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Meet the editors

Thalita Peixoto Basso received her Bachelor's degree in Agriculture Engineering (2008) from Londrina State University (PR-Brazil). During this period she studied fermentation characteristics of *Saccharomyces cerevisiae* isolated from the ethanol industrial process.
She obtained her Master's degree in Science from the Agri-food Industry, Food and Nutrition Department at the University of Sao Paulo (ESALQ/USP, SP-Brazil) in 2010. During this time she isolated and selected fungi with enzymatic activity able to hydrolyze sugarcane bagasse. She received her PhD in Science from the Soil Science Department (Agricultural Microbiology Program) at ESALQ/USP (2015), with a period of one year as visiting scholar at the University of California Berkeley and Energy Bioscience Institute. Meanwhile, she worked on improvement of *S. cerevisiae* by hybridization for increased tolerance towards inhibitors from second-generation ethanol substrates. Currently, she is postdoctoral working with metabolomics and proteomics of fermentation processes in the Genetics Department at ESALQ/USP.

Luiz Carlos Basso holds a Bachelor's degree in Agriculture Engineering (1969) from the University of Sao Paulo (ESALQ/USP), a Master's degree (1973) in Soil and Plant Nutrition (ESALQ/USP), a PhD in Biological Science from UNESP, and a Postdoctoral degree from the Institut des Produits de la Vigne, Montpellier (1989), and from the Superior Technical Institute, Lisbon. Since 1980 he has been involved with yeast biochemistry and physiology, aimed at increasing the ethanol yield using the fed-batch industrial process. During the last 15 years he has been conducting a yeast selection program resulting in the most widely used *Saccharomyces cerevisiae* strains (PE-2 and CAT-1) in the Brazilian ethanol industry. Currently, he is a senior professor at the University of Sao Paulo (ESALQ/USP), teaching biochemistry (for undergraduates), biochemistry and physiology of yeast fermentation (for graduates), and selecting tolerant strains for lignocellulosic inhibitors.

Contents

Preface

The world of bioethanol production reached 100 billion liters in 2017, with the United States (from corn) and Brazil (from sugarcane) being the main producers. Both countries produced 85% of total ethanol, and Brazil contributed with approximately 30% of world production. Brazil is the greatest sugarcane producer and the resulting ethanol represents 50% of total fuel used for transportation by light vehicles. Sugarcane is also the main crop for ethanol production in developing countries.

Sugarcane feedstock contributes to the highest energy balance of the industrial first-generation bioethanol process and additional gains could be achieved when lignocellulosic substrate from this crop is used for second-generation ethanol production.

This book offers a broad understanding of bioethanol production from sugarcane, although a few other substrates, except corn, will also be mentioned. The 10 chapters are grouped in five sections. The Fuel Ethanol Production from Sugarcane in Brazil section consists of two chapters dealing with the first-generation ethanol Brazilian industrial process. In these chapters the reader is guided on the implantation of the first world national effort to change the energy matrix aimed at dependency on fossil fuel. Many important lessons were learned regarding politics and economic and technological difficulties related to this successful implantation. Undoubtedly, this experience would be useful for other countries that wish to use biomass for energy conversion. In this section the industrial process for ethanol production is also depicted in detail related to the physiological and technological traits of fermentation, stressing conditions imposed on fermenting yeast, and the limiting factor of ethanol productivity. Special attention is paid to bacterial contaminants, the major challenge of industrial ethanol production.

The Strategies for Sugarcane Bagasse Pretreatment section deals with emerging physicochemical methods for biomass pretreatment, and the non-conventional biomass source for lignocellulosic ethanol production addresses the potential of weed biomass as alternative feedstock.

In the Recent Approaches for Increasing Fermentation Efficiency of Lignocellulosic Ethanol section, potential and research progress using thermophile bacteria and yeasts is presented, taking advantage of microorganisms involved in consolidating or simultaneous hydrolysis and fermentation processes. Finally, the Recent Advances in Ethanol Fermentation section presents the use of cold plasma and hydrostatic pressure to increase ethanol production efficiency. Also in this section the use of metabolic-engineered autotrophic cyanobacteria to produce ethanol from carbon dioxide is mentioned.

The first-generation industrial process is based on the cell recycling procedure (using both fed-batch and continuous versions), attaining high ethanol titers and productivities. As far as second-generation ethanol is concerned, three industrial Brazilian initiatives are being used for cellulosic ethanol production, and technical, mechanical, and microbiological problems are been tackled, allowing a valuable opportunity to learn about this forthcoming new industrial bioprocess.

This book offers a broad understanding of bioethanol production from sugarcane. It covers a multitude of issues such as feedstock suitability for ethanol production, industrial fermentation processes, limiting factors (temperature, acidity, yeast inhibitors, osmotic stress, yeast tolerance and efficiency, yeast selection, and bacterial contamination). Other aspects related to second-generation ethanol production are also described, such as pretreatment, enzymatic cellulose hydrolysis, selection of microorganisms, and pentose fermentation from sugarcane bagasse and other biomasses.

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Fuel Ethanol Production from Sugarcane in Brazil

Chapter 1

Assessment of Sugarcane-Based Ethanol Production

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Additional information is available at the end of the chapter

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Abstract

This chapter aims to explain how bio-ethanol has been drawn to become a successful alternative to partially replace petroleum as a source of liquid fuels in Brazil. A brief historical analysis about the production of bio-ethanol from sugarcane is presented. The motivation to start the production of the ethanol as biofuel in the 1970s and how the governmental policies have contributed to the ups and downs, successes, and failures of the sugarcane industry is shown. Then, the efficiency of the sector is addressed; firstly, the increasing efficiency of the agricultural sector is discussed, showing how the productivity per hectare has increased in the last decades and which improvements are further expected in a near future. Finally, the industrial process is discussed: the current efficiency in processing sugarcane to produce ethanol and the emerging technologies, not only to process sugarcane juice, but also to harness bagasse, vinasse, and sugarcane straw.

Keywords: Brazilian ethanol fuel, *Proálcool*, ethanol production, sugarcane ethanol, bio-ethanol

1. Introduction

The beginning of sugarcane cultivation in Brazil is related to the Portuguese occupation during the colonial period. Sugarcane crop met ideal soil and climate conditions, and it was used by the Portuguese to establish their settlement in Brazil. With the production of sugar, alcoholic beverages were produced by alcoholic fermentation of sucrose. The first studies on ethanol, as a fuel for internal combustion vehicles, started in the 1920s [1]. The characteristics of ethanol (liquid

fuel, high-energy density, and relatively safe handling) made it an important substitute for liquid fuel from petroleum in the Brazilian energetic matrix. In fact, the world overwhelming dominance of gasoline, diesel, and jet fuel for transportation clearly shows the preference for liquid fuels due to their high-energy density. Except for the ethanol, most of the liquid fuels in the world are petroleum based. As petroleum is not renewable, in the long term, it must be substituted by other kind of energy. Aside from that, the use of fossil energy results in the releasing of greenhouse gas emission, which contributes for global warming. Hence, society in general is looking for alternatives to avoid global warming and thus replace petroleum. Biomass, like the sugarcane, clearly represents a sustainable and low-cost resource that can be converted into liquid fuels on a large scale to have a meaningful impact on petroleum use.

2. Why has bio-ethanol become a successful alternative to partially replace petroleum fuels? A short history

The beginning of the development of the ethanol fuel in Brazil is related to the petroleum shortage in Brazilian territory and the worldwide oil crisis. Brazil had few oil wells in 1970s, and the country was extremely vulnerable to international oil crisis. In 1973, during the first oil crisis, prices increased by 400%, which greatly affected the Brazilian economy in this way, and the Brazilian government began to seek an alternative to reduce its international dependence on oil. At that time, anhydrous ethanol, produced from sugarcane, had already been mixed to gasoline at a ratio of 5%, since 1931. In 1975, government created the Brazilian ethanol program, *Proálcool*, which involved many economical sectors to develop bio-ethanol as fuel to replace gasoline [2]. This program had massive governmental funds to develop feedstock and industry. In 1979, during the second oil crisis, Brazil presented the first ethanol fuel-powered car. At that time, Brazil had active state intervention over the price and the production of ethanol [3], which dictated the amount of sugar and ethanol to be produced. The ethanol price paid to the producers was a function of the sugar price. The price of ethanol and gasoline was established by the government at the fuel station. Therefore, the lower price of ethanol compared to gasoline led the population to choose ethanol-powered car instead of gasoline-powered one. As shown in **Figure 1**, the sales of ethanol cars skyrocketed, and in 1984, about 76% of the sales of cars using Otto cycle engines were ethanol fuel-based. At that time, most fuel stations in Brazil could offer ethanol as fuel.

At this point, it is worth defining "ethanol fuel" compared to anhydrous ethanol. "Ethanol fuel" is also known as "hydrous ethanol," and it is basically composed of ethanol (92.5–94.6%wt) and water. Ethanol fuel is used straightly into car engines without any blend. Anhydrous ethanol consists of at most 0.7% water by weight, and it has been used mixed with gasoline in different blend levels. **Figure 2** shows the fraction of anhydrous ethanol mixed with gasoline over the years. Anhydrous ethanol is also used as anti-knock agent, substituting the additive added to gasoline to avoid getting ignited early before spark occurs. Many countries still use methyl tert-butyl ether (MTBE) as a gasoline additive instead of ethanol, despite the environmental and health concerns. In the United States, MTBE has been replaced by corn ethanol since 2005 [4]. In Europe, part of the MTBE has been substituted by ethyl-tertiary-butyl-ether (ETBE) which is an additive obtained by the reaction of isobutene with ethanol [5]. In Europe, the amount of ETBE used instead of MTBE is dependent on the price of ethanol.

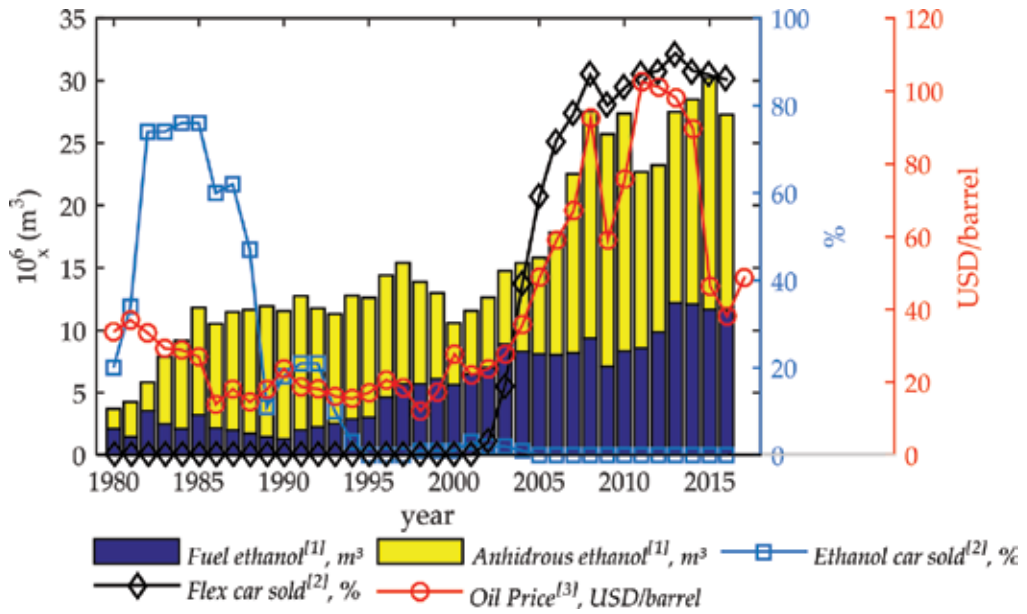

Figure 1. Fuel and anhydrous ethanol production; ethanol-powered car and flex fuel car sold in Brazil, and the price of the oil barrel. Source: [1] UNICA União da Indústria de Cana de Açúcar (2017); [2] ANFAVEA Associação Nacional dos Fabricantes de Veículos; Automotores; [3] eia U. S. Energy Information Administration.

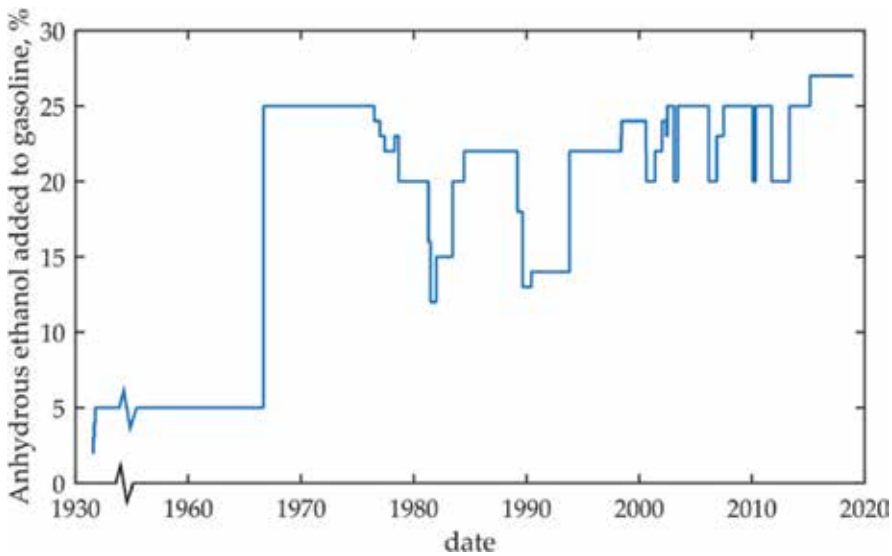

Figure 2. Fraction of anhydrous ethanol added to gasoline. Source: MME Brazilian Ministry of Mines and Energy.

In 1985, after government system changed to democracy, the congress changed the rules of public policy concerning ethanol to include stakeholders on the government decision. As a result, the government moved away from the sector and the bio-ethanol fuel development faced more challenges to overcome. Firstly, the oil price decreased and ethanol became

economically noncompetitive compared to gasoline. Oil should cost more than U$ 45.00/barrel in order to let sugarcane ethanol to be competitive [6]. Then, in 1990, the government suspended the quote requirement on the mill to produce ethanol [7, 8]. In 1996, the price control on the fuel sector ended [9]. In 1999, government completely deregulated the sugarcane sector [10]. As a result, the ethanol consumption stopped rising, and the ethanol fuel sector suffered without government regulations and incentives.

Besides the end of the many subsidies, ethanol car technology had to deal with lack of consumer confidence, and so the sales of ethanol fuel-based car decreased. In 1984, ethanol car was still under development and, at that time, many problems were still unsolved such as the engine cold start. In 1989, due to the sugar price raising and the low oil price, sugarcane mills started to produce more sugar than ethanol. This resulted in a shortage of ethanol fuel, which led ethanol car users to stop using it. Besides, the ethanol engine, due to technical reasons, could not be easily converted to gasoline engine. For this reason, as shown in **Figure 1**, the ethanol car sales dropped from 76% to about 11% in Brazil, and 6 years later, no car manufacturer had ethanol fuel cars in its production lines. From 1995 to 2003, the ethanol demand was basically to supply fuel to the ethanol cars which had been sold before.

By 2003, due to the rising of oil price, ethanol fuel regained its competitiveness. At this time, as a consequence of the ethanol production/demand occurred after 1985, automakers started manufacturing cars using flex fuel technology and, as a result, the demand for ethanol as fuel rose again. Due to the flex technology, the customers can decide whether to fuel their cars with ethanol or gasoline. So, there were no more customers concerns about purchasing ethanol-powered cars. Hence, it became a self-regulating market; for instance, during the sugarcane crop season, the ethanol fuel price decreases, which motivates the preferential use of ethanol instead of gasoline. By analogy, when the stock of ethanol fuel is low, the price of ethanol would rise and it could be preferable for customers to use gasoline instead. This also corrected the problems related to the possibility of ethanol shortage due to climate changes that would affect the sugarcane crop and the amount of sugarcane diverted to produce sugar instead of ethanol. Consequently, the flex technology seems to have solved most of the problems related to the use of ethanol as fuel.

Flex fuel technology consists in adjusting the engine to operate using both kinds of fuel, ethanol or gasoline, and their blend in any concentration. In an Otto cycle engine, each fuel has different operation characteristics such as air/fuel ratio, compression ratio, and ignition timing [11]. The air/fuel ratio issue has been solved by measuring the oxygen content of the exhaust gas by the lambda sensor, which supplies the necessary information for optimal air/fuel mixture to the engine control unit. Electronic ignition timing controller adjusts the ignition time for maximum torque and fuel conversion efficiency [12]. However, the compression ratio, which is the ratio of the volume of the combustion chamber from its largest to its smallest capacity, cannot be easily changed in an engine. Ethanol engine requires a higher compression ratio than the gasoline one; thereby, the commercial flex fuel car has an intermediate compression ratio, which is intermediate to the ideal one for both fuels. Automakers have worked in variable compression ratio engines, which would result in an increment of engine efficiency [13].

Nowadays, most of the cars sold in Brazil are flex fuel, and ethanol is easily found in every fuel station; thus, the customers are able to choose which fuel they want to use. It is worth noting that most Brazilians customers do not choose to use ethanol because it is environmentally friendly, but due to economic reasons. A survey carried out by the Brazilian Sugarcane Industry Association (UNICA) [14] shows that Brazilian consumers in general are not willing to pay more for ethanol than for gasoline even though the majority recognizes its environmental benefits. Even when ethanol has the same cost per driving kilometers (about 70% of the price of gasoline), 55% of the consumers choose to fuel the car with gasoline due to its higher autonomy. This behavior may be explained by "The Tragedy of the Commons" [15] in which the rational man finds that his share of the cost of the CO_2 he discharges into the commons is less than the cost of not releasing it individually. Consequently, the majority of the consumers choose the fuel taking into account only their own benefits. This means that ethanol can survive as an alternative fuel only if it is economically competitive when compared to gasoline or by law regulations.

Environmental and social concerns also have a beneficial impact on the fuel ethanol program: pressures from nongovernmental organization (NGO) and the United Nations (UN) about reduction of greenhouse gas emissions (GHGs), and some civil society organizations about the social impact of ethanol supply chain on the society. One action taken to support the claim for reducing GHG was the creation of a tax on the nonrenewable fuel [16], which aims to support environmental programs and natural disasters caused by GHG. This is based on the "beneficiary pays principle," whereby when purchasing fossil fuel, the beneficiaries should pay the bear costs on the environment, which are believed to contribute to climate change. This seems suitable; however, it is very difficult to precisely evaluate the impact on the environment. Moreover, in 2018, the Brazilian government created the *RenovaBio* [17]—a national biofuel policy to set rules to allow sustainable expansion of the Brazilian biofuel market. In fact, nowadays, ethanol supply chain is responsible for 950,000 direct jobs and 70,000 farmers [18] in the country. For each direct job, 2.39 indirect ones [19] are estimated, resulting in over 2.4 million jobs. For this reason, the ethanol fuel environmental and social benefits cannot be left at the mercy of the variations in petroleum price.

Besides its use as fuel, ethanol is used as a raw material to produce biopolyethylene. Polyethylene is one of the most popular plastics in the world. It is a polymer of ethylene and consists of a carbon backbone chain with pendant hydrogen atoms. Biopolyethylene is a polyethylene made from ethanol. The process consists in dehydrating ethanol to obtain ethylene prior to polymerization. The properties of this bioplastic are identical to the fossil-fuel based polymer. The main advantage of the polyethylene made from ethanol is that it captures and fixes CO_2 from the atmosphere.

Through this brief ethanol history, it is possible to infer that biofuel ethanol has undergone two different expansion phases: the first one is the *Proálcool* policy and the second one is the flex fuel car (concerning ethanol as liquid fuel). In these two expansion phases, the main claim was not the environmental one but an alternative fuel to the high price of petroleum. However, due to current global warming concerns, the world is looking for a renewable fuel

to replace petroleum and reduce emissions. A number of alternatives are under development, and the question that arises is if the bio-ethanol is going to be "the fuel." In case of a positive answer, one may expect a new expansion phase in the ethanol production sector. Further, in this new expansion phase, not only an alternative fuel is expected, but also a fully environmentally friendly one. Thus, the process might be highly efficient in all steps of the production chain, from the crop to its final use. Therefore, the efficiency of the production of ethanol and some opportunities to improve the efficiency will be addressed subsequently in this chapter.

3. Efficiency of the sugarcane ethanol production and what is expected in a near future

Since the beginning of the ethanol fuel program in Brazil, an improvement in all production chain has been observed. With the emerging technologies, new improvements are expected. Hence, in this section, the recent enhancements in productivity and efficiency of the sector and what is expected in a near future are analyzed.

3.1. Sugarcane crop

During the last decade, the principal change in crop management was the mechanization. One of the main reason for mechanizing the sugarcane crop is concerned environment protection. The traditional harvest was done manually and the sugarcane leaves had to be burned in the field. The consequence was high particle and CO_2 emissions, which led the Brazilian legislation to prohibit the burning of sugarcane leaves in the field [20]. This provided an opportunity to the sector to use this leaves (straw) as an additional feedstock to the ethanol process, producing electricity or second-generation ethanol. Yet this has also increased the amount of pesticides needed to control sugarcane bugs and diseases [21, 22] that are kept in the field for the next crop. It is important to mention that sugarcane is a semi-perennial crop, which means that the same plant may be harvested (without uprooting the plant) and re-grown for up to 5 years. In addition, mechanical harvest and crop have reduced the production cost; however, the amount of dirt brought with the cane to the refinery has increased, thus affecting the industrial process [23].

The productivity in Brazil is uneven concerning the region of the country, for example, in the 2016/2017 crop, the average productivity in the south, southeast, and central regions was 75.3 tonne/ha while in the north and northeast it was 48.6 tonne/ha [24]. In addition, some regions in Brazil, such as the southeast, can reach an avarange production yield higher than 100 tonne/ha [25]. Nowadays, in a good climate condition scenario, a national average productivity of at least 80 tonne/ha is expected [26]. The average sugarcane productivity and planted area have increased since 1980. There was a rapid acceleration in productivity growth in the 1980s, which is mainly due to the investments from the *Proálcool* program. **Figure 3** shows the productivity and planted area from 2005 to 2015 [24]. Nyko [26] studied the recent drop in the sugarcane productivity (2010/11 harvest) and concluded that mechanization of sugarcane planting and harvesting were the main cause, besides climate change and the lower

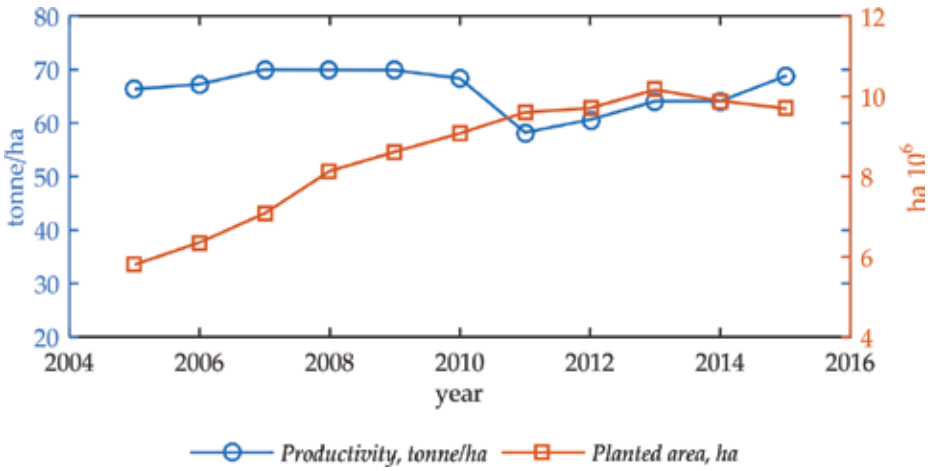

Figure 3. Productivity of sugarcane and planted area. Source: UNICA União da Indústria de Cana de Açúcar.

investment in the agricultural field due to the lower price of sugar and alcohol in this crop season. In fact, mechanization is a relatively new technology for some industries in Brazil, and they might have to adapt to the mechanical crop management and a learning curve is required. In addition, some researches in genetic-modified sugarcane have been carried out to increase the yield, and pest and disease resistance [27]. Consequently, the average productivity is expected to increase in the near future.

3.2. Sugarcane transportation

Transportation plays a crucial role in the cost of sugarcane production, owing to the multiple transport facilities and time-consuming activities involved in the delivery process. For instance, the total average cost of sugarcane production in São Paulo, in 2016/2017, was R$ 49.56 (U$ 14.57) per tonne of sugarcane [28]. In order to evaluate how much the delivery represents on the total cost, Françoso et al. [29] studied two cities in the same state and in the same crop season, and the cost of cutting, loading, and transportation of sugarcane from the farm to the mill gate 25-km away varied from R$ 26.77 (U$ 7.87) to R$ 37.25 (U$ 10.96) which represents 54–75% of the total production cost, respectively. The great variation in the transportation costs of sugarcane is due to the region topography, quality of roads, and technology employed in the transportation. So, the role of sugarcane transportation on the cost of bio-ethanol cannot be overlooked.

The most economical way of transporting sugarcane from field to the industry is the two semi-trailers attached to a tractor unit. The distance from the farm to the sugar mill is about 25 km. Different ways to transport sugar have been tested, from railroad, rivers, and road [30]. Until 2017, the largest truck licensed was nine axles with the total length of 30 m and a load of 74 tonne, which was the most economical way of bringing sugarcane from the field to the industry [31]. This kind of transport allows drivers to disconnect the tractor from the full trailer on arrival in the mill and then connect to an empty trailer and get back to the field without

waiting to unload. As from 2017, the department responsible for monitoring the road traffic has authorized 11 axles, two semi-trailers attached to a tractor unit with the same length, and a total load of 91 tonne [32]. To the best of our knowledge, there are no studies on the viability of this transport mode; however, this might be the most efficient transport mode since this is a claim made by sugarcane producers.

3.3. Cane reception preparation and extraction

The farmers are rewarded according to the quality of the sugarcane supplied to the industry. When the sugarcane arrives at the mill by a truck, it is weighed, and then the load is drilled in order to collect a sample. The quality of the sugarcane undergoes standardized analysis of the sample. The responsible for the standard is *Consecana* and *ABNT NBR 16271*. The payment is made in accordance with a coefficient called "total recoverable sugar," which is proportional to the sugarcane sucrose content. With the recent use of bagasse to produce electricity and the possibility to produce second-generation ethanol, the possibility of rewarding the producer for the amount of fiber in the sugarcane is under discussion by the agricultural and by the industrial sector [33]. Besides, new varieties, aiming to produce more fiber than sugar, have been developed by BioVertix®. Consequently, the sugarcane payment is expected to soon take into account the sugarcane fiber in addition to the amount of sugar.

An appropriate sampling method is fundamental to correctly evaluate the shipment. The collection of samples is usually done by drilling the shipment with mechanical oblique probe samplers. This kind of sampler was introduced in 2007 and has undoubted advantages over the method formerly used because it allows the sample to be taken from the top to the bottom of the truck load. Before the oblique probe, sugarcane was sampled using a horizontal probe or randomly samples were taken at three different points of the shipments.

Mechanization has increased the level of dirt brought with the sugarcane to the industrial process, so a cleaning process has become necessary. When sugarcane was harvested manually, it was possible to wash it before its being forward to an industrial-cutting shredder and milling process. However, because of mechanization, sugarcane arrived at the industry in small stalks since the harvester already cut the sugarcane. Sugarcane in small pieces cannot be washed due to the fact that a lot of sugar would be lost by the stalk-cutting edge. For this reason, a dry-cleaning technology has been adopted by many industries to avoid dirt from entering the industrial process. Increasing 1 kg of dirt per 100 kg of sugarcane is expected to a decrease in the sugar recovery at the industrial process by 0.1% [23]. The loss of sugar occurs with bagasse and filter cake during the sugar juice treatment step. However, the dry-cleaning system consumes about 0.5–1.0 kW per tonne of cane. Because electricity and sugar are products sold by the refinery, there is a feasible balance between profit and loss, that is, the cost of the electricity used to clean the sugarcane should be lower than the cost of the sugar lost due to the dirt. In fact, as shown by some suppliers [34], the dry cleaning system would be feasible when the sugarcane leaves (straw) are intentionally brought with the sugarcane to be burnt in the boiler. In this case, straw is easily collected with cane by lowering the speed of the harvester clean blower. Thus, the dry-cleaning process would separate straw from stalks before the extraction process and then would mix the straw and bagasse after milling. In fact, this is not a consolidated technology, since some industries prefer to harvest the straw on the field and bring it separately. Thus, the implementation of the sugarcane dry cleaner method will depend on the manner of straw handling.

The next process applied to the sugarcane is the extraction, which is done by the mill or diffuser. The aim is to separate fiber, a solid stream, from sugar in a liquid stream. In this process, sugarcane is first reduced into small pieces and the sugar-bearing cells are ruptured to facilitate the subsequent extraction process. This is basically a mechanical process whereby size reduction is generally achieved through the use of rotating knives and swing hammer shredder in the cane-conveying system. In the case of billeted cane, mechanically harvested, it can be fed directly into a shredder without any additional cutting. For cane juice extraction, there are many studies comparing milling and diffuser [30, 35, 36]. The main advantage of a diffuser over mill is the greater extraction of sugar; however, a diffuser uses more imbibition water and steam than a mill. As a result, there is a dilemma to the industrials: to increase sugar extraction, more thermal energy is spent. In Brazil, the preference has been for the use of mills, which consists of a set of four to six mill units. A new extraction technology called "Hydrodynamic Extraction" or "Rivière Juice Extractor" is under development and aims to achieve the same level of diffuser extraction using less imbibition water with a lower cost of installation and maintenance when compared to both technologies [37]; however, to the best of our knowledge, there is no commercial plant using this technology.

Sugarcane milling did not change much during the last two decades, except for the driving system. Two driving systems are the mostly used in Brazil: steam turbine and electric motor. Even when an electric motor is used, the electricity is produced using a steam turbo-generator, that is, in both cases, the primary energy to drive the extraction process is the bagasse, which is burned in a boiler to produce steam. The driving system with steam turbine is the most widely used, mainly in old industries. This system consists of a low-efficiency steam turbine working around 22 bar and 350°C admission, and 2.5 bar exhaust, so, in this system, steam energy is converted into a mechanical energy to the mill. The electric drive system consist of an electric motor attached to each roll of the mill unit. Even with the double transformation of energy, the overall efficiency of the electric drive is higher than the work done by a single-stage turbine. These become an issue for the sugar mill, since the surplus electricity becomes a profitable product for the mills. As from 2002 when the government has regulated the commercialization of electricity by the private sector using biomass [38], many sugar mills have invested in higher-pressure boilers (65–100 bar) and high-efficiency cogeneration systems.

3.4. Juice treatment

After being milled, the juice contains several impurities, which must be removed prior to fermentation or concentration. These impurities are removed using a set of unit operations, which basically consist of heating, adjusting the pH, settling the precipitate formed in the body juice, and filtering.

Only small changes on these processes were adopted during the last decades. In the heating, most industries prefer vertical shell-and-tube steam heaters. Despite the higher overall heat transfer coefficient in the horizontal one, the vertical one is easier to clean. The extracted sugarcane juice has a pH of about 5.3, and needs to be adjusted to 7 before clarification. For this, lime Ca(OH) is added, which is the most widespread process used. For refineries that produce sugar in addition to ethanol, processes such as sulfitation, phosphatation, and carbonation [36] are also used, aiming to lower the color and turbidity. After the pH adjustment, the juice is sent to a clarifier to settle the insoluble part of the juice. Before the use of chemical products

(flocculants and polymer) and instruments to control the flow and dosing, the most popular installation used multi-tray clarifiers. The main disadvantage of multi-tray is the retention time of about 3 h. Single-tray clarifiers, known as "rapid clarifiers," became possible with the development of chemicals that promotes the mud coagulation and settling. The retention time in this case is about 1 h. The main advantages of the rapid clarifiers over the multi-tray ones are the lower installation costs, and the small retention time, which reduces the degradation of sugars [30]. There are not many research works carried out recently about juice treatment, and consequently, great changes in this process are not expected in a near future.

3.5. Juice concentration

Ethanol can be produced in an autonomous distillery, producing only ethanol, or in an attached distillery, which produces sugar and ethanol. In the second case, ethanol can be produced only from molasses, a residue from the sugar production process, or a mixture of molasses and juice, depending on the amount of juice diverted to produce ethanol. When sugar is produced, the juice destined to the sugar process must be concentrated to achieve a suitable brix to start the crystallization process at about 60% sucrose by weight. This concentration is obtained using multiple-effect evaporators (MEVs), which reduces the required steam to concentrate the juice, since each effect produces lower-pressure steam, which is used in the next effect to evaporate more water and so on. In this way, only the first-effect evaporator uses the exhaust steam from turbines. Saving the exhaust steam is crucial for industries that have condensation turbine installed or for refineries that want to save bagasse for other purposes, such as second-generation ethanol or just selling bagasse as a product.

When sugar and ethanol are produced in a refinery, there is a synergistic effect that reduces the total consumption of steam. Since large amount of water must be taken off from the juice in order to concentrate it in the crystallization process, this water is withdrawn as steam in an MEV. This steam from MEV is used in the ethanol process. In this manner, steam from MEV replaces the necessity of using exhaust steam. Combining ethanol and sugar production, results in an energy efficient refinery.

3.6. Fermentation

Alcoholic fermentation is a biological process, which converts sugars such as glucose, fructose, and sucrose into cellular energy, producing ethanol and carbon dioxide as a side effect. The overall chemical reaction for alcoholic fermentation is as follows:

$$\underset{\text{sucrose}}{C_{12}H_{22}O_{11}} + H_2O + enzyme \rightarrow 2\,\underset{\text{fructose and glucose}}{C_6H_{12}O_6} \rightarrow 4\,\underset{\text{ethanol}}{C_2H_5OH} + 4\,CO_2 \quad (1)$$

Sucrose is a dimer of glucose and fructose molecules. In the first step of alcoholic fermentation, the enzyme invertase cleaves the glycolic linkage between the glucose and fructose molecules. When the fermentation finishes, the fermented liquor is centrifuged to remove east (*Saccharomyces cerevisiae*), which is recycled to fermentation. The product from the centrifugation (a stream with about 8º GL ethanol content) is sent to the distillation process.

There are two types of process for alcoholic fermentation commonly used to produce ethanol. The majority of the sugar mills use a fed-batch fermentation process. Continuous process is also used by some industries; however, despite the lower installation cost, continuous fermentation results in lower efficiency in the production of ethanol. The lower efficiency is a result of bacterial contamination since, in a continuous process, the fermenter cannot be as frequently cleaned as in a fed batch, in which cleaning can be carried out after each batch.

The main disadvantage of the fed-batch process is related to the large size of the fermenters. Many technologies are under development to reduce this size. There are many problems in the operation of such large equipment: high cost of installation, difficulty to control parameters, such as contamination, mixing, and temperature, which can cause temperature gradients and dead zones inside the fermenter. To reduce these problems, the total volume of broth under process must be reduced. Therefore, the proposed technologies aim to increase the concentration of reactants and products. Removing ethanol during the fermentation process is one possibility, since a high concentration of ethanol is toxic to yeast. There are some studies of pervaporation [39] and stripping [40] to take ethanol off the fermentation broth during the ethanol fermentation. The high cost of pervaporation membranes and the difficulty in recovering ethanol from CO_2 make this technology unfeasible nowadays. Reducing the temperature of the fermentation broth using a chiller is also an option under development, and there are some commercial-scale units [41]. At a lower temperature, yeast would resist a higher concentration of ethanol, but reducing the temperature would also reduce the reaction rate. By now, the best available technology continues to be the fed batch cooled by cooling towers.

3.7. Downstream processing

The recent development in downstream process did not aim to improve the ethanol recovery efficiency but to save the energy demand by the process. Downstream consists of the separation of ethanol from the other components in the fermented wine; the first step is the centrifugation of wine, which recovers yeast to the next fermentation fed batch. There are two main components at the centrifuged wine: water and ethanol. Fuel ethanol also called "hydrous ethanol" (ethanol 92.5–94.6 wt%) is obtained by distillation. Due to the azeotropic point in the mixture ethanol-water, anhydrous ethanol cannot be obtained using a common distillation process. Anhydrous ethanol (99.3 wt%) must be produced in order to be used in a mixture with gasoline. There are two common dehydration systems used in Brazil: azeotropic distillation with cyclohexane or monoethyleneglycol, and, more recently, absorption on molecular sieves. The main advantage of using molecular sieves is that steam consumption is about one-third of those in the azeotropic distillation. Pervaporation is a process that could significantly reduce the energy demand; however, the high cost of membranes makes it unfeasible to be used in a commercial scale.

3.8. Vinasse and biogas

Vinasse is the final by-product of the ethanol distillation and is the main effluent of the ethanol process. About 12 liters of vinasse are produced per liter of hydrous ethanol. Most industries

use the vinasse without any treatment as a fertilizer and, it is simultaneous used for irrigation due to its high amount of water (fertirrigation). The vinasse produced in a distillery is a stream composed basically of water, organic matter, and inorganic salts. Therefore, there are many possibilities to utilize this vinasse: as biogas obtained by conversion of the organic matter into gas, and as fertilizer through using the inorganic salts (phosphorus, potassium, and nitrogen) to partially replace synthetic fertilizers derived from the petroleum industry.

Biogas has a great potential opportunity for using the vinasse. Many studies have been carried out about the bio-digestion of vinasse [42–44]. Biogas is an easy to handle fuel since it can be transported in high-pressure cylinders, or by pipeline, and can fuel farm machinery and trucks to partially replace diesel [45]. Biogas can also be obtained from sugarcane trash (bagasse and straw) [46] in a bio-digester blended with vinasse. This is also an opportunity to the use of bagasse, that is, bagasse, as well as straw, can be converted into biogas instead of producing electricity or 2G ethanol. There are also many sugar mills close to the gas pipeline network in Brazil, which would raise the feasibility of implementing a biogas facility. Besides the high cost of installation, a great challenge to implementing biogas facilities is to deal with an unstable vinasse supply. Vinasse is not produced continuously, since industry interrupts its operation relatively frequently due to the rain, which stops the harvest, or due to the maintenance or the braking of equipment.

Another possibility is to concentrate vinasse and to recover water to be used in the process. In this case, the concentrated vinasse can be transported to longer distances to be used as liquid fertilizer. Concentrated vinasse can also be burned into the boiler; in this case, the higher the concentration, the higher its net calorific value. The main disadvantage in this process is the steam demand to concentrate vinasse.

After biodigestion, water can be withdrawn from vinasse using commercial technologies such as evaporator or ultra-filtration membrane. Reducing the use of water has a positive environmental impact, but the cost of these processes may be higher than the intake of water from natural sources (mainly rivers). So, only few refineries in Brazil are withdrawn water from vinasse to be used in the process.

3.9. Combined heat and power

What makes ethanol from sugarcane superior to that from other feedstocks (e.g., corn) is the bagasse that comes with the sugarcane. First-generation ethanol processes from sugarcane have a positive energetic balance, which means that it is not only self-sufficient on energy, but it can export the surplus energy usually as electricity. Using the combined heat and power (CHP) process is the most efficient way to produce electricity. In the CHP, high-pressure steam (between 65 and 100 bar) is expanded in turbines coupled with electric generators, and the exhaust steam from the turbines is used as thermal energy for the process. A high-efficient process, that is, a process which consumes small amount of thermal energy, results in surplus bagasse that can be used as feedstock to another process, such as second-generation ethanol, or to produce surplus steam—the steam produced by the boiler and not condensed in the process. The surplus steam can be expanded in condensation steam turbines to allow maximizing the electricity production.

The condensation turbine used to produce electricity with the steam competes with the cellulosic ethanol. The condensation turbine cannot be classified as CHP, since only power is

produced and the exhaust steam is condensed by cooling water, that is, heat is not used in the process. Despite the fact that it maximizes the production of electricity, an energetic analysis shows that the larger enthalpy jump occurs in the condenser and not in the turbo-generator expansion. Thus, the energy to condense the steam is released to the surrounding cooling tower.

Sugarcane bagasse has become a valuable product for sugarcane refineries, and it is really an important source of energy for the Brazilian economy. Before the possibility of exporting electricity to the grid [38], most sugarcane mills had low pressure and inefficient boiler and turbo-generator (about 22 bar 350°C). This allows the refinery to be self-sufficient in electricity, however, without the possibility to export to the grid. Some sugarcane mills are still running using this old technology. High-pressure and high-efficiency boiler and turbines allow the refinery to export electricity. For instance, in a scenario in which a refinery has a higher-efficiency boiler, counter-pressure and condensation turbine, and electrified mill, it is possible to export about 79.7 kW·h per tonne of sugarcane. The parameter and efficiency of this scenario are shown in **Table 1**. To verify the potential of the bagasse in Brazil, in 2016, the country produced 666.8 million tonne of sugarcane [47] and in the same year produced 35,236 GW·h of electricity from sugarcane bagasse [48]. If every sugar mill was prepared to export electricity as described in this scenario, this number could have been 53,135 GW·h. Further, considering the possibility of bringing 50% of the sugarcane straw (leaves and tips) which yields about 140 kg per tonne of sugarcane (15% humidity) [49], it would be possible to export 135,470 GW·h per year. For an idea of the order of magnitude, Itaipu, the biggest hydroelectric power plant in Brazil, in the same year, produced 103,098 GW·h.

3.10. Second-generation ethanol

The conventional ethanol production utilizes a fermentation process to convert sugars, such as starch, sucrose, glucose, and fructose, into ethanol. Second-generation biofuels, also commonly known as advanced biofuels, utilize agricultural residues or other feedstock that cannot be straightly used as food for humans. Cellulose is an important structural material for plants (along with lignin), and it is made up of many repeating sugar units. These repeating sugar units can be broken down by various processes into the component sugars, which can finely be fermented into ethanol.

Many investments on the development of ethanol from cellulosic material have been made, and some industrial-scale plants have been built; however, it has been taken longer than expected for cellulosic ethanol to succeed. In the United States, for instance, there are at least four commercial plants (DuPont Cellulosic Ethanol, Poet Project Liberty, Abengoa Bioenergy Biomass, Alliance Bio-Products INEOS) with an installed capacity of 121, 88, 110, and 35 million liters per year, respectively. In Brazil, there are two commercial plants, Granbio and Raizen, with an installed capacity of 82 and 40 million liters per year, respectively. In addition, in Italy, the first cellulosic plant, Crescentino, a Mossi & Ghisolfi group company, has an installed capacity of 31 million liters per year. Most of them started their operation in 2014, but not all has been well: in 2017, DuPont decided to close its plant and announced that it will sell the company's ethanol facility in Nevada, Iowa. Abengoa announced bankruptcy and financial restructuring in 2016 and, in the same year, the cellulosic biofuel plant was bought by Sinatra-Bio. In the end of 2017, Crescentino also applied for *concordato preventivo*

Bagasse produced per kg of sugarcane	0.276 kg
Bagasse losses due to degradation and boiler startup	5%
Net calorific value of bagasse	7300 kJ/kg
Boiler temperature	520°C
Boiler pressure	68 bar
Boiler efficiency	85%
Counter-pressure turbo-generator efficiency	83.5%
Condensation-pressure turbo-generator efficiency	78.3%
Steam consumption in the first-generation process per kg of sugarcane	0.4 kg
Electricity consumption per tonne of sugarcane	32 kW
Sugarcane straw brought with sugarcane per kg of sugarcane	0.140 kg
Net calorific value of sugarcane straw	12,900 kJ/kg

Table 1. Parameters used to obtain the electricity production from sugarcane bagasse.

in accordance to local bankruptcy Law. Granbio, in 2016, stopped producing ethanol and it is only burning bagasse to produce electricity. In January 2018, Frankens Energy LLC bought INEOS cellulosic plant in Florida, which had been closed at the end of 2016 [50]. Conversely, Poet announced in 2017, on its website press release, the achievement of the major breakthrough in cellulosic biofuels production and its intention to build an onsite enzyme manufacturing facility to directly pipe DSM enzymes into the process. Also, Ek Laboratories, Inc., a subsidiary of Alliance BioEnergy and owner of the CTS® patent whose process makes the pretreatment without using enzymes, started the operation of a demonstration plant processing 2.5 tonne/day, in 2015 [51]. In fact, by 2018, the cellulosic ethanol process has not been shown to be completely commercially feasible yet, but it has still a great potential to convert low-value feedstocks for increasing the production of biofuel.

