carbon dioxide and hydrogen also requires strict cooperation with hydrogenotrophic methanogens [4].

Members of the orders *Clostridiales* (*Clostridiaceae, Eubacteriaceae, Peptococcaceae, Peptostreptococcaceae* families), *Fusobacteriales* and phylum *Synergistetes* (*Aminobacterium colombiense*) are capable of amino acid fermentation [4, 7]. However, the transformation of amino acids to produce methane is only energetically possible in syntrophic association with hydrogenotrophic methanogens that scavenge hydrogen.

The *Cloacimonetes*, including Waste Water of Evry 1 (WWE1), are a sub-dominant group of bacteria found in mesophilic anaerobic digesters and gut microflora. So far, all attempts to cultivate representatives of the *Cloacimonetes* have failed, probably due to their need for obligatory symbiotic relationships with other microorganisms. However, the genome of a representative bacterium *Candidatus* Cloacimonas acidaminovorans has been reconstructed using metagenomic sequence data and genomic assembly procedures [93]. The candidate division WWE1 bacteria are regarded as syntrophs capable of amino acid fermentation, propionate and butyrate oxidation as well as cellulose degradation [7, 66].

Actinobacteria, *Chloroflexi* and *Plantomycetes* are often among the bacterial phyla detected in methane-producing anaerobic digesters and wastewater treatment plants. Their functional activities in methanogenic communities have not been well characterized. *Actinobacteria* and *Chloroflexi* are thought to hydrolyse and ferment carbohydrates. The contribution of *Chloroflexi* and *Plantomycetes* to butyrate oxidation was identified in experiments performed with [^{13}C]-labelled butyrate [62].

We have examined the microbial community processing an acidic effluent from molasses fermentation to methane in a UASB bioreactor [15]. Total DNA isolated from the methanogenic community formed in the reactor was sequenced by 454-pyrosequencing. The results revealed that the biodiversity of methanogenic sludge is significantly higher than that of the hydrogen-producing community. The ratio of *Bacteria* to *Archaea* in the methanogenic community was 4:1. The domain *Bacteria* was dominated by *Firmicutes* (~24%), *Bacteroidetes* (~21%), *Proteobacteria* (~9%), *Cloacimonetes* (~7.5%) and *Spirochaetes* (~7%). The *Firmicutes* were dominated by *Clostridia*, which constituted approximately 14% of all bacterial reads. The *Proteobacteria* were mostly represented by the delta and gamma subdivisions (~9 and ~1.5%, respectively), whereas the alpha and beta subdivisions were poorly represented (~0.5%). Other minor groups were *Actinobacteria* (~2%), *Chlamydiae* (~1%), *Synergistetes* (~1%) and *Chloroflexi* (~0.5%). A small number of reads were sequences from *Armatimonadetes, Negativicutes* and *Plantomycetes*. The low level of unfermented sugars and the abundance of *Clostridia* and *Bacteroidetes* suggested that these bacteria play a previously unrecognized role in acetogenesis, involving syntrophic oxidation of non-gaseous products of hydrogen-yielding fermentation. Moreover, an analysis of short-chain fatty acids revealed that butyric and lactic acids were the main substrates utilized in the methanogenic step.

Some of the aforementioned bacterial phyla are capable of oxidizing other compounds, including 1-propanol, benzoate, hydroxybenzoate, phenol and phthalates.

5. Methane formation

Methane formation, Stage IV of anaerobic digestion, is a complex process requiring specific enzymes and cofactors not found in other microorganisms. The course of the reaction depends on the substrates utilized by the methanogens. Three groups of substrates are recognized: (i) acetate, (ii) CO_2 and H_2 or formate, and rarely ethanol or secondary alcohols and (iii) methylated compounds including methanol, methylated amines and methylated sulphides. These substrates are, respectively, processed through three recognized pathways of methanogenesis: aceticlastic/acetotrophic, hydrogenotrophic and methylotrophic (hydrogen-dependent and hydrogen-independent) (**Figure 6**) [67]. Irrespective of the substrate, the final step in each methanogenic pathway is the reaction of methyl-coenzyme M (CH_3-S-CoM) and coenzyme B to produce heterodisulphide CoM-S-S-CoB and methane:

$$CH_3 - S - CoM + Co\,B \rightarrow CoM - S - S - CoB + CH_4$$

This reaction is catalysed by methylcoenzyme M reductase (Mcr), the key enzymatic complex of the methanogenic process. It possesses a unique prosthetic group, coenzyme F_{430}, containing nickel. CoM-S-S-CoB acts as the final electron acceptor during anaerobic respiration and is the key compound for energy gain by methanogens. Methane is a by-product of methanogen metabolism. The pathways of methanogenesis are in fact pathways of CoM-S-S-CoB synthesis.

Splitting of acetate (acetotrophic methanogenic pathway) involves the formation of acetyl-CoA, the transfer of methyl groups to tetrahydrosarcinopterin (H_4SPT) and the formation of methyl tetrahydrosarcinopterin CH_3-H_4STP. CH_3-S-CoM is formed in the reaction of CoM

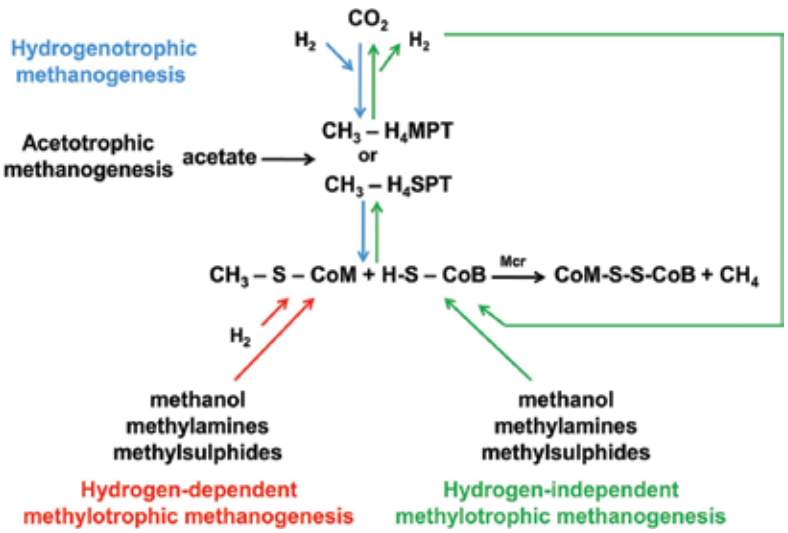

Figure 6. Pathways of methanogenesis. H-S-CoM, coenzyme M; H-S-CoB, coenzyme B; H_4MPT, tetrahydromethanopterin; H_4SPT, tetrahydrosarcinapterin.

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with CH_3-H_4STP. The electrons required to reduce CH_3-S-CoM to methane come from oxidation of the carboxyl group of acetate.

The formation of CH_3-S-CoM by the reduction in CO_2 with H_2, formate or alcohols constitutes the hydrogenotrophic methanogenic pathway. This pathway is comprised of the following steps: (i) the formation of formylmethanofuran (formyl-MFR) from methanofuran (MFR) and CO_2, (ii) the reaction of formyl-MFR and tetrahydromethanopterin (H_4MPT) to produce formyl tetrahydromethanopterin (formyl H_4MPT), (iii) the formation of methylene H_4MPT that in reaction with F_{420}, a derivative of 5′ dezaflavin, produces methyl H_4MPT and (iv) the reaction of methyl H_4MPT with CoM to generate CH_3-S-CoM. The electrons required to reduce CH_3-S-CoM to methane come from hydrogen, formate or alcohols.

In the methylotrophic pathway of methanogenesis, CH_3-S-CoM is formed by the direct transfer of methyl groups from methylated compounds to CoM. One methyl group bound to CoM is oxidized to CO_2 and hydrogen (in the form of $F_{420}H_2$ and reduced ferredoxin) to reverse the hydrogenotrophic pathway. The reducing equivalents are used to reduce CH_3-S-CoM to methane.