3.11. Second-generation ethanol versus CHP

Surplus bagasse can be used to produce more electricity or second-generation ethanol. Both fuels can be used in light vehicles. For instance, take two commercial cars where car "A" being sold in the USA and uses electricity and car "B" being sold in Brazil and using a flex fuel engine (it can use ethanol or gasoline). Knowing that surplus bagasse can be converted into electricity or second-generation ethanol, it is possible to draw two hypothetical scenarios where scenario 1 consists in a refinery processing 1 tonne of sugarcane to produce ethanol in the first-generation process and electricity using a condensation turbine as described in **Table 1**, and scenario 2, in which the same 1 tonne of sugarcane is used to produce second-generation ethanol from surplus bagasse besides first-generation ethanol. The parameters and efficiency adopted for second-generation ethanol is described in **Table 2**. **Table 3** compares both scenarios side by side to obtain the distance driven in each scenario.

Looking through these results, it would be possible to infer that, considering these parameters and efficiency, it is better to produce electricity instead of ethanol since the distance driven in scenario 1 is higher than in scenario 2. However, this is not a conclusive result since to reach a reliable best scenario, studies such as live cycle analysis, return on investment, energy storage method, concentrated versus dispersed emissions, autonomy, and the preferable fuel by customers are needed.

Parameter and efficiency adopted	Second-generation ethanol plant
Steam consumed per tonne of bagasse at second-generation process.	451 kg [52]
Electricity consumed per tonne of bagasse at second-generation process.	155 kW·h [52]
Lignin per kg of bagasse (dry basis).	0.264 kg [53]
Glucan group per kg of bagasse (dry basis).	0.405 kg [53]
Xylan group per kg of bagasse (dry basis).	0.197 kg [53]
Net calorific value of lignin (35% moisture).	12671.8 kJ/kg [54]
Pretreatment efficiency.	90%
Sucrose and glucose fermentation efficiency.	90%
Xylose fermentation efficiency.	80%
Distillation efficiency.	97%

Table 2. Parameters used to obtain the consumption rating of two vehicles.

Parameter	Scenario	
	First scenario	Second scenario
Surplus electricity	79.7 kW·h	38 kW·h
Ethanol	90 L	104.3 L
City consumption ratings car "A"	6.20 km/kW·h	
Highway consumption ratings car "A"	4.90 km/kW·h	
City consumption ratings car "B"	8.34 km/L	
Highway consumption ratings car "B"	9.9 km/L	
Distance driven in a city using car "A"	494 km	237 km
Distance driven in a highway using car "A"	390 km	186 km
Distance driven in a city using car "B"	750 km	870 km
Distance driven in a highway using car "B"	891 km	1072 km
Total distance driven in a city	1245 km	1107 km
Total distance driven in a highway	1281 km	1258 km

Table 3. Parameters used to obtain the consumption rating of two vehicles.

4. Conclusion

A new era with a clean worldwide energy matrix is expected nowadays. Ethanol has been shown to be a fuel with great potential to meet this world's aspiration. In this new phase, the fuel needs to be recognized by its environmental benefits and not only by the energy that it contains. Consequently, it has to be rewarded according to the benefits it brings to the society. For this in recent years, the sugarcane industry has positioned itself not only as a food industry but also as an energy industry. Having a look into the sugarcane feedstock, bringing a different viewpoint, one could say that it produces three different kinds of energy: sugar—energy for human beings; ethanol—energy for transportation; and electricity—energy for a variety of uses. As an energy company, the process itself cannot be energetically wasteful. So, recent improvements in the process have aimed to maximize its efficiency; meaning that using less energy in the process itself results in more energy left to be sold as a product. However, many questions, such as the destination of the use of straw, bagasse, and vinasse, are still unanswered and will depend on the next technology improvement. This new era will result in increasing the demand for ethanol, which must be met not only by the increase in the production but also in the productivity and efficiency. Nevertheless, many technologies, with notorious performances, are not applied to the production of ethanol nowadays because of their low feasibility. They would become feasible, however, if ethanol was rewarded for its environmental benefits.

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Chapter 2

Clash of Kingdoms: How Do Bacterial Contaminants Thrive in and Interact with Yeasts during Ethanol Production?

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Additional information is available at the end of the chapter

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Abstract

Brazilian fuel ethanol production from sugarcane is one of the largest industrial biotechnological processes in the world. However, in view of the complex chemical nature of this feedstock, as well as the non-aseptic conditions of the process, various stress conditions are imposed to the fermenting yeast. In this chapter, we deemed to elaborate a brief overview of the ethanol production process, and to dissect the chemical nature of sugarcane-based worts, as well as their physiological effects on the fermenting yeasts. Finally, the interplay between yeast and lactic acid bacteria, the two main players in the ethanol fermentation process, is generally discussed.

Keywords: ethanol, yeast, bacteria, chemical composition, stresses

1. Introduction to ethanol fermentation in Brazil

Traditionally, ethanol production in Brazil has been coupled to sugar production. Sugarcane is initially pressed to separate the sugar-containing broth (sugarcane juice) from the fibrous solid residue (bagasse). Sucrose crystals are obtained by crystallization of the concentrated broth, and as a result a dark and viscous sucrose-rich residue (molasses) is obtained. Molasses is mixed with either water or sugarcane juice (sugarcane must) in different proportions and used for fermentation, normally in a fed-batch process with cell recycle (for an overview, please refer to [1]).

In short, fermentation is initiated by the addition of the wort (also referred to as must), containing 18–25% (w/v) sugars, to a high-density yeast cell suspension, which represents 1/3 of the vat volume. Due to its large volumes, the feeding takes 4–6 h, and the fermentation is completed within 10 h. At the end of the fermentation, ethanol titres between 8 and 12% (v/v) are obtained, with a final cell density of 10–14% (w/v). Yeasts cells are then separated from the wine by centrifugation, which goes for distillation. The yeast slurry is diluted with equal volume of water and treated with sulfuric acid to reduce bacterial contamination, and reused in a subsequent fermentation cycle (**Figure 1**). This process configuration, using high cell densities and operating with cell recycling, is quite peculiar and allows two fermentation rounds per day during the harvest season that spans for almost 250 days. The reuse of cells reduces the need for yeast propagation, therefore diverting less sugar to yeast growth and saving it to ethanol formation.

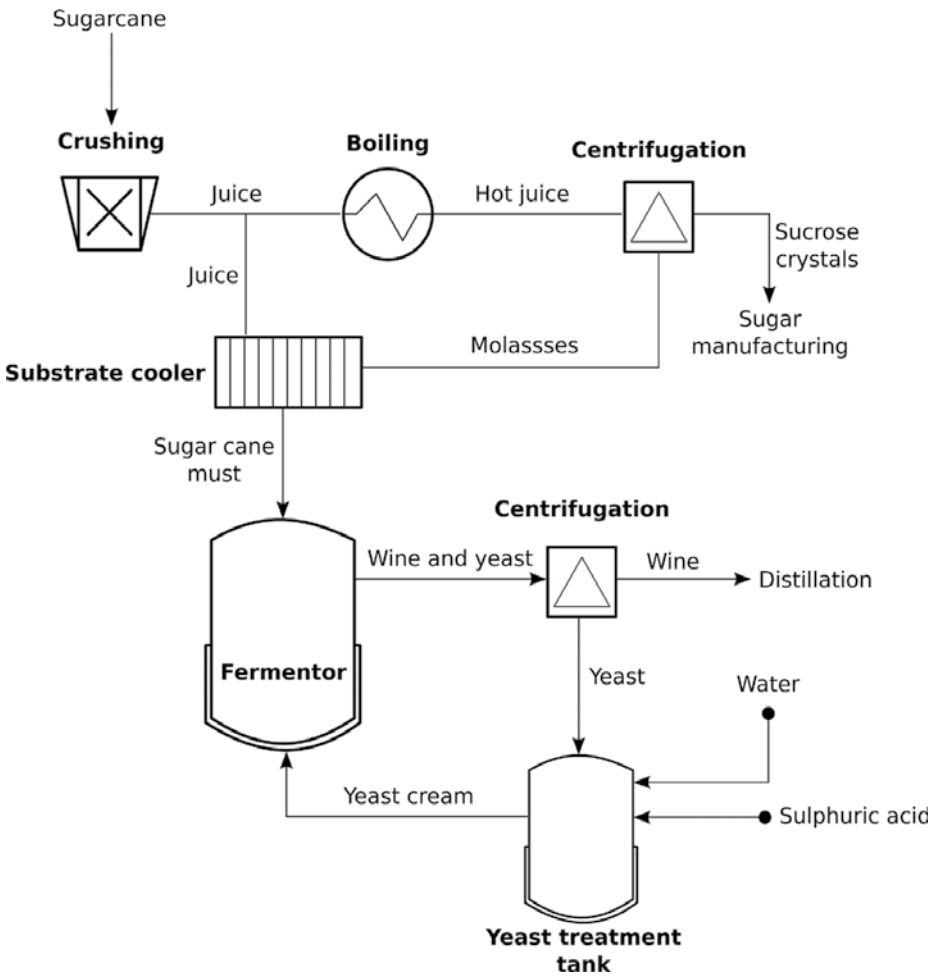

Figure 1. Simplified process flow diagram of the Brazilian ethanol production process (Courtesy of Jens C. F. Nielsen).

2. Influence of wort composition on fermentation performance

2.1. The chemical nature of sugarcane substrates and its by-products

As mentioned above, ethanol can be produced via direct fermentation of sugarcane juice, a mixture of juice and molasses, or molasses diluted in water [2, 3]. After shredding, cane is crushed in a milling tandem, constantly mixed with water, resulting in juices containing ca. 10–15% sucrose content [3]. This process results in two types of juice: the primary – which leaves the first set of miller and is richer in nutrients – and the secondary – coming from the subsequent millers. The primary juice is commonly used for sugar production, whereas the secondary juice can either be used for sugar or ethanol production [3].

Sugarcane juice is passed through clarification (reducing impurities to less than 2%), decantation, and concentration (up to 18–25% total sugars) steps before sugar or ethanol production. These steps help reducing wild yeast and bacterial contaminations during the subsequent fermentation step, allowing for higher ethanol titre and yield [2].

During sugar production, the juice is clarified with lime, and concentrated through repetitive steps of evaporation and centrifugation. The concentrated juice later passes through a crystallization step, which catalyzes the formation of sucrose crystals that are later removed via centrifugation. The remaining sugar is left in the spent, dark and viscous liquid called molasses [4]. Molasses can be further recycled back into the sugar production process, resulting in several other types of molasses (i.e. A, B, C). The higher the number of recycles molasses is subjected to, the poorer its quality as a fermentation substrate [2].

Sugarcane juice and molasses are a complex mixture of carbohydrates, proteins, inorganic salts and organic acids [5], and wort prepared with either juice, molasses mixed with juice, or molasses diluted with water will have different nutrient composition, which will ultimately impact fermentation performance [1]. A comparison between sugarcane juice and molasses composition is shown below (**Table 1**).

Asparagine, glutamine and aspartic acid are the most abundant amino acids in sugarcane-based worts, while the disaccharide sucrose is by far the major sugar. Minor amounts of the monosaccharides, glucose and fructose, and the oligosaccharide, kestose, are also found [5]. Lipids are represented by a mixture of n-alkanes and ethyl and methyl esters of fatty acids (palmitate and oleate are the predominant), as well as of phytosterols (stigmasterol, β-sitosterol, and campesterol) [6, 7].

After fermentation cells are separated from the fermented wort (wine) by centrifugation, and this stream is sent for distillation. The distillation of ethanol generates a considerable amount of a wastewater stream named vinasse (stillage) [8].

Vinasse is currently applied in soil as a fertilizer (fertirrigation), due to its high potassium titre [4, 9]. However, this high organic matter load shows deleterious impacts on soil, water and groundwater [10]. A possible solution to this environmental issue is the anaerobic digestion of vinasse, reducing the organic matter of vinasse for later fertirrigation use, and generating a new stream of revenue (biogas) for the sugarcane ethanol plant [11].

Composition	Sugarcane juice (g/L)	Sugarcane molasses (g/kg)
Total solids	140–190	735–875
Total sugars	105–175	447–587
Sucrose	98–167	157–469
Reducing sugars	6–11	97–399
Nitrogen	0.08–0.3	0.25–1.5
Phosphorous	0.02–0.1	0.3–0.7
Potassium	0.7–1.5	19–54
Calcium	0.1–0.5	6–12
Magnesium	0.1–0.5	4–11

Based on data compiled by [2].

Table 1. Composition of sugarcane juice and molasses.

Wort preparation influences final vinasse composition, which will have an impact on the performance of anaerobic digesters, and should be taken in consideration when designing such system. Vinasse can be defined as a mixture of various compounds, including organic acids, mainly fermentation-derived, such as succinic and malic acids, as well as lactic and acetic acids, derived from bacterial contamination. Glycerol is a substantial compound in vinasse, and non-distilled minor amounts of ethanol are also found [11, 12]. A general characterization of vinasse originated from different substrates is depicted in **Table 2**.

2.2. Substrate related stresses on ethanol fermentation

Even being successfully used as substrate for ethanol production for decades, sugarcane based-worts present many challenging conditions for the fermenting yeast *S. cerevisiae* [1]. Besides nutrients, industrial worts used in the fermentation process, also carry inhibitors which can be both feedstock- or process-related [13]. During heating steps of juice pretreatment, some fermentation inhibitors are produced, from sugar degradation (e.g. furfural) and Maillard melanoidins [14].

Furfural, for instance, has been shown to reduce the specific growth rate, the biomass yield on ATP, and both ethanol yield and productivity [15], and is lethal to cells in concentrations above 84 nmol/gDW [16]. During fermentation, furfural is reduced to furfuryl by NADH-dependent alcohol dehydrogenase (ADH) [17, 18], in a NAD+ regenerative manner, resulting in lower glycerol formation, and higher ethanol titres, when furfural concentration is kept under 29 nmol/l [16].

Tauer et al. (2004) have investigated the effect of Maillard derived products during fermentation of different beverages (i.e. Tequila, Mezcal, whiskey and beer). In their study they observed a reduction in the formation of ethanol of up to 80%. Also, it was observed that the inhibition of these Maillard products is pH dependent, showing little inhibition at pH 4, and increasing at higher pH values.

Wort composition	Parameters (in g/L)									
	COD[a]	BOD[b]	N	P	K	SO_4^{2-}	Ca	Mg	TS[c]	VS[d]
Sugarcane juice	27.7 ± 7.3	14.7 ± 4.9	0.5 ± 0.2	0.11 ± 0.1	1.5 ± 0.8	0.7 ± 1.0	0.4 ± 0.4	0.2 ± 0.1	22.5 ± 1.5	17.9 ± 1.8
Molasses	69 ± 24.7	28.6 ± 13.8	0.9 ± 0.6	0.13 ± 0.21	4.1 ± 2.4	2.3 ± 1.9	1.9 ± 1.4	0.6 ± 0.2	82	60
Mix	37.8 ± 5.8	15.7 ± 4.4	0.5 ± 0.1	0.03 ± 0.04	2.3 ± 1.1	0.9 ± 0.5	1.4 ± 1.1	0.3 ± 0.04	105 ± 52.5	8.4 ± 4.7

Based on data compiled by [11].
[a]Chemical oxygen demand.
[b]Biochemical oxygen demand.
[c]Total solids.
[d]Volatile solids.

Table 2. Vinasse composition from different sugarcane-based worts.

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Sugarcane juice also presents potentially toxic metal ions on its composition. Walford (1996) presented a compilation of mineral values from sugarcane juices (**Table 3**).

Aluminum shows high toxicity under acidic conditions (being present in Al^{3+} form) and is particularly deleterious to yeast cells, reducing cell viability, trehalose content, and ethanol yield [1]. Its deleterious effects can be alleviated by magnesium concentration in the broth, or completely abolished by mixing sugarcane juice with molasses. This might be related to some chelating property of molasses [2].

Other metal ions also play an important role in yeast fermentation inhibition. Sugarcane molasses showed inhibitory effect towards invertase activity of a laboratory *S. cerevisiae* strain. This inhibition could be further replicated in laboratory media when copper ions ($CuCl_2$) where added in the media at a concentration of 0.04 M [19].

Another important factor is the osmotic stress that is caused by elevated concentration of salts. Cations such as Ca^{2+}, Mg^{2+}, K^+, Na^+, and anions, like Cl^- and SO_4^{2-}, can all have severe effects on yeast growth and ethanol production [20].

Sugarcane juice and molasses have a complex composition of organic acids. Even though they do not respond to a major fraction of the composition of these raw materials, they are responsible for their pH values (ca. 5–6) and their buffering capacity [5]. The composition, and concentration, of such acids depend on several factors, such as the maturity stage and variety of the plant, weather, soil and health state [21]. The most common organic acids found in sugarcane are trans-aconitic (5000–8000 ppm/Brix), malic (1200–1800 ppm/Brix) and citric

Component	Concentration (% on total solids)
K_2O	0.77–1.31
Na_2O	0.01–0.04
CaO	0.24–0.48
MgO	0.1–0.39
Al_2O_3	0.005–0.17
Fe_2O_3	0.006–0.04
CuO	0.002–0.003
ZnO	0.003–0.012
MnO	0.007
CoO	0.00007
SO_3	0.017–0.52
P_2O_5	0.14–0.4
Cl	0.16–0.27
SiO_2	0.016–0.101

Based on data compiled by [5].

Table 3. Inorganic composition of sugarcane juice.

(900–1800 ppm/Brix) acids. Other acids found in lower concentrations are succinic, oxalic, tartaric and glycolic acids [5]. During fermentation, lactic and acetic acids are also formed via contaminating bacteria.

Undissociated organic acids are lipid permeable and thus enter yeast cells. Due to the higher intracellular pH as compared to the environment, dissociation into the corresponding anion, leads to intracellular acidification and ATP expenditure, a mechanism known as weak acid uncoupling [22], which is described in more details in the following sections. Moreover, the anion accumulated inside the cells may reach toxic concentrations that impair essential metabolic functions.

3. The interplay between yeast and bacteria in the fermentation process

Fuel ethanol fermentation performed in Brazil is one of the largest industrial biotechnological processes in the world, with the most favorable energy balance as compared to other similar processes for ethanol production [13, 23]. However, in view of the nature of the process and the large volumes processed, aseptic conditions are never achieved. Therefore, bacterial contamination is a concurrent problem in industrial fermentations.

This is regarded as a major drawback that deviates sugars away from ethanol formation and lead to detrimental effects upon yeast fermentative performance, such as reduced ethanol yield, yeast cell flocculation, and low yeast viability [24–27].

3.1. Homo- and heterofermentative lactic acid bacteria

Bacterial contaminants found present in the fermentation step of ethanol production comprise mainly lactic acid bacteria (LAB) [28], probably because of their higher tolerance towards acidic pH and ethanol titres when compared to other microorganisms [29, 30]. Studies that investigated the identity of these contaminants during yeast fermentation in Brazilian ethanol plants found that *Lactobacillus* was the most abundant genus [28].

Contaminating lactic acid bacteria are traditionally classified in two major metabolic subgroups according to the pathway used to metabolize hexose sugars: homo- and heterofermentative [29]. In general, bacteria isolates from industrial fermented sugarcane substrates have shown to include both types [31].

Homofermentative bacteria catabolize hexoses via the so-called Embden-Meyerhof-Parnas (EMP) pathway, in which 1 mol of hexose results in the formation of 2 mol of lactic acid and 2 mol of ATP. In comparison, in heterofermentative bacteria another pathway is active, 6-phosphogluconate/phosphoketolase (6-PG/PK) pathway [29], and hexoses are converted to equimolar amounts of lactic acid, ethanol or acetate, and carbon dioxide, yielding 1 mol of ATP per mol of hexose fermented [32, 33]. With the conversion of acetyl phosphate to acetate instead of ethanol, an additional ATP can be produced. Then, regeneration of surplus NAD+ must be achieved by an alternative electron acceptor. Under aerobic conditions, oxygen may serve as the electron acceptor [34], but under anaerobic or even oxygen-limited conditions, fructose is reduced to mannitol, serving as an electron sink [35]. There is a third classification

group that, differently from the homofermentative strains that cannot metabolize pentose sugars, they can ferment these sugars using an inducible phosphoketolase pathway, producing lactate and acetate [36].

3.2. LAB effects on the fermenting yeast

Contaminating bacteria found in ethanol fermentations are often fastidious organisms which compete for nutrients against the fermenting yeast, negatively impacting its fermentation performance [25]. These nutrients are often several growth factors like nucleotides, amino acids and vitamins [25]. Due to the fast-growing nature of these bacterial strains, wort can become rapidly depleted for such nutrients [37]. This nutritional deficiency might result in lower ethanol titres, lower yeast crop viability and budding, longer fermentation periods and higher contents of residual sugars, a phenomenon called stuck or sluggish fermentation [27].

Concomitantly to nutritional competition, these contaminants also deviate carbon to their catabolic pathways and produces organic acids and polyalcohols [27]. These weak organic acids also have a deleterious effect on yeast metabolism. In low pH conditions, usually found in fermentations (i.e. 6.5–4.5) they are found mostly in their protonated form, due to their high pKa values (3.86 for lactic acid; 4.75 for acetic acid) [27]. In this form, these uncharged molecules can permeate the plasma membrane and, when encounters the cytosolic pH (7.0), rapidly dissociates releasing its proton and acidifies the intracellular pH (pHi) [38].

This acidification of the pHi affects many cellular processes, by influencing the ionization states of acidic and basic side chains of amino acids in important enzymes, changing their tertiary structure and activity [39]. Among the several vital functions that are inhibited by pHi acidification is the glycolysis [40], thus inhibiting the cell's ability to recover ATP.

Saccharomyces responds, partially, to this acidification via the plasma membrane H^+ -ATPase pump, Pma1p. It pumps out H^+ using ATP hydrolysis, at a 1:1 ratio [41]. This response mechanism comes with a cost: almost 20% of all ATP formed during normal conditions is drained by this process40; at starvation conditions, when exposed to weak acid stress, the amount of consumed ATP can go as high as 60% [42].

Other responses are also triggered, when yeast cells are exposed to weak acid stress. The plasma membrane ATP binding cassette (ABC) transporter Pdr12p is induced when cells face this stressful condition [43], and it is believed to play an important role on yeast cells adaptation to grow in the presence of weak organic acids by pumping out H^+ ions, under the expense of either ATP or proton gradient [42].

When protons and anions are pumped out of cells, they re-associate. Once protonated, these molecules can permeate again to the interior of yeast cells, forcing them to pump out these ions repeatedly. This process is defined as a futile cycle36 and is a major energetic drain in industrial processes. These energetic drains increase cells maintenance coefficient (m) and reduce their fitness, inhibiting their growth and reducing their viability [44].

Despite these universal cellular responses, yeast might be more or less susceptible to a given contaminant, depending on its metabolism and also on the physiological context these organisms are exposed to. Growth conditions such as temperature, pH, sugar content, nutrients

availability and biological factors like yeast strain, population density and bacterial metabolism should all be considered when analyzing the impacts of contaminant species in industrial fermentations.

For instance, previous studies have demonstrated that, under certain fermentation conditions, competition towards nutrients is the most probable cause for yeast cells inhibition. *S. cerevisiae* in steady-state pure cultures withstood high concentrations of added lactic acid, without losing much of cell viability nor ethanol production/productivity, when compared to co-cultivated *S. cerevisiae* and the facultative heterofermentative *L. paracasei* [26]. This shows that organic acid production by bacterial metabolism might not be, in some circumstances, directly involved in yeast cells stress.

3.3. Co-cultivation studies

Co-cultivations experiments, in the context of ethanol production, are rather scarce in the literature. Very recently, a quite interesting investigation was performed using two common contaminant microorganisms in industrial ethanol plants, the heterofermentative bacterium *L. fermentum*, and the contaminating yeast, *Dekkera bruxellensis* [45]. The authors found that during co-cultivation experiments with *S. cerevisiae* strain PE-2 in the presence of both contaminants, the growth of the contaminating yeast was stimulated by the presence of the bacterium. This condition resulted in a more pronounced effect on the fermentation parameters than the effects observed in binary combinations of the fermenting yeast (PE-2) with each contaminant.

Wild contaminants of *S. cerevisiae* were also evaluated in co-cultivations experiments [46]. They were found to be more detrimental to fermentative performance (resulting in lower ethanol production and higher residual sugars) when compared to co-cultivations with a heterofermentative bacterium.

Bacteria showing different metabolic pathways may also impact differently yeast physiology and ethanol fermentation, on a context-dependent manner. Homofermentative bacteria (i.e. *L. plantarum*) was more inhibitory to yeast cells than heterofermentative bacteria (i.e. *L. fermentum*), when these strains where co-cultured with an industrial *S. cerevisiae* strain (CAT-1), under laboratory conditions (i.e. in equal cell concentrations). When the context of sugarcane ethanol fermentations is put in perspective (i.e. short fermentations catalyzed by high cell densities) *L. fermentum* were more deleterious, outcompeting yeast cells in the fermentation process. In these conditions, the fastidious metabolism of *L. fermentum*, and its faster uptake of fructose – a sugar typically not consumed as fast as glucose by *S. cerevisiae* – may have given *L. fermentum* a competitive advantage, when compared to *L. plantarum*, which had to compete with *S. cerevisiae* for the available glucose [27]. Therefore, it was concluded that under conditions similar to those used in the industrial production of fuel ethanol, heterofermentative strains have a more detrimental effect over yeast performance, in terms of ethanol yield and yeast viability.

3.4. Monitoring bacterial contamination

Mannitol is a suitable indicator of sugarcane deterioration and bacterial contamination during industrial fuel ethanol fermentation [47, 48]. Glucose and fructose normally present a 1:1 ratio,

since sucrose is the prevalent sugar in this feedstock. Therefore, mannitol titres can be used to predict sucrose losses due to bacterial contamination as well as dextran synthesis, which results in problems such as viscosity, evaporation, crystallization and, to a lesser extent, poor filterability in sugarcane factories [49].

Another interesting indicator of bacterial contamination is lactic acid. This organic acid is considered by many a suitable indicator of bacterial contamination during industrial ethanol fermentation. However, because D- and L-lactic acids is formed by industrial lactobacilli isolates [50], as a result of varying proportions of racemases [51, 52], conflicting results are expected depending on the technique employed to quantify this by-product. This is because most commercial enzymatic kits usually employed in the routine analysis of lactic acid in ethanol plants, normally detect only the L-form.

4. Concluding remarks

Industrial yeast cells are exposed to several stresses during sugarcane ethanol production. These stresses might be related to media composition, but also to the microbial community composition found in industrial fermenters. The understanding of the physiological responses of yeasts towards these stresses is paramount for improving current and future *S. cerevisiae* catalysed sugarcane-based bioprocesses.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Section 2

Strategies for Sugarcane Bagasse Pre-treatment

Chapter 3

Emerging Physico-Chemical Methods for Biomass Pretreatment

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Additional information is available at the end of the chapter

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Abstract

A major challenge to commercial production of cellulosic ethanol pertains to the cost-effective breakdown of the complex and recalcitrant structure of lignocellulose into its components by pretreatment methods—physical, chemical, physico-chemical, biological and various combinations thereof. The type and conditions of a pretreatment impacts both upstream processes such as size reduction as well as downstream processes such as enzymatic hydrolysis and enzyme loadings, and as such the choice of a pretreatment method for a specific biomass (or mix of materials) is influenced by several factors such as carbohydrate preservation and digestibility, sugar and ethanol yields, energy consumption, equipment and solvent costs, lignin removal and quality, formation of sugar/lignin degradation products, waste production, and water usage, among others. This chapter reviews both well-known and emerging physico-chemical methods of biomass fractionation with regards to process description and applications, advantages and disadvantages, as well as recent innovations employed to improve sugar yields, environmental sustainability and process economics.

Keywords: lignocellulose, ethanol, pretreatment, physico-chemical pretreatment

1. Introduction

Pretreatment and enzymatic hydrolysis present the most practical challenges (technical, environmental and economic) in the attempt to commercialize cellulosic bioethanol. Pretreatment is costly since it represents about 20% of total cost [1]. However, without pretreatment, enzymatic degradation of native biomass is generally below 20% yield [2], making pretreatment a crucial process of bioethanol production. In general, the selection of a pretreatment method

for a material or mix of feedstocks is influenced by factors that include carbohydrate preservation and digestibility, sugar and ethanol yields, energy consumption, equipment and solvent costs, lignin removal and quality, formation of degradation products from sugars and lignin, waste production, and water usage.

Pretreatment may be categorized as physical (e.g., size reduction, autoclaving, irradiation, popping, ultrasonication, steaming and extrusion), chemical (use of acids, alkali, solvents, etc.), biological (white-rot fungi, brown rot fungi, etc.), and physico-chemical which combine both physical and chemical processes to ensure the digestibility of the lignocellulosic material. Physical pretreatment usually demands high-energy consumption (e.g., side reduction) and is undertaken before chemical or biological pretreatment. While chemical methods offer benefits such as efficient fractionation of biomass and good sugar yields during enzymatic hydrolysis, their environmental impacts are higher than physical and biological methods due to biomass degradation into enzyme-inhibiting compounds, corrosion of reactors, solvent recycling issues, and generation of waste [3, 4].

The well-known physico-chemical pretreatment include liquid-hot water (LHW), steam explosion (SE), ammonium fiber explosion (AFEX), soaking in aqueous ammonia (SAA) and irradiation-chemical method. This chapter reviews and present novel findings as well as process innovations in physico-chemical processing of recalcitrant biomass to sugars and ethanol.

2. Liquid hot-water (LHW) pretreatment

2.1. Description

This hydrothermal process involves cooking of biomass in liquid water at high temperatures (150–240°C) and short times (≤ 50 min). Pretreatment causes pressurized water to rupture and penetrate the cell structure, resulting in fractionation of biomass into two product streams—liquid hydroxylate containing hemicelluloses sugars, minerals, and degradation products such as furfural and acetic acid, and a solid fraction comprising most of the cellulose and lignin and some residual hemicellulose. Pretreated solid substrates have increased surface area and pore volume, and consist of separated individual cellulose fibers, with large particles of repolymerized lignin on the surfaces of the cellulose matrix [5]. The harshness of the process is described by a severity factor (R_o) that allows for the determination of combined effects of temperature and reaction time on sugar yields and degradation products. It is expressed as: $R_o = t \times exp[(T - 100)/14.75]$, where t = reaction time (min), and T = temperature (°C) [6].

Generally, the carbohydrate content of pretreated substrates increases with temperature until a maximum temperature is reached where further temperature increase result in substantial degradation. Thus, high temperatures greater than 230°C disrupt pretreated particles and reduce the surface area and pore volume, which in turn limit enzymatic digestibility [5].

Based on the work of Mosier et al. [2], it is observed that at reaction conditions of 200–230°C and ≤ 15 min, biomass dissolution ranged from 40 to 60%, comprising 4–22, 35–60, and 100% of cellulose, lignin, and hemicellulose, respectively. Much of the hemicellulose dissolve into

poly- and oligosaccharides even at high severities which is beneficial since the minimization of monomer formation reduces the chance of further degradation into aldehydes (2-furaldehyde, 5-hydroxymethylfurfural, etc.), which are known inhibitors of enzymatic hydrolysis. However, oligomers also possess inhibitory tendencies to cellulase activity [7]. It has been established that digestibility of pretreated substrates depended more on partial removal of hemicellulose and relocalization of lignin than the modification of crystallinity and rupture of the cell wall [5, 8].

2.2. Applications

LHW pretreatment has been applied to pretreat various feedstocks including agricultural residues, woods and industrial waste. Archambault-Leger et al. [9] applied both batch and flowthrough pretreatment to corn stover, bagasse, and poplar and observed higher hemicellulose recovery, removal of non-carbohydrate carbon, and glucan conversion under simultaneous saccharification and fermentation (SSF) in the flowthrough reactor. Some authors have included additives to positively influence pretreatment outcomes. For example, by adding $AlCl_3$ to pretreatment solution of microcrystalline cellulose, low concentration of degradation products and high glucose yields were obtained [10]. Optimum glucose yield of 80% was also observed when solid carbon dioxide was used in hydrothermal fractionation of *Eucalyptus* [11]. In another study, an alcohol-water mixture was used to overcome challenges due to deposition of lignin particles on pretreated materials, and thus achieved increased pore volume and higher sugar yields [12].

Combinations of LHW and other methods have also been employed to overcome inherent drawbacks and to improve sugar yields. Low degradation products and higher sugar yields—xylose (91.62%) and glucose (88.12%)—was observed when LHW treatment (180°C, 20 min) of *Eucalyptus* was followed by wet disk milling before saccharification [13]. Alkaline-assisted LHW treatment of rice straw was found to improve glucose recovery and yield under enzymatic hydrolysis, caused by increased removal of hemicellulose and lignin [14].

At demonstration and industrial scale, one notable application of LWH is in the Integrated Biomass Utilization System (IBUS) platform where biomass is converted into ethanol, C5 molasses, and lignin pellets using uncatalyzed steam in an energy efficient manner underscored by high dry matter content in all process routes [15]. It was developed as a three-stage, pilot-scale process for treating wheat straw—by soaking at 80°C for 20 min, hemicellulose recovery at 170–180°C for 7.5–15 min, and cellulose hydrolysis at 195°C for 3 min. Under these conditions both ethanol production and lignin recovery for power production are maximized [16]. High glucose yield required the avoidance of water addition to the third stage while high hemicellulose yield (83%) required water addition. It was improved by Petersen and team [17] who used a two-stage procedure to achieve high cellulose recovery (over 90%) on wheat straw: soaking of biomass at 80°C for 5–10 min followed by pretreating at temperatures and residence times ranging from 185 to 198°C and 6–12 min respectively. The optimum pretreatment temperature was observed at 195°C at which cellulose and hemicellulose recovery reached 93–94 and 70% respectively at lower water/biomass ratio compared to the three-stage process. However, the two-step process was found to present economic challenges in

the recovery of C5 sugars after the first pretreatment in a commercial-scale plant, prompting Inbicon to settle for a simpler, one-stage treatment processs [15]. Currently, the Inbicon demonstration plant, which is based in Kalundborg (Denmark), processes about 4 tonnes straw/h and at yields greater than 198 L ethanol/tonne of wheat straw.

2.3. Positive attributes and drawbacks

LHW offers improved digestibility of cellulose by enzymes due to the solubilization of hemicelluloses and avoidance of inhibitors. Compared to steam explosion, LHW gives lower concentrations of solubilized hemicellulose and lignin products due to higher water input as well as higher pentosan recovery. Generally, catalysts/chemicals are avoided resulting in no/low neutralization demands and byproduct/precipitate generation, with additional benefits such as reduced risk of reactor corrosion and explosion. Reactor cost is lower compared to methods such as AFEX [18]. The effect of particle size reduction on hydrolysis is low, thus, large biomass flowrates can be handled effectively.

There are however drawbacks in LHW related to hemicellulose fractionation into large fractions of oligomers, and xylose yields are generally low, which affect sugar and ethanol yields. There is a risk of sugar degradation into byproducts such as carboxylic acids and furans at severe conditions [19, 20]. A major cost involved in LHW pertains to high energy used to generate saturated liquid water. Consequently, solid loadings are restricted to about 20% [21].

3. Steam explosion (SP)

3.1. Description

In steam explosion, biomass is exposed to saturated steam at high pressure (0.5–4.8 MPa) for a maximum period of 60 min followed by sudden reduction of pressure to atmospheric or lower, resulting in explosive decompression of biomass into component fiber and fiber bundles. The explosion is triggered by evaporation within biomass cells and sudden drop of pressure around the biomass. Exploded materials experience increase in water retention and pore size and specific surface area. Consequently, the bulk density is decreased. To improve penetration efficiency and swelling, biomass is pre-soaked before pretreatment. While the buffering effects of free moisture reduce heat transfer and increase energy demand, bound moisture softens fibers and increase pretreatment efficiency [22]. Thus, by carefully regulating water content of feedstock, substantial gains in sugar yield can be obtained during enzymatic hydrolysis, with collateral benefits in reduced energy demand [23].

The pretreated solids comprise unhydrolyzed cellulose, chemically-transformed lignin, and residual hemicelluloses. The liquid hydrolysate, on the other hand, contains solubilized hemicelluloses in oligomeric forms, with concentrations of monomers usually exceeding similar situations under LHW. Hemicellulose is hydrolyzed via the breakdown of both glycosidic and hemicellulose-lignin bonds. Hydrolysis of parts (acetyl groups and uronic acid substitutions) of hemicelluloses—via the catalytic actions of protons generated from the autoionization of

water—occurs to form acetic and other acids which enhance further fractionation of hemicellulose [24], and trigger the release of carbonium ions from benzyl alcohol structures in lignin which cause the breakdown of some of the β-O-4 structures in lignin leading to reduced molecular weight [25]. Simultaneously, condensation reactions may take place in the presence of electron-rich carbon atoms, resulting in lignin repolymerization [25, 26], with the composition affected by pretreatment severity [27].

The process is affected by temperature, reaction time, material size, moisture content and efficient mixing of biomass. The explosion mechanism and time which are independent of the severity factor are also known to affect yields [28]. Increasing reaction time and temperature decreases the degree of polymerization of cellulose [29]. Though severe conditions contribute to reduction in crystallinity and increase in moisture retention, they do not necessarily lead to increased hydrolysis rates due to possibility of thermal degradation of cellulose. Similarly, xylose recovery is reduced for longer pretreatment times due to formation of degradation products. Further, severe conditions increase the intensity of repolymerization and condensation reactions from byproducts of lignin, hemicellulose, and extractives leading to increased molecular weights of lignin [30]. This development reduces substrate amenability to enzymatic hydrolysis caused by the covering of cellulose surface with the repolymerised lignin-like materials (pseudo-lignin). The problem of lignin repolymerization was overcome by Li et al. [31] who used a carbonium ion scavenger (2-napththol) to achieve solubilize lignin, resulting in improved recovery (91%) as against 51% for steam pretreated aspen wood without the additive.

3.2. Applications

SE has been applied in combination with additives and pretreatment methods to improve yields and overall process economics. The major variations include the use of acids and bases as catalysts.

3.3. Acid-catalyzed steam explosion (ACSE)

In this process, SE is undertaken after the biomass is soaked with dilute acid or impregnated with SO_2 or CO_2 at low or atmospheric pressures for 0.5–25 h depending on the temperature (5–100°C). It favors solubilization of hemicelluloses into monomer units, making substrates more reactive while improving enzymatic hydrolysis of cellulose. Compared to dilute acid, SO_2 impregnates biomass substrates better and more uniformly but requires harsher conditions to remove hemicellulose [32]. Both SO_2- and CO_2-based SE create the formation of pores of different sizes and shapes in the outer region of the cell wall of pretreated substrates, with the effect more noticeable in SO_2-based applications due to its higher combined severities under similar conditions [33]. Though CO_2 has a lower solubility compared to SO_2, CO_2 is highly available, less toxic and corrosive, and thus safer to apply.

A major positive attribute about ACSE is that most glucan and lignin are untouched and remain in solid form after pretreatment [34] though lignin presence hinders enzymatic hydrolysis [35]. Nonetheless, high sugar yields are generally obtained. Yields obtained by some investigators are given in **Table 1**.

Agent/catalyst	T (°C), t (min)	Biomass	Observation	Reference
CO_2	205, 15	Sugar cane bagasse and leaves	High glucose yield of 86.6%	[36]
	220, 5		High glucose yield of 97.2%	
SO_2	190, 5	Sugarcane bagasse	Moderately high glucose yield of 79.7%	
		Sugarcane leaves	High glucose yield of 91.9%	
SO_2	205–225, 5–10	Spruce, pine, birch and aspen	High fractionation efficiency of alkaline extractable lignin for hard woods, but low for softwoods.	[31]
H_2SO_4	185, 2	Rice straw	Overall saccharification yield of 73% in a pilot plant	[37]
H_2SO_4	190, 10	Wheat straw	Glucose and xylose yields of 102 and 96% of theoretical. Ethanol yield of 67% based on glucose content of raw material in SSF.	[38]
Acetic/ethanol	180–225, 3–60	Wheat straw	Sugar yield after enzymatic conversion was found higher than treatment without additive, with maximum yield of 264 g/kg DS obtained for ethanol/SE.	[39]

Table 1. Results of acid-catalyzed SE of selected biomass.

The main disadvantages include the toxicity of SO_2 in SO_2-catalyzed applications and the unavoidable release of degradation products. The acidic nature of pretreatment requires expensive reactors that can withstand corrosion. SO_2 may be costly and as such on-site production could be an alternative for improving the financial viability [18]. The efficient use of co-products such as lignin and hemicellulose in process integration improves the economic health of the process considerably.

3.4. Alkaline-catalyzed steam explosion

Alkaline-catalyzed SE has received less attention compared to acid-based SE. The alkaline solution improves delignification of biomass, giving higher enzymatic degradability. Park et al. [40] pretreated *Eucalyptus* under alkaline environment and observed enzymatic digestibility (relative to uncatalyzed SE), leading to a maximum glucose recovery of 66.55%.

3.5. Double-stage pretreatment involving SE

The major target of the two-step process is to achieve higher delignification and increase biomass digestibility. In many cases, significant increase in glucose yields relative to SE application only, have been observed as outlined in **Table 2**.

3.6. Industrial application

SE is among leading pretreatment methods in terms of cost effectiveness and has been implemented at demonstration (e.g., BioGasol plant in Denmark; Green Plains's plants in USA) and industrial scale (e.g., Crescentino, Italy; Raízen and Iogen's plant in São Paulo, Brazil).

First stage	Second stage	Biomass	Results	Reference
SE	Organosolv	Poplar	Improved lignin removal; over 98% recovery of cellulose; glucan digestibility >88%	[32]
SE	O_2 in alkaline solution	Douglas-fir	84% removal of lignin left in exploded substrates	[41]
SE	H_2O_2 + stabilizers	Douglas-fir	Effective lignin removal	[42]
SE	Laccase	Wheat straw	Effective removal of lignin phenols; high ethanol yields	[43, 44]
SE	Fungi	Wheat straw	75% of lignin degraded	[45]
SE	WO	Pine	96% cellulose yield; ~100% hemicellulose yield	[46]
Dilute acid	SE	Rice straw	Reduced inhibitor formation; enhanced xylose yield degradability	[47]
SE	Alkaline	Sugarcane straw	Enzymatic conversion of 85% in an industrial (SE) reactor	[48]

Table 2. Examples of combined pretreatment including SE.

3.7. Positive attributes and drawbacks

SE is among the most cost-effective methods for and agricultural residues and hardwoods since it does not require external catalysts. It offers the possibility of pretreatment at high solids loading due to the high-energy content of steam and low water requirements which reduce capital expenditure. Moreover, excessive dilution of sugars in pretreated liquor is reduced while the downstream processing of waste solution is minimized or eliminated. Another advantage relates to the possibility of using large biomass sizes which can lead to lower energy intensity. Though particles smaller than 2 cm are usually used, a recent study using larger biomass size (2.5 cm) was found to improve saccharification yield and overall process economics more than smaller sizes (0.5–1 cm); however, smaller particles recorded higher pretreated sugar recovery [49]. Corrosion is reduced due to the non-usage/low-use of chemicals.

Despite the advantages, there are inherent drawbacks associated with SE. The formation of inhibitory products, especially furan derivatives, weak acids and phenolic compounds, negatively affect enzymatic hydrolysis and fermentation [50]. Severe conditions cause increased degradation of cellulose and hemicellulose. There is also a risk of condensation and precipitation of soluble lignin components which leads to reduced digestibility of the biomass substrates [41, 51], while disrupting the lignin structure. SE is less effective on softwood and unexploded materials are common. Further, pretreatment at high temperatures and pressures creates additional challenges in material handling, reactor operation, energy management and heat recovery [52]. Thus, scaling-up is a challenge since large volumes of biomass must be heated to high temperatures in short times.

4. Ammonium fiber explosion (AFEX)

4.1. Description

In AFEX, liquid (anhydrous) ammonia at moderate-to-high temperatures (60–200°C) and pressures (6.5–45 bar) is mixed with moist biomass for about 5–30 min, followed by a sudden drop in pressure to atmospheric. Ammonia is usually fed at less than 2 kg/kg of dry biomass. AFEX leads to the removal of lignin and some hemicelluloses, in addition to the decrystallization of cellulose, partly due to the strong affinity of ammonia for such biomass components. According to Chundawat et al. [53], pretreatment causes morphological and physicochemical changes to cell walls of the material, by creating nanoscale network of interconnected tunnels within the cell wall structure through the cleaving of lignin-carbohydrate ester bonds, and the partial removal and subsequent deposition of extractives on cell wall surfaces, leading to enhanced enzymatic access to cellulose. Further, Maillard reactions between ammonia and carbonyl-based aldehydic groups give rise to several intermediate products [54].

AFEX is generally affected by the moisture content and particle size of biomass, ammonia loading and process conditions including temperature and residence time. Higher temperatures cause more ammonia to flash causing greater disruption of the fibrous structure. Both glucan and xylan conversion (at fixed temperature and ammonia loading) was found to increase with moisture content of switchgrass [55]. In another study, particle size reduction increased the conversion of cellulose and xylan during pretreatment of corn stover [56].

4.2. Applications

AFEX has been widely applied to various class of lignocellulosic materials. Some results obtained from AFEX pretreatment of some biomass are given in **Table 3**.

4.3. Positive attributes and drawbacks

AFEX is a dry-to-dry process since no liquid stream is produced, making it potentially less costly compared to steam explosion [63] and dilute acid methods [64]. The process is simple as it reduces requirements of post-pretreatment washing, stream separation and nutrient supplementation, and produces intermediates that are of value in developing advanced bioproducts. Reaction temperatures are moderate and energy requirements are low. Large solids (up to 5 cm) can be fractionated with good yields. Moreover, desired solid loadings are easily obtained, and high solid loadings are easier to implement due to low water demands. High glucose and xylose yields are both obtained under similar process conditions which simplify the optimization of process parameters. Moreover, except for some phenolic fragments of lignin and cell wall extractives that may form on the surface of pretreated solids, no enzyme-inhibitors are produced [50]. AFEX give high sugar yields at low enzyme loadings of 1–10 FPU cellulase/g of dry biomass [1]. Klason lignin and carbohydrates are preserved and pretreated substrates possess high fermentability. Recently, process improvements bordering on ammonia loading and recovery, ammonia recycle concentration, and enzyme loadings have been developed and shown to reduce the cost of operation of AFEX-based biorefinery [65].

Reaction conditions	NH_3 loading, g/g dry mass	Biomass	Results	Reference
102°C, 30 min, 2.24 MPa	2[a]	Agave bagasse	~100% carbohydrate preservation; 42.5 g glucose and xylose/100 g native biomass	[57]
40–110°C, 1.4 MPa	1	Rice and wheat straw, sorghum and maize stovers	60–85% glucose recovery, 50–85% xylose recovery	[58]
165.1°C, 69.8 min, 14.3% NH_3, 2.2 MPa of CO_2		Rice straw	93.6% glucose yield; 97% theoretical ethanol yield	[59]
170°C, 10 min	5	Giant weed	94.2% glucan conversion; 84.4% xylan conversion	[60]
150°C for 30 min	1.5	Switchgrass	98% xylose yield	[61]
70°C, 350–430 psi, 14–18 min	0.8	Dry distillers' grains	90% cellulose conversion to glucose	[62]

[a]Wet-basis.

Table 3. Results of AFEX pretreated biomass.

Its main demerit is its unsuitability for handling materials with high lignin content such as wood. Much of the hemicellulose is fractionated to oligomers making it more challenging during fermentation. High pressures are usually required due to high ammonia loadings and high vapor pressure of ammonia. Moreover, ammonia is expensive and recovery of all feed ammonia for reuse is challenging. Safety issues arising from the corrosive and toxic nature of ammonia present additional challenges in process operation at industrial level. Compared to soaking in aqueous ammonia (SAA), AFEX requires expensive reactors and equipment.

5. Soaking in aqueous ammonia (SAA)

5.1. Description

SAA involves treatment of biomass with aqueous ammonia (5–50%w/w) at low temperatures (25–90°C) under ambient pressure in a batch reactor. Pretreatment is undertaken for residence times ranging from about 1 h to 3 months. Pretreatment efficiency is depended on variables such as temperature, reaction time and ammonia concentration. Lignin dissolves in the aqueous solution without appreciable decrease in the carbohydrate content, and high levels of solubilization are observed with high temperatures and times. In addition, severe conditions also cause release of acetyl groups, hemicelluloses, extractives and ash into pretreatment liquor [66]. In other aqueous ammonia treatment, moderate temperatures (≥100°C) are used to achieve high delignification of biomass using pressure vessels [67]. Higher temperatures are compensated using lower reaction times.

Biomass	Optimal pretreatment		DL, %	X/H, %	Hydrolysis	Yield, %		Reference
						Glu	Eth	
Rice straw	27 wt% NH_3, 25°C, 2 wk		42			71	44–49	[75]
Rice straw	21 wt% NH_3, 69°C, 10 h		60.6[a]		15 FPU/g-glucan, 30 CBU/g-glucan	71.1	83.1	[76]
	15 wt% NH_3, 130°C, 325 psig, 20 min	No acid treatment	69.8	77	50°C, 15 FPU/g-glucan, 15 CBU/g-glucan	83.2		[77]
		+ acid treatment				90.8		
	60°C, 15 wt% NH_3, 24 h	PBI: 3 kGy, 45 MeV			50°C, 60 FPU/g-glucan, 10 CBU/g-glucan	90		[78]
Corn fiber (destarched)	15 wt% NH_3, 65°C, 8 h			76–78	50°C, 72 h, 15.57 FPU/g-glucan, 30 CBU/g-glucan	85.4		[79]
Corn stover	29.5 wt% NH_3, 10–60 days, RT		56–74	85	50°C, 72 h, 15 FPU/g-glucan, 30 CBU/g-glucan	86–89	73–77	[80]
	15 wt% NH_3, 60°C, 12 h		62	85	15 FPU/g-glucan	85	77	[81]
	50 wt% NH_3, 30°C, 4 weeks		55		15 FPU/g-glucan, 30 CBU/g-glucan	86.5	73	[82]
	15 wt%NH_3, 69°C, 12 h			>80			84	[70]
	15 wt% NH_3, 60°C, 8 h	Hot water, 10 min	68		50°C, 24 h, 15 FPU/g-glucan, 30 CBU/g-glucan	96		[83]
	12.5 wt% NH_3, 60°C, 24 h, O_2	+ TiO_2, UV	70		50°C, 24 h, 15 FPU/g-glucan, 30 CBU/g-glucan	85		[84]
		+ ZnO, UV				82		
Switchgrass	29.5 wt% NH_3, 10 days, RT		40–50	50			72	[85]
	30 wt% NH_3, 5 days (pilot-scale)	Aseptic conditions					73	[86]
		Semi-aseptic					52–74	
	15 wt% NH_3, 40°C/24 h, 60°C/8 h		40.8–46.9		50°C, 72 h, 22–25 FPU/g-glucan, 44–50 CBU/g-glucan, + xylanase	>85		[87]
	15 wt% NH_3, 120°C, 24 h	No H_2O_2	65		15 FPU/g-glucan, 30 CBU/g-glucan	53.7		[88]
		+ 5% H_2O_2	77			74.3		

Biomass	Optimal pretreatment	DL, %	X/H, %	Hydrolysis	Yield, %		Reference
					Glu	Eth	
Oil palm trunk	80°C, 8 h and 7 wt% NH_3	40–50		50°C, 96 h, 60 FPU/g-glucan	95.4	78.3	[89]
Oil palm empty fruit bunch	60°C, 12 h, and 21 wt% NH_3	40.9		60 FPU/g-glucan, 96 h	41.4	65.6	[90]
Miscanthus	150°C/30 wt% NH_3, 180°C/10 wt% NH_3, 1 h (not optimum)	>65	39.3–77.1	50°C, 96 h, 20 FPU/g-glucan	53.4		[91]
News paper	4 wt% NH_3 + 2 wt% H_2O_2, 40°C, 3 h			50°C, 72 h, 60 FPU/g-glucan	90		[92]

DL: delignification; RT: room temperature; PBI: proton beam irradiation; X/H: percentage of xylan/hemicellulose retained in the solids after pretreatment; Glu: maximum theoretical glucose yield after enzymatic hydrolysis; Eth: ethanol yield after fermentation, SSF, SSCF, etc.

[a]Conditions: 70°C, 10 h, 20 wt% NH_3.

Table 4. Sugar and ethanol yields from selected SAA pretreated biomass.

5.2. Applications

Chen et al. [68] used aqueous ammonia to pretreat silvergrass, napiergrass and rice straw at room temperature, resulting in over 90% of cellulose recovery in 4 weeks. On destarched barley hull, SAA pretreatment (15w/w NH_3, 75°C, 48 h) produced zero glucan loss and 83% saccharification yield using 15 FPU/g-glucan; and with the addition of a xylanase in simultaneous saccharification and co-fermentation (SSCF), a high ethanol yield of 89.4% of the maximum theoretical was obtained [69]. High ethanol concentration and yields from SAA-pretreated corn stover followed the use of a two-phase SSF involving pentose and hexose conversion with the help of *S. cerevisiae* and a recombinant bacterium, respectively [70]. Recently, the addition of surfactants such as Tween 80 and PEG 400 was found to improve sugar and ethanol yields [71]. In a similar study Raj and Krishnan [72] obtained high sugar yield by adding laccase and a mediator to enhance enzymatic hydrolysis of pretreated biomass. Nahar and Pryor [73] also found out that pelleting of samples before SAA application required less harsh pretreatment conditions and lower costs.

Two-stage processes targeting separate removal of hemicelluloses and lignin have also been investigated. Kim et al. [74] employed acetic acid medium to remove hemicelluloses followed by aqueous ammonia at elevated temperatures. Results obtained from other studies are given in **Table 4**.

5.3. Positive attributes and drawbacks

SAA retains most of the hemicelluloses in the solid, eliminating the need to separately process hemicellulose and cellulose sugars. It leads to efficient delignification, producing low levels of enzyme inhibitory compounds. The reactor configuration is simpler and less costly, while ammonia recovery is easier compared to AFEX [18]. It can be adapted to small-scale production. Further, neutralized salts from liquid hydrolysates could be used as nutrient source in fermentation.

There are few disadvantages associated with SAA pretreatment. Since pretreated solids contain high fractions of hemicellulose, a high demand for C5 conversion enzymes is needed to produce xylose and other pentose monomers [18]. Post-treatment washing usually result in carbohydrate losses.

6. Irradiation-chemical pretreatment

6.1. Description

In irradiation-chemical pretreatment, the biomass is typically soaked in a solvent (water, acid, or alkali) before undergoing irradiation via microwaves, gamma radiation, proton and electron beam, or radio frequency. In some cases, irradiation is performed before the chemical or other pretreatment, with advantages that include solubilization of lignin and hemicellulose, minimization of cellulose degradation, use of lower doses of chemical and less severe conditions. Further, undertaking irradiation before milling of biomass can reduce energy consumption (from size reduction) significantly [93].

Dielectric heating of biomass causes more energy absorption by the more polar part which creates a hot spot, resulting in generation of high internal steam pressure that induces an explosive effect, disrupting the biomass structure [94, 95]. The disruption is underpinned by radiolytic reactions that cause release of free radicals, triggering cross-linking and chain scission [96]. Cross-linking reactions are believed to happen within the cellulose structure and as such when they predominate over chain scission reactions, sugar yields are not affected.

In general, pretreatment results in degradation of hemicellulose and lignin, and the alteration of cellulose structure. There is an increase in the specific surface area and a reduction in the degree of polymerization [97], as well as a change in the crystallinity of cellulose to amorphous pattern [98]. In general, higher radiation intensities and lower biomass moisture content lead to higher rates of increase in final temperatures; however longer radiation time causes higher average final temperature and lower rate of temperature increase [99]. Increases in irradiation strength have been found to affect hemicellulose more than lignin or cellulose [96, 100].

6.2. Applications

Microwave-assisted pretreatment has been applied to various materials. In a comparative study of the efficacy of mild sulfuric acid (5% v/v) application in combination with various heating modes—hot plate (100°C, 30 min), autoclave (121°C, 30 min), and microwave (200°C, 700 W, 15 min) on the biodegradability of garden biomass, microwave heat treatment was found to produce 53.95% cellulose recovery, leading to reducing sugar yield of 46.97%, which was about 10% higher than the other two modes [101]. Application of microwaves on alkali pretreated wheat straw [102] and coconut husk fiber [103] was found to produce

higher ethanol concentration and yield than substrates that did not receive any radiation. In another study, yields of 25.3, 21.2, and 46.5 g/100 g biomass, respectively, was obtained during radio frequency-assisted NaOH pretreatment (27.12 MHz, 0.20–0.25 g NaOH/g biomass; 90°C) of switchgrass at solids content of 20% [94]. In an investigation to ascertain the effects of microwave chemical pretreatment on sweet sorghum bagasse (12% moisture, 1–2 mm), lime was found to enhance lignin removal, with sugar yields reaching 23.2 g/100 g biomass (38% of theoretical yield) for lime concentration of 0.1 g/10 ml of water. Microwave has also been used in conjunction with eutectic solvent, with enhanced lignin and hemicellulose removal and improved cellulose digestibility [104].

Under electron beam application, Karthika et al. [105] obtained 79% sugar yield from the saccharification (30 FPU/g-biomass, 144 h) of a hybrid grass exposed to 250 kGy of radiation, while Bak et al. [106] realized 52.1% from rice straw when it was exposed to 80 kGy and saccharified using 60 FPU/g-glucan for 132 h. Prior removal of hemicellulose using dilute acid and alkaline before irradiation exposes cellulase to enzymatic action during hydrolysis, and culminates in higher sugar yields [107]. Electron beam has also been applied together with other physico-chemical methods such as SE with good results [108]. The main challenge regarding the use of electron beam pertains to its low energy and as such some interest are focusing on proton beam.

6.3. Positive attributes and drawbacks

The mode of heating is uniform, energy efficient and offers rapid processing of biomass. Pretreatment is performed at low temperatures and at shorter period. It has the potential to be used for effective isolation of hemicelluloses. Irradiation generates no/low levels of inhibitors and by carefully controlling the chemical pretreatment, inhibitor levels are reduced.

Irradiation-chemical methods do not come without disadvantages. Microwave-assisted pretreatment comes with the risk of causing extensive degradation of hemicelluloses and contamination of dissolved lignin at severe conditions, releasing toxic compounds that inhibit enzymatic hydrolysis. Hu and team [94] argue that practical issues with scaling-up is more of a challenge in microwave than in radio frequency which can be used on large quantities of biomass, and at relatively high solids loading (20–50%) with uniform temperature profile when combined with chemical methods.

7. Conclusion

Among the three main stages of cellulosic ethanol production, namely, pretreatment, hydrolysis and fermentation, pretreatment presents the most practical and economic challenges in the attempt to produce ethanol at industrial-scale due its influence on both upstream and downstream processes. Thus, emerging and promising pretreatment methods that rely on physico-chemical fractionation of biomass are discussed, with prominence given to process description, advantages, drawbacks, and innovations employed to counteract inherent

technical, economic and environmental challenges. The methods reviewed include liquid hot-water (LHW), steam explosion, ammonium fiber explosion (AFEX), soaking in aqueous ammonia (SAA), and irradiation-based pretreatment. Size reduction operations have been well integrated with other chemical and physico-chemical methods at the pilot and demonstration levels though energy consumption remains the rain challenge and as such research is shifting in favor of relatively low-energy methods such as wet disc milling as well as post-pretreatment size reduction. Irradiation-based methods have also shown promise at the industrial-level as demonstrated by burgeoning research interest around the world. With regards to physico-chemical methods, steam explosion and LHW-based methods have already been developed for industrial application.

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Chapter 4

Sugarcane Bagasse Pretreatment Methods for Ethanol Production

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Additional information is available at the end of the chapter

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Abstract

Lignocellulosic biomass such as sugarcane bagasse (SCB) is a renewable and abundant source for ethanol production. Sugarcane bagasse is composed of cellulose, hemicellulose, lignin, extractives, and several inorganic materials. Pretreatment methods of SCB are necessary for the successful conversion of SCB to ethanol. Each pretreatment process has a specific effect on the cellulose, hemicellulose, and lignin fraction. The conversion of SCB to ethanol typically consists of four main steps: pretreatment, enzymatic hydrolysis, fermentation, and distillation. Hence, different pretreatment methods should be chosen according to the process design for the following hydrolysis, fermentation, and distillation steps. There are many types of pretreatments such as physical, chemical, physico-chemical, and biological pretreatments. This chapter reviews the chemical and physico-chemical pretreatment methods of SCB which are often used by many researchers for ethanol production. Different chemical and physico-chemical pretreatment methods of SCB are introduced and discussed based on relevance to the sugar yield, lignin removal, and cellulose content after pretreatment.

Keywords: sugarcane bagasse, pretreatment, ethanol

1. Introduction

According to the latest report produced by the United Nations Food and Agricultural Organization, there are 10 largest sugarcane producing countries in the world in 2018. The 10 countries are Brazil, India, China, Thailand, Pakistan, Mexico, Colombia, Indonesia, Philippines, and United States. About 540 million metric tons per year of sugarcane bagasse are produced globally [1]. **Table 1** presents sugarcane bagasse production annually for several

countries. Sugarcane bagasse is the solid residue obtained after extraction of the juice from sugar cane (*Saccharum officinarum*) and can be a potential substrate for ethanol production since it has high sugar content and is a renewable, cheap, and readily available feedstock.

Sugarcane bagasse is mainly composed of cellulose (33–36%), hemicellulose (28–30%), and lignin (17–24%). Cellulose is the most abundant polysaccharide polymer which comprised of a linear chain of β(1 → 4) linked D-glucose units that generates crystalline regions and consequently increases resistance to the hydrolytic process. Hemicellulose is the second most abundant polysaccharide after cellulose and is a short and highly branched polymers which comprised of pentose (xylose and arabinose) and hexose (mannose, glucose, and galactose) sugars. It possesses a heteropolysaccharide composition that varies according to the source. Sugarcane bagasse hemicellulose is composed of heteroxylans, with a predominance of xylose. Hence, it can be chemically hydrolyzed more easily than cellulose. Lignins are complex phenylpropanoid polymers formed by the polymerization of aromatic alcohols. The combination of the cellulose-hemicellulose-lignin matrix is conferring resistance to enzymatic and chemical degradation [10, 11]. Bagasse could represent the main lignocellulosic biomass in many tropical countries since it is available at the sugar factory without additional cost and contains high sugar and low lignin content [12].

Production of bioethanol from SCB has a major advantage, like its less carbon intensive, than fossil fuel which reduces air pollution [13]. The bioethanol produced from lignocellulosic materials is named as second-generation (2G) ethanol or cellulosic ethanol, while the first generation ethanol is produced from sucrose (juice extracted from sugarcane, sugarbeet, or sweet sorghum) or starch (typically extracted from grains) [14]. The second-generation ethanol production from lignocellulosic biomass has been considered to be the biofuel with the greatest potential to replace oil-based fuels ([15, 16], and it can be produced from various lignocellulosic biomasses such as wood, agricultural, or forest residues. Typically, bioethanol can be produced in a four-step process, that is, pretreatment, enzymatic hydrolysis, fermentation, and distillation (**Figure 1**), where hydrolysis and fermentation may be combined. Currently, bioethanol is produced mostly in U.S and Brazil (**Table 2**) [17].

Country	Sugarcane bagasse production (million metric ton/year)	References
Brazil	181	[2]
India	101.3	[3]
China	80	[4]
Thailand	20	[5]
Mexico	15	[6]
Colombia	7	[7]
Philippines	5.1	[8]
United States	3.5	[9]

Table 1. Sugarcane bagasse production annually for several countries.

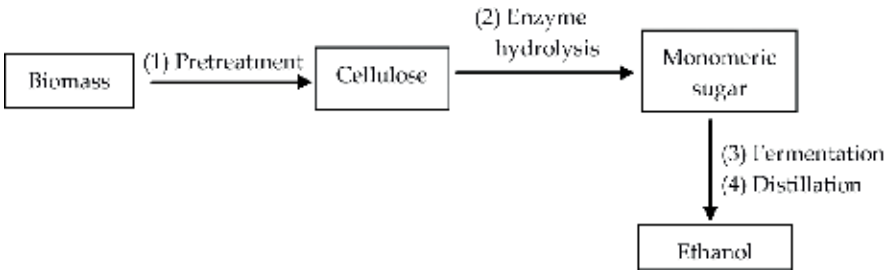

Figure 1. A four-step process for ethanol production from biomass.

Country	Bioethanol production (million gallon)
United State	15,250
Brazil	7295
European Union	1377
China	835
Rest of World	490
Canada	436
Thailand	322
Argentina	264
India	225

Table 2. Bioethanol production by country, million gallons, 2017 [17].

2. Pretreatment

The main objective of the pretreatments is to break down the lignin structure and disrupt the crystalline structure of cellulose for enhancing enzymes accessibility to the cellulose during the hydrolysis step [18]. These pretreatments may be biological, chemical, and physical processes that are used individually, combined, and/or sequentially [19, 20]. The natural structure of lignocellulosic material is extremely recalcitrant to enzymatic hydrolysis. Therefore, the pretreatment step is required for efficient enzymatic hydrolysis of cellulose by removal of lignin and hemicellulose, reduction of cellulose crystallinity and increase the porosity of the biomass [21]. Each pretreatment has a different effect on the cellulose, hemicellulose, and lignin fraction.

It is necessary to choose suitable pretreatment methods for SCB since different lignocellulosic materials have different physico-chemical characteristics [22]. An efficient pretreatment should (1) improve the formation of fermentable sugars, (2) avoid the loss or degradation of carbohydrates, (3) avoid the formation of inhibitory by-products, and (4) be cost-effective [23]. According to Puligundla et al. [24], an ideal pretreatment should be economically efficient,

low energy consumption, and producing less or no residues. High digestibility of cellulose and versatility of feedstock are also important in the pretreatment process. In addition, other factors such as low sugar decomposition, low water or high solids, and low chemical consumption during the process should be considered. Besides that, the pretreatment should be performed at low operational risk and safe.

2.1. Chemical pretreatment

2.1.1. Dilute acid pretreatment

There are two types of acid pretreatments either using concentrated acid or diluted acids. Concentrated acid hydrolysis can be performed at a low temperature (30–60°C) using acid with the concentration around 40–80%. High sugar yield can be obtained using this method, however, requires large volumes of acid which are toxic and corrosive. Thus, corrosion resistant reactors are needed if concentrated acid is employed. Furthermore, the acid concentration must be recovered after hydrolysis to make the process economically feasible [10]. The development of effective acid recovery technologies has made this process renewed its interest [25]. On the other hand, dilute acid hydrolysis is the most widely used and has been considered to be one of the treatment methods with greater potential for wide-scale application. This process can be performed using diluted acids in the range of 0.5–6% and high temperatures from 120–170°C, with variable treatment times from minutes up to an hour.

Dilute acid pretreatment has received numerous research interests, and it has been successfully developed for pretreatment of lignocellulosic biomass. Dilute acid pretreatments are normally used to degrade the hemicellulosic fraction and increase the biomass porosity, improving the enzymatic hydrolysis of cellulose. The dilute acid pretreatment is important to weaken the glycosidic bond in the hemicellulose and lignin-hemicellulose bond and the lignin bond. This will lead to the dissolution of the sugar in the hemicellulose and also increase the porosity of the plant cell wall for effective enzyme digestibility [26]. Acid pretreatment is a very commonly used technology for biomass to ethanol conversion due to its low cost and the fact that the used acids are easily available. However, acid pretreatments can cause side effects such as the formation of furan and short chain aliphatic acid derivatives, which are considered strong inhibitors in microbial fermentation [27, 28].

Several different acids used in pretreatments of SCB, including dilute sulfuric acid [29–35], dilute hydrochloric acid [36], dilute phosphoric acid [32, 37], and dilute nitric acid [38], have been reported. High hydrolysis yields have been obtained when lignocellulosic biomass was pretreated with dilute sulfuric acid compared with hydrochloric, phosphoric, and nitric acid [22]. Sulfuric (H_2SO_4) and phosphoric (H_3PO_4) acids are widely used for acid pretreatment since they are relatively inexpensive and efficient in hydrolyzing lignocellulose. H_3PO_4 also gives less negative impact on the environment compared to H_2SO_4, meanwhile hydrochloric (HCl) acid had better penetration to biomass and more volatile and easier to recover than H_2SO_4 [39]; similarly, nitric acid (HNO_3) possesses good cellulose to sugar conversion rates [40]. However, both acids are expensive compared to H_2SO_4. Sulfuric acid is the most commonly used acid in the pretreatment of SCB [41, 42]. **Table 2** shows the yield of sugar at different types of acid pretreatment of SCB.

According to **Table 3**, the acid concentration used in the range of 0.5–6.0%, temperature 120–170°C and time is around 10 to 300 min. Dilute acid at moderate temperature effectively removes most of the hemicelluloses and recovers as dissolved sugars.

2.1.2. Alkali pretreatment

Beside acid pretreatment, alkaline pretreatment is also one of the chemical pretreatment technologies receiving numerous attention for SCB pretreatment. It employs various bases, including sodium hydroxide (NaOH) [43–53], calcium hydroxide ($Ca(OH)_2$) [54, 55], potassium hydroxide (KOH) [56], aqueous ammonia (NH_3) [57], ammonia hydroxide (NH_4OH) in combination with hydrogen peroxide (H_2O_2) [58], NaOH in combination with $Ca(OH)_2$ (lime) [59], and NaOH in combination with H_2O_2 [60]. Alkaline pretreatment is basically a delignification process. It disrupts the cell wall of SCB by (1) dissolving hemicelluloses, lignin, and silica, (2) hydrolyzing uronic and acetic esters, and (3) swelling cellulose under mild conditions. This process results in two fractions, a liquid (hemicellulose oligomers and lignin) and a solid fraction (cellulose). **Table 4** depicts the composition of lignin in SCB and pretreated SCB with NaOH. It shows that the lignin content decreased when SCB was pretreated with NaOH for all different pretreatment conditions.

The physical structure and chemical composition of the substrate as well as the treatment conditions are important factors for the effectiveness of alkaline pretreatment. In general, alkaline pretreatment is more effective on hardwood, herbaceous crops, and agricultural residues with a low lignin content than on softwood with a high lignin content [61]. Although hydroxides are not expensive, the drawback of this process is that it consumes a lot of water for washing the sodium (or calcium) salts that incorporate into the biomass so that the treatment of a large amount of

Type of acid	Pretreatment conditions	Yield of sugar		References
		mg/g	g/L	
Sulfuric acid	1.5% H_2SO_4, 170°C, 15 min	350		[29]
	0.5% H_2SO_4, 120 °C, 120 min	452.27		[30]
	2.0% H_2SO_4, 155°C, 10 min		22.74	[31]
	0.5% H_2SO_4, 130°C, 15 min	414.9		[32]
	1.25% H_2SO_4, 121°C, 2 h		59.1	[33]
	0.5% H_2SO_4, 121°C, 60 min		24.5	[34]
	2.5% H_2SO_4, 140°C, 30 min		30.29	[35]
Hydrochloric acid	1.2% HCl, 121°C, 4 h	37.21		[36]
Phosphoric acid	3.5% H_3PO_4, 130°C, 180min	404.5		[32]
	4% H_3PO_4, 122°C, 300 min		23.2	[37]
Nitric acid	6% HNO_3, 122°C, 9.3 min		23.51	[38]

Table 3. Yield of sugar at different types of acid pretreatment of SCB.

Lignin (% w/w)		Pretreatment conditions	References
SCB	**Pretreated SCB**		
21.5	10.6	1.0% NaOH, 120°C, 10 min	[43]
27.9	9.2	0.9% NaOH, 80°C, 2 h	[44]
25.4	7.8	2% NaOH, 121°C, 30 min	[45]
18.0	1.8	15% NaOH, 175°C, 15 min	[46]
17.8	4.3	4% NaOH, 121°C, 30 min	[47]
25.0	9.0	2.5% NaOH, 126°C, 45 min	[48]
30.1	18.5	1.0% NaOH, 120°C, 60 min	[49]
23.4	5.2	5% NaOH, 121°C, 60 min	[50]
25*	6	1% NaOH, 100°C, 30 min	[51]
34.3*	5.7	1% NaOH, 100°C, 1 h	[52]
22.0	9.5	2.0% NaOH, 120°C, 40 min	[53]

*Lignin content of SCB pretreated by steam explosion.

Table 4. Composition of lignin in SCB and pretreated SCB.

salts becomes a challenging issue for alkaline pretreatment. In addition, some enzyme inhibitors can be generated during lignin depolymerization [62]. In comparison with other pretreatment technologies, alkali pretreatment usually uses lower temperatures and pressures, even ambient conditions. Pretreatment time, however, is recorded in terms of hours such as 24 hours or days that are much longer than other pretreatment processes [63].

Alkaline pretreatments differ from acid pretreatments so that they are more efficient in lignin removal, substantially increasing cellulose digestibility, even after removing only part of the lignin. The hydrolysis of ester linkages between hemicellulose residues and lignin promotes an increase of porosity in the biomass, and as a result, cellulose and hemicellulose become more accessible to enzyme action [10, 64]. As this pretreatment results in a large fraction of both cellulose and hemicellulose to remain intact, it has the potential for hydrolysis of a much larger fraction of the pretreated biomass, releasing glucose from cellulose and additional pentose sugars from hemicellulose. In addition, this occurs in an environment free of strong acids and fermentation inhibitors. Under these conditions, the degradation of sugars is minimal [65]. Sodium hydroxide shows the greatest lignin degradation when compared to other alkalis, such as sodium carbonate, ammonium hydroxide, calcium hydroxide, and hydrogen peroxide.

Lime (calcium hydroxide) pretreatment is another attractive alkali pretreatment technology due to the low formation of fermentation inhibitors, which increases pH and provides a low-cost alternative for lignin solubilization where the process is removing approximately 33% of lignin and 100% of acetyl groups. Even though the action of lime is slower than other pretreatments, lime is much cheaper than other alkalis and has low toxicity to the environment and safe handling [66]. The effectiveness of lime pretreatment in improving sugarcane bagasse susceptibility to enzymatic hydrolysis was studied by Rabelo et al. [54]. The result showed that lime pretreatment improved the enzymatic digestibility of SCB.

2.1.3. Organosolv pretreatments

The organosolv process is a delignification process, with varying simultaneous hemicellulose solubilization. The organosolv process uses organic or aqueous organic solvent mixtures with or without an acid or alkali catalysts to extract lignin from lignocellulosic biomass. Numerous organic solvent mixtures including methanol, ethanol, acetone, ethylene glycol, triethylene glycol, and tetrahydrofurfuryl alcohol have been used. The advantages of ethanol as a solvent are that it is produced in many biorefineries. It is easily replenished and recycled as a solvent for the pretreatment process. Ethanol is also inexpensive and less toxic to humans compared to other solvents such as methanol [67].

The ethanol organosolv process is among the chemical pretreatment being studied for the conversion of SCB to ethanol. In this pretreatment, high degrees of delignification can be achieved for SCB following ethanol organosolv pretreatment using formic acid as a catalyst. The degree of delignification increased with increasing pretreatment temperature. The maximum degree of delignification of sugarcane bagasse reached 80% at 210°C [68]. Mesa et al. [69] reported that the combination of a dilute-acid pretreatment followed by the organosolv pretreatment with NaOH at a temperature of 195°C for 60 min using 30% (v/v) was an efficient technique for SCB fractionation for the subsequent use on the enzymatic hydrolysis process, since yielded a residual solid material containing 67.3% (w/w) glucose. Novo et al. [70] showed that one of the best pretreatment conditions for lignin removal from SCB by the organosolv method could be achieved at 190°C and 150 min.

Beside ethanol, glycerol is an excellent solvent for organosolv pretreatment [71]. Glycerol, a high-boiling-point organic solvent derived from the oleochemical industry as a by-product has become very attractive. Martín et al. [72] studied the effect of glycerol pretreatment on the main components of SCB. The result shows that the glycerol acted more selectively on lignin than on xylan where cellulose was almost completely recovered in the pretreated solids, accounting for 72% (g/g) of the pretreated substrate. Meanwhile, Novo et al. [70] reported that the glycerol pretreatment attained good cellulose preservation (>91%) and 80% lignin removal. However, Zhang et al. [73] found that >96% of the cellulose was recovered, whereas the lignin and hemicellulose removal were almost 60 and 80%, respectively, when SCB was treated with an acid-catalyzed glycerol organosolv pretreatment.

2.2. Physico-chemical pretreatment

2.2.1. Steam explosion pretreatment

Steam explosion is one of the most efficient methods to deconstruct the plant cell wall macromolecular organization [19, 74]. This process occurs both chemically and physically by revealing the lignocellulosic materials to high temperatures ranging from 160 to 260°C for reaction times varying from 2 to 30 min in the saturated steam either in the absence or presence of an exogenous acid or basic catalyst. The steam is able to expand the cell wall of the polysaccharide fiber and destroys cell structure into small pieces and breaks down the lignin network. This process would increase the accessibility of the enzyme to cellulose by exposing internal cellulose surface, which acetyl groups of hemicellulose can be hydrolyzed to acetic acid [75, 76]. The physical forces cause partial hemicellulose solubilization and lignin reorganization. The

major variables that affect steam explosion pretreatment efficacy include biomass origin, particle size, temperature, residence time, and moisture content [77, 78].

When pretreatment is performed in the presence of an acid catalyst such as sulfuric (H_2SO_4) or phosphoric (H_3PO_4) acids, the need for time and temperature decreases substantially depending on the strength of the acid and its actual concentration in relation to the dry mass of the biomass. In addition, this process can remove hemicelluloses almost completely, whereas lignin is modified to a deeper extend, thus making the cellulosic materials more susceptible to enzymatic or acid hydrolysis [27, 74, 79]. There are several advantages of steam explosion pretreatment which includes lower environmental impact, cost-effectiveness, greater energy efficiency, and less or no chemical usage [22]. Also, to obtain the same particle size of the substrate, steam explosion method requires a 70% lower energy consumption compared to the conventional mechanical process [10]. The main drawbacks of steam explosion pretreatment are the partial degradation of hemicelluloses and the formation of toxic components that could affect the enzymatic hydrolysis and fermentation process [76].

2.2.2. Liquid hot water

According to Sánchez et al. [80], liquid hot water (LHW) pretreatment is performed at various temperatures from 160 to 240°C in the liquid state with water instead of steam. The LHW process primarily maximizes the solubilization of hemicellulose, partial removal of lignin, and making cellulose more accessible to the enzyme. In addition, the formation of the undesirable side products in liquid fraction can be reduced due to solubilized hemicellulose mostly appears in oligomers forms [18]. The LHW pretreatment cleaves hemicellulose linkages and liberates various acids during the process. These acids help to hydrolyze hemicellulose to monomeric sugars, which can be subsequently degraded to aldehydes (i.e., furfural from five carbon sugars and HMF from six carbon sugars). LHW has a great potential to be chosen as a pretreatment step in the biorefinery process as it can be considered as a green technology [81].

During high temperature pretreatment processes, water molecules penetrate the biomass cell wall and hydrate cellulose, with the partial removal of hemicellulose and minor amount of lignin [82]. The advantage of using the neutral method compared to the dilute-acid and alkaline catalyzed pretreatments is to avoid the chemical use in excess, because pH close to neutral does not cause corrosion from occurring, and the formation of excess furans during sugar degradation reactions can be eluded. [83]. However, sugar release yields from LHW pretreated biomass are lower than diluted acid pretreated biomass, otherwise higher pretreatment temperature and longer residence time are required for comparable performance [84]. The LHW has a few advantages compared to other pretreatment methods such as no additional catalysts or chemicals, operates at relatively moderate temperature, high hemicelluloses recovery, low levels of inhibitory by-products and cost-effective [85].

Table 5 presents the comparison between the cellulose content before and after pretreatment of LHW and steam explosion. The temperature range used in LHW is around 170–200°C, whereas in steam explosion the temperature is in the range of 180–195°C. Compared to the untreated SCB, cellulose content increased in pretreated SCB for both LHW and steam explosion pretreatments. The LHW pretreatment of SCB led to an excellent preservation of glucan (cellulose) fraction [88]. Meanwhile, steam explosion with and aid of H_2SO_4 acid during

Physico-chemical pretreatment	Pretreatment conditions	Cellulose content of SCB (%)		Reference
		Before pretreatment	After pretreatment	
Liquid hot water	Temp. 200°C, time 10 min, LSR 4	39.5	41.7	[86]
	Temp. 200°C, time 30 min, LSR 10	37.53	53.02	[87]
	Temp. 180°C, time 20 min, LSR 9	43.43	66.53	[88]
	Temp. 170°C, time 60 min, LSR 3	42.6	48.5	[89]
Steam explosion	Temp. 180°C, time 5 min, LSR 20	42.8	49.1	[90]
	Temp. 190°C, time 10 min, LSR 10, impregnated with 4%(v/v) H_2SO_4	50.7	61.4	[91]
	Temp. 195°C, time 7.5 min	36.9	62.8	[92]
	Temp. 190°C, time 15 min	43.1	57.5	[93]

LSR: liquid solid ratio.

Table 5. Cellulose content of SCB before and after pretreatment by LWH and steam explosion.

pretreatment also increases the cellulose content in the pretreated SCB [91]. The increment of cellulose in pretreated SCB is related to the lignin removal during the pretreatment process either in LHW or steam explosion.

2.3. Biological pretreatment

Biological pretreatment of lignocellulosic biomass is considered as an efficient, ecofriendly, and cheap alternative [94]. The biological pretreatment of lignocellulosic biomass is usually performed using cellulolytic and hemicellulolytic microorganisms. The commonly used microorganisms are filamentous fungi which are ubiquitous and can be isolated from the soil, living plants or lignocellulosic waste materials [95]. White-rot fungi have been reported as the most effective microorganisms for the pretreatment of most of the lignocellulosic materials [96]. These microorganisms degrade lignin through the action of lignin-degrading enzymes such as peroxidases and laccases [97]. Brown-rot fungi mainly attack cellulose, while white and soft rot fungi attack both cellulose and lignin [10]. **Table 6** shows the type of fungal species commonly used in biological pretreatment. The biological pretreatment appears to be a promising technique and has very apparent advantages, including low-capital cost, low energy requirement, no chemical requirement, and mild environmental conditions. However, the main disadvantages are the long incubation time, low efficiency, considerable loss of carbohydrate requirement of careful control of growth conditions, and space restrain its applications [98].

Jiraprasertwong et al. [99] investigated the effect of different microbial strains on biological pretreatment of SCB for enzymatic hydrolysis. The results showed that the pretreatment with the white-rot fungus gave the highest glucose concentration around two-fold higher when compared with the others. Hernández et al. [100] reported that SCB pretreated with *Pycnoporus sanguineus* promotes better lignin decay, glucose release, and hydrolysis yields. Studies by Khuong et al. [101] have shown that the initial moisture content of the bagasse was found to affect biological delignification by MG-60, and the 75% moisture content was

Type of fungus	Fungal species
White rot	*Phanerochaete chrysosporium*
	Pleurotus ostreatus
	Cyathus stercoreus
	Penicillium sp.
Brown rot	*Aspergillus niger*
	Fomitopsis palustris
	Gloeophyllum trabeum
Soft rot	*Trichoderma reesei*

Table 6. Type of fungal species commonly used in biological pretreatment.

suitable for selective lignin degradation and subsequent ethanol production when white-rot fungus *Phlebia* sp. MG-60 was applied to sugarcane bagasse.

3. Conclusions

There are several pretreatment methods available for SCB; however, the final choice for the selection of pretreatment methods depends upon the effective delignification or hemicellulose removal, low sugar loss, time savings, being economic, and causing less environmental pollution. Each pretreatment method has its own advantages and disadvantages. Instead of performing the chemical pretreatment alone, it is good to combine the pretreatment with other physico-chemical pretreatment such as steam explosion in order to improve the sugar yield and increase the lignin removal from SCB. The combination of pretreatment is a promising method to improve enzymatic hydrolysis and ethanol production from SCB.

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Section 3

Non-conventional Biomass Source for Lignocelullosic Ethanol Production

Chapter 5

Potential of Weed Biomass for Bioethanol Production

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Additional information is available at the end of the chapter

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Abstract

Lignocellulosic biomass from weedy plants represents a potential alternative feedstock for economic production of bioethanol. Large numbers of weedy plant species are growing all over the world. Characteristics such as high dry matter yield, low water and nutrient requirements for growth, and cellulose contents make weedy plants very attractive as feedstock for bioethanol production. However, like other lignocellulosic feedstock, the complex structure presents resistance and recalcitrance to processes of conversion to bioethanol. Several weedy plants have been studied to determine their physical characteristics and suitability for bioethanol production. Different conversion techniques have been employed to increase monomer sugars and hence bioethanol yield. This chapter discusses processes and current research activities in bioconversion of weed biomass to bioethanol.

Keywords: bioethanol, fermentation, lignocellulosic biomass, pretreatment, weedy plants

1. Introduction

Rapid economic and population growth have resulted in drastic increase in energy consumption especially in the transportation sector. To meet growing demand for fuel energy, most countries around the world depend heavily on imported petroleum fuel [1]. However, concerns have been raised about gradual depletion of fossil fuels and environmental pollution as a result of its combustion [2]. This has necessitated the search of alternative sustainable and eco-friendly source(s) of fuel energy. As part of the search, many governments worldwide are promoting the use of biofuels such as bioethanol and biodiesel as alternative transportation fuel [3].

Bioethanol is currently the most widely used liquid biofuel [4]. It is an eco-friendly and renewable fuel produced from plant-based starches and sugars [5]. Global production of bioethanol is mainly from food-related crops such as corn, cassava, sugarcane, rice, and sweet potatoes [3]. However, these feedstock are directly consumed by humans as food or as animal feed. Continuous use of these crops for bioethanol production may put pressure on productive agricultural lands and result in higher food prices [6]. Concerns about sustainability of bioethanol production from food-related crops have raised attention to the potential of lignocellulosic biomass for bioethanol production [7].

Lignocellulosic biomass is inexpensive and abundant worldwide. It includes agricultural and forestry waste, grasses, and other nonfood plants [8]. This type of biomass is a rich source of biopolymers, chemicals, and sugars [9]. Current research into bioethanol production is mainly focused on assessing the potential of nonfood crops as feedstock and improving the efficiency of their conversion [10]. Lignocellulosic biomass from invasive weeds is a good feedstock for the economic production of bioethanol [2]. These weedy cellulosic substrates do not need extra expenses as they grow on agriculturally degraded land or water bodies [11]. Large numbers of such invasive species are found all over the world. The potential of weed biomass for the production of bioethanol has been explored and discussed in this chapter.

2. Lignocellulosic biomass from weedy plants: chemical composition and potential for bioethanol production

The major components of lignocellulosic biomass are cellulose, hemicellulose, and lignin. Cellulose and hemicellulose are the main carbohydrates in lignocellulosic biomass. The contents of these components vary significantly depending on the type of biomass and source [6]. Cellulose is a crystalline and linear structure made up of units of glucose strongly linked together by β-1-4-glycosidic bonds. These linkages give cellulose very high crystalline structure making it resistant to degradation. It is the most abundant organic polymer on earth. Hemicellulose on the other hand, consists of linear and highly branched mixture of pentoses (xylose and arabinose) and hexoses (glucose, galactose, and mannose). Lignin is a highly branched polyphenolic polymer, which gives stability to biomass structure [12]. Cellulose and hemicellulose, the major substrates for bioethanol production, form the main components of the total dry weight of lignocellulosic biomass [7]. These fractions are linked together by covalent and hydrogen bonds, which are further strongly bonded to lignin. This gives lignocellulosic biomass a very complex structure, which is very resistant to degradation. Digestibility of lignocellulosic biomass is therefore affected by the degree of complexity and composition [11]. The structure and composition of different lignocellulosic biomass differ and this greatly affects the efficiency of their conversion to bioethanol.