In the recently discovered process of hydrogen-dependent methylotrophic methanogenesis, CH_3-S-CoM is also formed through the direct transfer of methyl groups from methylated compounds to CoM. However, the electrons required to reduce CH_3-S-CoM to methane come from externally supplied hydrogen. Genomic analysis revealed that organisms generating methane by this process lack genes encoding the enzymes of hydrogenotrophic methanogenesis [50, 51].

The known cultured methanogens are strict anaerobes and comprise seven orders in the class *Euryarchaeota* of the domain *Archaea*: *Methanobacteriales*, *Methanococcales*, *Methanomicrobiales*, *Methanosarcinales*, *Methanopyrales* [2, 48], *Methanocellales* [49] and *Methanomassiliicoccales* [50, 51]. Surprisingly, only two known genera, *Methanosarcina* and *Methanosaeta*, members of the order *Methanosarcinales*, are capable of methane production from acetate. Moreover, only *Methanosaeta* is strictly acetoclastic, whereas *Methanosarcina* is able to produce methane from acetate, CO_2 and H_2 and from methylated compounds. The recognized methylotrophic methanogens belong to the order *Methanosarcinales*. All other known methanogens produce methane by the reduction in CO_2 [1, 2, 48, 49, 67]. The known members of the *Methanomassiliicoccales* order are H_2-dependent methylotrophs. They use methylated compounds (mono-, di-, tri-methylamine and dimethylsulphide) as substrates for methanogenesis, and the methyl group is reduced by hydrogen [50, 51].

It has been estimated that 70% of methane is produced from acetate. When biomass is transformed into methane under mesophilic conditions in anaerobic digesters or natural environments, it is first fermented to acetate, carbon dioxide and hydrogen and formate, as well as short-chain fatty acids during acidogenesis. The theoretical maximum hydrogen yield during dark fermentation occurs with the conversion of one-third of the substrate to hydrogen and carbon dioxide and two-thirds of the substrate to acetate. Therefore, it follows that two-thirds of methane originates from acetate and one-third is from hydrogen, formate and carbon dioxide [2].

Culture-independent analyses of methanogenic communities (mainly from anaerobic digesters) based on cloning and sequencing of 16S rRNA and *mcrA* gene fragments or high-throughput DNA sequencing technologies have revealed that the contribution of methanogens performing the aceticlastic or hydrogenotrophic pathways depends on the substrate and the process conditions. *Methanomicrobiales*, represented by *M. marisnigri*, often predominate in methanogenic communities in biogas plants, indicating that methane is produced through the hydrogenotrophic pathway. This finding does not support the thesis that methane is produced primarily from acetate through the acetoclastic pathway [68]. We found that the hydrogenotrophic pathway of methane production was predominant in the bioreactor processing an acidic effluent from molasses fermentation to methane, and the order *Methanomicrobiales* dominated the archaeal community, constituting about 59%. The most abundant genus within this order was *Methanoculleus* represented by *M. marsigni* and *M. bourgensis*, while the second and the third most abundant genera were *Methanocorpusculum* and *Methanofollis*. Other representatives of this order were members of the genus *Methanoplanus* including the species *Methanoregula formicica, Methanosphaerula palustris* and *Methanospirillum hungatei*. Among the identified hydrogenotrophic methanogens were representatives of the *Methanobacteriales* including the genera *Methanobacterium, Methanococcales* and *Methanocellales*. *Archaea* conducting the aceticlastic pathway of methane production included the *Methanosarcinales* (~3.5%), represented by the genera *Methanosaeta* and *Methanosarcina*. Metagenomic analysis revealed a relatively large contribution of sequences assigned to the genus *Methanomassiliicoccus*, including *Methanomassiliicoccus luminyensis, Candidatus* Methanomassiliicoccus intestinalis and *Candidatus* Methanomethylophilus alvus.