Lignocellulosic biomass from weedy plants is one of the most sustainable alternative feedstock for bioethanol production [12]. Annual and perennial weedy plants are found all over the world at all seasons. They invade large areas of land and water bodies causing environmental and socioeconomic problems [2]. They grow rapidly on marginal lands under extreme conditions such as drought, low nutrient and high temperatures, hence requiring no

additional economic input such as fertilizer and pesticides [7]. Weed biomass contains large amounts of chemicals and materials, which can be extracted for several industrial applications [13]. These plants have been reported to produce high dry matter yield and contain high and low percentages of cellulose and lignin contents, respectively [14]. The high dry matter

Scientific name	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Ash (%)	EtOH TY (L/Ton)
Imperata cylindrica	44.4 ± 0.1	31.1 ± 0.0	6.7 ± 0.0	6.9 ± 0.0	548.4 ± 1.4
Amaranthus viridis	37.4 ± 0.1	34.2 ± 0.0	5.1 ± 0.1	16.9 ± 0.2	521.0 ± 0.9
Sida acuta	56.0 ± 0.3	16.0 ± 0.4	6.8 ± 0.1	5.1 ± 0.1	520.3 ± 5.4
Rottboellia cochinchinensis	41.6 ± 0.7	28.6 ± 0.4	7.5 ± 0.1	10.5 ± 0.3	509.7 ± 8.1
Sorghum halepense	44.4 ± 0.1	25.8 ± 0.2	6.6 ± 0.5	8.8 ± 0.3	508.8 ± 2.6
Eragrostis amabilis	39.7 ± 0.4	29.6 ± 0.2	7.2 ± 0.2	5.5 ± 0.1	502.9 ± 4.7
Cyperus imbricatus	35.6 ± 0.1	32.3 ± 0.3	4.7 ± 0.3	7.4 ± 0.1	493.6 ± 3.2
Cenchrus echinatus	35.8 ± 0.6	31.8 ± 0.4	6.3 ± 0.3	13.9 ± 0.2	491.4 ± 7.4
Cyathula prostrata	50.0 ± 0.3	17.0 ± 0.3	10.9 ± 0.1	10.3 ± 0.1	484.8 ± 4.4
Eriochloa procera	37.0 ± 0.0	29.5 ± 0.1	5.3 ± 0.3	13.1 ± 0.0	483.2 ± 1.1
Brachiaria mutica	37.7 ± 0.01	28.8 ± 0.8	5.6 ± 0.8	10.9 ± 0.2	482.8 ± 6.7
Sporobolus indicus	35.6 ± 0.0	29.9 ± 0.1	6.6 ± 0.0	9.1 ± 0.3	476.2 ± 1.0
Leucaena leucocephala	55.2 ± 0.0	10.1 ± 0.1	16.1 ± 0.1	2.6 ± 0.6	471.9 ± 1.2
Echinochloa crus-galli	34.7 ± 0.2	30.1 ± 0.1	4.6 ± 0.0	8.9 ± 0.5	470.8 ± 2.0
Cyperus iria	33.4 ± 0.2	31.0 ± 0.0	6.3 ± 0.0	5.4 ± 0.1	468.9 ± 1.3
Typha angustifolia	47.1 ± 0.1	16.9 ± 0.4	10.0 ± 0.3	11.3 ± 0.1	462.9 ± 3.9
Dactyloctenium aegyptium	32.0 ± 0.1	31.6 ± 0.1	7.7 ± 0.0	9.5 ± 0.4	462.4 ± 0.3
Achyranthes aspera	53.7 ± 0.1	10.2 ± 0.1	8.5 ± 0.1	11.7 ± 0.3	461.0 ± 1.5
Pennisetum polystachyon	40.0 ± 0.0	23.3 ± 0.1	6.2 ± 0.2	7.5 ± 0.3	459.2 ± 0.6
Cyperus compactus	32.8 ± 0.3	29.0 ± 0.8	4.6 ± 0.5	11.2 ± 0.1	448.9 ± 8.5
Aeschynomene Americana	48.3 ± 0.2	13.4 ± 0.0	15.4 ± 0.3	7.4 ± 0.4	446.2 ± 1.3
Celosia argentea	44.3 ± 0.3	17.2 ± 0.2	9.7 ± 0.9	10.0 ± 0.1	445.3 ± 3.2
Dicliptera roxburghiana	41.9 ± 0.3	17.5 ± 0.3	8.7 ± 0.4	15.2 ± 0.0	429.8 ± 4.3
Crotalaria pallida	49.6 ± 0.2	9.1 ± 0.2	11.7 ± 0.1	4.3 ± 0.2	423.6 ± 2.7
Scoparia dulcis	36.5 ± 0.3	19.1 ± 0.1	6.6 ± 0.0	4.5 ± 0.6	402.6 ± 2.9
Urena lobata	43.5 ± 0.3	11.4 ± 0.7	9.6 ± 0.1	7.5 ± 0.3	396.7 ± 4.4
Cyperus cyperoides	29.7 ± 0.6	24.6 ± 0.2	10.9 ± 0.6	8.8 ± 0.1	394.0 ± 5.3

Source: [14] EtOH TY = Theoretical ethanol yield.

Table 1. Chemical composition and theoretical ethanol yields of weed biomass.

yield and cellulose contents of weedy plant species make them ideal feedstock for bioethanol production. They also have an added advantage as feedstock for bioethanol production since they do not compete with food crops for productive agricultural lands [15]. Moreover, due to seasonal nature of agricultural wastes, lignocellulosic biomass from weed species is very important in ensuring continuous production of bioethanol throughout the year [16]. A wide range of weedy species are grown naturally on marginal lands all over the world that can be used as feedstock for bioethanol production. Perennial grasses and short rotation forest plants are among these weedy species growing worldwide [17]. The possibility of converting biomass from invasive weeds to fuel bioethanol is currently an area of great research interest around the world. The physical characteristics and bioethanol production potential of several weedy species have been studied.

Parthenium hysterophorus, a common invasive weed species was studied in India as a potential feedstock for bioethanol production. Chemical composition analysis of this weed species revealed 53.63% holocellulose and 10.44% lignin contents, making it an attractive feedstock for production of bioethanol [18]. *Cannabis sativa*, a versatile weedy plant, grows naturally in large areas in Pakistan. It produces large amount of biomass due to its rapid growth rate. *Cannabis sativa* contains 55% cellulose and only 5% lignin. It has been reported as a potential cheap and eco-friendly feedstock for bioethanol production in Pakistan [19]. *Pennisetum purpureum*, commonly known as Napier grass or elephant grass, *Vetiveria zizanioides* also known as vetiver grass, *Digitaria decumbens*, *Paspalum atratum*, *Cynodon* sp., and *Pennisetum polystachyon* are all weedy species found in Asia that have been studied and proposed as feedstock for bioethanol production [12].

In an earlier research, different types of weedy plants were identified in six provinces in lower Northern Thailand (**Table 1**). Majority of these weed biomass were found to contain high cellulose but low lignin contents. The cellulose contents of most of these weed biomass is higher or similar compared to well-known lignocellulosic materials from agricultural residues including corn stalk bagasse (43.4%) [20], corncob (31.5 ± 1.2%) [21], wheat straw (35.2 ± 0.3%) [22], paddy straw (32.6%) [23], soybean straw (34.40%) [24], and sugarcane bagasse (27.3%) [25]. High theoretical bioethanol yields were also estimated for these weed biomass based on the contents of cellulose and hemicellulose. Bioethanol yield of between 548.4 ± 1.4 and 394.0 ± 5.3 L/ton was realized from some of the weed species [14]. Majority of these weed species are potential substrate for bioethanol production.

3. Biological conversion of weed biomass to bioethanol

Bioethanol is produced from three main renewable resources namely starch, sugars, and lignocellulosic biomass. The production of bioethanol from starch and sugar (first generation bioethanol production) differs significantly from that of lignocellulosic biomass. The process of bioethanol production from sugar-related crops involves direct extraction of sugars followed by fermentation to bioethanol. However, starch carbohydrates are extracted from starch-based crops and hydrolyzed into monomer sugars with subsequent fermentation of

sugars to bioethanol [26]. Unlike first generation bioethanol production where carbohydrates are easily converted to bioethanol, carbohydrate portions in weed biomass are not freely available for the conversion to bioethanol. Biological conversion of weed biomass to bioethanol involves various processes (**Figure 1**). The major steps involved in the conversion process include pretreatment of biomass to make it easily digestible in subsequent processes. The cellulose and hemicellulose contents are then hydrolyzed to monomer sugars followed by the fermentation of sugars to bioethanol. Finally, bioethanol is purified through distillation or other processes such as dehydration to conform to world bioethanol specifications [27].

3.1. Pretreatment of weed biomass

Like most lignocellulosic biomass, the recalcitrance of weed biomass is a major problem in their conversion to bioethanol. This is due to the crystalline structure of cellulose coupled with lignin and hemicellulose strongly bonded to each other and serving as a protective cover to cellulose. The pretreatment of weed biomass is thus very important in releasing fermentable sugars for bioethanol production [6]. It helps to break the bond between lignin and hemicellulose, hence destroying the protective cover of cellulose. It also helps to decrease cellulose crystallinity making it more susceptible to enzymatic hydrolysis and fermentation [12]. Different pretreatment methods can be used on various types of weed biomass for bioethanol

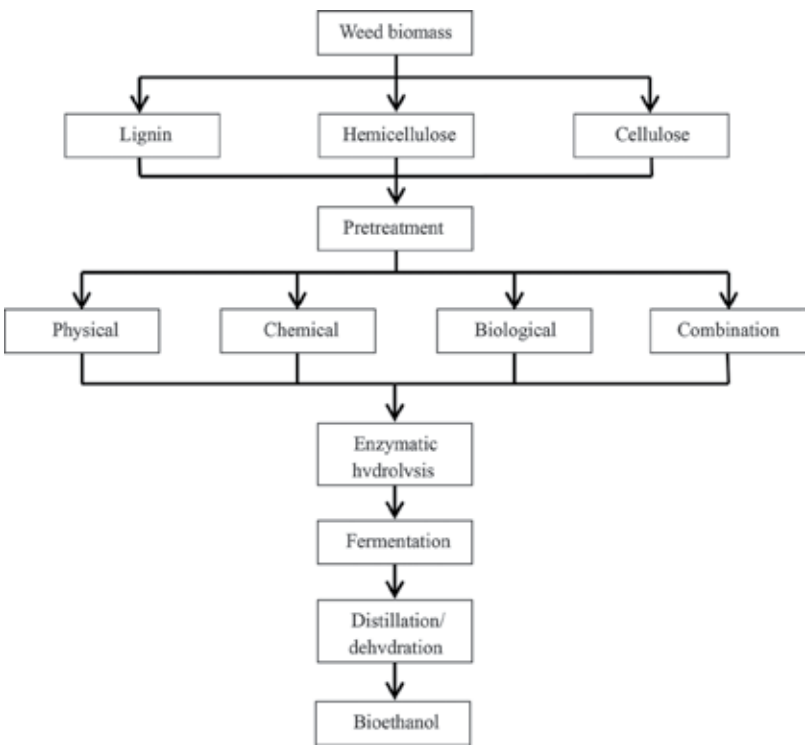

Figure 1. Schematic diagram of major steps in weed biomass conversion to bioethanol.

production. However, the cost of pretreatment, production of inhibitors, type of weed biomass, energy requirements, and efficiency are major factors that need to be considered in the choice of pretreatment method [28]. Pretreatment may be physical, chemical, biological, or a combination of these [29].

3.1.1. Physical pretreatment

Physical pretreatment includes methods aimed at reducing particle size of biomass. These methods consist of mechanical operations such as chipping, milling, and grinding. These processes help to increase the porosity and surface area of biomass to enhance its conversion to bioethanol [9]. Mechanical operations are usually carried out as a preparatory step during the conversion process [12]. Other methods including different kinds of irradiation and ultrasonic pretreatment have been developed to physically enhance accessibility to cellulose during the conversion process. Physical pretreatment, however, requires high amount of energy contributing to high cost of bioethanol production [9].

3.1.2. Chemical pretreatment

Chemical pretreatment is the most common and studied pretreatment method for the conversion of lignocellulosic biomass to bioethanol. Different chemicals including alkali, ionic liquids, organic solvents, oxidizing agents, and acids can be used [30]. Acid pretreatment is one of the most promising methods and has been extensively studied. It mainly results in solubilization of hemicelluloses but less effective in lignin removal [27]. The type of acid, concentration, volume, and pretreatment temperature are some factors that affect the efficiency of this technique [9]. Acid pretreatment may be carried out with either concentrated or dilute acid. However, dilute acid is normally preferred as concentrated acid, which is toxic and corrosive, and results in the production of high levels of inhibitors including furfural derivatives, acetic acid, phenolics, and other aromatic compounds [31]. Pretreatment with acid may be conducted at high temperature for a short time or low temperature for a longer period [32]. Various types of acids including hydrochloric, phosphoric, nitric, oxalic, formic, acetic, and maleic have been studied as chemicals for pretreatment of lignocellulose biomass. Despite its effectiveness, acid pretreatment is toxic and generates inhibitory compounds that negatively affect enzymatic hydrolysis and fermentation processes [9]. It is therefore crucial to remove these compounds, a process that adds to the cost of bioethanol production.

Alkaline pretreatment on the other hand breaks the intermolecular bonds between lignin and hemicelluloses and reduces cellulose crystallinity [33]. During alkaline pretreatment, biomass is treated with alkali chemicals such as sodium, calcium, ammonium, and potassium hydroxides at varying temperatures with or without pressure [5]. Alkaline pretreatment enhances accessibility of enzymes to cellulose by mainly solubilizing lignin contents of biomass. It results in less sugar degradation and produces low inhibitors compared to acid pretreatment [20]. However, alkaline pretreatment results in the production of salts are very difficult to recover [6].

Ozone, a strong oxidizing agent is very effective for the removal of lignin in lignocellulosic biomass. This type of chemical pretreatment is normally done at room temperature and results in

no inhibitor formation [30]. Organic solvents such as methanol, ethanol, ethylene glycol, glycerol, acetic acid, formic acid, phenol, and dioxane are also very effective in extracting lignin and hemicellulose [29]. Ionic liquids have been identified as promising solvents for pretreatment because of their ability to dissolve lignin and carbohydrates. A variety of ionic liquids including those containing cholinium cations and linear carboxylate anions have been identified for their ability to enhance digestibility of lignocellulosic biomass. An advantage of ionic liquid is the recovery of separate lignin and carbohydrate fractions after pretreatment. However, ionic liquids are very expensive and can inhibit enzymatic hydrolysis and fermentation processes [34].

3.1.3. Biological pretreatment

Biological pretreatment of lignocellulosic biomass involves using different types of microorganisms including fungi, bacteria, and actinomycetes [9]. These organisms have the ability to produce ligninolytic enzymes such as peroxidases (lignin peroxidase and manganese peroxidase) and laccases. These two groups of enzymes play significant role in lignin degradation during biological pretreatment. The most common microorganism for biological pretreatment is filamentous fungi. White-rot fungi have been identified as the most effective microorganism for the biological pretreatment of lignocellulosic biomass [35]. A number of white-rot fungi including *Phanerochaete chrysosporium*, *Pleurotus ostreatus*, *Cyathus stercoreus*, *Pycnoporus cinnabarinus*, *Ceriporia lacerata*, and *Ceriporiopsis subvermispora* are able to produce lignin degrading enzymes for the effective delignification of lignocellulosic biomass. Biological pretreatment does not generate toxic substances, is mild, requires low energy, and more environmentally friendly compared to other pretreatment techniques [23]. Nonetheless, the process is very slow and requires carefully controlled conditions as well as large space making it not attractive for commercial bioethanol production. Some microorganisms also tend to degrade cellulose and hemicellulose in addition to lignin [31].

Biological pretreatment may also be carried out with ligninolytic enzyme extracts. This has been reported to prevent degradation of carbohydrates that is associated with microbial pretreatment [31]. These enzymes are extracted from lignin degrading microorganisms, purified and used for the pretreatment process. Crude enzyme extracts have, however, been reported to contain other factors such as proteins and mediators. The presence of these factors enhance the activity of these enzymes making them more effective compared to purified ones. The major problem associated with enzymatic delignification is low enzyme production and activity. Enhancing the culturing conditions may however help to increase the activity and the yield of these enzymes [36].

The effect of pretreatment on biomass varies depending on the method and type of lignocellulosic biomass. Development of effective pretreatment conditions is thus crucial for converting weed biomass to bioethanol. To release monomer sugar units from weed biomass, researchers have studied the effect of different kinds of pretreatment on different types of weed biomass (**Table 2**). Ratsamee [10] pretreated purple guinea grass (*Panicum maximum* cv. TD 53) with dilute sulfuric acid (H_2SO_4) and calcium hydroxide ($Ca(OH)_2$) to improve cellulose digestibility. Pretreatment with the two chemicals resulted in a significantly higher glucose contents in the biomass after enzymatic hydrolysis. However, purple guinea grass biomass

pretreated with calcium hydroxide yielded slightly higher glucose concentration after hydrolysis. Wongwatanapaiboon [17] assessed the potential of bioethanol production from different types of grasses by pretreating them with alkaline peroxide (H_2O_2 + NaOH). Following alkaline peroxide pretreatment and enzymatic hydrolysis with cellulase and xylanase, total reducing sugar in the range of 521–559 mg/g biomass was obtained. Chandel [37] reported maximum total reducing sugar yields of 310 ± 9.80, 541.2 ± 9.53, and 646.23 ± 8.99 mg/g biomass after enzymatic hydrolysis of wild sugarcane (*Saccharum spontaneum*) biomass pretreated with dilute sulfuric acid (H_2SO_4), dilute sodium hydroxide (NaOH), and aqueous ammonia (aq. Ammonia), respectively. In an earlier research, pretreatment of *Achyranthes*

Weed biomass	Pretreatment conditions	Enzymatic hydrolysis	Sugars after pretreatment/ hydrolysis	Reference
Panicum maximum cv. TD53	3% H_2SO_4, autoclave at 121°C for 30 mins	Accellerase™ 1000 (9FPU/g)	10.1 g/L glucose	[10]
	4% $Ca(OH)_2$, autoclave at 121°C for 5 mins		11.9 g/L glucose	
Paspalum atratum	7.5% H_2O_2 + NaOH	Cellulase (60 U/g) + xylanase (1200 U/g)	506 mg/g biomass	[17]
Pennisetum purpureum Schum.			529 mg/g biomass	
Pennisetum purpureum cv. Mott			559 mg/g biomass	
Pennisetum purpureum × *Pennisetum americanum*			556 mg/g biomass	
Saccharum spontaneum	1.5% H_2SO_4 (v/v)	Cellulase (15 FPU/g)	310 ± 9.80 mg/g biomass	[37]
	1.0 M NaOH	Cellulase (25 FPU/g)	541.2 ± 9.53 mg/g biomass	
	15% aq. ammonia	Cellulase (25 FPU/g)	646.23 ± 8.99 mg/g biomass	
Achyranthes aspera	80% H_3PO_4	Cellulase (30 FPU/g) + β-glucosidase (60 U/g)	8.0 g/L glucose	[38]
Sida acuta	75% H_3PO_4		8.6 g/L glucose	
Arundo donax	1% (v/v) H_2SO_4, autoclave at 121°C for 30 mins followed by 1.5% NaOH + ultrasound irradiation	Cellulase (135 FPU/g) + Cellobiase (75 FPU/g)	724.0 mg/g biomass	[2]
Saccharum spontaneum			851.7 mg/g biomass	
Mikania micrantha			592.0 mg/g biomass	
Lantana camara			662.2 mg/g biomass	
Eichhornia crassipes			758.6 mg/g biomass	
Leucaena leucocephala	*Phanerochaete chrysosporium*	Cellulase (30 FPU/g)	1.2 g/L glucose	[39]

Table 2. Pretreatment and enzymatic hydrolysis of weed biomass.

aspera and *Sida acuta* with different concentrations of phosphoric acid (H_3PO_4) helped to increase glucose concentration (8.0 and 8.6 g/L, respectively) of the biomass after enzymatic hydrolysis with a combination of cellulase and β-glucosidase [38]. Preliminary studies on biological pretreatment of *Leucaena leucocephala* with *Phanerochaete chrysosporium* also resulted in an increase in glucose concentration (1.2 g/L) of pretreated biomass after hydrolysis with cellulase enzyme [39]. Borah [2] carried out acid hydrolysis with sulfuric acid (H_2SO_4) followed by delignification with sodium hydroxide (NaOH) and ultrasound irradiation of five weed species as feedstock for bioethanol production. After enzymatic hydrolysis, the average yield of total fermentable sugars (hexose and pentose) from all five weed species was reported to be 43.85 g/100 g of biomass, representing 27.36 g theoretical bioethanol yield. It can be inferred from **Table 2** that the optimum conditions of pretreatment differ significantly for each weed biomass.

3.2. Enzymatic hydrolysis

Pretreatment of lignocellulosic biomass is followed by acid or enzymatic hydrolysis to break down cellulose and sometimes hemicellulose into fermentable sugars such as glucose and xylose [12]. Enzymatic hydrolysis is however eco-friendly and preferred to the noneco-friendly harsh acid hydrolysis [33]. The total amount of fermentable sugars produced is dependent on the type of lignocellulosic biomass and efficiency of pretreatment process [12]. Enzymatic hydrolysis of biomass is carried out in different forms. In some cases, pretreated biomass is initially hydrolyzed by enzymes followed by fermentation of sugars to bioethanol in a process called, separate hydrolysis, and fermentation (SHF). This process requires two separate distinct process conditions for both enzymatic hydrolysis and fermentation. A major setback back to this process is the accumulation of sugar during enzymatic hydrolysis step, which can inhibit enzymatic activities [12]. The production of monomer sugars and fermentation of these sugars may also be carried together in a process known as simultaneous saccharification and fermentation (SSF) [11]. The tendency of monomer sugar accumulation is as less as individual sugars released are converted to bioethanol at the same time. This process may however be very complex with respect to process conditions, which can lead to a decrease in bioethanol yield. Specific operating conditions must therefore be established to enhance both enzymatic hydrolysis and fermentation processes [12]. An emerging method is consolidated bioprocessing (CBP) in which a microorganism or group of microorganisms are used to convert untreated biomass to bioethanol. The microorganism(s) have special inherent abilities to secret enzymes that degrade biomass and ferment sugars released to bioethanol. This method is very promising, however, research activities is still at an infant stage [12].

Cellulase enzymes are used for enzymatic hydrolysis of cellulose after pretreatment. Enzymes for hydrolysis may be obtained from commercial enzyme producers. In some cases, the enzymes may be produced, harvested, and use for hydrolysis. These enzymes are produced by both bacteria and fungi; however, most commercial cellulases are produced from fungi [33]. Cellulases are made up of three set of enzymes including endoglucanase (1,4-β-D-glucan glucanohydrolase, EC 3.2.1.3), exoglucanase (1,4-β-D-glucan cellobiohydrolyase, EC 3.2.1.91), and cellobiase (β-glucosidase; EC 3.2.1.21). Endoglucanase cuts cellulose chains into fragments

of glucose, cellobiose, and cellotriose while exoglucanase cleaves it into cellobiose units [11]. Cellobiase, however, breaks cellobiose units into glucose that can be fermented to bioethanol. Majority of cellulases obtained from fungi lacks β-glucosidase and must be supplemented with β-glucosidase during enzymatic hydrolysis to enhance efficiency [33]. Cellulase activity is dependent on the concentration and source. Different dosages of cellulases are used during enzymatic hydrolysis. This may depend on the composition of pretreated biomass as well as the type of pretreatment technique used. Enzymatic hydrolysis of cellulose requires mild conditions including pH of between 4.8 and 5.0 and temperature of approximately 50°C. High hydrolysis efficiency is however achieved with an optimized temperature, time, pH, enzyme load, and biomass concentration [4].

The hemicellulose component may also be hydrolyzed with hemicellulases into monomer sugars for fermentation to bioethanol [7]. Compare to cellulose, hemicellulose hydrolysis is very complex because of its composition (mixture of pentoses and hexoses). Multiple enzyme system including endo-xylanase, exo-xylanase, and β-xylosidase together with auxiliary enzymes α-arabinofuranosidase, α-glucuronidase, acetyl xylan esterase, and ferulic acid esterase are involved in hemicellulose hydrolysis [26].

Enzymatic cocktails comprising cellulases and hemicellulases have been used to hydrolyze various pretreated weed biomass for bioethanol production (**Table 2**).

3.3. Fermentation

Following enzymatic hydrolysis, the supernatant containing various sugars (pentoses and hexoses) is fermented to bioethanol. Different types of microorganisms including fungi and bacteria can be used to ferment sugars from weed biomass to bioethanol. *Zymomonas mobilis* [40], *Kluyveromyces* sp. [41], and *Saccharomyces cerevisiae* [4] are common microorganisms for fermentation of glucose to bioethanol. *S. cerevisiae* is the most common microorganism for commercial bioethanol production. However, *Pachysolen tannophilus*, *Pichia stipitis*, and *Candida shehatae* are well-known for their ability to ferment xylose to bioethanol [33]. However, the activity of *S. cerevisiae* is affected by several factors including high temperature, osmotic stress, bioethanol concentration, and contamination from bacteria [41]. These conditions inhibit microbial growth during fermentation process, thus affecting the yield of bioethanol production. Furthermore, the inability of *S. cerevisiae* to ferment pentoses also affects bioethanol yield during fermentation. However, studies are continuously being conducted to isolate and identified *S. cerevisiae* strains that are able to tolerate these stress conditions to improve bioethanol yield during fermentation. Microbial strains from *Pichia* sp., *Candida* sp., *Schizosaccharomyces* sp. and *Pachysolen* sp. have also been identified for fermentation of pentoses to bioethanol. Recombinant DNA technologies have been exploited to develop strains that are resistant to stress and also have the ability to ferment pentoses, all aimed at increasing bioethanol yield [4].

Fermentation of bioethanol is normally undertaken in a bioreactor with three major different processes namely batch, fed-batch, and continues [4]. During batch process of bioethanol production, the fermentation ingredients including substrate, culture medium, and nutrients

are fed to the bioreactor only at the start of the process. No feeding is done till the process is over after which bioethanol is harvested. The substrate, medium, and nutrients may however be fed and bioethanol removed continuously during continues fermentation process. The fed-batch process is a combination of the batch and continues processes. During this process, fermentation ingredients are continuously fed to the bioreactor but bioethanol is only harvested at the end of the process [26]. Bioethanol produced after fermentation is further purified through distillation and other cutting-edge processes such as pervaporation [7]. Different types of microorganisms have been studied for their ability to ferment weed biomass to bioethanol (**Table 3**).

Wongwatanapaiboon [17] reported a significantly higher bioethanol yield from alkaline peroxide pretreated *Vetiveria zizanioides cv.* Sri Lanka and *Vetiveria zizanioides* cv. Ratchaburi. Using the fermenting organisms *Saccharomyces cerevisiae* TISTR 5339 and *P. stipitis* CBS 5773, 32.72 and 30.95% of theoretical ethanol yield was reported for pretreated *Vetiveria zizanioides* cv. Sri Lanka and *Vetiveria zizanioides* cv. Ratchaburi biomass respectively. Tavva [18] reported similar bioethanol yield for *Torulaspora delbrueckii* R3DFM2, *Schizosaccharomyces*

Weed biomass	Pretreatment	Fermenting microorganism	EtOH production	Reference
Vetiveria zizanioides *cv.* Sri Lanka	Alkaline peroxide	*Saccharomyces cerevisiae* TISTR 5339 + *P. stipitis* CBS 5773	0.14 ± 0.01 g/L	[17]
Vetiveria zizanioides cv. Ratchaburi			0.14 ± 0.01 g/L	
Parthenium hysterophorus	Sulfuric acid	*Torulaspora delbrueckii* R3DFM2	0.24 g/g biomass	[18]
		Schizosaccharomyces pombe R3DOM3	0.27 g/g biomass	
		Saccharomyces cerevisiae R3DIM4	0.27 g/g biomass	
Saccharum spontaneum	Aqueous ammonia	*Pichia stipitis* NCIM3498	0.40 ± 0.01 g/g biomass	[37]
	Sulfuric acid		0.38 ± 0.02 g/g biomass	
	Sodium hydroxide		0.39 ± 0.02 g/g biomass	
Lemna minor	Alkaline	*Saccharomyces cerevisiae*	0.218 g/g biomass	[13]
Lemna gibba			0.197 g/g biomass	
Pistia stratiotes			0.215 g/g biomass	
Eichhornia sp			0.189 g/g biomass	
Pennisetum polystachion	Sodium hydroxide	*Saccharomyces cerevisiae* (TISTR 5596)	16.0	[42]
Panicum maximum cv. TD 53	Calcium hydroxide	*Saccharomyces cerevisiae* TISTR 5596	5.9 g/L	[10]

Table 3. Ethanol production from weed biomass.

pombe R3DOM and *Saccharomyces cerevisiae* R3DIM4 fermentation of sulfuric acid pretreated *Parthenium hysterophorus*. The efficiency of bioethanol production by the three microbial strains was reported as 78.84, 87.82, and 87.17%, respectively. Chandel [37] used *Pichia stipitis* NCIM3498 to ferment hydrolyzate obtained from aqueous ammonia, sulfuric acid and sodium hydroxide pretreated *Saccharum spontaneum*. The results show maximum bioethanol production from hydrolyzate for all the pretreated biomass. Gusain and Suthar [13] converted alkaline pretreated aquatic weeds into bioethanol using *Saccharomyces cerevisiae*. Bioethanol yields of between 0.189 and 0.218 g/g biomass were reported for the four different species of aquatic weeds. Prasertwasu [42] fermented hydrolyzate from sodium hydroxide pretreated *Pennisetum polystachion* with *Saccharomyces cerevisiae* (TISTR 5596) and reported high bioethanol yield after 24 hours. Ratsamee [10] also reported maximum bioethanol yield after fermenting hydrolyzate from calcium hydroxide pretreated *Panicum maximum* cv. TD 53 with *Saccharomyces cerevisiae* TISTR 5596 for 48 hours.

4. Conclusion and future perspectives

Weed biomass is a promising feedstock for economic bioethanol production. The abundance of weed biomass worldwide is an assurance of its sustainability as a feedstock. Current research on the conversion of weed biomass to bioethanol is focused on pretreatment techniques. Different pretreatment techniques have been explored to convert weed biomass into bioethanol. Maximum bioethanol yields have been reported after fermentation of hydrolyzates from pretreated weed biomass. However, current technologies are still inadequate for bioethanol production from weed biomass to compete with starch and sugar based bioethanol in terms of production yield and cost. Production of cellulosic bioethanol from weedy plants is only at the laboratory scale. Further research to establish cost effective and efficient conversion processes including pretreatment technique(s) for a wide range of weed biomass is needed. Predictive models will also aid in the selection, design, optimization, and process control pretreatment technologies that match biomass feedstock with appropriate method and process configuration. On the other hand, active research is going on to ensure commercial production of bioethanol from weed biomass. This includes improvements in pretreatment technologies, specific activities of enzymes as well as isolation of new fermentation microorganism from natural environment. With strong support from various governments, bioethanol production from weed biomass will play a major role in meeting energy demand globally.

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Conflict of interest

The author has no conflict of interest to declare.

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Section 4

Recent Approaches for Increasing Fermentation Efficiency of Lignocellulosic Ethanol

Chapter 6

Progress in Second Generation Ethanol Production with Thermophilic Bacteria

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Additional information is available at the end of the chapter

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Abstract

Thermophilic bacteria have gained increased attention as prospective organisms for bioethanol production from lignocellulosic biomass due to their broad substrate spectra including many of the hexoses pentoses, and disaccharides found in biomass and biomass hydrolysates, fast growth rates, and high tolerance for extreme cultivation conditions. Apart from optimizing the ethanol production by varying physiological parameters, genetic engineering methods have been applied. This review focuses upon those thermophilic anaerobes recognized as being highly ethanologenic, their metabolism, and the importance of various culture parameters affecting ethanol yields, such as the partial pressure of hydrogen, pH, substrate inhibition, and ethanol tolerance. Also, recent developments in evolutionary adaptation and genetic engineering of thermophilic anaerobes are addressed.

Keywords: thermophilic bacteria, biofuel, bioethanol, lignocellulosic biomass, bioprocessing, genetic engineering

1. Introduction

The production of sustainable biofuels have increased in recent years because of a driving need for highly renewable and environmentally friendly energy carriers with bioethanol, biobutanol, biomethane, and biohydrogen being the most widely investigated. In order to meet international obligations to address climatic and geopolitical issues, many governments have set production targets as a response to meet these mandates. Ethanol production has been the main aim of many authorities as a suitable biofuel, for instance, a target set by the EU necessitates that 20% of energy production must be from renewable sources and energy

efficiency must increase by 20% while greenhouse gases must decrease by 20% by 2020 [1]. This has led to a dramatic increase in the production of bioethanol from 48 billion liters in 2007 to 2.6 billion liters in 2017 [2]. Both the United States and Brazil are by far the largest producers of bioethanol although the vast majority of ethanol produced is from first generation biomasses such as sucrose-rich sugarcane and easily fermentable starch-rich crops such as corn. However, there is a growing concern over the use of these feedstocks because they are food and feed related and thus in a direct competition with food production [3–5]. In addition, increased concern has been regarding the negative impact on agricultural areas used for the production of this biomass.

Production of bioethanol by second-generation non-food (lignocellulosic) biomass, such as agricultural residues, addresses some of the above mentioned environmental concerns although poses several challenges as a raw material for bioprocessing. Second generation biomass requires extensive and costly chemical or physical pretreatment in addition to enzymatic treatment processes which negatively impacts its industrial feasibility. Lignocellulosic biomass is often difficult to degrade due to the lignin sheath and the highly crystalline nature of cellulose [6]. In addition to cellulose, lignocellulose is also composed of lignin and hemicelluloses of which the latter contains a plethora of monosaccharides with various connectivities and varying degrees of branching. Therefore, processing lignocellulosic biomass and subsequent fermentation of the liberated sugars to ethanol has proven to be a major complication for large-scale production [3–5].

To address the challenges posed by lignocellulosic biomass, fermentative organisms that can meet these process needs will help improve the feasibility of bioethanol production from lignocellulosic biomass. At present, the majority of bioethanol is produced using well-established mesophilic organisms despite some of the inherent advantages to the use of thermophilic microorganisms such as higher operating temperatures and utilizing a non-glucose hexoses and pentoses such as xylose and arabinose. This work focuses on the physiology of ethanol-producing thermophiles with an emphasis on their salient features relevant to the utilization of lignocellulosic biomass as well as the use of genetic engineering to improve their potential for bioethanol utilization.

2. Selected aspects of ethanol production from lignocellulosic biomass

For the fermentative production of ethanol from biomass to be commercially successful, several key processes and organisms need to be considered [3, 4, 7–9]. These process requirements needed to simultaneously consider two viewpoints: the physiological properties of the ethanologen used and process requirements. Concerning organism requirements, an ideal strains should be homoethanologenic, with high productivity (> 1/g/L/h), have broad substrate spectra and high tolerance of ethanol, inhibitory compounds and high initial substrate concentrations. Other key factors include high cellulolytic activity, simple nutritional needs, low biomass production and ease of genetic manipulation. Ideally, a single organism that

is both highly ethanologenic and cellulolytic would be ideal for consolidated bioprocessing (CBP) although co-cultures of organisms together fulfilling these requirements may also be considered in a simultaneous saccharification and fermentation (SSF) setup. To adequately meet the process requirements, ethanol yields should reach a minimum of 90% of theoretical, achieve high ethanol titers (> 5% v/v), have a minimum number of process steps, and require minimal or no process cooling. Additionally, cells should be robust enough to be recyclable and substrates co-fermented and pretreatment should be limited or excluded.

No single wild type organism possesses all these features. Although genetic manipulation has yielded only modest improvements for ethanologens although transformants are not always stable [9–11]. Many thermophilic clostridia have much broader substrate spectrum as compared to standard ethanologens such as *Saccharomyces cerevisiae* and *Zymomonas mobilis*. Additionally, cultivations of thermoanaerobes do not require extensive agitation or temperature control of the fermentation vessels and these often tolerate extremes of pH and salt concentrations during fermentation with minimal need for nutrient supplementation [4]. The mixing of reaction vessels and pumping of liquids are easier at elevated temperatures due to reduced viscosity as well increased substrate solubility [12].

3. Ethanol producing thermophilic anaerobes

While more than 300 species of thermophilic anaerobic bacteria have described as of 2008 from a wide range of environs with new species being continuously discovered. Thermophilic anaerobes have been isolated from a diverse range of environments [13] including deep-sea vents [14], geothermal areas [15–17], compost piles [18], municipal solid waste or sewage sludge [19], oil wells [20], and canned goods [21]. Most thermophilic microorganisms are either obligatory or facultative anaerobic, likely due to the limited availability of oxygen and highly reducing nature of geothermal features [22]. The majority of the those that are highly ethanologenic that have been described in the literature are often strict anaerobes within the genera of *Clostridium*, *Caloramator*, *Caldanaerobacter*, *Thermoanaerobacter*, or *Thermoanaerobacterium* [3, 23].

The highly polyphyletic genus *Clostridium* within the class *Clostridia* (family *Clostridiaceae*, order *Clostridiales*) currently has greater than 200 species with standing in nomenclature although only about 15 are strains within the genus are thermophilic [24, 25] usually with temperature optima for growth between 45 and 65°C although several strains reportedly grow at temperatures as high at 75°C. All species within the genus are strictly anaerobic and can typically be isolated from a broad range of nutrient-rich environments [26]. Many members within the genus can hydrolyze cellulose and produce ethanol, making them target of extensive research on biofuel production from complex [27, 28].

C. thermocellum is a thermophilic species that degrades crystalline cellulose using a cellulosome which is comprised of a complex arrangement of glycohyldrolases attached to a scaffold-like matrix [6]. Several other members of *Clostridium* have glycohydrolases including *C. acetobutylicum* [29, 30] and *C. cellulovorans* [31]. Ethanol yields by *Clostridium* species are often moderate and vary depending on environmental conditions with other organic acids,

including lactic acid, being common end-products. Examples of ethanol production from sugars by members of the genus include *Clostridium thermocellum* [32, 33] and *Clostridium* strain AK1 with 1.5 mol ethanol/mol glucose [34].

The genus *Thermoanaerobacterium* is comprised of thermophilic anaerobes which fall within Cluster V of *Clostridia* [35]. Currently, the genus currently consists of nine species and *T. thermosulfurigenes* is the genus type species [36]. Species within *Thermoanaerobacterium* are usually amylo- and xylanolytic with a T_{opt} between 55 and 65°C and have been isolated from a diverse range of environments including geothermal features and from heat-treated canned foods [21, 37, 38]. They catabolize a broad range of hexoses, pentoses, and disaccharides to a mixture of ethanol, acetate, lactate, hydrogen, and CO_2. One challenge for these organisms is achieving good ethanol yields from high initial substrate concentrations which considerably lower ethanol yields. Examples of ethanol production from sugars by members of the genus include *Thermoanaerobacterium saccharolyticum* with 1.18 mol ethanol/mol glucose [37] and *Thermoanaerobacterium* strain AK17 with 1.50 and 1.33 mol ethanol/mol glucose and xylose, respectively [39].

Thermoanaerobacter species have similar physiological characteristics as *Thermoanaerobacterium* species; all species within the genus are highly saccharolytic and produce end-products which include ethanol, acetate, lactate, alanine, CO_2, and H_2. Nineteen species and five subspecies belong to the genus [24, 25]. The main difference between *Thermoanaerobacter* and *Thermoanaerobacterium*, is that the majority of *Thermoanaerobacter* species produce H_2S from thiosulfate whereas *Thermoanaerobacterium* produces sulfur [37]. Additionally, the temperature optima for *Thermoanaerobacter* species (65–75°C) are higher as compared to *Thermoanaerobacterium* species (55–65°C). The type species, *Thermoanaerobacter ethanolicus* and several other species within the genus, have been extensively studied for ethanol production [40–43]. High ethanol yields have been observed by several members of the genus including *T. pseudoethanolicus*, *T. mathranii*, *T. pentosaceus*, *Thermoanaerobacter* strain AK5, and *Thermoanaerobacter* strain J1 [17, 38, 44–46]. The ethanol yields, however, vary extensively depending on culture conditions [17, 38]. Recently, *Thermoanaerobacter subterraneous* was moved to the genus *Caldanaerobacter* that currently comprises two species: *Caldanaerobacter subterraneous* (and its four subspecies) and *Caldanaerobacter uzonensis* [24, 25]. Other representative examples of thermophilic ethanologenic bacteria can be found within the genera of *Caldicellulosiruptor* [47], *Caloramator* [48], *Geobacillus* [49], *Caloramator boliviensis*, for example, produces 1.53 mol ethanol/mol xylose [50].

4. Culture parameters

Most saccharolytic thermophiles use the Embden-Meyerhof-Parnas (EMP) pathway [5, 51] but do not use pyruvate decarboxylase for converting pyruvate to acetaldehyde as do yeasts. The theoretical yields of ethanol from 1 mol of hexose and pentose are 2.0 and 1.66 mol, respectively [5]. There are several routes from pyruvate to other end-products than ethanol. The following equations show the most common end-products from glucose with anaerobic bacteria:

1. 1 Glucose → 2 Ethanol +2 CO_2
2. 1 Glucose → 2 Lactate
3. 1 Glucose → 2 Acetate +2 CO_2 + 4H_2
4. 1 Glucose → 1 Butyrate +2 CO_2 + 2 H_2
5. 1 Glucose +2 NH_4^+ → 2 Alanine

Butyrate is not a commonly observed end-product with thermophilic anaerobes and alanine is not commonly assayed. The distribution of end products are known to be influenced by various factors which can be of direct relevance for the production of ethanol; these conditions include the substrate types and concentrations, the partial pressure of hydrogen, pH, and temperature. Some of these factors are discussed in detail below.

4.1. Partial pressure of hydrogen

Early observations of the influence of hydrogen concentrations on the end-product formation of *Thermoanaerobacter ethanolicus* were reported in 1981 [15]. Higher partial pressure of hydrogen (pH_2) leads to increased ethanol production and less acetate production from glucose fermentations [15, 38, 46]. Strict anaerobes produce H_2 either via pyruvate ferredoxin oxidorecutase or NAD(P)-dependent oxidoreductase [52]. It has been well established that the high concentrations affects mesophilic bacteria more severely than thermophiles because the NADH ferredoxin oxidoreductase (FNOR) that converts NADH to Fd_{red} is more strongly inhibited. The reduction potential is −400 mV for the Fd_{red}/Fd_{ox} couple but −320 mV for the $NADH/NAD^+$ couple [52, 53]. Therefore, at low temperatures, elevated hydrogen concentrations inhibit H_2 evolution at much lower concentrations as compared to at high temperatures. Microorganisms respond to this by directing their reducing equivalents to other more favorable electron acceptors and consequently produce reduced products such as ethanol and lactate. In nature, hydrogen accumulation usually does not occur because of hydrogen-utilizing organism such as methanogens and sulfate-reducers, allowing for a complete catabolism of glucose to end-products. However, batch fermentation with monocultures allows hydrogen to accumulate leading to a change in end production profile in some *Thermoanaerobacter* species [15, 38, 41]. For instance, during degradation of glucose and xylose, the major end-product for *Thermoanaerobacter brockii* was ethanol [54]. Under hydrogen scavenging conditions, however, the flow of electrons from glucose degradation was directed away from ethanol but towards acetate with extra ATP produced. Several experiments using different liquid-to-gas ratios have revealed that changes in end-product formation occur during hydrogen accumulation among species of *Clostridium, Thermoanaerobacter,* and *Caloramator*. Hydrogen accumulation in these cultures can either change the carbon flow to more reduced end-products or inhibit substrate degradation. The inhibition observed can be either direct, inhibiting the hydrogenases, or indirect by productions of acids, lowering the pH in a closed system, and thus stopping further degradation of the substrates.

4.2. Substrate loadings

In natural environments of thermophilic bacteria, the concentration of sugars is relatively low. It is thus not surprising that most thermophilic bacteria are inhibited at relatively low (often between 10 and 30 mM) initial substrate concentrations as compared to yeasts and *Z. mobilis* [4, 38, 39, 46]. This inhibition may be caused by accumulated hydrogen or by acid accumulation and pH drop, or it could also be an intriguing factor for thermophiles. *Thermoanaerobacter*, strain J1, has been shown to be very tolerant towards high sugar concentrations [17]. This high ethanol producing thermophile produces up to 1.7 mol ethanol/mol glucose at 100 mM initial glucose concentration. Recent work on *Thermoanaerobacter pentosaceus* showed a complete removal of xylose at 13.3 mM initial concentrations but only about 30% removal at 10 times higher concentrations [55]. Additionally, the ratio of ethanol to acetate and lactate decreased by a factor of more than six resulting in dramatic decrease in ethanol yields.

4.3. Ethanol tolerance

One of the most important traits for good ethanol producers is their ethanol tolerance. For an economic ethanol recovery to occur, using classical downstream processes, the microorganism should grow and produce ethanol in the presence of at least 4% (v/v) ethanol [56]. It is well known that growth rates of many organisms decrease markedly with increasing ethanol concentrations because of leaky membranes resulting in loss of energy during cellular metabolism and finally cell lysis. Yeasts and *Z. mobilis* tolerate much higher ethanol concentrations as compared to thermophiles mainly due to their composition of fatty acid in their cell membrane.

Studies on ethanol tolerance of wild-type species of thermophiles show tolerance between 0.5 and 3.0% (v/v) [4, 46, 57, 58]. Substantial efforts to increase ethanol tolerance of wild type thermophiles, have been done. The highest ethanol tolerance observed for a thermophile has been with a mutant strain of *Thermoanaerobacter ethanolicus* (12.7% v/v) [28]. However, later studies with one of its mutant derivatives, JW200 Fe 4, showed much less tolerance [59]. *Thermoanaerobacter* BG1L1 showed 8.3% (v/v) tolerance in continuous culture studies [43] on xylose. Increased ethanol tolerance was also observed with *Thermoanaerobacter thermohydrosulfuricus* 39E by successively sub-culturing the strain to higher ethanol concentrations [57]. The resulting mutant strain 39EA tolerated 10.1% (v/v ethanol) at 45°C but only 2.6% (v/v) at 68°C. Additionally, the ethanol yields dropped considerably.

4.4. Other culture parameters

Other environmental factors of importance for thermophilic bacteria is their pH and temperature growth optimum, their tolerance towards inhibitory compounds like furfuraldehyde and 5-hydroxymethyl-furfuraldehyde (5-HMF) and their need for trace elements and vitamins often originating from complex medium supplements like yeast extract.

5. Production of ethanol from lignocellulose

Production of bioethanol from lignocellulosic biomass by wild type thermophilic bacteria has been widely reported in the literature where the focus has been mostly on *Clostridium*

thermocellum and species within the genera *Thermoanaerobacterium* and *Thermoanaerobacter*. However, there is a large variation in the type and concentration of biomass used, fermentation processes (batch, semi-batch, continuous), pretreatment methods as well as whether pure or mixed cultures are used.

The theoretical maximum yield of ethanol obtained from glucose fermentation is 0.51 g ethanol/ g glucose (2 mol ethanol/mol glucose or 11.1 mM/g). Unsurprisingly, considering the complex structure of lignocellulosic biomass, ethanol yields are usually considerably lower from such substrates as seen in **Table 1**.

Organisms	Substrate	Fermentation mode	Pre-treatment	Ethanol yields (mM/g)	Temp (°C)	References
Clostridium thermocellum	Avicel (2.5 g/L)	Batch	A	5.00	60	[60]
Clostridium thermocellum	Whatman paper (8.0 g/L)	Batch	None	7.20–8.00	60	[61]
Clostridium thermocellum	Paddy straw (8.0 g/L)	Batch	None	6.10–8.00	60	[61]
Clostridium strain DBT-IOC-C19	Avicel (10.0 g/L)	Batch	None	3.26	60	[62]
Clostridium strain AK1	Hemp (5.0 g/L)	Batch	A/Alk	3.5	50	[34]
Thermoanaerobacter pentosaceus	Rapeseed straw (5 0 g/L)	Con	Alk	1.40	70	[55]
Thermoanaerobacter mathranii	Wheat straw (6.7 g/L)	Batch	WO/E	2.61	70	[63]
Thermoanaerobacter ethanolicus	Beet molasses (30.0 g/L)	Batch	None	4.81	65	[64]
Thermoanaerobacter BG1L1	Corn stover, wheat straw (25.0–150.0 g/L)	Batch	WO/E	8.50–9.20	70	[42]
Thermoanaerobacter BG1L1	Wheat straw (30.0–120.0 g/L)	Batch	WO/E	8.50–9.20	70	[65]
T. ethanolicus	Wood HL (8.0 g/L)	Batch	E	3.30–4.50	70	[66]
Thermoanaerobacter strain AK5	Grass (4.5 g/L)	Batch	A/E	4.31	65	[38]
Thermoanaerobacter strain J1	Hemp (4.5 g/L)	Batch	A/E	4.3	65	[17]
T. saccharoylticum	Xylan (10.0 g/L)	Batch	WO	6.30	60	[67]
Thermoanaerobacterium strain AK17	Grass (2.5 g/L)	Batch	A/Alk/E	5.5	60	[39]

Cultivation were either in batch or continuous (con). Ethanol yields given in mM/g substrate degraded as well as substrate concentrations and incubation temperature are also shown. A—acid; Alk—alkaline; E—enzymatic; and WO—wet oxidation.

Table 1. Examples of ethanol production from lignocellulosic biomass by thermophilic bacteria.

Early experiments on ethanol production from lignocellulose included as the ethanol-producing organisms *Thermoanaerobacter ethanolicus* and *Clostridium thermocellum* with hemicellulose from birch- and beechwood as a feedstock [66]. *Clostridium thermocellum* produced between 7.2 mM ethanol /g and 8.0 mM/g from avicel and Whatman paper, respectively. Studies of ethanol production from paddy straw, sorghum stover and corn stubs, pretreated with alkali showed similar results [68]. However, these results were obtained with relatively low substrate loadings (8.0 g/L) but later studies showed that increased substrate loadings lowered the ethanol yields considerable [69]. The highest ethanol yields reported from lignocellulose are by *Thermoanaerobacter* BG1L1 grown on corn stover and wheat straw [42, 43] that were pretreated with acid or wet oxidation, or 9.2 mM/g for biomass hydrolysates. *Thermoanaerobacterium* strain AK17 showed ethanol yields of 2.0 (paper) mM/g, 2.9 (grass) mM/g and 5.8 (cellulose) mM/g biomass [23]. Optimization experiments showed an increase in ethanol yields on grass and cellulose up to 4.0 and 8.6 $mM \cdot g^{-1}$, respectively. The main culture factors increasing ethanol yields was obtained by lowering of the substrate concentration from 7.5 to 2.5 g/L [39]. Recent investigations on two *Thermoanaerobacter* strains, AK5 and J1, showed promising results from various types of hydrolysates made from chemically and enzymatically pretreated lignocellulosic biomass [17, 38] (**Table 1**).

6. Evolutionary adaptation and genetic engineering of thermophiles

The thermophilic anaerobes mentioned in the previous sections make logical targets for genetic improvement due to their ability to produce ethanol from a wide range of substrates as evidenced by acceptable yields on lignocellulosic biomass. There are two general strategies for enhancing characteristics for ethanol production by wild type microorganisms: evolutionary adaptation or genetically modify the organisms. Early work often used classical methods such as the selection of clones and nonspecific mutagenesis to improve ethanol production [70]. These methods are time-consuming, and genetic modification is not without drawbacks as modified strains can exhibit poor growth and unexpected shifts in end-product formation. More recent work has focused more on modern techniques in molecular biology discussed herein.

6.1. Evolutionary adaptation

One of the major drawbacks of using thermophiles for the production of ethanol is their low substrate and ethanol tolerance. The use of classical evolutionary adaptation methods, such as non-specific mutagenesis and artificial selection, to enhance specific traits of microorganisms for industrial bioethanol production have been applied to thermophilic anaerobes on a limited basis. Examples of adaptation methods on three new mutant strains of *Thermoanaerobacter ethanolicus* were obtained by selection of pyruvate and iron deprivation [51] leading to enhanced ethanol tolerance (10% v/v) at substrate concentrations above 10 g/L. *Clostridium thermocellum* showed increased ethanol tolerance (up to 5% v/v) by stepwise increasing and transferring cultures to increased ethanol concentrations [71]. *Thermoanaerobacter pentosaceus* has been gradually adapted

to higher substrate concentrations and demonstrated higher ethanol tolerance and substrate utilization [72]. Thus, evolutionary adaptation, may still be used for evolving of wild type strains and further improving GM strains to meet requirements for tolerance to high ethanol titers, improve substrate utilization, and potentially resistance to inhibitory compounds generated during biomass pretreatment such as 5-HMF and fufuraldehyde.

6.2. Genetic engineering

Despite other promising features, one of the main drawback of most wild type thermophiles is their production of mixed end-products resulting in lower ethanol yields and the fact that highly ethanologenic organisms are not natively cellulolytic and *vice versa*. Two main strategies have been used to metabolically engineer thermophilic organisms for consolidated bioprocessing (CBP). The first strategy is to increase the ethanol yields of cellulase-producing organisms while the other is to express enzymes for biomass deconstruction in highly ethanologenic microorganisms [73, 74]. The first approach involves increasing ethanol yields by redirecting the flow of carbon and electrons which involves eliminating other potential fermentation products. Obvious targets include knocking out acetate and lactate pathways. The second approach involves addition of cellulolytic genes to the genome of a good ethanol producing bacterium.

The first thermophilic bacterium to be genetically modified to increase ethanol yields was *Thermoanaerobacterium saccharolyticum* in 2004 [75]. Since then, several other ethanologenic thermophiles have been genetically modified to increase ethanol titers and minimize the formation of other end-products (**Table 2**).

Deletion of genes involved to the production of various end-products to increase ethanol production capacity is the most obvious way to increase ethanol titers. This has been done by knocking out lactate dehydrogenase in *Thermoanaerobacterium saccharolyticum* [73, 82], *Thermoanaerobacter mathranii* [79], *Clostridium thermocellum* [83] and *Geobacillus thermoglucosidasius* [78].

Wild type *Clostridium thermocellum* produces a mixture of ethanol, acetate, lactate, hydrogen, and carbon dioxide [84] from cellulose and other substrates. The first successful transformation of the species was performed in 2006 [85], later on leading to the development of genetic systems to knock out the *pta* gene and thus acetate formation [85]. However, growth of the resultant strain was abnormal although cellulase active remained intact. Later work on *C. thermocellum* showed improved ethanol yields in an adapted strain (Δ*hpt*, Δ*ldh*, Δ*pta*) lacking acetate and lactate pathways and was successfully used in co-culture with *Thermoanaerobacterium saccharolyticum* [85].

Early work on *Thermoanaerobacterium saccharolyticum* were performed by using electroporation and shuttle vectors [86], but later on this strain has been further modified by inserting a cellobiohydrolase gene from *Clostridium thermocellum* into its genome [77]. Also a *ldh* gene knock out was done using *Thermoanaerobacterium saccharolyticum* [75] and then a double knock out of both *ldh* and *ak* [73]. The knocking out of acetate production led to less available energy,

Strain	Genotype	Substrate	Mode	Ethanol yields (mol/mol)	References
C. thermocellum	Δ*pyrF*, Δ*pta*::gapDHp-cat	Cellobiose (5.0 g/L)	Batch	0.59	[76]
C. thermocellum	Δ*pyr*F, Δ*pta*::gapDHp-cat	Avicel (5.0 g/L)	Batch	0.71	[76]
C. thermocellum adhE*(EA) Δldh	Δ*hpt*, Δ*ldh*	Cellobiose (5.0 g/L)	Batch	0.37	[77]
C. thermocellum	Δ*hpt*, Δ*ldh*, Δ*pta* (evolved)	Avicel (19.5 g/L)	Batch	1.08	[77]
C. thermocellum/*T. saccharolyticum*	Δ*hpt*, Δ*ldh*, Δ*pta* (evolved) and Δ*pta*, Δ*ack*, Δ*ldh*	Avicel (19.5 g/L)	Batch	1.26	[77]
T. saccharolyticum TD1	Δ*ldh*	Xylolse (5.0 g/L)	Batch	0.98	[77]
T. saccharolyticum ALK2	Δ*pta*, Δ*ack*, Δ*ldh*	Cellobiose (70.0 g/L)	Con	ND	[73]
T. saccharolyticium HK07	Δ*ldh* , Δ*hfs*	Cellobiose (1.8 g/L)	Batch	0.86	[74]
T. saccharolyticium M0355	Δ*ldh* , Δ*ack*, Δ*pta*	Cellobiose (50.0 g/L)	Batch	1.73	[74]
T. saccharolyticum M1051	Δ*ldh* , Δ*ack* Δ*pta*, *ure*	Cellobiose (27.5 g/L)	Batch	1.73	[74]
G. thermoglucosidasius TM242	Δ*ldh*-, *pdh* up, *pfl*B-	Glucose (34.0 g/L)	Batch	1.73	[78]
G. thermoglucosidasius TM242	Δ*ldh*-, *pdh* up, Δ*pflB*-	Glucose (34.0 g/L)	Batch	1.84	[78]
G. thermoglucosidasius TM242	Δ*ldh*-, Δ*pdh* up, Δ*pflB*-	Xylose (29.0 g/L)	Batch	1.37	[78]
T. mathranii BG1L1	Δ*ldh*	Wheat straw (30-120 g/L)	Con	1.53–1.67	[65]
T. mathranii BG1G1	Δ*ldh*, *GldA*	Glucose + glycerol (5.0 g/L)	Batch	1.68	[79]
T. mathranii BG1G1	Δ*ldh*, *GldA*	Xylose + glycerol (5.0 g/L)	Batch	1.57	[79]
T. mathranii BG1G1	Δ*ldh*, *GldA*	Xylose + glycerol (12.8 and 7.2 g/L)	Con	1.53	[79]
Thermoanarobacter Pentocrobe 411	Δ*ldh*, Δ*ack*, Δ*pta*	Wheat straw (65 g/L)	Con	1.84	[80]
C. bescii JWCB018	Δ*ldh*-	Celo (10 g/L)	Batch	0	[81]
C. bescii JWCB032	Δ*ldh*-, *adhE*+	Celo (10 g/L)	Batch	0.66	[81]
C. bescii JWCB049	Δ*pyrFA*, Δ*ldh*-	Celo (10 g/L)	Batch	0.54	[81]
C. bescii JWCB054	Δ*pyrFA*, Δ*ldh*-	Celo (10 g/L)	Batch	0.28	[81]

ack—acetate kinase; *GldA*—glycerol dehydrogenase A; *hfs*—hydrogenase; *hpt*—hypoxanthine phosphoribosyl transferase; *pdh*—pyruvate decarboxylase; *pyrF*—orotidine-5-phoshate decarboxylase; *pfl*—pyruvate formate lyase; and *ure*—urease.

Table 2. Ethanol yields of genetically engineered thermophilic bacteria from different substrates and fermentation conditions.

thus less cell biomass and increased ethanol yields, both from glucose and xylose. Another double knock out of *Thermoanaerobacterium saccharolyticum* focused on the electron transfer system of the bacterium [74]. The *hfs* gene cluster, which codes for hydrogenase, and the *ldh* gene were knocked out resulting in a considerable increase in ethanol (44%) production as compared with the wild type.

Thermoanaerobacter mathranii has been modified and used in several investigations. The first mutant generated was BG1L1 by knocking out *ldh* resulting in a more than two-fold increase in ethanol production as compared with the wild type [87]. This strain showed good ethanol yields from undetoxified pretreated corn stover and wheat straw [42, 43]. Further manipulation of this strain involves overexpression of NAD(P)H-dependent alcohol dehydrogenase, resulting in the strain BG1E1. Clearly, this enzyme is of great importance for ethanol production and its overexpression resulted in higher ethanol yields [79]. The electron balance for sugar degradation was additionally focused upon with this strain when mannitol, which is more reduced than glucose and xylose, was used as a substrate [87] and this resulted in higher ethanol yields. The BG1G1 strain of *Thermoanaerobacter mathranii* was developed which included the insertion of a NAD^+-dependent glycerol dehydrogenase which increased ethanol yields by 40% greater than the type strain. Additionally, the strain utilized the highly reduced glycerol and co-metabolism of glycerol and sugars.

Recently, the highly ethanologenic strain *Thermoanaerobacter* BG1 "Pentocrobe 411" was genetically engineered by knocking out lactate dehydrogenase, phosphotransacetylase, and acetate kinase [80]. Pentocrobe 411 achieved very high ethanol titers (1.84 to 1.92 mol ethanol/mol hexose equivalent) nearing the maximum theoretical yield from hexoses and pentoses on various pretreated biomass in continuous culture.

Thermophilic bacteria within the genus of *Geobacillus* have also attracted increased interest due to their ethanol production capacity. *Geobacillus* strains are facultative anaerobes and can ferment various sugars to pyruvate by pyruvate dehydrogenase to acetyl-Coenzyme A) [78]. Under aerobic conditions, however, pyruvate formate lyase is used and a variety of end-products are formed. A research group led by Cripps manipulated *Geobacillus thermoglucosidasius*, producing variant with upregulated pyruvate dehydrogenase expression under anaerobic conditions in a strain lacking lactate dehydrogenase activity [78]. Several mutants were developed (TM89; *ldh* knockout; TM180; *ldh* knockout and upregulated *pdh*; TM242; *ldh*, upregulated *pdh* and *pfl*). The TM180 strain produced 1.45 mol ethanol/mol hexose (the wild type produced 0.39 mol ethanol/mol hexose and TM89 produced 0.94 mol ethanol/mol hexose). The triple mutant TM242 produced 1.65 mol ethanol/mol hexose. This mutant also showed good yields on xylose (1.33 mol ethanol/mol xylose) and good productivity rates. *Geobacillus thermoglucosidasius* has recently been genetically modified by expressing pyruvate decarboxylase from *Gluconobacter oxydans* [88]. Ethanol yields obtained were as high as 1.37 mol ethanol/mol glucose.

A natural target for the strategy of converting a cellulolytic organism into a good ethanol producer would be members of the genus of *Caldicellulosiruptor* which has several cellulolytic members although none are good ethanol producers. Recent work with *Caldicellulosiruptor bescii*, a naturally cellulolytic organism, has produced ethanol producing strains [89–93].

The type strains of *C. bescii* typically yield a mixture of lactic and acetic acid in addition to hydrogen and CO_2 as end-products although other strains within the genus of *Caldicellulosiruptor* have been noted to produce low ethanol titers. Work by Cha [89] deleted the gene coding for lactate dehydrogenase by introducing a non-replicating plasmid via marker replacement. The resultant knockout strain did demonstrate increased biomass yield as well as acetate and hydrogen production with no lactate production when grown on cellobiose and lactose as well as switch grass hydrolysates. Subsequent work by Chung [81] inserted a NADH-dependent *adhE* gene (from *Clostridium thermocellum*) into the *ldh* mutant (JWCB018) resulting in strain *C. bescii* JWCB032. The resultant *ldh*$^-$ *adhE*$^+$ strain yielded less acetate (4.3 mM) but produced 14.8 mM of ethanol from 29.2 mM cellobiose or 12.7% of the theoretical yield. It should be noted that this strain only used a small portion (4.4 mM of 29.2 mM cellobiose) provided and not produce ethanol above 65°C. Work by Cha [89] and Chung [93] introduced the alcohol dehydrogenase genes (*adhB* and *adhE*) from *Thermoanaerobacter pseudoethanolicus* into the *ldh* deficient strain. The two resultant strains yielded ethanol at temperatures greater than 65°C although titers were lower than the aforementioned strain JWCB032 (*ldh*$^-$ *adhE*$^+$). The *C. thermocellum* strain with *adhB* only produced 1.4 mM ethanol on avicel and 0.4 mM on switch grass while a strain with *adhE* gave 2.3 and 1.6 mM of ethanol on avicel and switch grass, respectively. One of the reasons for suggested for the low ethanol titers is the availability of cofactors and it should be noted that *T. pseudoethanolicus* ADHs utilize NADPH while the gene products from *C. thermocellum* use NADH as a source of reducing potential. Additional work is therefore needed to more carefully mimic the complex NAD(P)H system of multiple ADHs in *Thermoanaerobacter pseduoethanolicus*.

Overall, efforts to engineer thermophilic anaerobes to increase ethanol titers has resulted in modest gains in yields while minimizing or eliminating the formation of unwanted end products. Future targets for genetic manipulation might include the inclusion of the cellulolytic machinery of *C. thermocellum* into highly ethanologenic *Thermoanaerobacter* and *Thermoanaerobacterium* strains.

7. Conclusions

Bioethanol production from lignocellulosic biomass with thermophilic bacteria needs robust microbes regarding several aspects. One of the main advantages of thermophilic bacteria is their broad substrate spectra with many strains capable of simultaneous pentose and hexose degradations. Additionally, some thermophiles degrade complex carbohydrates like cellulose and hemicellulose although many of these strains are not highly ethanologenic. Recent advantages in genetic engineering have improved ethanol yields, mostly by knocking out pathways of undesired end-products. On the back side is the fact that yields and ethanol tolerance as well as low tolerance for high initial substrate concentrations still limits the use of thermophiles for large scale operations. The use of stable co-cultures where on microbe hydrolyses the sugar polymers and the other one ferments the sugars released to ethanol is an attractive way to go forward but warrants further investigations.

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Chapter 7

Potential of Thermotolerant Ethanologenic Yeasts Isolated from ASEAN Countries and Their Application in High-Temperature Fermentation

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Abstract

Thermotolerant ethanologenic yeasts receive attention as alternative bio-ethanol producers to traditionally used yeast, *Saccharomyces cerevisiae*. Their utilization is expected to provide several benefits for bio-ethanol production due to their characteristics and robustness. They have been isolated from a wide variety of environments in a number of ASEAN countries: Thailand, Vietnam, Laos, and Indonesia. One of these yeasts, Kluyveromyces marxianus has been investigated regarding characteristics. Some strains efficiently utilize xylose, which is a main component of the 2nd generation biomass. In addition, the genetic basis of *K. marxianus* has been revealed by genomic sequencing and is exploited for further improvement of the strains by thermal adaptation or gene engineering techniques. Moreover, the glucose repression of *K. marxianus* and its mechanisms has been investigated. Results suggest that *K. marxianus* is an alternative to *S. cerevisiae* in next-generation bio-ethanol production industry. Indeed, we have succeeded to apply *K. marxianus* for bio-ethanol production in a newly developed process, which combines high-temperature fermentation with simultaneous fermentation and distillation under low pressure. This chapter aims to provide valuable information on thermotolerant ethanologenic yeasts and their application, which may direct the economic bioproduction of ethanol and other useful materials in the future.

Keywords: thermotolerant yeast, high-temperature fermentation, genomic aspects

1. Introduction

Worldwide economic growth with the related increase in CO_2 emissions from fossil fuels causes global warming. Utilization of renewable energy with low CO_2 emission therefore has been getting increased attention. Renewable energy is generated from renewable natural resources, such as sunlight, wind, rain, tides, waves, geothermal heat, as well as biomass. One such important source of renewable energy, *bio-ethanol*, has been highlighted due to the characteristics of its production from biomass, which is generated by plants using sunlight for CO_2 fixation, resulting in carbon neutrality. Bio-ethanol is the name for ethanol produced from biomass by fermentation. This bio-process is thoroughly researched and well-established, and to-date, it produces the most prominent and cost-effective biofuel [1]. Although bio-ethanol production is increasing worldwide and the production of biofuels including ethanol in 2022 is forecasted to be more than 126 billion L [2], biofuels are still more costly than fossil fuels [3]. Therefore, several industrial companies and researchers aim to develop new technologies, enabling the cost-effective production of bio-ethanol from biomass. Since microorganisms are essential for material production through bio-processing, their characteristics and traits are crucial for the production process efficiency. Ethanologenic yeast, *Saccharomyces cerevisiae*, has been traditionally and widely utilized for the production of alcoholic beverages and bio-ethanol [4, 5]. Industrially common problems in bio-ethanol production related to *S. cerevisiae* strains are temperature level (35–45°C) and high ethanol concentration (over 20%) [6]. These two factors inhibit yeast proliferation and fermentation activity if they reach the upper limit. In addition, for cost-effective bio-ethanol production, the production source must be changed from 1st generation biomass (sugarcane, corn, and wheat, which are important food sources) to 2nd generation biomass (lignocellulosic biomass or woody crops, which are agricultural residues or waste) [7]. Lignocellulosic biomass is composed of hemicellulose, cellulose, and lignin, and the first consists of six (e.g., glucose) and five (e.g., xylose) carbon sugars. However, the low efficiency of ethanol production by *S. cerevisiae* from lignocellulosic biomass hydrolyzates is mainly due to its little ethanol productivity from xylose [8]. Although the *S. cerevisiae* genome encodes all components necessary for xylose utilization, most of them are rarely expressed [9]. In addition, *S. cerevisiae* preferably utilizes glucose while repressing the uptake and catabolism of alternate carbon sources by a mechanism such as glucose repression [10]. This results in the reduction of ethanol production rates from several kinds of biomass. For economically feasible bioethanol production from lignocellulosic biomass, the efficient co-fermentation of glucose and other sugars is also necessary. Therefore, genetic engineering of *S. cerevisiae* strains has been extensively performed, and metabolically engineered strains were developed [11], which have showed higher stress tolerance and/or improved xylose utilization [12, 13]. However, the utilization of genetically recombinant strains in industry has been very limited, especially due to the instability of the desirable phenotype and the necessary confinement to a closed system to prevent their leakage into the environment, which can eventually endanger public health or biodiversity. Therefore, the development of new feasible strains for next-generation bio-ethanol production is under way, and new yeast strains have been isolated that may have advantages compared to *S. cerevisiae*.

Recently, thermotolerant microorganisms were found among mesophiles with optimum growth temperatures that are 5–10°C higher than those of the typical mesophilic strains

belonging to the same genus or even to the same species [14]. These thermotolerant mesophiles are mainly and widely distributed in foods, plants, soils, and waters from tropical environments in ASEAN countries [15]. In these environments, relatively high temperature presumably becomes a selective pressure to enrich thermotolerant strains. These thermotolerant strains are expected to provide a benefit for the industries because they are more robust and resistant to many stressors [14]. In addition, some of these thermotolerant microorganisms can produce distinctive enzymes that function under relatively high temperature conditions [16–18]. Thermotolerant yeasts have been found and isolated from a number of countries [19–28]. Of these, *K. marxianus* is a haploid, homothallic, thermotolerant, and hemiascomycetous yeast [29, 30]. One such yeast, *K. marxianus* DMKU 3-1042 isolated in Thailand, shows relatively high ethanol productivity and fermentation ability at high temperatures [31], assimilates various sugars including xylose and/or arabinose [32], and exhibits relatively weak glucose repression on utilization of some sugars including sucrose [33]. Therefore, *K. marxianus* is, in comparison to *S. cerevisiae,* a promising candidate for next-generation bio-ethanol production. In addition, the genomic sequences of *K. marxianus* are available [34, 35], and genetic technology and tools have also been developed [36]. Moreover, *K. marxianus* has been a platform for next-generation protein production for structural and biochemical studies [18, 29]. However, it is possible that unidentified and more beneficial thermotolerant yeasts exist in ASEAN countries, especially, thermotolerant high xylose-utilizing and ethanol-producing yeasts, which are needed for 2nd generation biomass utilization. None of the isolated *K. marxianus* strains, however, are able to more efficiently convert xylose to ethanol than strains of other xylose-utilizing yeasts, such as *Pichia stipitis* (*Scheffersomyces stipitis*) [32, 37].

Thermotolerant strains allow the development of high-temperature fermentation (HTF) technology, which enables fermentation at 5–10°C higher than the traditional fermentative process [38, 39]. HTF is thus expected to reduce cooling costs, running costs at the simultaneous saccharification and fermentation (SSF) stage, and contamination risks [6, 31, 38–40], therefore offering a promising technology for bio-ethanol production. Moreover, thermotolerant yeast can also be applied for temperature-uncontrolled fermentation, hence offering another economical advantage. A combination of efficient bioreactors and robust hosts, such as thermotolerant strains, leads to lowest energy consumption and emission of CO_2 in biofuel production [41].

In this chapter, we outline a number of thermotolerant yeasts including *K. marxianus* species isolated in Thailand and their characteristics, including utilization of various sugars, glucose repression, and genetic information, that are beneficial for high-temperature fermentation. In addition, new strains of thermotolerant yeasts that have been isolated in Indonesia, Vietnam, and Laos are summarized. Subsequently, the trial results of HTF with some of these strains for ethanol production are presented.

2. Various ethanologenic thermotolerant yeasts and their characteristics

Increasing global energy demand that exceeds the finite supply of fossil fuel has spurred scientific research to deliver alternative fuels. Microbial fermentation and efficient conversion

technologies now allow the extraction of biofuels from biomass, such as wood, crops, and waste materials. Supplies of ethanol have increased tremendously and are expected to continue rising rapidly in both developed and developing countries [41]. A variety of feedstocks from the 1st, 2nd, and 3rd generation have been used in bioethanol production [42]. First-generation bioethanol involves feedstocks rich in sucrose (sugar cane juice, molasses, and sweet sorghum) and starch (corn, wheat, cassava, and potato). Second-generation bioethanol comes from lignocellulosic biomass such as wood, straw, and other agricultural wastes. Third-generation bioethanol is derived from algal biomass including microalgae and macroalgae [43, 44]. The process of ethanol production depends on the types of feedstocks used. Generally, there are three major steps in ethanol production: decomposition of biomass, fermentation, and product recovery. During fermentation, the cooling of fermenters is one of the major energy consuming steps because the metabolism of yeast releases a large amount of heat. Therefore, the application of thermotolerant yeasts can significantly reduce the cooling cost and help prevent contamination [38]. High-temperature ethanol fermentation will also benefit a simultaneous saccharification and fermentation process.

Many thermotolerant yeasts have been isolated from various natural habitats and tested for their capability to produce ethanol at high temperatures (**Table 1**). Many strains of *K. marxianus*, *Pichia kudriavzevii*, and *S. cerevisiae* were often isolated as ethanol-producing yeasts at high temperatures. Of these, *K. marxianus* was found to be the most thermotolerant yeast. Limtong et al. [31] isolated *K. marxianus* DMKU 3-1042 in Thailand and found optimum ethanol production at 40°C. The strain was compared with other *K. marxianus* strains including NCYC587, NCYC1429, and NCYC2791 and found to be the best ethanol producer at 45°C [36]. Kumar et al. [45] isolated *Kluyveromyces* sp. IIPE453 from a soil sample in a sugar mill, which showed high ethanol production rate at 45–50°C. Yanase et al. [46] reported that *K. marxianus* NBRC1777 efficiently produced ethanol corresponding to 92.9% of the theoretical yield. *K. marxianus* DBKKUY-103, that was recently isolated, achieved the maximum ethanol concentration of 83.5 g/L, corresponding to 96.6% of the theoretical yield [47]. Nitiyon et al. [37] reported that *K. marxianus* BUNL-21 is a highly competent yeast for high-temperature ethanol fermentation with lignocellulosic biomass. When compared with the strain DMKU 3-1042, the strain BUNL-21 had stronger ability for conversion of xylose to ethanol and tolerance to various stresses including high temperature and hydrogen peroxide.

Recently, there have been several reports on ethanol production at high temperatures using *P. kudriavzevii* (formerly known as *I. orientalis*). Several *P. kudriavzevii* strains were reported to grow and produce high levels of ethanol at high temperatures. The strain DMKU 3-ET15 was isolated from traditional fermented pork sausage in Thailand by an enrichment technique in a medium supplemented with 4% ethanol at 40°C. The strain produced 78.6 g/L ethanol from 180 g/L glucose at 40°C [20]. The strain KVMP10 that was isolated from soil located beneath apple trees for ethanol production from orange peel achieved 54 g/L ethanol at 42°C [48]. Strain RZ8-1 that was recently isolated from various samples collected from plant orchards in Thailand produced 33.8 g/L ethanol from 160 g/L glucose at 40°C [49].

Yeast strain	Temp. (°C)	P (g/L)	Q*p* (g/L/h)	T.Y (%)	Refs.
Kluyveromyces marxianus					
DMKU 3-1042	40	67.8	1.13	60.4	[31]
IIPE453[a]	50	82.0	nd	nd	[45]
NBRC1777	40	47.4	nd	92.9	[46]
DBKKUY-103	40	83.5	1.39	96.6	[47]
Pichia kudriavzevii					
DMKU 3-ET15	40	78.6	3.28	85.4	[20]
KVMP10	42	54.0	2.25	nd	[48]
RZ8-1	40	33.8	1.41	77.9	[49]
Saccharomyces cerevisiae					
VS3	40	60.0	nd	nd	[50]
C3867	41	38.8	nd	nd	[51]
DBKKUY-53	40	85.0	2.83	—	[52]
KKU-VN8	40	89.3	2.48	96.3	[53]

[a]*Kluyveromyces* sp.
P, ethanol concentration; Q*p*, volumetric ethanol productivity; T.Y, fraction of theoretical yield; nd, no data.

Table 1. Thermotolerant yeasts used in bioethanol production.

Several *S. cerevisiae* strains were also isolated for high-temperature ethanol fermentation. Sree et al. [50] reported a strain VS3 that could grow at 40°C and produced ethanol up to 60 g/L. Auesukaree et al. [51] reported a strain C3867 that produced 38.8 g/L of ethanol at 41°C. Recently, Nuanpeng et al. [52] and Techaparin et al. [53] isolated *S. cerevisiae* DBKKUY-53 and KKU-VN8, respectively, in Thailand. The former strain produced the maximum ethanol concentration and volumetric ethanol productivity of 85.0 g/L and 2.83 g/L h, respectively, at 40°C, and the latter strain produced the maximum ethanol concentration of 89.3 g/L with a productivity of 2.48 g/L h and a theoretical ethanol yield of 96.3% from sweet sorghum juice at 40°C.

Table 1 shows a number of ethanologenic thermotolerant yeasts. A temperature of 40°C was found to be the best condition for most strains to produce ethanol.

3. Utilization of various sugars in thermotolerant yeasts

Bioethanol significantly contributes to the reduction of crude oil consumption and environmental pollution. Thus, it has been identified as the mostly used biofuel worldwide [42]. Feedstocks for biofuel currently seem to be the option for sustainable development in the

context of economical and environmental considerations. There are various types of feedstocks for ethanol production [54], and accordingly, different processes including biomass pretreatment are required. Feedstock rich in sugar that mainly contains sucrose is readily fermented to ethanol. Feedstock rich in starch must first be hydrolyzed to glucose monomers by the action of enzymes [55]. Lignocellulosic and algal biomass needs further pretreatment and hydrolysis before liberating simple sugars, which can be readily converted to ethanol by microorganisms [56–58]. The resulting hydrolysates of these raw materials contain various sugars depending on the type of biomass [59]. In case of algal biomass, the sugar composition varies largely, based not only on algal species but also on their environmental and nutritional conditions [43, 56]. Lignocellulosic biomass is a complex mixture of carbohydrate polymers, and the biomass hydrolysate mainly contains hexoses (D-galactose, L-galactose, and D-mannose) and pentoses (D-xylose and L-arabinose) [60]. Glucose and xylose are the most abundant monosaccharides in this biomass taking up 60–70% and 30–40% of the total hydrolysate, respectively [61, 62]. Predominant pentose sugars derived from the hemicellulose of most feedstocks are xylose and arabinose. Like in higher plants, algae biomass is comprised of rigid cellulose-based cell walls and various complex polysaccharides, which can be hydrolyzed to sugars and subsequently fermented to ethanol [43, 63]. However, algae biomass contains a low percentage of lignin and hemicellulose compared to other lignocellulosic plants [64].

Microorganisms are the key factor in the conversion of sugars to ethanol. One of their several desired characteristics is thermotolerance. Ethanol production at high temperatures by thermotolerant yeasts has earned much interest due to several advantages as described above [38]. There are several ethanologenic yeasts that have been characterized and classified as thermotolerant yeasts such as *K. marxianus* [31, 37, 47], *P. kudriavzevii* (formally known as *I. orientalis*) [20, 48, 49, 65, 66], *Hansenula polymorpha* [67], and some strains of *S. cerevisiae* [21, 52, 68–70]. However, for cost-effective and efficient ethanol production, not only thermotolerance but also a broad spectrum in sugar assimilation and fermentation capability is beneficial for the conversion of a variety of raw materials containing various sugars to ethanol, especially xylose, which is the most common pentose sugar and the second most abundant after glucose in lignocellulosic biomass and algal biomass [71, 72].

S. cerevisiae is commonly employed in ethanol production due to its high ethanol productivity and high ethanol tolerance [73]. It is capable of converting different types of sugars, such as glucose, mannose, galactose, fructose, sucrose, and maltose to ethanol via the glycolysis pathway under anaerobic conditions [55]. Unfortunately, it is not able to ferment other carbon sources from plant or algal hydrolysates such as D-xylose, L-arabinose, and L-rhamnose [59]. A few types of yeasts can ferment both glucose and xylose but their performance regarding the rate of ethanol production from xylose, and the yield is lower than those from the main hexose sugars (for example, *S.* (*Pichia*) *stipitis* [74], *Scheffersomyces* (*Candida*) *shehatae* [75], *Pachysolen tannophilus* [76], *H. polymorpha* [67], and *K. marxianus* [32, 37]). Among these xylose-fermenting yeasts, it seems that *K. marxianus* has the potential for practical application in high-temperature ethanol fermentation because of its thermotolerance and ability to utilize a variety of sugars.

Feedstock	Substrate	Organism	Temp. (°C)	P (g/L)	T.Y (%)	Refs.
Sugar containing materials	Sugar cane juice	*K. marxianus* DMKU 3-1042	40	67.8	60.4	[31]
	Jerusalem artichoke	*K. marxianus* DBKKU-Y102	40	97.5	92	[77]
	Sweet sorghum juice	*K. marxianus* DBKKUY-103	40	83.5	100	[47]
	Palm sap	*K. marxianus* TISTR 5925	40	45.4	92.2	[39]
	Jerusalem artichoke	*K. marxianus* PT-1	40	73.6	90	[21]
Starchy materials	Taro waste	*K. marxianus* K21	40	43.8	94.2	[78]
Lignocellulosic biomass	Kanlow switchgrass	*K. marxianus* IMB3	45	22.5	86	[79]
	Switchgrass	*K. marxianus* IMB4	45	16.6	78	[80]
	Solka-floc	*K. marxianus* L. G.	42	37.6	98	[81]
	Rice straw	*K. marxianus* NRRLY-6860	45	21.5	86	[82]

P, ethanol concentration; T.Y, fraction of theoretical yield.

Table 2. Ethanol production of *K. marxianus* from various substrates at high temperatures.

K. marxianus's most important characteristics in this respect are thermotolerance to temperatures between 45 and 52°C, efficient ethanol production at temperatures between 38°C and 45°C, and a rapid growth rate that is twice as high as that of *S. cerevisiae* in rich media. Moreover, it has a broad spectrum of sugar assimilation, which includes glucose, mannose, galactose, fructose, arabinose, xylose, xylitol, sucrose, raffinose, cellobiose, lactose, and inulin [32, 36]. However, there has been little ethanol production from xylose and none from arabinose [32]. This strain can utilize a wide variety of industrially relevant substrates and efficiently converts substrates to ethanol. Especially, with lignocellulosic raw materials, it resulted in 78–98% of the theoretical ethanol yield (**Table 2**).

4. Complete genome sequence of thermotolerant yeast *K. marxianus* DMKU 3-1042 and transcriptomic analysis

High-temperature fermentation technology with thermotolerant microbes has been expected to reduce the cost of bioconversion of biomass to fuels or chemicals. *K. marxianus* was included in GRAS (FDA) and QPS (EU) lists of safe microorganisms for use in foods [83, 84]. The capacity of *K. marxianus* to utilize a wide variety of sugars reflects its potential for biotechnological applications [29, 84], which has been indicated by many studies with diverse substrates such as whey permeate, crop plants, and lignocellulosic biomass [32, 33, 78, 85, 86]. *K. marxianus* is also distinguished by its thermotolerance [36, 87] and the highest growth rate

in eukaryotes [88]. In recent years, interest also increased in several new applications such as production of biomolecules [89, 90], biocatalysts [91, 92], and heterologous protein expression [93, 94].

Genomic and transcriptomic studies have started to shed light on *K. marxianus*, and a growing number of genome sequences of *K. marxianus* strains are now available. Those include KCTC 17555 [34], DMB1 [95], CCT 7735 [96], NBRC1777 [97], DMKU 3-1042 [35], B0399 [98], UFS-Y2791 [99], and other nine strains: L01, L02, L03, L04, L05, CBS397, NBRC0272, NBRC0288, and NBRC0617 [100].

4.1. Genomic information and comparative genomics

The genome sequence of *K. marxianus* DMKU 3-1042 as one of the most efficient thermotolerant strains was determined, and the complete genome sequence of 11.0 Mb including all centromeric regions and boundary regions containing up to one to several sequence repeats (GGTGTACGGATTTGATTAGTTATGT) of telomeres was obtained [35]. The genome was composed of eight chromosomes in total, including mitochondrial DNA. Annotation of the genome of DMKU 3-1042 revealed a total of 4952 genes. UniProt and KAAS assignments led to the assignment of homologous genes of about 86.4% of predicted genes and KEGG Orthology numbers of 50.5% respectively.

A total of 202 tRNAs and 8 rDNAs were identified. According to the optical mapping experiment, 140 rDNA copies were observed on chromosome 5 instead of 6 rDNA copies found in the genome sequence in the database. The rDNA copy number and the thermotolerance were expected to positively relate. However, there was no such correlation among 10 *K. marxianus* strains, which exhibited different growth at different temperatures, and at least 31 copies of rDNA are sufficient to support its thermotolerance [35].

The yeast shares 1552 genes with other hemiascomycetous yeasts, including *K. lactis, Ashbya gossypii, Candida glabrata, S. cerevisiae, Ogataea parapolymorpha, Debaryomyces hansenii, S. stipitis, Clavispora lusitaniae, Yarrowia lipolytica,* and *Schizosaccharomyces pombe* [101–105]. *K. marxianus* was found to be phylogenetically closest to *K. lactis*. There are 193 genes specific to *K. marxianus*, which may be responsible for its species-specific characteristics [35]. The 422 genes shared between *K. marxianus* and *K. lactis* may be related to their genus-specific characteristics, such as production of β-galactosidase [106], assimilation of a wide variety of inexpensive substrates [84], efficient productivity of heterologous proteins [107–109], and synthesis of a killer toxin against certain ascomycetous yeasts [110, 111].

The two attractive traits of *K. marxianus* for fermentation applications were the thermotolerance and pentose assimilation capability. The thermotolerant ability was also found in *O. parapolymorpha*, and 30 genes were found to be shared between the two thermotolerant yeasts, including genes for three siderophore-iron transporters and three vacuolar proteins. For pentose assimilation capability, there are 27 putative genes for sugar transporters in the *K. marxianus* genome, and some of them (*KLMA_60073, KLMA_70145 and KLMA_80101*)

were induced by xylose. The initial xylose catabolism after its uptake in *K. marxianus* is accomplished by three reactions catalyzed by enzymes, xylose reductase (*XYL1*), xylitol dehydrogenase (*XYL2*), and xylulokinase (*XKS1*), which are involved in the conversion of xylose to xylulose-5-phosphate as an intermediate in the pentose phosphate pathway (PPP). Genes for utilization of various other sugars and alcohol dehydrogenases were also found [35, 112, 113].

4.2. Ploidy variation in *K. marxianus*

K. marxianus showed a high level of phenotypic variation. Recently, the single nucleotide polymorphisms (SNIPs) in 14 strains of *K. marxianus* were analyzed [100]. On the basis of SNIP analysis and flow cytometry, it was found that the isolates included haploid, diploid, and triploid strains. All isolates from dairy environments were diploid or triploid, whereas most isolates (6 out 7 isolates) from nondairy environments were haploid.