It should be noted that the acetoclastic pathway provides only a small amount of energy available for growth:

$$CH_3COO^- + H^+ \rightarrow CO_2 + CH_4 \left(\Delta G^{0\prime} = -36\ \text{kJ / mol}\right)$$

In comparison, the hydrogenotrophic pathway produces fourfold more energy:

$$4H_2 + CO_2 \rightarrow CH_4 + H_2O \left(\Delta G^{0\prime} = -131\ \text{kJ / mol}\right)$$

$$4HCOO^- + 4H^+ \rightarrow CH_4 + 3CO_2 + H_2O\ (\Delta G^{0\prime} = -144.5\ \text{kJ / mol})$$ [67]

Thus, the hydrogenotrophic pathway is much more energetically effective, and this may be one of the reasons for the dominance of the *Methanomicrobiales* order in the analysed communities. Moreover, as it was mentioned previously, acetate oxidizers such as *Synergistetes* successfully compete with acetoclastic methanogens belonging to the *Methanosaeta* for acetate [64].

An analysis of the substrate preferences of the recognized methanogenic *Archaea* revealed that hydrogen and carbon dioxide, methyl compounds and acetate are utilized by 74.5, 33 and 8.5% of the methanogens, respectively [69].

http://dx.doi.org/10.5772/64645

In all methanogenic microbial communities examined by high-throughput DNA sequencing, the contribution of unidentified sequences is usually high. As phylogenetic analyses are dependent on comparison with DNA sequences present in databases and the majority of the recognized genera of methanogens produce methane through the hydrogenotrophic pathway, it is possible that acetoclastic methanogens are hidden among the unidentified sequences. Therefore, the apparent dominance of hydrogenotrophic methanogens such as *Methanomicrobiales* may only be due to our limited knowledge of methanogenic *Archaea*.

Recently, Dziewit and co-workers [18] described four novel molecular markers—other than 16S rRNA and *mcrA*—for the metagenomic analysis of methanogenic communities, with a particular focus on methylotrophic methanogens. These are the *mcrB, mcrG, mtaB* and *mtbA* genes encoding beta and gamma subunits of the methyl-CoM reductase, methanol-5-hydroxybenzimidazolylcobamide Co-methyltransferase and methylated [methylamine-specific corrinoid protein]:coenzyme M methyltransferase, respectively.

It is commonly recognized that methanogenic granular sludge is rich in minerals, mainly ferric sulphide and Ca-, Mg-, Na-, K- or Al-containing compounds. They constitute between 10 and 90% of the dry mass, depending on the composition of the wastes and nature of the methanogenic process [70]. The inorganic components of the extracellular matrix of methanogenic granules may inhibit some metabolic pathways and thus determine the processes leading to methane production by the microbial community. Both Al and K are undesirable elements in the methanogenic sludge due to their competition with other essential metals, inhibiting microbial growth and consequently their adverse effect on the methanogenic process. In contrast, Ca and Mg have a positive effect due to their promotion of the granulation process. Sodium plays a role in the formation of ATP and oxidation of NADH and then is essential for the growth of methanogens. However, high concentrations of Ca^{2+}, Mg^{2+} and Na^{+} ions cause inhibitory effects on methanogen activity. The optimum concentration of Ca^{2+} and Na^{+} ions for methane synthesis from acetate was found to be 200 and 230 mg/L, respectively, whereas a concentration of 8000 mg/L of either ion inhibited the process [71]. Interestingly, the combination of various elements can mitigate the toxicity of others, e.g., magnesium, sodium and ammonium counteract potassium toxicity. It is noteworthy that the acetoclastic pathway of methanogenesis and the oxidation of propionate are particularly sensitive to raised levels of certain minerals [71]. Moreover, it has been observed that inhibition of the acetotrophic pathway of methane formation is usually accompanied by inhibition of propionate oxidation [61].