4.3. Transcriptomic analysis

A major potential future application of *K. marxianus* may be ethanol production from lignocellulosic biomass, which is an anaerobic or oxygen-limited process where both glucose and xylose may be present. Detailed transcription start site sequencing (TSS Seq) to explore the response of *K. marxianus* DMKU 3-1042 was reported for four different conditions: shaking condition in rich medium at 30°C (30D) or 45°C (45D), static condition in rich medium at 30°C (30DS), and shaking condition in xylose-containing rich medium at 30°C (30X) [35].

Under the 30DS condition, there were 159 and 154 significantly upregulated and downregulated genes, respectively. In brief, *K. marxianus* may increase the turnover of RNAs and proteins in addition to suppression of transporters that depend on mitochondrial respiratory activity. Most genes for several oxygen-dependent biosynthetic pathways (**Figure 1**), such as those for heme, sterols, unsaturated fatty acids, pyrimidine, and deoxyribonucleotides [114], are crucial for the cellular metabolism under the static condition.

Under the 45D condition, there were 199 and 508 significantly upregulated and downregulated genes, respectively. *K. marxianus* seems to drastically change metabolic pathways under the 45D condition, that is, the enhancement of PPP and the attenuation of TCA cycle after the fumarate-producing step (**Figure 2**). Several genes for homologous recombination and nonhomologous end joining, which function in the repair of DNA-double stranded breaks, were also upregulated. As expected, heat shock proteins and chaperones, such as Hsp26, Hsp60, Hsp78, Hsp82, Ssa3, and Cpr6, are crucial for survival at high temperatures. The thermotolerance of *K. marxianus* is likely achieved by systematic mechanisms consisting of various strategies. The yeast prevents reactive oxygen species (ROS) generation by minimizing mitochondrial activity and mainly acquires ATP from glycolysis rather than from TCA cycle at high temperatures.

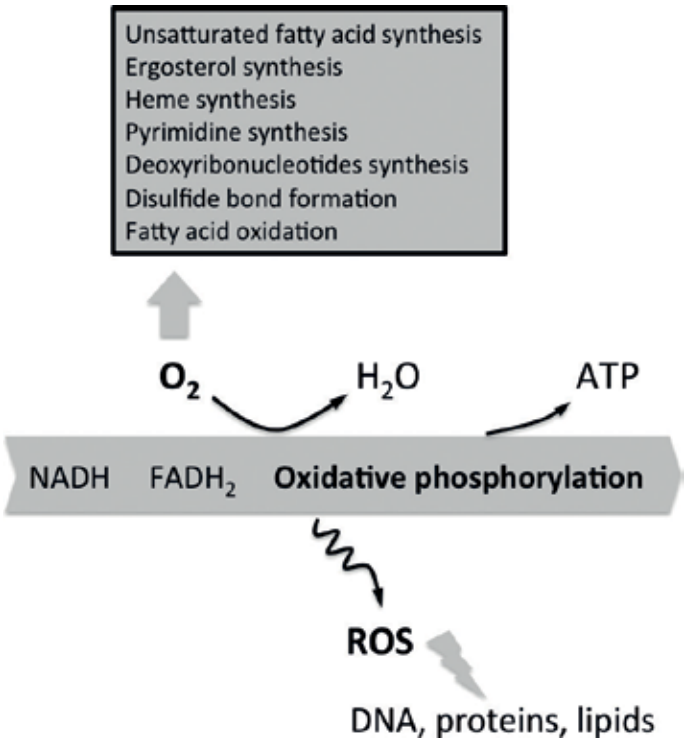

Figure 1. Oxygen-related metabolism in budding yeast. Oxygen is used for the biosynthesis of unsaturated fatty acids, ergosterol, heme, pyrimidine, and deoxyribonucleotides, as well as during disulfide bond formation and fatty acid oxidation. Oxygen is also the final electron acceptor for the electron transport chain, which oxidizes reduced equivalents of nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$) for the synthesis of ATP. However, ROS are produced as a by-product during some of these processes. The ROS can cause damage to DNA, proteins, and lipids.

Under the 30X condition, there were 89 and 79 significantly upregulated and downregulated genes, respectively. This condition may stimulate the degradation of lipids in the peroxisome and keep a low level of amino acid synthesis, indicating the possibility that fatty acids could be a subsidiary intracellular carbon source in xylose medium (**Figure 3**). Similarly, Schabort et al. [99] also reported that peroxisomal fatty acid catabolism was dramatically upregulated in a defined xylose mineral medium without fatty acids, along with mechanisms to activate fatty acids and transfer products of β-oxidation to the mitochondria. It is known that *K. marxianus* tends to suffer from cofactor imbalance in xylose medium [115, 116]. Redox balancing mechanisms between the cytoplasm and mitochondria are probably used to resolve the NADH/NADPH imbalance owing to lack of transhydrogenases [117]. In *S. cerevisiae*, five cytosolic-mitochondrial redox shuttles have been proposed [118]. Of these, genes for enzymes related to ethanol-acetaldehyde, citrate-oxoglutarate, and oxaloacetate-malate shuttles were relatively upregulated under the 30X condition, which were different from those found in *S. cerevisiae* and *S. stipitis* [103, 119].

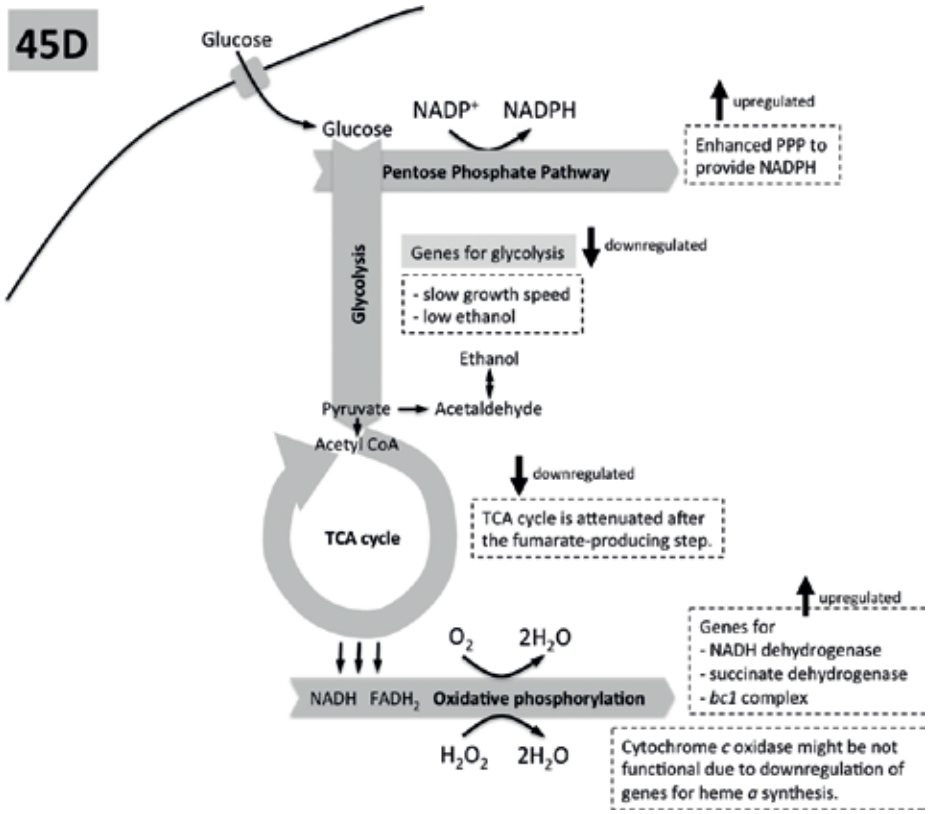

Figure 2. Difference of metabolism under the 45D condition from that under the 30D condition in *K. marxianus* DMKU 3-1042 (see more detail in Ref. [35]).

TSS seq analysis revealed that the oxidative stress-response genes were highly induced under the three conditions tested, indicating that ROS is accumulated in the cytoplasm, mitochondria, and peroxisome under the 30DS and 30X conditions and in the cytoplasm and mitochondria under the 45D condition.

Moreover, *K. marxianus* has been exploited as a cell factory to produce valuable enzymes, showing retention of the activity in a broad temperature range [120]. The 30X condition showed high expression of *INU1* for inulinase, which is useful for the production of recombinant proteins [108, 109, 121]. These useful characteristics may allow simultaneous production of ethanol and valuable proteins, thus generating additional revenue from ethanol production.

In conclusion, the transcriptome analyses clarified distinctive metabolic pathways under three different growth conditions, static culture, high temperature, and xylose medium, in comparison to the control condition of a glucose medium under a shaking condition at 30°C. Interestingly, the yeast appears to overcome the issue of ROS, which tend to accumulate under all three conditions. Nicotinamide adenine dinucleotide phosphate (NADPH) synthesis from several reactions is the key for cells to cope with ROS (**Figure 4**).

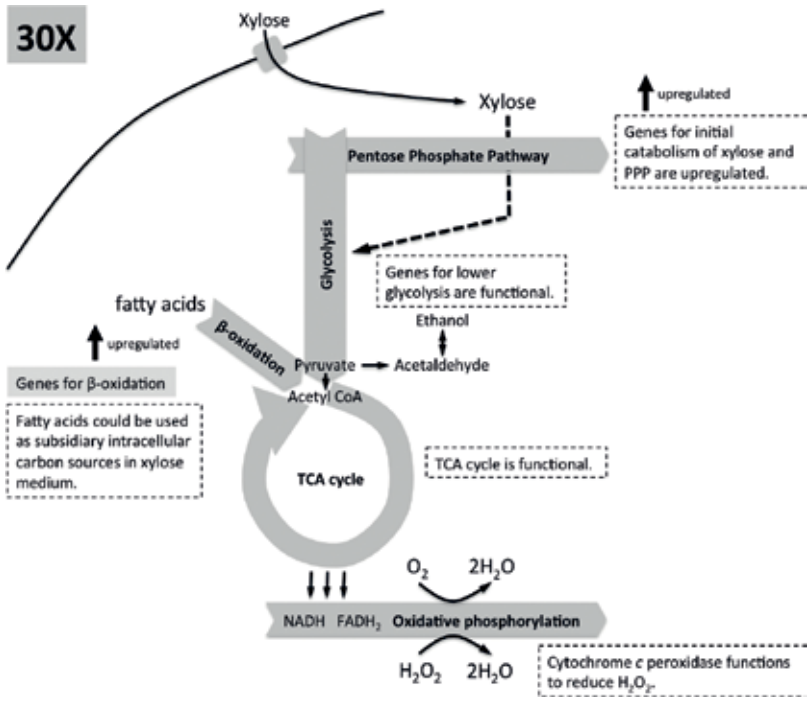

Figure 3. Difference of metabolism under the 30X condition from that under the 30D condition in *K. marxianus* DMKU 3-1042 (see more detail in Ref. [35]).

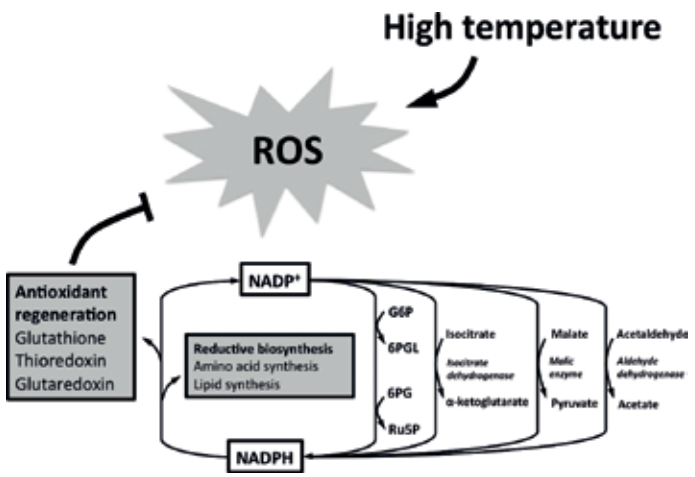

Figure 4. Generation and utilization of NADPH in budding yeast. A major source of cellular-reduced NADPH is thought to be produced via the oxidative branch of the pentose phosphate pathway. Oxidation of isocitrate, malate, and acetaldehyde generates NADPH. NADPH is consumed during the synthesis of amino acids and lipids. The reducing power of NADPH is also used to regenerate a variety of antioxidants and antioxidant enzymes, which protect the cell from ROS and engage in deoxyribonucleotide triphosphate (dNTP) synthesis. Abbreviations: G6P, glucose-6-phosphate; 6PGL, 6-phosphogluconolactone; 6PG, 6-phosphogluconate; Ru5P, ribulose-5-phosphate.

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5. Glucose repression in thermotolerant yeast *K. marxianus*

Glucose repression is a general phenomenon in organisms including yeasts, by which glucose prevents the assimilation of other sugars [122, 123]. This process will disturb the fermentation of mixed sugars like hydrolysate of cellulosic biomass. As mentioned in the previous sections, *K. marxianus* is a well-known budding yeast, which has potential for production of bioethanol, hydrolytic enzymes, food biomass, and food additives [29, 31, 124]. *K. marxianus* DMKU 3-1042 is a thermotolerant yeast from Thailand and efficiently produces ethanol at high temperatures [31]. Although the strain can utilize various sugars including xylose [32, 35, 125], it has an intrinsic system of glucose repression like other microbes. In this section, we describe glucose repression in thermotolerant yeast, *K. marxianus*, and in conventional yeast, *S. cerevisiae*.

5.1. Mechanism of glucose repression in *S. cerevisiae*

Glucose repression in *S. cerevisiae* has been well studied. Mig1 and Hxk2 play as the main regulator of glucose repression in this species [126]. The former is a C_2H_2 zinc finger protein [127], and the latter is a bi-functional protein acting as a hexokinase and transcriptional regulator, which is localized in both the cytoplasm and the nucleus [128, 129]. Hxk2 activity in glucose repression mechanism is influenced by the concentration of glucose. Under high concentrations of glucose, Hxk2 in the cytoplasm moves to the nucleus and, as a complex with dephosphorylated Mig1, Cyc8, and Tup1 [126], represses the transcription of several genes including respiratory and gluconeogenic genes. As a result of Hxk2 binding to Mig1, serine 311 in Mig1 is dephosphorylated, resulting in maintenance of repressive conditions [130]. On the other hand, in the presence of a low concentration or absence of glucose, Hxk2 and Mig1 remain in the cytoplasm, where neither Mig1 nor Hxk2 can repress Mig1-regulated genes [126]. In this situation, Hxk2 does not interact with Mig1 but still interacts with Snf1. No interaction between Hxk2 and Mig1 facilitates phosphorylation of serine 311 in Mig1 by the Snf1 kinase. Snf1 is phosphorylated by Sak1 and forms a complex with Snf4 and Gal8 to become activated. The Snf1 complex inhibits formation of a complex of Mig1-Hxk2-Cyc8-Tup1. In this situation, since Mig1 is also phosphorylated or inactive and absent in the nucleus, Mig1-regulated genes are de-repressed [130].

5.2. Mechanism of glucose repression in *K. marxianus*

K. marxianus DMKU 3-1042 exhibits almost no glucose repression on sucrose assimilation unlike *S. cerevisiae* [33]. To acquire glucose repression-defective strains in *K. marxianus*, some researchers performed spontaneous isolation on 2-deoxyglucose (2-DOG) plates or random insertion of *kan*MX4 [131, 132]. According to the characteristics of sugar consumption abilities, cell growth and ethanol accumulation along with cultivation time, only one of 33 isolates of 2-DOG-resistant mutants showed enhanced utilization of xylose in the presence of glucose. Further analysis revealed that this isolate had a single nucleotide mutation to cause amino acid substitution (G270S) in *RAG5* encoding hexokinase and exhibited very low activity of the enzyme [132]. Another technique for obtaining glucose repression-defective strains showed

one group of 2-DOG-resitant mutants with intragenical insertion of *Kan*MX4. This group also exhibits enhanced utilization of xylose in the presence of glucose, presumably due to a defect in the glucose-repression mechanism [131].

On the other hand, Zhou et al. focused on the function of Mig1 in *K. marxianus* and showed that the *MIG1* mutation increased hydrolysis of lactose [133] and production of inulinase [134]. Nevertheless, information on the function of Rag5 as a transcriptional regulator is hardly available, and thus construction of the complete disrupted mutation of *RAG5* and its analysis become a challenge. Thus, disrupted mutants of genes for Mig1 and Rag5 were constructed, and their characteristics were compared with those of the corresponding mutants of *S. cerevisiae*. *MIG1* and *RAG5* mutants exhibited more resistance to 2-DOG in YP plates containing sucrose. *RAG5* and *HXK2* mutants showed more resistant to 2-DOG than the corresponding *MIG1* mutants [135].

Several attractive characteristics of *MIG1* and *RAG5* mutants of *K. marxianus* DMKU 3-1042 were uncovered. *MIG1* mutants consumed almost two times faster xylose and accumulated glycerol and xylitol much more than those of the parental strain and the *RAG5* mutant in the liquid media YPX (containing 20 g/L of xylose) and YPDX (containing 20 g/L of glucose and 20 g/L of xylose) at 30°C. The accumulation of glycerol and xylitol may be due to accumulation of NADH. *RAG5* mutants exhibited very slow utilization of glucose in the liquid media of both YPD (containing 20 g/L of glucose) and YPDS (containing 20 g/L of glucose and 20 g/L of sucrose). However, with this mutant, high amounts of fructose (about 11.9 g/L in YPDS at 30°C for 96 h) were accumulated. *MIG1* and *HXK2* mutants of *S. cerevisiae* also accumulated high amounts of fructose in the same medium, but after 12 h, fructose was consumed.

The fructose accumulation in *RAG5* mutants is probably due to the inability of this mutant to uptake fructose or the lack of kinase activity. To further analyze this phenomenon, Enzyme activities[a] and gene expression levels of inulinase and kinase in *MIG1*- and *RAG5*-disrupted mutants and the parental strain were measured (**Table 3**) [135]. *RAG5* mutants showed very high activities of inulinase, about 77 times higher than those of the parental strain, but almost no activities of hexokinase and glucokinase that are encoded by *RAG5* and *GLK1*, respectively. The inulinase activity in *RAG5* mutant was consistent with the gene expression level of *INU1*, being about 22 times higher than that of the parental strain. However, the expression level of *GLK1* in this mutant was higher, which was inconsistent with glucokinase activity. It is thus likely that there is a post-transcriptional regulation for glucokinase. *MIG1* mutants showed no significant increase in inulinase activity, but *INU1* transcriptional expression was eight times higher than that of the parental strain. This inconsistence may also be due to post-transcriptional regulation for inulinase. These results suggest that Mig1 and Rag5 are related to the glucose repression mechanism in *K. marxianus* and share some functions with Mig1 and Hxk2, respectively, in *S. cerevisiae*.

In conclusion, Mig1 and Rag5 in *K. marxianus* share some functions with Mig1 and Hxk2, respectively, in *S. cerevisiae*. Mig1 and Rag5 in *K. marxianus* may form a complex similar to that consisting of Mig1 and Hxk2 in *S. cerevisiae.*

Strains	Enzyme activities[a]			Gene expression levels		
	Inulinase (U/mg DCW)	Gluco-hexokinase (U/mg)	Hexokinase (U/mg)	*INU1/ACT1*	*GLK1/ACT1*	*RAG1/ACT1*
DMKU 3-1042	127.38	1.107	0.662	0.087	0.136	0.916
MIG1 mutant	160.1	1.466	0.774	0.696	0.141	0.266
RAG5 mutant	9838.16	0.007	0.005	1.927	1.495	0.051
RAG1 mutant	4229.23	0.203	0.027	1.234	0.606	0.091

[a]The data are from Ref. [135].

Table 3. Comparison of enzyme activities and gene expression levels in *MIG1*- and *RAG5*-disrupted mutants of *K. marxianus* in YPD liquid medium.

6. Thermotolerant and ethanologenic yeasts in Vietnam

In Vietnam, ethanol is a compound in many different products from fermentation technology including alcoholic drinks and biofuel. In the national strategy with a vision to 2025 designed by the government, the technology of biofuel production in Vietnam using the various raw material resources that are abundantly available, e.g., pineapple, cassava, sugarcane, etc., will reach the advanced worldwide level. For the scheme on the development of Vietnam's alcoholic beverages with a vision to 2025, the Mekong Delta is one of the top national areas for the improvement of such products. In addition, nowadays due to global warming, the exploration of thermotolerant yeasts for ethanol fermentation at high temperature also falls in the potential priorities in Vietnam.

6.1. Characteristics of thermotolerant and ethanologenic yeasts

Recent research studies under international programs, such as the Asian Core Program (2008–2012) and the Core-to-Core Program (2014–2018), have addressed the exploration of useful thermotolerant ethanologenic yeasts isolated from Vietnam and their applications for fermentation technology at high temperature. The diversity of yeast isolates with high capacities and stability for the controlled processing of alcoholic winemaking and ethanol production from cheap and available raw materials in the region has been studied.

A total of 712 yeast isolates were purified from many different kinds of raw material sources in the Mekong Delta, Vietnam, such as ripe fruits, flowers of fruit-tree, cocoa, fermented products, alcoholic fermentation starters, sugarcane, molasses, sawdust, agricultural by-products, and soil samples. All of these yeast isolates could grow well at 37°C and about 80, 45 and 10% of these yeasts could grow at 40, 43 and 45°C, respectively. More than 80% of yeasts were able to grow in a medium containing 9% (v/v) of ethanol, this number decreased to about 40% of yeasts growing in a medium supplemented with 12% (v/v) of ethanol. For conservation, all pure yeast isolates have been stored at −20 and −80°C in stock culture of glycerol freezing broth.

A bank collection of genetically diverse yeasts with thermotolerant ethanologenic capacity at high temperatures was developed and systemized. The full data of morphological,

physiological, and biochemical characteristics, as well as the nucleotide sequencing analyses of the 88 selected yeasts, have been established. Some predominantly abundant identified species include *Candida tropicalis*, *S. cerevisiae*, *P. kudriavzevii*, and *C. glabrata* (**Table 4**). Besides, a number of other species was also characterized, such as *Torulaspora globosa*, *Candida nivariensis*, *Pichia manshurica*, *C. lusitaniae*, *Hanseniaspora opuntiae*, and *Meyerozyma caribbica*.

With the aim to pave the way for the application of useful thermotolerant ethanologenic yeasts toward industrial fermentation technology, ethanol production, and winemaking by using the selected thermotolerant yeasts, investigations at laboratory-scale and pilot-scale were performed. The optimum fermentation conditions at different temperatures (37, 40, and 43°C) were also tested in a factorial design with three factors including yeast inoculum, initial sugar concentration, and fermentation time. For wine manufacture, different kinds of fruits were employed as raw materials such as: pineapple, watermelon, dragon fruit, guava, jackfruit, rambutan, tangerine, and three-leaved wild vine. The highest ethanol concentration of the final wine product reached about 12% (v/v) and up to 7% (v/v) during the fermentation at 37 and 40°C, respectively. For ethanol production, a number of raw materials were tested including molasses, sugarcane juice, sugarcane waste, and pineapple waste hydrolysate. The highest ethanol concentration could be found at about 7% (v/v) and up to 4% (v/v) during the fermentation at 37 and 40°C, respectively.

No	Isolated yeast species	Vietnam	Laos	Indonesia
1	*Blastobotrys adeninivorans*		2	
2	*Candida glabrata*	7	2	
3	*Candida manshurica*		2	
4	*Candida nivariensis*	4		
5	*Candida stellimalicola*		1	
6	*Candida tropicalis*	16	26	16
7	*Clavispora lusitaniae*	1		
8	*Cyberlindnera rhodanensis*		2	
9	*Hanseniaspora opuntiae*	1		
10	*Issatchenkia orientalis*			1
11	*Kluyveromyces marxianus*		6	3
12	*Meyerozyma caribbica*	1		
13	*Meyerozyma guilliermondii*			2
14	*Pichia kudriavzevii*	35	47	1
15	*Pichia manshurica*	2		
16	*Saccharomyces cerevisiae*	19	1	
17	*Torulaspora globosa*	2		
	Not identified	624	70	56
	Total	712	159	79

Table 4. Isolated yeast strains from Vietnam, Laos, and Indonesia.

The research findings on the diversified collection of thermotolerant ethanologenic yeasts isolated from Vietnam and the high ethanol yields as well as and fermentation efficiencies by using the selected yeast isolates indicate the promising application of such newly isolated functional thermotolerant yeasts for the controlled ethanol production at high temperatures from agricultural by-products and the winemaking manufacture from different available fruit resources in the region. Further advanced research on the expression levels of the selected genes and the metabolic pathways will be performed to explore the regulation of these genes to get maximum benefits of the superior thermotolerant yeasts for high-temperature ethanol production.

7. Thermotolerant and ethanologenic yeasts in Laos

Ethanol production in Lao PDR is generally used for human consumption and household use, rather than for small or large-scale industries. Until now, no ethanol as a substitute of energy in Lao PDR is produced in the industry. The raw material used to make ethanol for drinking is mostly sticky rice and the starter culture used for fermentation contains sticky rice and many other herbs. Drinking alcohol in Lao PDR is available in all provinces, mainly for consumers in their own province. Currently, alcoholic beverages are still very productive and the most popular products to customers are produced in the Saravan province in Meuangkhong district. High quality ethanol used for medicine, hospitals or laboratories are imported from neighboring countries.

The National Economic Research Institute under the Ministry of Planning and Investment reported that production of ethanol in 2010–2011 was increased 3.2 times compared to 2001. Lao government plans to develop other sources of renewable energy, which have been investigated by the private sector. Demonstration projects including a bio-diesel oil from Jatropha plant and biofuel (bio-gasoline and bioethanol) from Palm and Carmelina plants have been developed. In 2011, the Savannakhet sugar factory has been established by a Thai company to produce biogas and biomass energy. In 2013, a Vietnam company started a biomass power and ethanol production plant in Phouwong District, Attapeu Province.

7.1. Characteristics of thermotolerant and ethanologenic yeasts

Isolation of yeasts was first attempted from fruits, vegetables, leaves and soils in four provinces, Louang Phrabang, Xayaburi, Xiengkhouang, and Vientiane of Lao PDR. The attempt was carried out at 37°C by an enrichment culture. Samples (5–10 g) of fruits pressed in small pieces, leaves cut in small portions, and mashed soil were transferred into 100-mL Erlenmeyer flasks containing 10 mL of YPD (1% yeast extract, 2% peptone and 2% glucose) medium and incubated at 37°C for 3 days with occasional shaking. The cultures were then streaked on YPD agar plates and incubated at 37°C for 24–48 h. As a result, 43 strains were isolated, and their ethanol fermentation ability was characterized under various conditions including different sugars and different temperatures. A second isolation was attempted from similar kinds of samples described above in four provinces, Bolikhamxay, Champasak, Louang Phrabang, and Oudomxay, and 116 strains were obtained after enrichment culture as described above except that 4% ethanol was added in YPD medium. Of a total of 159 strains, 89 were identified by nucleotide sequencing of D1/D2 domains and analysis on MALDI-TOF/MS [28]. Fermentation experiments allowed to classify them into two groups: the first bears

an ethanol-fermenting ability at high temperature (116 strains) and the second the converting ability of xylose to ethanol at 37°C or more (43 strains). In fermentation of ethanol, the first group can use glucose, sucrose, sugar cane juice, and molasses as carbon sources, producing a maximum of ethanol concentrations of 7.9% (w/v), 6.7% (w/v), 7.3% (w/v), and 4.0% (w/v) from 16% sugar concentration, respectively. The second group produced 1.2–1.7% (w/v) ethanol from 4% xylose at 37°C. Species identification revealed that isolates include nine species including *C. tropicalis*, *P. kudriavzevii*, and *K. marxianus* (**Table 4**).

7.2. Characteristics of newly isolated *K. marxianus* strains

Out of six isolated *K. marxianus* strains, BUNL-17 was found to be the most efficient ethanol producer at high temperature [28]. Comparison with DMKU 3-1042, which is one of most thermotolerant *K. marxianus* strain isolates from Thailand, revealed that BUNL-17 possesses an efficient conversion activity of xylose to ethanol, resistance to 2-deoxyglucose and tolerance to various stresses including temperature, high sugar concentration, and hydrogen peroxide [37]. Compared to *S. stipitis* the fermentation activity toward xylose of BUNL-21 is slightly lower at around 30°C and much higher at higher temperatures. BUNL-21 is thus a highly competent yeast for high-temperature ethanol fermentation with lignocellulosic biomass. Interestingly, the fermentation activity was shown to be significantly enhanced by over-expression of *KmADH2* for alcohol dehydrogenase 2 [37].

8. Thermotolerant and ethanologenic yeasts in Indonesia

Ethanol production in Indonesia is generally performed for medical, industrial processes, and beverages. Several potential biomass resources for bioethanol production in Indonesia are (1) sugar-based materials including sugar cane (molasses), (2) starch-based including root (cassava and sweet potato) and grain (corn and sorghum), and (3) lignocellulosic-based including bagasse, straw, stalk, wood waste, corn cob, and sap of several plants or trees. The main biomass used for bioethanol production in Indonesia is molasses [136] probably because Indonesia is one of the largest sugarcane producers in the world. Annual cane production in Indonesia is about 32–35 million tons with an average cane productivity of 70–85 ton/ha. Sugar production is about 2.2–2.7 million tons, including molasses with about 1.3–1.5 million tons. Molasses are mainly used for monosodium glutamate production in the ethanol industry and for export to other countries [137].

Bioethanol development for fuel in Indonesia was started from 2006. Its road map until 2010 showed production of 99.5% ethanol as a fuel grade ethanol (FGE), which can be mixed with petroleum for gasohol E10 (10% ethanol and 90% petroleum). For the first period, biomass used for bioethanol production was molasses and cassava and bioethanol supply was about 1.48 mil kL (million kiloliters) or equal to 10% of total gasoline consumption. In the period 2011–2015, bioethanol supply was estimated to increase to 2.78 mil kL or equal to 15% of total gasoline consumption. Until 2025, bioethanol supply is predicted to be 6.28 mil kL or 20% of total gasoline consumption [138]. The application of bioethanol for fuel in Indonesia is E5, and only two bioethanol filling stations are operating in two cities, Malang and Semarang [139]. However, because of

some obstacles such as limitation of fuel grade ethanol market, inconsistency supply, insufficient demand, and price volatility, there is almost no fuel ethanol production since 2010 [136].

8.1. Characteristics of thermotolerant and ethanologenic yeasts

In international programs including the e-ASIA Joint Research Program, yeast strains were isolated from various samples such as soils, waters, flowers, fruits, vegetables, and fermented foods. The isolation method for thermotolerant and ethanol-producing yeast was similar to that applied in Lao PDR. The enrichment culture was carried out in YPD medium without the addition of ethanol. Most of the isolates can grow at relatively high temperatures ranging from 37 to 48°C. Of those, 52 yeast isolates grow well at 37°C on agar plates containing different types of sugar, such as glucose, xylose, and sucrose. Some can produce around 6% ethanol in a rich medium containing 16% (w/v) glucose at 40°C. These prominent characteristics are important for the development of bioethanol production in Indonesia.

Most yeast strains isolated from Indonesia are able to grow at relatively high temperatures not only in glucose medium but also in xylose and sucrose. However, their growth gradually decreases as temperature increases and is very weak at more than 45°C. Indonesian yeast isolates from fruits and fermented foods seem to be more thermotolerant than those from soils and waters. Most of the isolates grow very well at 40°C. These isolates include *C. tropicalis, K. marxianus* and *P. kudriavzevii* (**Table 4**).

9. High-temperature fermentation technologies with thermotolerant yeast

Currently, biofuel-aimed ethanol fermentation in industry is performed at around 30°C because the most frequently applied yeast is nonthermotolerant *S. cerevisiae*. In the fermentation process, the temperature in the fermenter increases close to a nonpermissible level for the yeast by metabolic and mechanical heat sources. A cooling system with a large amount of water and/or by a cooling unit is equipped for effective fermentation. The cooling cost tends to be higher in tropical countries or increases in summer time in other many countries, and the electricity problem largely affects productivity of ethanol. The HTF using a thermotolerant microbe is expected to provide several advantages. First, it can reduce the cooling cost. Second, the amount of enzyme used for saccharification can be reduced in the simultaneous saccharification and fermentation at higher temperature. Third, higher temperature causes lower contamination by various germs. Fourth, when the distillation under reduced pressure is applied at around 40°C, fermentation and distillation can be performed by one tank, which reduces the manufacturing time and the cost of equipment. Here, we introduce a fundamental research for an energy-saving fermentation technology using thermotolerant yeast.

9.1. Temperature-noncontrolled fermentation with thermotolerant yeast

For development of the fermentation technology, *K. marxianus* DMKU 3-1042 was used, which efficiently produces ethanol at high temperatures as mentioned above [32, 33]. The utilization

of the thermotolerant yeast is favorable to fermentation in a tropical country because it can be performed under temperature-noncontrolled conditions. When a bench-scale fermentation, 2 L of 9% glucose medium, was tested, DMKU 3-1042 produced ethanol equivalent to that under the temperature-controlled condition at 30°C [39]. In a fermenter-scale fermentation with 4000 L of 18% sugarcane, 7% ethanol production was achieved [39].

9.2. Distillation-connected fermentation with thermotolerant yeast

As an additional challenge, distillation-connected fermentation was attempted. Because the saturated vapor pressure of ethanol is 177.8 mbar at 41°C, where a thermotolerant microbe can grow well, ethanol can be collected from the fermenting culture when pressure is reduced to less than the saturated vapor pressure. The system shown in **Figure 5** was constructed and tested, which consists of a fermentation and a distillation tank, the primary and secondary ethanol recovery units, a vacuum pump, and a drain unit. In this system, ethanol is concentrated as the process proceeds from the primary to secondary ethanol recovery units. Due to the set-up of this system, the air in the tank was discharged outside during the vacuum distillation, and some ethanol was trapped in the drain unit. When fermentation with *K. marxianus* DMKU 3-1042 and distillation at 70 mbar and 41°C was applied, about 35 and 60% were recovered in the primary and secondary bottles [39]. The process of the simultaneous fermentation and distillation under a low pressure was continuously repeated three times, with 12% rice-hydrolysate [39]. Similar performance was achieved with a thermo-adopted strain of *Zymomonas mobilis* TISTR548, an ethanologenic bacterium [39].

That system provides some benefits: (i) microbes avoid exposure to high concentrations of ethanol or acetic acid or strong oxidative stress and (ii) fermentation can be continued during distillation increasing ethanol yields. Although further experiments for its evaluation are required, the system including HTF is expected to be one of next-generation fermentation technologies.

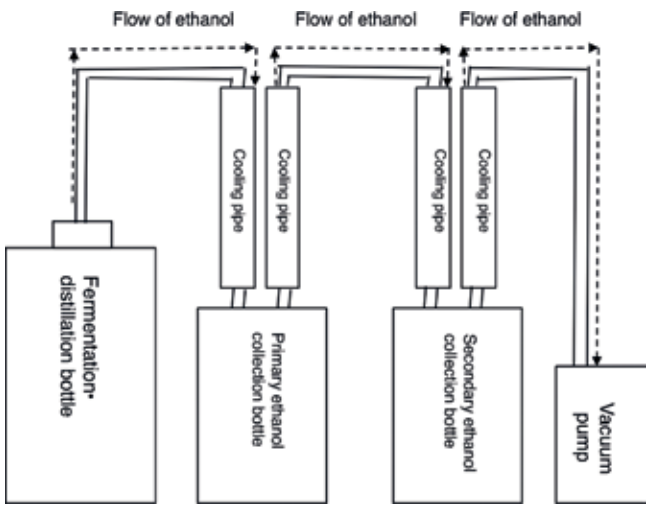

Figure 5. Apparatus for fermentation and distillation under a low pressure. This apparatus consists of a fermentation and distillation tank, primary and secondary recovery bottles, a drain unit, and a vacuum pump.

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Section 5

Recent Advances in Ethanol Fermentation

Chapter 8

Enhanced Ethanol Production of *Saccharomyces cerevisiae* Induced by Cold Plasma at Atmospheric Air Pressure

Xiao-Yu Dong

Additional information is available at the end of the chapter

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Abstract

In this study, cold plasma at atmospheric pressure, as a novel approach of bioprocess intensification, was used to induce yeast for the improvement of ethanol production. Response surface methodology (RSM) was used to optimize the discharge-associated parameters of cold plasma for the purpose of maximizing the ethanol yield achieved by cold plasma-treated *S. cerevisiae*. The resulting yield of ethanol reached to 0.48 g g^{-1} under optimized parameters of plasma exposure time of 1 min, power voltage of 26 V, and an exposed sample volume of 9 mL, which represented an increase of 33% over control. Compared with non-exposed cells, cells exposed with plasma for 1 min presented a notable increment in cytoplasmic free Ca^{2+}, when these exposed cells showed the significant increase in membrane potential. At the same time, ATP level decreased by about 40%, resulting in about 60% reduction in NADH. Taken together, these data suggested that the mechanism that air cold plasma raised plasma membrane potential, which led to increases in cytosolic Ca^{2+} concentration. Furthermore, the cofactor metabolism, such as ATP and NADH, was subjected to regulation that was mediated by Ca^{2+}, ultimately improving yeast productivity. This may have a underlying and broad utilization in enhancing bioconversion capability of microbe in the next few years.

Keywords: ethanol, *Saccharomyces cerevisiae*, cofactor metabolism, bioprocess intensification, cold plasma at atmospheric pressure

1. Introduction

Bioethanol is currently being commercially produced as an alternative to petroleum-based transportation fuels, since it is clean, renewable, carbon-neutral and environmentally friendly [1–3].

Saccharomyces cerevisiae is one of dominant strains of bioethanol production. During fermentation, various factors such as cell membrane barrier, intracellular enzyme activity, the multiple inhibitions of products and substrates, limit the yeast growth and reduce microbial viability, and consequently cause a decrease in ethanol yield [4]. Among those factors, cell membrane permeability is main influence factor that restricts the rates of substrate uptake and release of metabolic products. It has become a focus of global attention to develop a novel method to control membrane permeability for improving yeast capacity in bioconversion of ethanol.

Pretreatment technologies have been developed to intensify bioethanol production, including physical, chemical, biological and physicochemical technologies [5–9]. Furthermore, the methods to control the membrane permeability have also been established, such as microwave, electric field, oxidative stress [10–12]. However, these methods have several drawbacks. For example, the chemical methods could generate enormous amounts of hazardous waste, while physical methods are difficult to apply at large scales. It is therefore necessary to develop a novel approach to change cell membrane permeability for improved bioethanol yield.

Cold plasma at atmospheric air pressure has recently been regarded as a new and advantageous pretreatment technology result from its superior features of high efficiency, low energy consumption and environmentally friendly. Air cold plasma could present various biological effects on the microbes, such as activation effect, sterilization effect and mutagenesis effect, due to the changes in the concentration of reactive species caused by different parameters associated with the plasma discharge [13]. Therefore, the discharge-associated parameters for improved ethanol yield need to be optimized. In this study, the response surface method (RSM) was performed to optimize experimental parameters that could cause the increase in the yield of ethanol generated by *S. cerevisiae*.

Saccharomyces cerevisiae has been widely used in the production of bioethanol by transforming glucose in industry. The glucose metabolic pathway in *S. cerevisiae* during anaerobic fermentation is shown in **Figure 1**. The tricarboxylic acid cycle (TCA) pathway occurs as two branches in the cytosol [14], but does not operate as a cycle in the mitochondrion as most of the earlier reports.

The cell membrane is the first barrier that the substrate enters into the cytoplasm. Thus the improved membrane permeability would promote the glucose utilization and even ethanol release. The rapid consumption of glucose could disturb the cofactor metabolism (such as ATP, NADH et al.) and the re-distribution of carbon flux in glycolysis pathway [15]. In addition, the open of ion channels is the one of mechanisms that the cell membrane permeability is improved. Especially, calcium ion channel administers the alterations of cytoplasm calcium ion concentration ($[Ca^{2+}]_{cyt}$). Ca^{2+}, as a key secondary messenger, is importantly responsible for cell metabolism and activities of some categories of ATPase [16]. As shown in **Figure 1**, a raise of $[Ca^{2+}]_{cyt}$ can be result of improved inflow of extracellular Ca^{2+} by Cch1 protein/Mid1 protein (Cch1/Mid1 p) on cell membrane or as a result of outflow of vacuolar Ca^{2+} into the cytoplasm through vacuole membrane-located Yvc1 protein (Yvc1p) channel [17–20]. Until now, little knowledge has been obtained on the relationship among air cold plasma, cell membrane permeability, cofactor metabolism and ethanol yield.

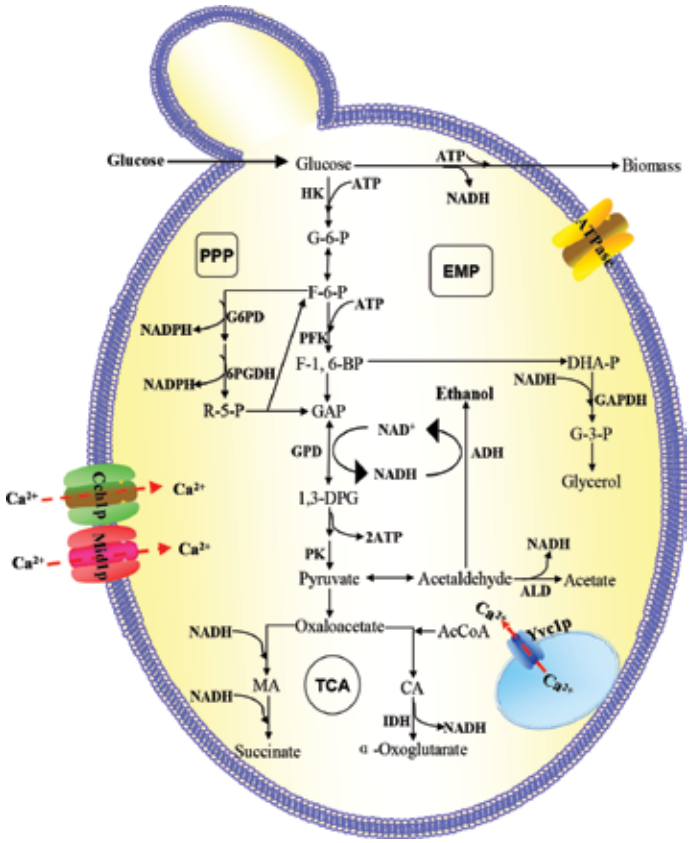

Figure 1. Major pathway of glucose metabolism in *S. cerevisiae* under anaerobic conditions.

The object of this study was to achieve the maximum yield of ethanol by optimizing parameters associated with plasma discharge. Moreover, the mechanism of intensified yield of ethanol produced by *S. cerevisiae* was explored. These data will provide the valuable theory base for developing a novel bioprocess intensification technology in biochemical engineering industry.

2. Results and discussion

2.1. Parameter optimization associated with plasma discharge for enhanced ethanol yield

2.1.1. Influence of plasma treatment time on ethanol yield

To achieve the maximum ethanol-yield, plasma treatment time was set at five different time intervals, from 1 to 5 min. Ethanol yield at 3 min reached to the maximum (0.45 g/g), and it presented an increase of 29% over the control (**Figure 2**). This indicated that a plasma treatment

time of 3 min was appropriate for maximal ethanol production. Thus, 3-min treatment time was chosen as the treatment time for studying the influences of various power supply voltages and volumes of yeast suspension on ethanol yield. In our earlier research, the highest yield of 1,3-propanediol produced by *Klebsiella pneumoniae* was got when the cell suspension was treated by dielectric barrier discharge for 4 min [21], proposing that different species microbes tend to respond differently to different times of plasma treatment. It was clear that 3-min is optimal for *S. cerevisiae* to obtain the maximum ethanol yield in glucose fermentation.

2.1.2. Influence of power supply voltage on ethanol yield

The influence of the power supply voltage in plasma treatment on ethanol yield is shown in **Figure 3**. Ethanol yield raised with raising power supply voltages, up to 0.42 g/g, then dropped with further increase in power supply voltage. The maximum yield of ethanol was achieved at 26 V.

It has been reported that charged particles in low-temperature plasma play a key role in the alterations of the outer structure of *Candida albicans* [22]. Raising the power supply voltages also causes an increase of the electric field in the gap distance. This might cause the microbial cell membrane to depolarize and become permeabilized, making it easier for the substrate to enter into the cells and for the products to release out the cells, which accordingly forming 27% increase in ethanol yield over the control. However, further increment in voltage results in a reduction of ethanol yield. This might be attributed to the neutralization of the negative charges, which could lead to cytoplasm leakage and cell death [13].

2.1.3. Influence of treated suspension volume on ethanol yield

The influence of various sample volumes on the ethanol yield was studied for the maximal ethanol yield. As shown in **Figure 4**, a sample volume of 5 mL enhanced ethanol yield by 28% for

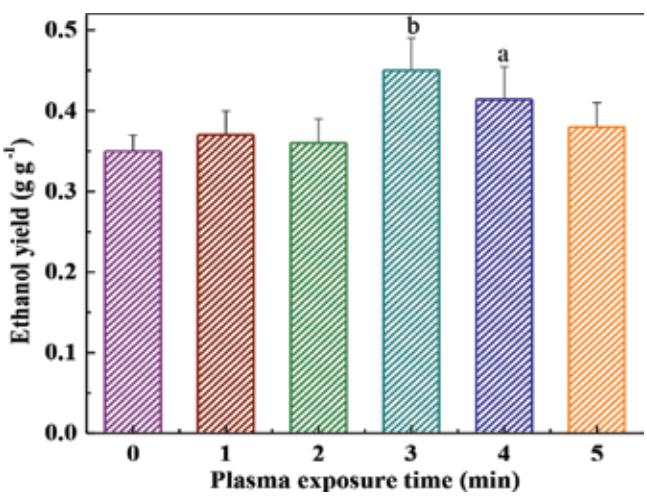

Figure 2. Influence of plasma treatment time on ethanol yield. Data are expressed as mean ± SES. 'a' and 'b' indicate $P < 0.05$ and $P < 0.01$, respectively.

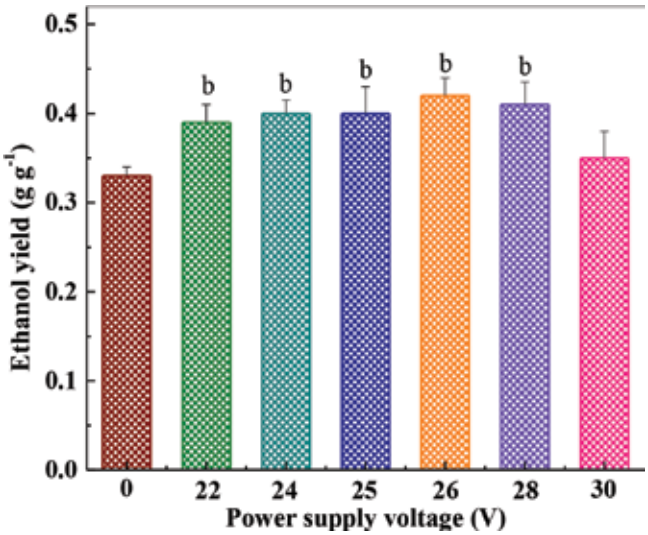

Figure 3. Influence of power supply voltage on ethanol yield. Data are expressed as mean ± SES. 'a' and 'b' indicate $P < 0.05$ and $P < 0.01$, respectively.

the plasma pretreated cells over the control. Cell suspension also constitutes a dielectric layer. A larger suspension volume means that the thickness of the dielectric layer would increase in a Petri dish of 60-mm diameter, and any alteration about dielectric properties would also caused a alteration in discharge characteristics, especially for the power voltage [23]. As a result, a sample suspension volume of 5 mL could show an impactful augment in ethanol yield.

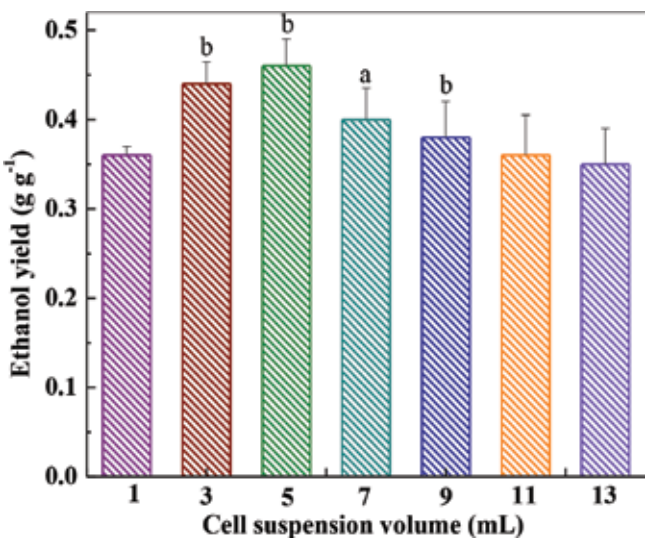

Figure 4. Influence of yeast suspension volume on ethanol yield. Data are expressed as mean ± SES. 'a' and 'b' indicate $P < 0.05$, $P < 0.01$, respectively.

2.1.4. Predictive response model

The design matrix and the corresponding experimental data were presented in **Table 1**. These values were fitted to the next second-order polynomial equation and the results were presented in **Table 2**.

$$Y = 0.22 - 0.12\,X_1 - 0.046\,X_2 + 0.11\,X_3 - 0.039\,X_1X_2 + 0.044\,X_1X_3 - 0.039\,X_2X_3 + 0.05\,X_1X_1 - 0.04\,X_2X_2 + 0.07\,X_3 \quad (1)$$

The adequacy of the model was checked using analysis of variance (ANOVA), which was tested using Fisher's statistical analysis [24]. The Model F-value of 6.09 indicated model significance. Value of "Prob > F" less than 0.05 indicated that the model terms were remarkable, whereas values greater than 0.10 indicated no significance. ANOVA resulted in a value of 0.85 for the coefficient of determination (R^2) and 0.71 for the adjusted coefficient of determination

Run	X_1	X_2	X_3	Y
1	−1	−1	−1	0.27
2	1	−1	−1	0.04
3	−1	1	−1	0.35
4	1	1	−1	0.03
5	−1	−1	1	0.48
6	1	−1	1	0.49
7	−1	1	1	0.47
8	1	1	1	0.27
9	−1	0	0	0.49
10	1	0	0	0.02
11	0	−1	0	0.31
12	0	1	0	0.02
13	0	0	−1	0.22
14	0	0	1	0.29
15	0	0	0	0.23
16	0	0	0	0.22
17	0	0	0	0.23
18	0	0	0	0.22
19	0	0	0	0.22
20	0	0	0	0.22

[Y] Observed Ethanol yield (g/g)

Table 1. Experimental design and results for the central composite design.

Source	Sum of squares	*df*	Mean square	*F*-value	*P*-value
Model	0.37	9	0.041	6.09	0.005
X_1	0.15	1	0.150	21.92	0.001
X_2	0.02	1	0.021	3.12	0.108
X_3	0.13	1	0.130	18.47	0.002
X_1X_2	0.01	1	0.012	1.77	0.213
X_2X_3	0.02	1	0.015	2.26	0.164
X_2X_3	0.01	1	0.012	1.77	0.213
X_1X_1	0.006	1	0.007	1.01	0.338
X_2X_2	0.003	1	0.004	0.65	0.440
X_3X_3	0.013	1	0.013	1.98	0.189
R^2	–	–	0.85	–	–
Adj-R^2	–	–	0.71	–	–

Table 2. AVOVE for response surface quadratic model for ethanol production.

(R^2_{adj}). The R^2_{adj} value was close to 1, which indicated a high degree of correlation between the observed and predicted values [25]. *P*-values were used to check the significance of each variant. Each of the *P*-values also indicated the interaction strength between any two of the independent variants; the smaller the *P*-value, the higher the significance of the corresponding variant [26]. As with the interaction between any of the two variants, the smallest *P*-value was seen with X_1X_3. This suggested that among the three parameters investigated, maximum interaction occurred between plasma treatment time and the volume of the induced sample.

2.1.5. Influence of various experimental parameters on ethanol yield

The influences of the independent parameters, including plasma treatment time, power supply voltage and induced-sample volume, on ethanol yield were analyzed by three dimensional response surface plots (**Figure 5**). **Figure 5(a)** presented the ethanol yield based on a combination of plasma treatment time and power supply voltage. The predicted ethanol yield showed to increases at 1 min and from 25 to 27 V. **Figure 5 (b)** presents the interaction between plasma treatment time and sample suspension volume on ethanol yield. The highest ethanol yield was achieved when 9-mL sample suspension was treated by dielectric barrier discharge (DBD) plasma for 1 min. The predicted ethanol yield of *S. cerevisiae* reached to a maximum when 9-mL sample was treated under the range of the power supply voltage from 22 to 26 V (**Figure 5(c)**). These three-dimensional plots offer a visual interpretation of the interaction between two parameters and promote the location of optimum experimental parameters. The optimized conditions for the three experimental parameters (as obtained from the maximal point of the model) were calculated by the Design expert software to be 1 min, 26 V and 9 mL, respectively, corresponding to plasma exposure time, power voltage, and volume of exposed cell suspension. The model forecasted a highest response of 0.49 g/g ethanol yield for this point.

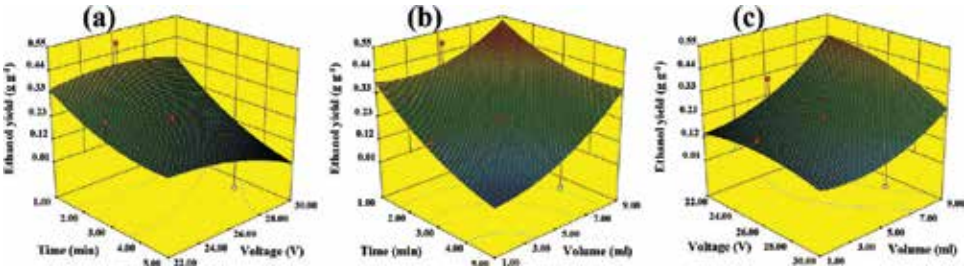

Figure 5. (a) Response surface plot of the interaction between plasma-treatment time and power supply voltage on ethanol yield; (b) response surface plot of the interaction between plasma-treatment time and induced sample volume on ethanol yield; (c) response surface plot of the interaction between power supply voltage and volume of induced sample on ethanol yield.

2.1.6. Confirmation of optimum parameters

Optimum conditions of the parameters achieved from the above analysis were verified by carrying out flask fermentation with *S. cerevisiae* from 9-mL sample suspension that had been exposed with plasma for 1 min and a power supply voltage of 26 V. As shown in **Table 3**, the ethanol yield reached to 0.48 g/g, which was very close to the predicted value of 0.49 g/g, and represented a 33% increase compared with the yield of the untreated sample (0.36 g/g). The outstanding correlation between the predicted and the measured values confirmed that the model was feasible and that an optimal point for increasing ethanol yield could be obtained. The ethanol concentration in the fermentation also raised by 42% and the biomass raised by 24% over those obtained from fermentation by untreated sample (**Table 3**).

To enhance the concentration of ethanol, different methods have been used to improve the productivity of the correlative microorganism strains, including construction of genetic engineering strain [27], mutagenesis and breeding [28], as well as metabolism control by changing the osmotolerance of the external environment [29]. Up to now, little study has been reported about the application of cold plasma at atmospheric pressure in intensifying ethanol yield of *S. cerevisiae*. It has been early found that growth of *K. pneumoniae* could be enhanced by air cold plasma, causing an increment in productivity of 1,3-propanediol [21]. In addition, the application of plasma discharge could also lead to the degradation of the biomacromolecules that constitute the cell-envelope, such as polysaccharides and protein [30]. Cell membrane permeability is influenced as a result of alterations in the cell envelope composition. This then leads to alterations in metabolic products as well as in the physiological activity of the cells. Yonson et al. has discovered that human hepatocytes (HepG2) cells could become provisionally permeabilized

Groups	Biomass (g/L)	Glucose consumption (g/L)	Ethanol (g/L)	Ethanol yield (g/g)
Control group	5.4 ± 0.9	132.0 ± 8.3	47.5 ± 2.7	0.36 ± 0.02
Optimized group	6.7 ± 1.1	141.0 ± 10.8	67.5 ± 4.2	0.48 ± 0.03

Table 3. Comparison of flask fermentation by ***S. cerevisiae*** under optimized and untreated conditions.

when they are induced by a miniature atmospheric-pressure glow-discharge plasma torch [31]. Therefore it is thought that the permeability of the cell membrane in *S. cerevisiae* might probably promote the diffusion of substrates into the cell as well as the export of products out of the cells, causing an alteration in the metabolic process. This could also be the reason why the improved ethanol yield could be observed with plasma treated *S. cerevisiae* in this research.

The optimized parameters (1 min, 26 V, 9 mL) achieved by the central composite design experiment were different from the optimized parameters (3 min, 26 V, 5 mL) achieved by single-factor experiment. This may be due to the following reasons. Firstly, response surface methodology reflected the influences of interaction among the three parameters employed with the other parameter maintained at its respective zero level on ethanol yield. In this research, the dielectric layer became thick when the volume of the test sample was increased in an unchanged 60-mm-diameter Petri dish, causing an alteration in the power voltage. Therefore, the three parameters (plasma exposure time, test sample volume, power supply voltage) underwent a simultaneous alteration. Secondly, the plasma discharge device was directly laid in air at room temperature, and the discharge was affected by various environmental factors, such as air humidity and ambient temperature. Finally, experimental errors were observed during the operation. For example, the gap distance between electrodes was widened again and again for putting the sample on the bottom electrode before every experiment, and then the distance between electrodes was recovered.

2.2. Mechanism study about enhanced ethanol yield of *Saccharomyces cerevisiae* with cold plasma

2.2.1. Plasma membrane permeability

The alterations in membrane permeability exhibited by *S. cerevisiae* cells following their exposure to plasma and subsequent culturing under fermentation conditions are shown in **Figure 6**. After plasma treatment for 1 min, the membrane permeability reduced compared with that of untreated cells, but raised when the samples were treated respectively from 2 to 4 min, and fell back to the level of untreated cells when the sample was induced for 5 min. The membrane permeability of the treated cells reached to a maximum when the sample treated for 4 min were cultured for 9 h, producing a 1.2-fold increase over that of untreated cells. As for sample that was cultured for 21 h, a significant increase in membrane permeability only occurred for those that were derived from samples treated to plasma for 1 and 5 min.

2.2.2. Plasma membrane potential

The membrane potential was measured with the aid of the fluorescence probe Rh123 (**Figure 7**). The fluorescence intensity of Rh123 was positively correlated with plasma membrane potential. These data indicated that the plasma membrane permeability was increased (20%) when the samples were treated for 1 min, but was decreased when they were treated for 2–5 min. When the treated samples were cultured for 9 h, only the membrane potential of the sample treated for 1 min reduced relative to that of non-treated sample. Other

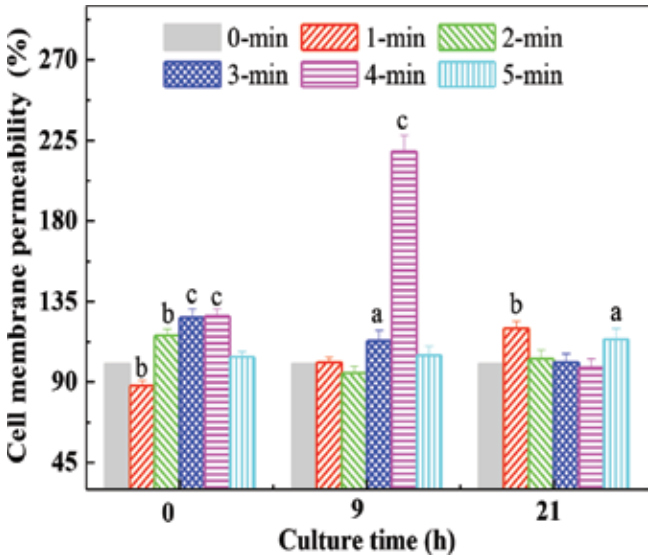

Figure 6. Influence of plasma treatment on cell membrane permeability of *S. cerevisiae* before and after fermentation. Data are expressed as mean ± SES. 'a', 'b', and 'c' indicate $P < 0.05$, $P < 0.005$ and $P < 0.001$, respectively.

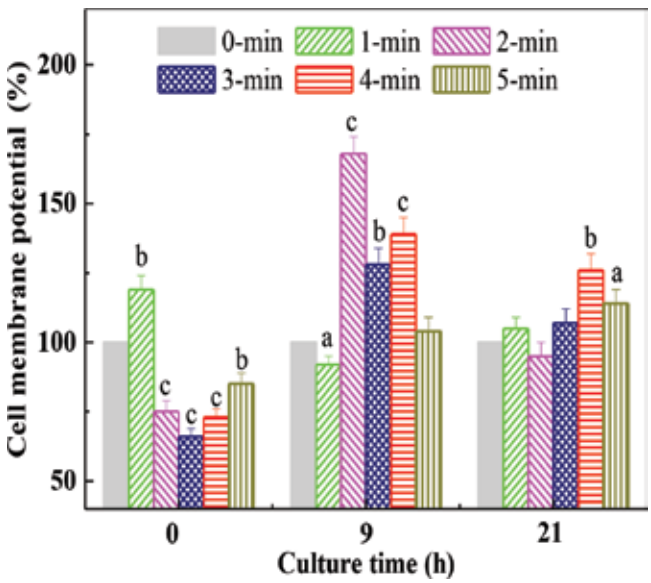

Figure 7. Influence of plasma treatment on cell membrane potential before and after fermentation. Data are expressed as mean ± SES. 'a', 'b', and 'c' indicate $P < 0.05$, $P < 0.005$ and $P < 0.001$, respectively.

exposure times gave various increases in membrane potential, among which 2 min exposure yielded the maximum increase (70%) compared with non-treated sample. In the case of 21-h fermentation, 4- and 5-min exposures gave remarkable improvements in membrane potential over non-treatment. These data seemed to show that cold air plasma discharge could either increase or decrease the plasma membrane potential of *S. cerevisiae* cells.

2.2.3. Cytoplasmic calcium concentration

The intracellular calcium concentration of plasma-treated samples was detected using the fluorescence probe Fluo-3 AM (**Figure 8**). The calcium concentrations in the cytoplasm were improved with plasma treatment time, with 5 min treatment giving the maximal increase, about 36% more than the concentration measured in the non-treated cells. After 9 h of fermentation, cytoplasmic Ca^{2+} concentrations were significantly increased in the sample of 1- or 2-min plasma treatment over non-treatment of plasma, but in the samples from 3- to 5-min plasma treatment, Ca^{2+} concentrations were less compared with non-treatment of plasma.

2.2.4. Extracellular ATP concentration

The influence of plasma treatment on extracellular ATP concentration was most significant prior to fermentation (0 h) and at the 9-h stage of fermentation following plasma exposure (**Figure 9**). Prior to fermentation, some significant reductions in extracellular ATP concentration were measured when *S. cerevisiae* cells were treated by plasma for 1 and 2 min, but the remarkable increases in ATP concentration happened when the samples were treated by plasma for 3–5 min over non-treated sample. At the 21-h period of fermentation, however, the extracellular ATP concentrations in 1- and 5-min treatments appeared to be somewhat lower than that of non-treated cells. Thus the data showed that the plasma treatment might change the concentration of extracellular ATP either immediately after treatment or when the treated samples were permitted to reproduce for a moderate stage of time under normal fermentation conditions.

2.2.5. Extracellular NADH concentration

Differences in extracellular NADH concentrations between non-treated and plasma-treated *S. cerevisiae* samples were less uniform for all the three periods of measuring. The differences were more noticeable between non-treated sample and treated samples before fermentation or at the 21-h stage of fermentation (**Figure 10**). Before fermentation, 1-min treatment induced a decrease of 60%, but 2- and 3-min treatments led to 0.8- and 1.8-fold increases, respectively, in extracellular NADH concentration. At the 9-h fermentation stage, the extracellular NADH concentrations of treated samples were either similar to or significantly lower than those of non-treated sample. However, the sample that were treated with plasma for 1 min represented a noticeably higher extracellular NADH concentration than that of non-treated sample at the 21-h fermentation stage, although it remained much lower than that of non-treated sample in the other two stages (0 and 9 h). In addition, the samples treated for 2 to 5 min also showed remarkably higher extracellular NADH concentration than non-treated sample at the 21-h fermentation stage. Taken together, these results indicated that plasma treatment can change the extracellular NADH concentration, either quickly after treatment or in subsequent fermentation, depending on the exposure time.

In this research, we have proved that remarkable decrease in membrane permeability of live cells were distinct after the sample was treated by plasma for 1 min (**Figure 6**). At the 21-h periods of fermentation, the membrane permeability was increased showing that the effect of air cold plasma on membrane permeabilization was temporary and non-inheritable. This

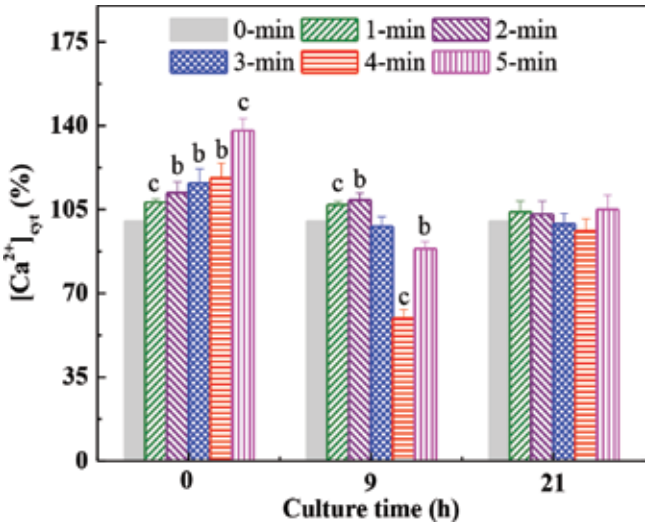

Figure 8. Influence of plasma treatment on $[Ca^{2+}]_{cyt}$ before and after fermentation. Data are expressed as mean ± SES. 'a', 'b', and 'c' indicate $P < 0.05$, $P < 0.005$ and $P < 0.001$, respectively.

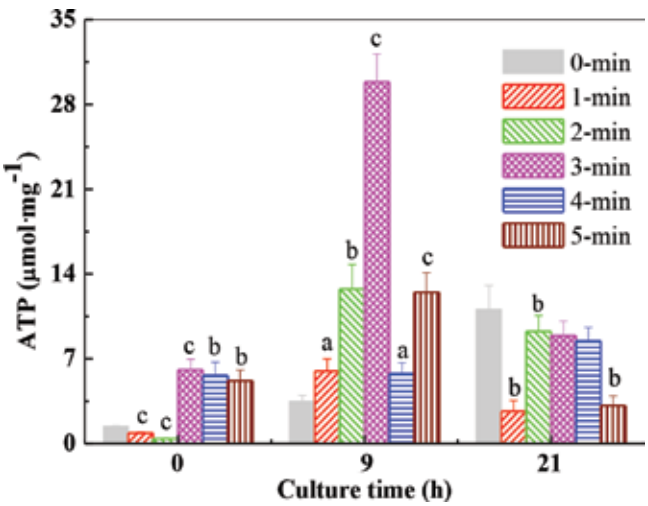

Figure 9. Influence of plasma treatment on extracellular ATP before and after fermentation. Data are expressed as mean ± SES. 'a', 'b', and 'c' indicate $P < 0.05$, $P < 0.005$ and $P < 0.001$, respectively.

result was in accordance with the study of Yonson *et al.*, who reported that cell membrane permeability is temporarily improved by a miniature atmospheric pressure glow discharge plasma torch [31].

Membrane potential is an important factor in cellular functions such as signaling and transport, which can eventually affect cell metabolism [32]. An alteration in membrane potential can be positively detected by an alteration in fluorescence intensity of Rh123. When discharge

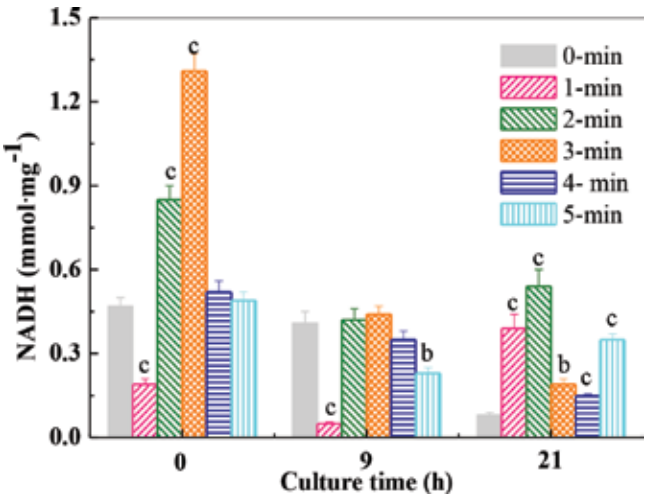

Figure 10. Influence of plasma treatment on extracellular NADH before and after fermentation. Data are expressed as mean ± SES. 'a', 'b', and 'c' indicate $P < 0.05$, $P < 0.005$ and $P < 0.001$, respectively.

plasma occurs over the solution surface, a variety of physical and/or chemical processes are activated. Many active species such as oxygen, hydrogen, hydroxyl and hydroperoxyl radicals are produced. These reactive species can diffuse in the surrounding liquid and induce the redistribution of charges on the inner and outer surfaces of the cell membrane, leading to an increase or reduction of membrane potential. Such change of the membrane potential would directly affect the plasma membrane permeability. After *S. cerevisiae* cells were treated by air cold plasma, the change in the membrane potential quickly contrasted with the change in membrane penetrability (**Figure 7** versus **Figure 6**). The cell membrane was depolarized due to the lowered potential, finally improving the permeability of the membrane. More inorganic and organic ions can then pass freely through the cell membrane as a result of this enhanced permeability [33]. After the 9- and 21-h stages of fermentation, the increase in membrane potential led to membrane hyperpolarization, and accordingly enhanced the membrane permeability.

The change of cell membrane potential could activate the voltage-dependent Cch1p channel, causing more influx of Ca^{2+} from the extracellular environment into the cytoplasm (**Figure 1**). Therefore, the calcium level in the cytoplasm of treated cells was enhanced after plasma treatment. Air cold plasma slightly improved the cytoplasmic calcium concentration of the sample following treatment for 1 min. This might result from the increase in plasma membrane potential (**Figure 7** versus **Figure 8**, at 0-h culture), causing cell membrane hyperpolarization and opening of Ca^{2+} channels. But the opening of Ca^{2+} channels did not cause an increase in cell membrane permeability (**Figure 6**). This result suggests that the increment in cell membrane permeability might be controlled by more than one channel modulator.

The alteration trend of ATP concentration was different from the alteration tend in membrane permeability with plasma discharge. This shows that change of extracellular ATP concentration is a direct consequence of alterations in intracellular ATP. Before fermentation, the

lower concentrations of ATP at 1 and 2 min plasma treatment might be due to 6.8 and 10% increments in calcium concentration, respectively. The increased calcium concentration promoted the hydrolysis of ATP to adenosine diphosphate (ADP) (**Figure 9**). A Ca^{2+} concentration gradient from 1 to 10 μM, could improve the cell function that regulates cell growth and metabolism to eventually enhance microbial productivity. However, the high concentrations of intracellular Ca^{2+} can induce cell injury or death [34, 35]. The higher concentrations of ATP in the samples treated by plasma for 3–5 min might be due to an inhibition of ATP hydrolysis caused by the higher cytoplasmic calcium concentration (**Figures 8** and **9**). In addition, any disturbance in environmental conditions would influence the activities of catabolic enzymes, thereby accelerating the accumulation of ATP or ADP [35]. Air cold plasma might lead to the accumulation of ADP in the treated samples within 1–2 min of treatment, and of ATP in the treated samples within 3–5 min of treatment, as suggested by the data in **Figure 9**. The accumulation of ATP or ADP might have immediately affected the glycolysis rate [36], producing different ATP concentrations at the 9- or 21-h period of fermentation, depending on the plasma treatment time (**Figure 9**).

Air cold plasma produces different reactive species in the gas phase [37]. These active species further react with water and produce a variety of biologically active reactive species (RS) in the liquid phase, including long-lifetime RS (ozone, hydrogen peroxide and nitrate ions) and short-lived RS (superoxide, hydroxyl radicals and singlet oxygen) [38]. In our research, these reactive species could increase or decrease the cell membrane potential and open Ca^{2+} channels, consequently improving $[Ca^{2+}]_{cyt}$ (**Figures 7** and **8**, at the beginning of culture). Ca^{2+} supplementations of 0.5 and 1.5 mM have been shown to induce the increment in ATPase activity [29]. The enhanced ATPase activity would then promote the generation of proton motive force through hydrolysis of ATP [29, 39]. A reduction in the intracellular ATP level can result in the up-regulation of the activities of phosphofructokinase (PFK) and pyruvate kinase (PK) [40]. This would accelerate the glycolytic flux and enhance the NADH level in the central metabolic pathway [41]. At the same time, NADH-dependent alcohol dehydrogenase (ADH) activity might be improved, leading to up-control of the oxidation of NADH to NAD^+ [40, 42] (**Figure 1**). Therefore, the NADH concentration obtained from 1 min treatment was reduced over the control because of the lower level of ATP (**Figure 10** 1 min versus **Figure 9** 1 min). The oxidation of NADH to NAD^+ would lower the activity of NADH-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPDH), causing decreased glycerol production and ultimately causing more carbon flux from glycolysis being funneled to ethanol [42–44].

3. Conclusion

Experimental parameters associated with cold plasma discharge at atmospheric air pressure for enhancing ethanol yield of *S. cerevisiae* has been successfully optimized in this research. The maximum theoretical ethanol yield of 0.49 g/g was predicted by the response model under three optimized parameters (1 min of exposure time, 26 V of power voltage and 9 mL of test sample volume), which was closely consistent with the experimental yield of 0.48 g/g. The model may be used as a reference for modulating the experimental parameters related with dielectric barrier discharge at air atmospheric pressure and a novel approach for improving ethanol yield in bio-manufacturing industry.

Furthermore, the potential mechanism that air cold plasma alters the cofactor metabolism of *S. cerevisiae* was explored by analyzing the changes in plasma membrane potential, cytoplasmic calcium concentration and the two cofactors of ATP and NADH. The sample of 1-min treatment presented a notable increment in plasma membrane potential, whereas the sample of 2-min treatment presented a distinct reduction in plasma membrane potential. In addition, the calcium concentrations for the samples treated by plasma for 1–5 min were remarkably improved prior to the beginning of the fermentation compared with that for the untreated sample. An increase of 7.0% in calcium concentration led to the remarkable reductions of 40% in ATP and 60% in NADH in the sample of 1-min treatment. At 9-h culture, the ATP concentration of treated sample for 1 min increased by 72%, whereas NADH concentration decreased by 88% relative to those of the control. Briefly, the mechanism that plasma promoted alterations in cofactor level in *S. cerevisiae* showed to be by improving the cell membrane potential, which then caused increases in cytosolic free Ca^{2+} concentrations within the cells, eventually enhancing microbial productivity. This may a potential and broad application in intensifying the biotransformation capability of microorganisms in the future.

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Conflict of interest

The author declares no financial or commercial conflict of interest.

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Chapter 9

High Hydrostatic Pressure Process to Improve Ethanol Production

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Additional information is available at the end of the chapter

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Abstract

The use of high hydrostatic pressure (HHP) is an interesting approach to optimize the production of both first- and second-generation ethanol. It may be applied on *Saccharomyces cerevisiae* cells to enhance the fermentation pathway and on the lignocellulosic biomass to increase sugar release. HHP has a wide effect on many biological processes, such as growth, division and cellular viability. Actually, conformation, stability, polymerization and depolymerization of proteins are affected by HHP as well as lipid packaging. Moreover, transcriptional profile analysis indicates an activation of the general stress response. In yeast, HHP higher than 100 MPa leads to significant morphological and physiological alteration, and loss of cellular viability occurs over 200 MPa. A yield rate increase in ethanol production occurs at pressures of 10–50 MPa, but over 87 MPa alcoholic fermentation is interrupted.

Keywords: *Saccharomyces cerevisiae*, high hydrostatic pressure, fermentation, stress, ethanol productivity

1. Introduction

Ethanol has a long history as an alternative fuel, and nowadays, it is the most widely used biofuel in the transportation sector [1]. Since the 1980s, the interest in using bioethanol has been increasing, and it is currently used in many countries. Bioethanol can be categorized into three groups depending on the feedstock used to obtain it. First-generation bioethanol is

produced from feedstock rich in sucrose (sugarcane, sugar beet, sweet sorghum, and fruits) or starch (corn, wheat, rice, potato, cassava, sweet potato, and barley). Second-generation bioethanol is obtained from lignocellulosic biomass such as wood, straw, bagasse, grasses and other agricultural residues. Third-generation bioethanol derives from algal biomass including microalgae and macroalgae.

Microorganisms such as yeasts play an essential role in bioethanol production by fermenting a wide range of sugars to ethanol. They have been used for thousands of years for beer brewing and are probably the oldest domesticated organism [2]. Current industrial ethanol fermentation is mainly carried out with the yeast *Saccharomyces cerevisiae* because of its hardiness, low pH, and high ethanol tolerance, thus making the process less susceptible to contamination [1, 3]. Wild *S. cerevisiae* strains are able to survive and dominate alcoholic fermentation vats, which pass through phases of high sugar content, high temperature, CO_2 pressure, being considered, therefore, inhospitable environments [4]. Thus, a yeast strain with multiple stress resistance is a desired attribute [5].

Many microbial communities are adapted to live and survive on extreme environmental conditions including high hydrostatic pressure (HHP). It is known that high hydrostatic pressure induces changes in proteins, enzyme conformation and aggregation, interaction between lipids and proteins, gene expression and cell structures that are composed of lipids such as biological membranes [2]. It has already been shown that high hydrostatic pressure exerts a broad effect in *S. cerevisiae* with results similar to those of other common stresses, such as temperature, ethanol, and oxidative stresses [6]. Moreover, *S. cerevisiae* produces ethanol faster at high pressure when compared to ambient pressure, proving HHP as a tool to enhance ethanol production [7].

In biotechnology industry, one of the oldest and most important fermentation processes used is the ethanol fermentation. Ethanol is the most consumed biofuel in the world, and Brazil was the first country that introduced it in its energy matrix, holding the most economically viable process for its production. It was for decades the largest producer, losing that position to the United States, but it remains the largest exporter of ethanol [4]. About 4.5 billion gallons of ethanol are produced annually from corn and used as a transportation fuel only in the United States. The annual bioethanol production in the U.S. is expected to grow to more than 7.5 billion gallons in the next few years and reach 30 billion gallons by 2025 [3].

This chapter approaches the interaction between HHP and ethanol production by *S. cerevisiae*, describing the main HHP effect in yeast, linking this knowledge to further improvement of ethanol production efficiency.

2. Fermentation process

2.1. Bioethanol production

S. cerevisiae cells under anaerobic conditions undergo alcoholic fermentation; a process that convert monosaccharides (sugars) to ethanol, carbon dioxide and heat. Basically, one molecule

of glucose yields two molecules of ethanol and two molecules of carbon dioxide as shown in **Figure 1** [8]. Baking, brewing and fuel industries rely on this ability of the yeast *S. cerevisiae* to convert glucose into ethanol and carbon dioxide. The fermentation process may present multiple stress conditions such as temperature, ethanol concentration, pressure, desiccation, acidity or alkalinity, osmotic and ionic stress and low oxygen levels (**Figure 2**). Therefore, *S. cerevisiae* has been chosen over the centuries for being physiologically adapted to them [9].

To enhance yeast growth for fermentation, usually, nutritional salts, vitamins, fermentation inducers and inhibitors, precursors, acids, antifoams are added. Then, in the bioreactor, time is given for yeast duplication until the desired cell concentration is reached. Bioreactor is a tool used in yeast bioprocesses, and it is, often, a stirred tank. It separates the internal environment from the external one so anything entering or leaving the fermentation is monitored. Therefore, its use leads to a higher production and productivity of the intended product due to its capacity to easily control and module the chemical and physical conditions [2].

2.2. Exposure and response to different stresses

Yeasts are free-living microorganisms and therefore need to have mechanisms for rapid adaptation to environmental changes. Upon fermentation in the bioreactor, yeasts are subjected to changes in temperature, ethanol concentration, osmotic pressure, pH, and oxygen level [10, 11]. *S. cerevisiae* presents a general response to changes in the environment disregarding the kind of stress, and there is also a gene expression regulation specific for each stress; therefore, it is controlled by each new condition [12].

Osmotic stresses occur at the beginning of the fermentation and decrease with the gradual reduction of sugar in the medium, leading to alterations in the cell metabolism and viability decrease [13]. Osmotic stress causes a rapid loss of the cell actin filaments, perturbation on the cell membrane structure, permeability and mechanical properties, besides the expected loss

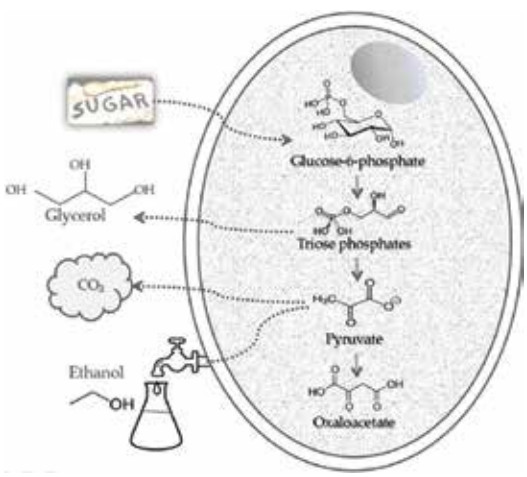

Figure 1. Carbohydrate or sugar or monosaccharide metabolism in yeast under anaerobic conditions.

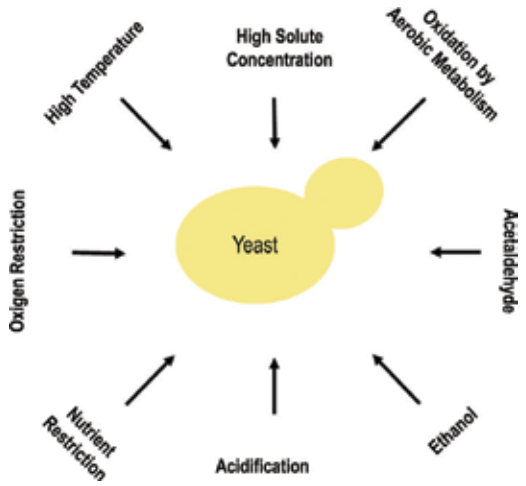

Figure 2. Stresses suffered by yeast during fermentation process.

of water and shrinking of the cell, and, as for most stresses, G1 arrest [9, 14]. Then, during the adaptation phase, these actin filaments are restructured, and the cell is repolarized and starts growing again [15, 16].

Temperature also has a great influence on the metabolic process and can serve as both an activator and a microbial development inhibitor, with lethal implications in some cases. Yeast optimum temperature ranges between 25 and 30°C. When cells are presented to temperatures below optimum they undergo a cold shock, while when grown in higher temperatures than the optimum leads to heat shock. Thermal stress can change proteins properties (chemical and physical), mostly protein aggregation, which triggers malfunctions in all cellular compartments. Cells submitted to thermal stress increase synthesis of the heat shock proteins (HSPs) in order to revert this situation. In *S. cerevisiae*, protein Hsp104 has a decisive role for thermotolerance, acting with Hsp70 and Hsp40 forming a protein complex, which is responsible for induction of partially denatured proteins by high temperatures to return to the native state. Yeast shows intrinsic tolerance when exposed to a sudden thermal shock (50°C) while induced thermotolerance appears when the cells are exposed to an initial moderate thermal shock followed by a severe thermal shock. Others factors can also influence thermotolerance like Ca^{2+} ions, trehalose and cellular growth phase [17].

Ethanol in low concentrations acts as an inhibitor of cell division, while in high concentrations it may lead to cell death [18]. The structure of the cell membrane is severely affected by ethanol, as well as hydrophobic and hydrophilic proteins and the endoplasmic reticulum [19]. Ethanol also causes changes in cellular metabolism, biosynthesis of macromolecules, increases DNA mutations and leads to intracellular protein denaturation, which in response induces the production of heat shock proteins (HSPs) [3]. Moreover, genes that respond to environmental stresses [environmental stresses response (ESR)] are overexpressed during ethanol stress [20]. Among those genes, a HSP group is positively regulated during ethanol stress, especially HSP12, HSP26, HSP78 and HSP104 [21].

Cellular response to damages produced by accumulation of reactive oxygen species (ROS) is known as oxidative stress [10]. ROS are produced in larger quantities during mitochondrial respiration process. Lipid peroxidation may lead to a decrease in membrane fluidity and permeability and enzymatic inactivation. Oxidative damage in proteins may lead to formation of hydrogen peroxide and changes in molecular structure by protein aggregation or fragmentation. Another effect is the damaging of DNA structure by ROS, showing a greater influence in mitochondrial DNA [9]. The adaptive response mechanisms to oxidative stress in *S. cerevisiae* are mainly regulated by transcription factors that collectively coordinate appropriate responses to distinct oxidative stresses by repressing or regulating the transcription of specific genes, which are related to antioxidant defenses. These transcription factors are Yap1, Skn7, Msn2 and Msn4 [20, 22–25].

2.3. High hydrostatic pressure and its physical effects

The force applied on a given surface, that is, in an area unit, is called pressure. Thus, the mathematical equation that represents this phenomenon is:

$$P = \frac{F}{A} \tag{1}$$

where P represents the pressure, F represents the normal force applied to the surface, and A is the surface area. Pressure can be determined as static or dynamic. The dynamic pressure is the one in which a super high pressure is applied for a short period of time and can be associated with temperature. On the other hand, static pressure is a constant pressure value maintained for a long time. Pressure can also be classified as isostatic or nonisostatic. In isostatic, the pressure value is the same in all directions of the given space (e.g., hydrostatic pressure) while the nonisostatic pressure corresponds to a gradient of normal forces in response to pressure applied by an equipment or when there is nonuniform compression due to the inhomogeneous composition of the material.

Atmospheric pressure greatly varies on Earth. In terrestrial habitats, pressure value decreases with increasing elevation and it is close to 1 atm (0.101325 MPa) at sea level, while in the oceans at an average depth of 3800 m, pressure reaches approximately 380 atm (~38 MPa). In addition, most living organisms are below 1000 m (**Figure 3**), those organisms tolerant to high pressures are named piezophiles [26, 27].

2.4. How high hydrostatic pressure affects microorganisms?

2.4.1. General effect of HHP in microorganisms

High hydrostatic pressure (HHP) is a unique type of stress since the effect it triggers is caused only by a change in the system volume. Therefore, when compared to thermal stress which involves temperature and volume changes, the results obtained by HHP are simpler. Moreover, it is important to consider that when HHP is applied, biochemical reactions are followed by volumetric changes; therefore, if a reaction is coupled to an increase in volume, it will be inhibited by the HHP, and when associated with a decrease in volume, it can be enhanced [28, 29].

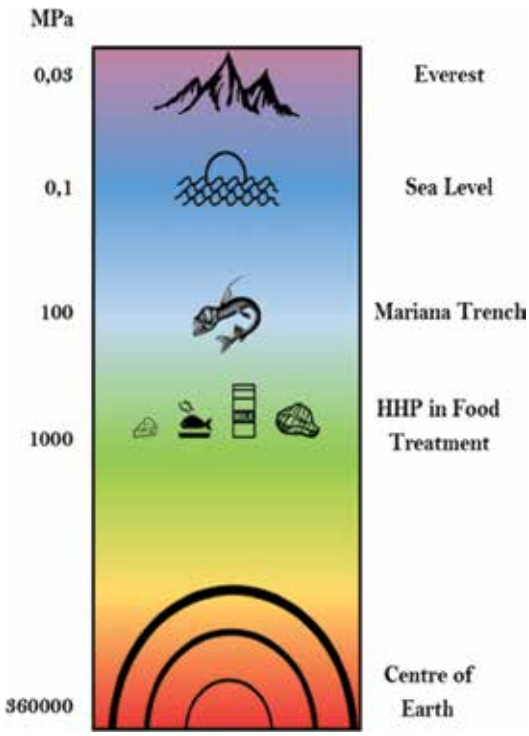

Figure 3. Pressure on Earth—variation and biotechnological use.