6. Hydrogen and methane production in a two-stage anaerobic digestion

There is currently great interest in the development of new technologies for the production of energy from renewable sources, of which fermentation processes generating methane and hydrogen show great promise. Hydrogen-yielding fermentation is considered to be one of the most attractive alternative biological methods of hydrogen production. However, there are two major drawbacks: low productivity of the process and the formation of large amounts of

environmentally unfriendly non-gaseous fermentation products [29, 72]. The theoretical maximum hydrogen yield during *Clostridium*-type fermentation is four moles of hydrogen per mole of glucose, when all of the substrates are converted to acetic acid according to the following equation:

$$C_6H_{12}O_6 + 2\,H_2O \rightarrow 4\,H_2 + 2\,CO_2 + 2\,CH_3COOH$$

This gives the highest possible yield of hydrogen during dark fermentation. The complete oxidation of glucose provides 12 moles of hydrogen per mole of glucose:

$$C_6H_{12}O_6 + 6\,H_2O \rightarrow 12\,H_2 + 6\,CO_2$$

Theoretically, only one-third of the biomass can be converted to hydrogen by the process of hydrogen-yielding fermentation. In practice, this value is lower due to the formation of non-gaseous products such as organic acids and alcohols. For example, when the glucose is converted to butyrate, the hydrogen yield drops to two moles. It is estimated that the efficiency of hydrogen production must reach 60–80% to be economically attractive [73, 74]. This level of efficiency may be attained by using two-stage systems to achieve the transformation of substrates into hydrogen and methane. In such systems, the hydrogenic (hydrolysis and acidogenesis) and methanogenic (acetogenesis and methanogenesis) steps are performed separately under controlled conditions to favour biohydrogen and biomethane production, respectively. In the first stage, hydrogen-rich fermentation gas is produced, while in the second, the non-gaseous products of hydrogen fermentation act as substrates for methanogenic consortia. These two processes are carried out in separate bioreactors that differ in design and have different pH conditions and hydraulic retention times.

A growing number of reports describe the use of two-stage systems for hydrogen and methane production. Such systems have shown promise at the laboratory and pilot scales using various substrates including organic wastes, plant biomass, by-products of the food industry and pure hydrocarbons [66, 75–90]. Increases in energy recovery of up to 20–30% have been achieved using these systems compared to one-stage biogas-producing bioreactors [76, 78, 85, 90]. Effective biomethane production from non-gaseous fermentation products could make biological production of hydrogen through fermentation economically attractive. It has been estimated that by 2040, biohydrogen may be produced on an industrial scale [91].

The idea of two-phase anaerobic digestion as a method for the effective degradation of biomass to methane and carbon dioxide is not new [92]. The novel aspect is the co-production of hydrogen and methane. Many studies on the production of both hydrogen and methane by the anaerobic digestion of biomass have focused on the performance and efficiency of the entire process, but they have lacked any in-depth analysis of the microbial communities in the bioreactors where the two steps are performed. Recognition of the structure and diversity of the microbial communities capable of syntrophic cooperation in the transformation of

http://dx.doi.org/10.5772/64645

substrate to the desired gaseous products should facilitate the optimization of hydrogen and methane co-production from organic matter in two-stage systems.

Research on two-stage anaerobic digestion has been conducted in our laboratory for several years. We have developed and described a laboratory-scale two-stage anaerobic digestion system that produces hydrogen (in Stage 1) and methane (in Stage 2) from sucrose-rich by products of the sugar beet refining industry as the primary energy substrate under mesophilic conditions [15, 43]. Initially, hydrogen is generated through processes of acidogenesis in a three-litre packed bed reactor (PBR) by a hydrogen-yielding microbial community fermenting molasses. Subsequently, non-gaseous organic products from this first stage feed a 3.5-litre UASB reactor in which methane (biogas) is produced by a methane-yielding microbial community. A detailed molecular characterization of this two-stage anaerobic digestion system producing hydrogen and methane from sugar beet molasses was achieved using optimized DNA extraction protocols and high-throughput pyrosequencing (454 Roche) [15, 43].

Recently, the two-stage system for hydrogen and methane production described above has been successfully scaled up 10 times and is currently being trialled in a Polish sugar factory.

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