Studies on the effects of HHP in microorganisms mostly use the yeast *S. cerevisiae* (**Figure 4**) and the bacteria *Escherichia coli* as models. Yeast cells that are on stationary phase are more resistant to pressure when compared to cells on proliferative phase [30]. This response also occurs in prokaryotic organisms, since *E. coli* presents a 70% resistance in stationary phase after 200 MPa when compared to cells on exponential phase, which present a decrease up to 0.01% of cellular viability under the same amount of HHP [31]. High pressure induces many physiological changes in *E. coli*, such as lag phase extension, cellular filamentation and DNA, RNA and protein synthesis interruption [10, 32–34]. Both in yeast and *E. coli*, changes in membrane lipids occur, as well as the reduction of its fluidity [35].

The wide effects of HHP influence many processes in biological systems, such as growth, division and cellular viability. Depending on the amount and time that HHP is applied, the pressure acts inhibiting or retarding cytokinetic and mitotic activities in dividing cells. The conformation, stability, polymerization and depolymerization of mitotic proteins are affected by high pressure. It also induces lipid packaging, which leads to a reduction in membrane fluidity [36].

2.4.2. HHP effect on Saccharomyces cerevisiae cells

The yeast *S. cerevisiae* is a unicellular fungus that can undergo asexual and sexual reproduction. The asexual reproduction is carried out through budding and the sexual reproduction is

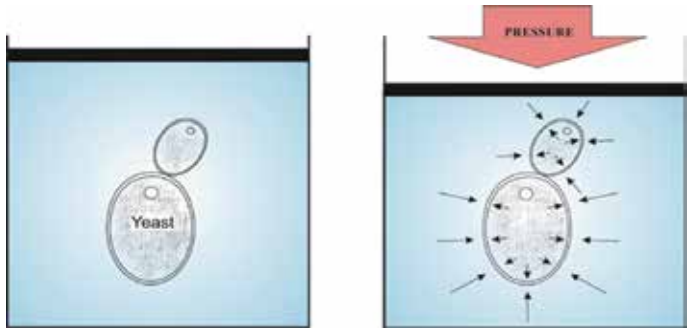

Figure 4. Yeast under high hydrostatic pressure.

through mating between cells of opposite mating type, *a* and α. Cells can grow as haploids or mate and grow in a vegetative form as diploid, or even form spores (meiosis) generating haploid gametes. *S. cerevisiae* was the first eukaryote to have its genome completely sequenced, generating the possibility to study many aspects of life [36].

In yeast, significant morphological and physiological effects are observed in pressures higher than 100 MPa, and loss of cellular viability occurs over 200 MPa [37, 38]. At 50 MPa, cell cycle arrest is induced, but it is a sublethal effect and does not affect the morphology of the cell [6, 39–41]. A yield rate increase for ethanol production is observed after 10 MPa pressurization, but higher pressure can lead to an opposite effect, interrupting the alcoholic fermentation when pressures over 87 MPa are applied (**Figure 5**) [42].

2.4.2.1. Yeast morphology under pressure

Pressure presents an interference in the structure of the cell by directly affecting the cell wall (**Figure 6**), cell membrane and its fluidity, as well as other intracellular organelles [44]. The yeast cell wall is conformed by polysaccharides (80–90%), mainly glucans and mannans and

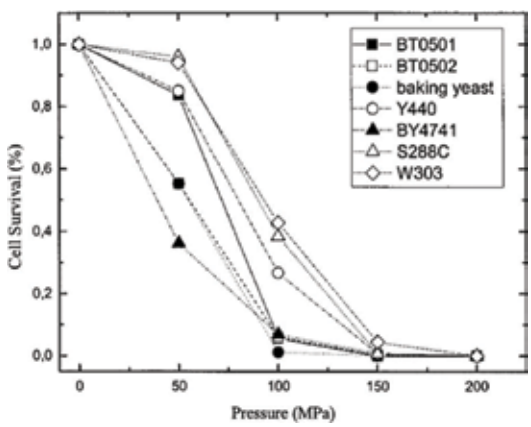

Figure 5. Effect of HHP on different wild-type yeast cells. *Saccharomyces cerevisiae* survival, expressed as percentage of viable cells, was measured on yeast cells at logarithmic phase submitted to various hydrostatic pressures for 30 min [43].

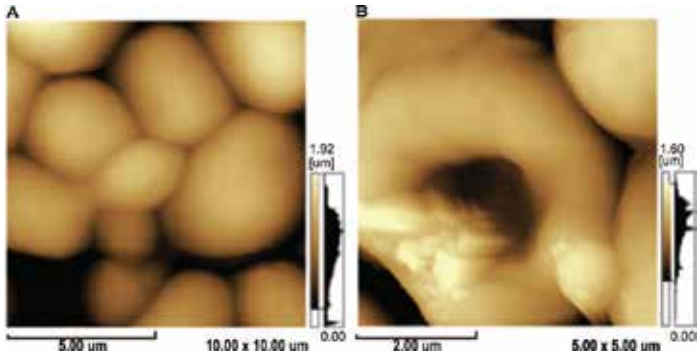

Figure 6. Atomic force micrograph of *Saccharomyces cerevisiae* wild-type cells Y440. (A) Yeast cells at atmospheric pressure. (B) Detail of a yeast cell after hydrostatic pressure treatment of 250 MPa for 30 min [43].

in a lower quantity by chitin. It presents a thickness around 100–200 nm. In nonstressed yeast cells, chitin can be seen in the neck and scars by using the fluorescence of calcofluor. Cells treated with HHP present abnormal distribution of the calcofluor fluorescence in the cell wall. Transmission electron microscopy images suggest that HHP induces alterations in the cell wall and cytoskeleton affecting the cell membrane and the dynamic of cell organelles (**Figure 7**) [41].

Another effect that pressure has is the upregulation of the gene HPS12 [45], which codifies a hydrophilic protein of 12 kDa that increases flexibility in the cell wall and the cell membrane [46, 47]. The suppression of HSP12 induces changes in the size of cells submitted to hypo and hyperosmotic stress and an increase in sensitivity to rapid pressure variations [48]. This characteristic of the HSP12 protein (HSP12p) can be observed using a model with agarose, which is a carbohydrate polymer, to represent the glucan found in the cell wall of yeasts. It was seen that adding known upregulating solutes of HSP12 to the agarose gel decreased its flexibility, but adding the HSP12p increased it. Atomic force microscopy studies suggest that HSP12p interrupts the hydrogen bond and ionic interactions between polysaccharide polymers found in the cell wall enabling more flexibility to the structure [48]. These findings suggest that high hydrostatic stress and osmotic stress affect the cell wall directly interfering with its flexibility and the cell responds by increasing the production of HSP12p.

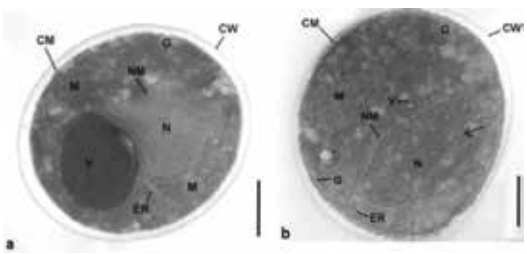

Figure 7. Transmission electron micrographs of a thin section through *Saccharomyces cerevisiae* Y440 wild-type cells. (a) Typical *S. cerevisiae* cell at atmospheric pressure. (b) Cell submitted to 200 MPa for 30 min. CM, cell membrane; CW, cell wall; NM, nuclear membrane; N, nucleus; V, vacuoles; M, mitochondria; G, Golgi apparatus; ER, endoplasmic reticulum. The bar in panel a represents 0.8 μm; the bars in panel b represent 0.5 μm [41].

HHP also interferes with the structure of the cell membrane by increasing the level of arrangement of the lipids (**Figure 8**), especially in surrounding molecules as a consequence of volume reduction. This characteristic induces a decrease in the cell membrane fluidity followed by an increase in thickness [29]. The effect of pressure over the cell membrane is explained by the fact that lipids are more compressible than proteins, reason why they are more sensible to pressure [49]. To compensate pressure, there is an increase in the unsaturation of fatty acids so there is more flexibility in the membrane, and it can stay in its functional liquid-crystalline phase as unsaturated membranes have a less ordered structure than saturated bilayers. This mechanism is used by organisms that live in the bottom of the sea to adapt to the increase in pressure [29, 50]. Fatty acid composition content also might play a role in the protection of the cell membrane from oxidative damage produced by HHP. A desaturase-deficient *Saccharomyces cerevisiae* mutant strain (OLE1 gene deletion) grown in media supplemented with fatty acids differing in size and number of unsaturations and submitted to pressure up to 200 MPa for 30 min shows different responses after the stress. Desaturase-deficient yeast supplemented with palmitoleic acid demonstrated increased sensitivity to pressure compared to cells supplemented with oleic acid or a proportionate mixture of both acids. In contrast, yeast cells grown with linoleic and linolenic acids were more piezoresistant than cells treated with oleic acid. Furthermore, growth with palmitoleic acid led to higher levels of lipid peroxidation [51].

Wild-type yeast cells submitted to 200 MPa for 30 min were observed using transmission electron microscopy and showed that they maintained their external shape, but the cell membrane presented an increase in ondulation, invaginations and evidences of a diluted nuclear membrane [41].

Scanning electron microscopy (SEM) analysis revealed that *S. cerevisiae* submitted to 300 MPa does not show apparent consequences in the cell surface, but at 500 MPa, there is a visible damage and disruption in the cell wall [52]. After pressures above 200 MPa, the nucleus and other organelles are no longer differentiated and membranous fragments can be detected [41]. There are no major visible external changes in the cell under pressure of 80–150 MPa, which might be related to the rigidity of the cell wall [53] The cell mortality as a result of a HHP treatment might be related to the mass transfer through the cell membrane, which causes a change in the permeability of the membrane leading to the intracellular solutes leakage.

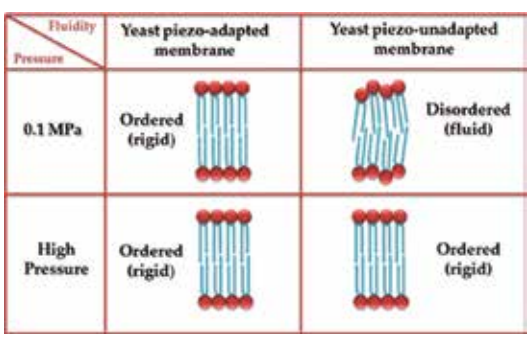

Figure 8. The effects of high pressure in yeast membrane cell (arrangement of lipids).

It seems that trehalose is also involved in cellular protection when HHP is applied. It was shown that trehalose acts inside of the cell as its effect was only observed when applied intracellularly. Actually, cells with a mutation on the trehalose-6-phosphate synthase gene present more sensitivity to high pressure compared to the parental strain [41, 51]. During stress caused by HHP, there is a compression of lipids and increase in ROS [54, 55]. Therefore, it is possible that trehalose acts in the internal bilayers protecting the cell from free radicals and inhibiting lipid peroxidation [51].

2.4.2.2. HHP influence on yeast physiology

HHP affects various structures and cellular functions [36]. Depending on its extent, cytokinetic and mitotic activities are delayed or inhibited, the reactivity of enzymes and other proteins are affected and cell viability decreases with the increase of pressure. This effect is more effective in pressures over 100 MPa and wild-type strains do not survive over 220 MPa. A pressure of 50 MPa is not high enough to kill the cell or modify its cellular morphology, but changes in gene expression and physiology can be observed. Yeast cells in stationary phase have various alterations in morphology and physiology and are more resistant to pressure than proliferative cells [38].

When S. cerevisiae is submitted to 50 MPa for 30 min, it presents an arrest in the log phase of the cellular cycle. If these cells are incubated at ambient pressure after the stress, they show a diminution in the formation of buds up to 45 min after pressurization. Cell recovery starts around 60 min after the stress and achieves full recovery after 2 h [6].

Studies showed that the sensibility of the strain to HHP is related to its genotypic background. The comparison of critical pressure for survival was studied with strains isolated from Brazilian distilleries and laboratory strains (Y440, BY4741, W303 e S228C), and it was seen that industrial strains were more sensitive to HHP (**Figure 5**). The critical pressure for the strains varied between 50 and 100 MPa. It was also observed that nonetheless of the variation in survival, all the strains share a universal mechanism for survival after HHP, which is related to cellular volume [36].

It was found that the cells have a higher tolerance to HHP during stationary phase and are capable of acquiring higher tolerance after a heat shock [36, 41]. A HHP treatment at 50 MPa for 30 min increases the production of ROS in yeast cells, dropping 15 min after the cells are taken out of the HHP and grown in ambient pressure. This showed that oxidative defense mechanisms are induced during cellular recovery after HHP to prevent the accumulation of ROS [55].

2.4.2.3. Alteration on gene expression upon HHP treatment

Gene expression profile in *S. cerevisiae* after 50 MPa HHP treatment (sublethal stress) [7], and 200 MPa treatment (lethal stress) [45] was assessed by microarray analysis. After the piezotreatment with 200 MPa for 30 min at room temperature, 5% of the 6200 known or predicted genes of *S. cerevisiae* are affected. From the 274 genes that shows more than twofold change in the expression, 131 are upregulated, while 143 are downregulated. The most upregulated

genes code small HSPs, HSP30 and HSP12 [45]. HHP, as well as other stresses, promotes cytoplasmic acidification in yeast cells increasing the activity of the H^+-ATPase [9]. HSP30 is important for ATPase activity regulation allowing preservation of the cell energy during stress [56].

Genes related to stress defense and carbohydrate metabolism are also upregulated after 200 MPa, while several genes related to cellular transcription, cell cycle regulation and protein synthesis and target are downregulated. Other response seen after the treatment with 200 MPa was that some gene categories related with transport, cellular organization control, and translation exhibited the same amount of upregulated and downregulated genes. However, other categories show a strict upregulated or downregulated profile. The amount of genes downregulated with strong inhibition were involved with protein regulation and destination, cell cycle progression [45], and this response justifies the cell cycle arrest displayed in cells after HHP treatment.

Some specific pathways are induced after the 200 MPa treatment as lipidic, fatty acid and carbohydrate metabolism, glycolysis, gluconeogenesis, respiration, while amino acid and nucleotide metabolism are repressed. Actually, contrasting with other stresses, the metabolism of trehalose does not show modulation in its expression after HH, even though trehalose plays an important role in the response to this stress. The gene ERG25, associated to ergosterol synthesis, and OLE1, that codes a Δ9-desaturase, which increases the unsaturation of fatty acids in the lipidic membranes, are both induced [45, 57].

The overall microarray analysis of *S. cerevisiae* exposed to HHP of 50 MPa also reveals transcriptional changes in a wide range of genes. Among 6200 known or predicted genes in yeast, mRNA levels for approximately 2.7% of genes were altered more than twofold after 30 min of pressurization when compared to untreated cells. From these 167 genes, 123 were induced and 47 were repressed. Gene expression after 15 min of incubation at atmospheric pressure (0.1 MPa) after 50 MPa treatment showed alteration in 12.9% of the genes, with 408 genes being over-regulated and 392 genes were downregulated more than twofold. This temporal profile of gene transcription presented by cells after HHP suggests that gene regulation follows a priority line. First, genes corresponding to repair and membrane modifications, mitochondria, vacuoles, as well as genes related to aggregation protection are regulated. Then, along the recovery period, other groups of genes, such as the ones encoding membrane proteins and chaperone proteins, genes related to cellular respiration and spore formation are regulated [58].

Transcriptional profile analysis indicates an activation of the general stress response, for instance cell cycle arrest and energy metabolism that is maintained after 15 min of recovery at ambient pressure. The comparison between the groups of genes altered immediately after the pressure and after 15 min of recovery demonstrates that the promoters of genes up or downregulated in response to HHP harbor different motifs governing transcriptional control. Analysis of gene expression and gene ontology made after 5 and 10 min postpressurization showed an effect in categories involved in the regulation of sulfur metabolism. After 15 min of incubation at atmospheric pressure, the affected categories are those related to amine transporter activity and cell cycle. Of the three motifs known to regulate gene expression, all are

identified within 15 min after piezotreatment. On the other hand, only one motif is found after 10 min and another one after 15 min [58].

Interesting enough, genes related to oxidative damage are also induced after HHP treatment [7, 45]. In addition, studies that submit yeast cells to HHP in the presence of glutathione exhibit piezoresistance. This confirms the importance of an oxidative defense mechanism to reduce the damage caused by hydrostatic pressure [6].

Moreover, genes associated with ATP synthesis through glycolysis were modified after pressure. HXK1, a cytosolic protein that catalyzes phosphorylation of glucose during glucose metabolism, were upregulated after 50 MPa for 30 min, increasing after 15 min of recuperation. Genes related to high affinity glucose transportation, HXT6 and HXT7, also were highly regulated after treatment with pressure. An increase in the expression of the ADH1 gene was observed 15 min after the treatment with hydrostatic pressure. This gene is responsible for coding the alcohol dehydrogenase enzyme that is required for the reduction of acetaldehyde to ethanol in the last step of the glycolytic pathway [*Saccharomyces* Genome Database (SGD)] [58]. Those results prove the interference of HHP in fermentation.

2.5. Improvement on ethanol production by HHP

Ethanol production may be based on direct access to sugar found in fruit extract (first-generation production) or access to sugar present in lignocellulosic biomass (second-generation production). Applying HHP in *S. cerevisiae* cells is a strategy to optimize both first-generation and second-generation ethanol production. However, for second-generation ethanol, HHP can also be used in previous steps, to treat the lignocellulosic biomass and obtain higher sugar concentrations.

The effects of high pressure in microorganisms and lignocellulosic biomass for bioethanol production differ according to the pressure value and duration of treatment. Therefore, the process used is case-specific, being differentiated when used for pretreatment, continuous pressure during the fermentation processes or applied in lignocellulosic biomass and enzymes.

2.5.1. Use of HHP on first-generation ethanol production

2.5.1.1. HHP as pretreatment

Positive effects on HHP treated *S. cerevisiae* cells can be observed during the fermentation process. The use of HHP can induce cross protection to other stresses in the fermentative vats. Therefore, HHP in mild conditions can be used to increase stress tolerance to high temperature, high pressure, and ultra-cold shock. The acquisition of stress tolerance by applying HHP occurs after the cells are incubated for 15 min in ambient pressure, but it is lost after 1 h [28, 38].

Yeast cells pretreated with 50 MPa already begins to produce ethanol after 4 h of being inoculated in the fermentation vat, reaching 0.3% of ethanol. After 10 h of fermentation, those cells produced up to 0.8% of ethanol, while nontreated cells produced 0.6% (**Figure 9**) [7].

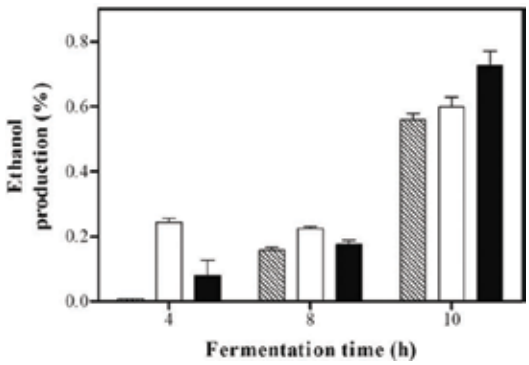

Figure 9. Ethanol production (in percent) after pressure treatment. Hydrostatic pressure of 50 MPa for 30 min (empty bars) and 50 MPa for 30 min and then incubated at room pressure (0.1 MPa) for 15 min (filled bars), and after that, the fermentative efficiency of this strain was evaluated. A nonpressurised sample was used as a control (striped bars) [5].

Other techniques described to induce piezotolerance in wild yeast strains used UV light and HHP. Two methods were compared trying to produce a tolerant strain. The first method treated the wild *S. cerevisiae* with UV light to induce a mutation and subsequently to test them in HHP at 200 MPa for 240 s. These cells proved to be piezotolerant. The other method consisted in using UV light on the strain and then putting them under pressure of 250 MPa for 240 s followed by 48 h of recovery with agitation. These medium was analyzed to determine the cells survival. Then, the medium with less viable cells was submitted to pressure (250 MPa for 240 s) until the survival rate stayed constant. These cells were grown in solid medium and the distinct colonies were put under pressure three more times to produce piezotolerant strains. One of these strains also presented tolerance to high pressure but showed a growth delay, which evidences piezotolerance and piezosensitivity. This delay was also seen in the wild strain but not in the cells treated with the first method. The use of pressure to obtain this delay and mutations proved to be effective to induce piezotolerance and piezosensibility improving the metabolism including ethanol production [59].

2.5.2. Continuous pressure for first-generation ethanol

When used during fermentation, the positive effects are not only described in literature relating to gene expression or cell morphology, but also relating to the increase of glucose flow for the production of ethanol and its relationship with the efficiency/deficiency of some enzymes. *S. cerevisiae* at room temperature produces about 90–95% of the maximum theoretical ethanol yield, since some nutrients are also used for cell maintenance biomolecules synthesis. The kinetic reactions for ethanol production are characterized as firstorder: [42].

$$[Eth] = 2 \times [Glc] \times (1 - e^{-kt}) \quad (2)$$

with [Eth] as the ethanol concentration expressed in $mol.L^{-1}$, [Glc] as the glucose concentration used to produce ethanol, expressed in $mol.L^{-1}$ (the factor 2 comes from the general equation of fermentation, 1 mole of glucose giving 2 moles of ethanol), k as the reaction constant in h^{-1} and t the time in h [42].

Differing from the findings that high pressure is useful as a pretreatment for cells to later produce more ethanol at atmospheric pressure, it was seen that applying low hydrostatic pressure (up to 10 MPa) show better results when used during the whole fermentation due to the acceleration of ethanol production. The optimum value the obtaining of higher ethanol yield (relationship between the ethanol produced and to the amount of sugar present in the medium) is 5 MPa [42]. The *S. cerevisiae* mortality shows significance starting at 25 MPa as described in **Figure 5** [43]. It is also noticed that mortality varies between strains, reason why the effects of high pressure may undergo changes, so the applied pressure amount must be adjusted individually to each strain [30]. As the best results of ethanol production under continuous pressure are around 5–10 MPa, it can be concluded that does not affect cell mortality in a relevant manner.

Low pressure does not produce a delay in fermentation as it is not reported to induce protein synthesis. These results are related to the enzymatic kinetics, that show that pressure up to 10 MPa shifts the equilibrium to the state of lower volume, which is ethanol. Even though maximum ethanol yield was presented at 5 MPa (100% at 30 min), at 10 MPa the reaction rate is considerably higher, being more than two times the rate shown at atmospheric pressure, with a yield of 99% at 30 min [42].

There is a divergence in the reported pressure that is necessary to interrupt ethanol production that ranges from 50 to 87 MPa. This can be explained by experimental variations or the use of different yeast strains between studies. It is known that HHP interrupts fermentation in that pressure range because over 20 MPa there is a decrease in cytoplasmic pH, which disturbs a crucial stage of fermentation by negatively affecting the action of phosphofructokinase, an important enzyme for the glycolytic pathway [3–6]. From 20 to 87 MPa the ethanol yield is reduced and after that it comes to a halt (**Figure 10**).

$$\text{Yield decrease: } 2 \pm 0.1 \times 10^{-3} \times mo\, l^{-1} \times MPa^{-1} \tag{3}$$

The pressure is chosen depending on the strategy that wants to be used in a specific process as it has distinct effects. In the case of pretreating cells with pressure before fermentation, it acts on gene modulation, but when continuous pressure is applied during fermentation, the goal is only to shift the equilibrium of the reaction to ethanol. When the best pressure used for pretreatment (50 MPa) was used continuously for fermentation ethanol yield drops to 45% [7, 42].

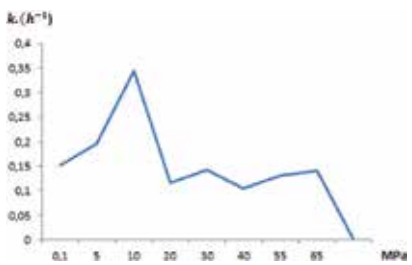

Figure 10. Relation between applied pressure and reaction constant in hours (modify from [42]).

2.5.3. Use of HHP for second-generation ethanol

Second-generation bioethanol characterizes for using lignocellulosic biomass, which are normally residues, so there is no competition with food production. The process to obtain fermentable sugars is more complex than for first-generation ethanol. First the biomass has to undergo a treatment to break the intricate structure and remove lignin. Then, cellulose and hemicellulose are degraded by enzymes to monosaccharides that can be used by the yeasts to produce ethanol.

One use of HHP for second-generation ethanol production is submitting the lignocellulosic biomass to pressure to open the structure, facilitating the access of digesting enzymes to complex sugars. Unlike the pressures reported for yeast cells, the pressure used in lignocellulosic biomass is much more aggressive, since it has the role of breaking cellulosic fibers facilitating the action enzymes. The values shown for lignocellulosic biomass use are higher than 300 MPa, showing a relevant efficiency in the breakage of these fibers. There is a release of fermentable sugars when the lignocellulosic biomass is treated with HHP even without enzymes. Also, an increase in phenols shown that the HHP mainly breaks lignin [60, 61].

In addition to the effects on biomass fibers, HHP also affects the efficiency of the enzymes used for hydrolysis to obtain fermentable sugars from cellulose and hemicellulose to produce ethanol. These enzymes are known as cellulases, and they are composed of different kinds of enzymes with specific functions. They are normally used as cocktails that contain these different enzymes but the proportions and individual activities may vary, affecting the overall performance of the cocktail. In a study using coconut husk as a substrate, cellulases produced by fungi isolated from the husk and commercial cellulases were tested under pressure. First, the commercial enzymes were studied by submitting just the enzyme or the husk to 300 MPa for 30 min and then doing the hydrolysis in atmospheric pressure at 50°C or by introducing both the enzyme and the substrate to the pressure and performing the hydrolysis under pressure (300 MPa) at 22 and 50°C for 30 min. The highest hydrolysis rate was found when the hydrolysis was performed under pressure at 50°C, followed by doing the hydrolysis under pressure at 22°C. This shows that the effects induced by pressure are reversible. Then a comparison between the activity of the commercial enzymes and enzymes produced from isolated fungi was made. In these tests, the activity of different cellulases and the overall cellulase activity were measured at atmospheric pressure at 50°C, 300 MPa at 50°C and 300 MPa at 22°C. Best results were shown for cellulases produced by Penicillium variabile, which was isolated from the coconut husk, at 300 MPa at 50°C. As a general result, all the enzymes tested presented the highest activity at 300 MPa and 50°C. It was seen that the activity of the enzyme cellobiase was especially enhanced by HHP for enzymes isolated from the two fungi tested [60]. This effect was also seen in another study that showed that cellulase structural and functional stability are not negatively affected by HHP from 300 to 400 MPa and HHP gives enzyme stability hydrolysis in a larger range of temperatures [62].

These benefits can be used to obtain higher sugar concentration, which leads to higher ethanol concentration, with a variety of lignocellulosic substrates. This technique was also in used *Eucalyptus globulus*, which showed similar results while pressurizing cellulolytic enzymes. In this case, the pressure applied varied from 200 to 400 MPa using different treatment times from 15 to 15 min. It was seen that higher pressure or time did not have best results, demonstrating that optimum conditions must be found to optimize the process. In this case, the best

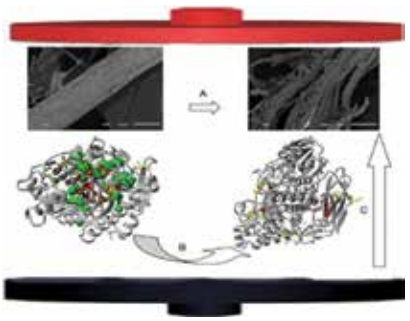

Figure 11. HHP effect on lignocellulosic matrix and enzyme.

results were found using 300 MPa for 45 min. This treatment promoted a better accessibility to xylan, and it was reflected in the sugar concentration after hydrolysis, which reached up to 35% with the HHP compared to the 10% obtained at atmospheric pressure.

The improvement in cellulase activity has been attributed to different factors that act at the same time. One is conformational change on enzyme by HHP, which leads to the exposition of hydrophobic amino acids that interact with the sugar through hydrophobicity creating new binding sites. Other is that, HHP causes a diminution in volume that brings the enzyme closer to the substrate (cellulose and hemicellulose). Finally, as mentioned earlier, HHP helps to break the lignocellulosic tight matrix, which facilitates its degradation by cellulases (**Figure 11**) [60].

These results demonstrate the importance of monitoring hydrostatic pressure, among other abiotic factors, so that ethanol production is maximized. Finally, it can be concluded that the use of high hydrostatic pressure can be used in different stages of fermentation processes and each stage will have its value and specific purpose.

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Chapter 10

Metabolic Engineering of the Model Photoautotrophic Cyanobacterium *Synechocystis* for Ethanol Production: Optimization Strategies and Challenges

J. Tony Pembroke, Patricia Armshaw and Michael P. Ryan

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.77271

Abstract

Photoautotrophic ethanol production using model cyanobacteria is an attractive technology that offers potential for sustainable ethanol production as a biofuel. Model strains of *Synechocystis* PCC6803 have been metabolically engineered to convert central metabolic intermediates such as pyruvate to acetaldehyde via cloned heterologous pyruvate decarboxylase and from acetaldehyde to ethanol via cloned homologous or heterologous alcohol dehydrogenase. While the technology is now proven, strategies are required to increase the ethanol levels through metabolic and genetic engineering and in addition, production and process strategies are required to make the process sustainable. Here we discuss both genetic and molecular strategies in combination with do wnstream strategies that are being applied while also discussing challenges to future application.

Keywords: *synechocystis*, ethanol metabolic engineering, challenges industrial production

1. Introduction

As an alternative to ethanol fermentation using carbohydrate substrates, the use of photoautotrophic cyanobacteria metabolically engineered to produce ethanol offers an interesting alternative for sustainable biofuel production. Cyanobacteria or Cyanophyta, the name deriving from their color, are a distinct phylum of bacteria, which are photoautotrophic getting energy from sunlight and carbon from carbon dioxide. They are the only photosynthetic bacteria that

Genetic construct	Strain	Rate per day ($g.L^{-1}.day^{-1}$)	References
ZmPDC and ADH1 P_{rbLS}	*Synechococcus* PCC7942	0.0082	[4]
ZmPDC and ADH1 P_{psbA2}	*Synechocystis* PCC6803	0.0766	[5]
ZmPDC and slr1192	*Synechocystis* PCC6803	0.097	[6]
JCC1581 B Isolate	*Synechococcus* PCC7002	0.41	[7]
ZmPDC and slr1192 P_{ziaA}	*Synechocystis* PCC6803	0.236	[8]
ZmPDC and slr1192 P_{corT}	*Synechococcus* PCC7002	0.235	[8]
ZmPDC and slr1192 P_{rbc}	*Synechocystis* PCC6803	0.202	[9]
ZmPDC and slr1192 P_{petJ}	*Synechocystis* PCC6803	0.261	[10]
TK504 Plasmid P_{co}	ABICyanol1	0.552	[11]

Table 1. Ethanol yields ($g.L^{-1}.day^{-1}$) as reported for various constructs using the Zymomonas mobilis (Zm) *pdc* gene, a variety of ADH genes and various promoter constructs to express these genes.

can evolve oxygen. Model species such as *Synechocystis sp.* PCC6803 have received considerable attention because they can be relatively easily manipulated genetically and metabolically engineered to produce a wide range of potentially valuable products of biotechnological interest [1]. Considerable attention has focused on the potential to utilize sunlight and CO_2 to produce ethanol as a biofuel at yields comparable to other biological production systems. Although there have been reports of natural ethanol production during dark metabolism, reported levels are far too low for exploitation [2].

The interest in utilizing cyanobacteria as cell factories for ethanol production has been stimulated via flux balance analysis on ethanol yields, which estimate that the stoichiometric energy yield for ethanol compares well with other potential fuel metabolites [3]. The earliest reports of photoautotrophic metabolically engineered ethanol production came in *Synechococcus elongatus* PCC 7942 [4] where heterologous genes encoding pyruvate decarboxylase and alcohol dehydrogenase were expressed from the ethanol producer *Zymomonas mobilis.* This was followed by expression of the same constructs in *Synechocystis sp.* PCC6803 [5] with reported higher yields (**Table 1**).

This was followed by reports in several patents from the US biotechnology companies Algenol and Joule Unlimited who further manipulated the system to improve yields (**Table 1**). The reported yields are represented as a daily yield and often the production cycle can last up to 20 days such that the yields would be multiplied by the production days. However, with potential evaporative loss and degradation of ethanol by contaminants in non-axenic culture these yields are lower than would be needed for commercial production. Thus, much effort has been focusing on improving this yield level by metabolic and strain engineering.

2. The model strain and production of key intermediates

The first *Synechocystis* strain was originally isolated in Oakland, California in 1968 [12] and placed in the Pasteur Culture Collection as *Synechocystis sp*. PCC6803 and the American Type

Culture Collection as *Synechocystis sp.* ATCC27184. Over the years, many sub-strains emerged from the original strain, such as the *Synechocystis sp.* GT (Glucose Tolerant) strain. This GT strain was sent to the Kazusa Research Institute in Japan and became known as the 'Kazusa' strain. Other sub-strain are known as the 'Vermaas' strain [13], the 'China' strain [5] and indeed others sub-strains have been reported [14] such as 'Moscow', 'Amsterdam' and 'New Zealand' depending on the location of the research laboratories using the so-called original *Synechocystis* sp. PCC6803 strain. Many of these strains have undergone microevolution, which may be a feature of cyanobacterial strains growing in high light conditions under laboratory conditions [15] with such genetic changes being detected by genome sequencing. Many sub-strains have interesting variations, which may be of biotechnological interest such as low transformation rates, buoyancy and variation in growth rate.

Yields of product such as ethanol are highly dependent on the biomass produced during growth of engineered strains. When growing photoautotrophically at 30°C doubling times of *Synechocystis sp.* PCC6803 can vary between 10 and 15 h, with optimal conditions observed at light intensities of 40–70 $\mu E.m^{-2}.s^{-1}$ [16]. In controlled photobioreactors higher growth rates can be achieved when optimal conditions are provided throughout a growth cycle. Because the flux of carbon is diverted in metabolic engineered strains, from pyruvate via many of the engineered pathways, this has the effect of lowering biomass yields and indeed the more ethanol as a product that is produced the greater the effect on biomass yield will be. In general, the relatively slow growth rates of *Synechocystis* may be attributed to many reasons, its photoautotrophic metabolism or its polyploid (multi-copy) genome; however, one of the key issues is its encoding genome optimized for photoautotrophy. *Synechocystis sp.* PCC6803 was the first cyanobacterium to have its genome sequenced [17] and since then many further sub-strains have been sequenced [15, 18, 19]. Analysis of genome data reveals that *Synechocystis* in the main does not possess transporters for vitamins, co-factors, amino acids or nucleotides and must encode synthesis pathways and synthesize essential building blocks from the energy of photosynthesis. The needs therefore for complex synthetic machinery for its photoautotrophic lifestyle coupled to polyploidy are key aspects of its relatively slow growth rate. This then may be exacerbated when this organism is used as a cell factory for products such as ethanol.

During photoautotrophic metabolism in *Synechocystis* an intermediate of the Calvin cycle, Ribulose 1,5-bisphosphate, is used to fix carbon dioxide to 3-phosphoglycerate. This can be converted to phosphoenolpyruvate (PEP) from 2-phosphoglycerate via enolase or travel back through the Calvin cycle. Pyruvate kinase (pk) is then used to convert PEP to pyruvate [20]. This central intermediate, pyruvate, can then be diverted via metabolic engineering to a number of potential biotechnological products including ethanol [1].

3. Key aspects of the engineered ethanol cassette in *Synechocystis*

To metabolically engineer *Synechocystis* as a cell factory a 'cassette' of genes and sequences are needed. A key ingredient of a functional ethanol cassette, suitable for expression in *Synechocystis*, is the functional expression of a pyruvate decarboxylase gene encoding the

enzyme pyruvate decarboxylase (PDC). The PDC produced converts the metabolic intermediate pyruvate to acetaldehyde, which is in turn converted to ethanol by engineered alcohol dehydrogenase (ADH) or by the native *Synechocystis* ADH.

Pyruvate decarboxylase (PDC, EC 4.1.1.1) carries out the decarboxylation of pyruvate to acetaldehyde in alcohol fermentations and requires thiamine diphosphate/pyrophosphate (ThDP) and the divalent cation Mg^{2+} as cofactors. Several other enzymes in various metabolic pathways also require these cofactors to function and it is believed that each of them use a similar mechanism of action. PDC can be found in fungi, plants and yeast and is not present in humans [21]. PDC genes have been observed and characterized from only a small number of bacterial species as it appears to be rather rare amongst prokaryotes. These include *Zymomonas mobilis*, [22], *Zymobacter palmae* [23], *Acetobacter pasteurianus* [24], *Gluconacetobacter diazotrophicus* [25], *Thermococcus quaymacensis* [26], *Geobacillus thermoglucosidasius* [27] and *Sarcina ventriculi* [28]. Although the *Zymomonas mobilis* PDC is the most extensively utilized in ethanol production there is much potential to utilize some of the other bacterial PDC's on the basis of pH optimum or lower Km (see **Table 2**). With model organisms, such as *Synechocystis,* using a PDC with a lower Km may increase the flux from pyruvate and couple the product acetaldehyde better with ADH resulting in higher ethanol yields. There is thus some scope for improvement of the ethanol cassette given that some of the newly characterized PDCs have better kinetics than the original *Zymomonas* PDC. All known PDC's have specific co-factor requirements and co-factor availability is an issue when expressing engineered cassettes. While there may be little problem with Mg^{2+} supply, the availability of ThDP will be limited as the host organism must synthesis it (as *Synechocystis* does not possess a thiamine transporter) [18]. Equally ThDP will be required for other cellular metabolic reactions and its availability will be squeezed by added engineered PDC. Hence if metabolic engineering were to result in high level expression of heterologous PDC, the limited availability of ThDP would pose limitations on its function

Bacterial host and enzyme	kM (mM) pyruvate	Optimum pH	Optimum temperature (°C)
Gluconacetobacter diazotrophicus	0.06 pH 5.0	5.0–5.5	45–50
	0.6 pH 6.0		
	1.2 pH 7.0		
Zymobacter palmae	0.24 pH 6.0	7.0	55
	0.71 pH 7.0		
Acetobacter pasteurianus	0.39 pH 5.0	3.5–6.5	65
	5.1 pH 7.0		
Zymomonas mobilis	0.43 pH 6.0	6.0–6.5	60
	0.94 pH 7.0		
Sarcina ventriculi	5.7 pH 6.5	6.3–6.7	N/A
	4.0 pH 7.0		
Gluconobacter oxydans	0.12 pH 5.0	4.5–5.0	53
	2.8 pH 7.0		

Table 2. Properties of known bacterial PDCs [23, 25, 27].

and perhaps also on other host enzymes that use ThDP as a co-factor. Thus this may affect competitiveness of engineered strains and in the long term, engineering a thiamine transporter may be needed in *Synechocystis* production strains to overcome such issues.

While there is a potential choice of PDCs to use, in practice most work so far has been carried out on the *Zymomonas mobilis* PDC. This enzyme is a homo tetramer of 240 kDa [29] and has an optimum pH of 6.0 [30]. Given the pH optimum for growth of *Synechocystis* is ~pH 8, full enzymatic function or co-factor binding [31] may be somewhat compromised by the pH difference between the enzyme optimum and the host pH optimum which may suggest looking at other potential PDC candidates.

4. *Zymomonas mobilis* and *Synechocystis* alcohol dehydrogenase (ADH)

In most reports on engineered ethanol cassettes the source of ADH has been *Zymomonas mobilis*. Two ADH isozymes are known to be present within the genome of *Zymomonas mobilis* - ADH I and ADH II (EC 1.1.1.1) [32]. For metabolic engineering of ethanol production the Fe^{2+} containing ADHII encoded by the *adh*B gene of *Zymomonas* has been utilized [5]. This enzyme has a pH optimum of pH 8.5 (as opposed to pH 6.5 for ADHI) and a cofactor requirement for Nicotinamide adenine dinucleotide (NADH) for the reduction of acetaldehyde to ethanol [33].

Unusually *Synechocystis* also encodes its own native ADH gene via the *adh*A gene (slr1192). This is a medium chain alcohol dehydrogenase, which catalyzes the reversible oxidation of alcohols to aldehydes or ketones [34]. The *Synechocystis* ADH encodes a 140 kDa zinc dependent enzyme with broad alcohol dehydrogenase activity and which interestingly is Nicotinamide adenine dinucleotide phosphate (NADPH) dependent as opposed to the *Zymomonas* activity, which is NADH dependent [34]. Indeed *Synechocystis* has been reported to possess multiple *adh* genes but does not contain an native *pdc* gene [35] suggesting that the native ADH may play an as yet unknown function in the cyanobacterium. Indeed the *Synechocystis adh* gene has been substituted for the *Zymomonas* gene [9] and functions very well. Recently we have reported [36] metabolic engineered cassettes with a copy of the *Zymomonas mobilis adh* gene and the native *Synechocystis adh* gene with increased ethanol producing activity. This may occur because the two activities rely on different co-factors NADH (*Zymomonas*) and NADP (*Synechocystis*) which may spread the co-factor requirement and availability within the cell [36].

5. Construction of functional ethanol cassettes in *Synechocystis*

In general, terms the construction of an ethanol cassette follows the basic components as reported [4, 5]. The *Zymomonas mobilis pdc* gene is amplified and fused with the *Zymomonas mobilis adh*B gene under the control of an inducible promoter. The light inducible P_{psbA2} promoter is often utilized but other promoters have also been evaluated [37]. There is then the need for a strong selection of the cassette encoded generally by an antibiotic resistance determinant such as kanamycin or zeocin [36]. Homology sequences are needed at both ends of the

cassette to allow homologous integration into a neutral site within the organism (see **Figure 1**). The cassette utilized by Dexter and Fu [5] utilized the *psb*A2 gene as a neutral site for integration but recently a number of other neutral sites have been discovered [38]. Indeed homologous integration has been used as a mechanism of integrating cassettes into functional competing genes, as a knock out mechanism also. This occurs where the cassette is integrated via use of homologous ends into genes such as the *pha* genes whereby integration knocks out the synthesis of polyhydroxyalkanoate (PHA) a competing pathway for pyruvate use [9, 36]. Using the *pha* genes as an integration site effectively increases the flux of pyruvate to ethanol by blocking alternative storage of photosynthetic products.

In attempts to increase ethanol production, gene dosage has been utilized such that two cassettes have been integrated at different sites giving potentially twice the gene copy number and protein expression level of PDC and ADH [9, 36]. While this strategy has been shown to increase the levels of ethanol produced it may be that given the polyploid nature of *Synechocystis* putting in and stabilizing two cassette copies which would be multiplied by some 50 copies (due to polyploidy) may be reaching the very limits of gene dosage with this metabolic engineering strategy.

Figure 1 illustrates the construction of an ethanol cassette pUL004 Kan. This cassette [36] consists of the Zm*pdc* coupled to the *Synechocystis adh*A gene with a kanamycin resistance determinant from the ICE R391. The genes are controlled via the P_{psbA2} light inducible promoter. The cassette contains 500 bp of DNA at each end with homology to a neutral integration site, in this case the *psb*A2 gene. This construct is housed in pUC18 and replicated in *Escherichia coli* and termed pUL004. For integration, the plasmid pUL004 is transformed into *Synechocystis* whereupon

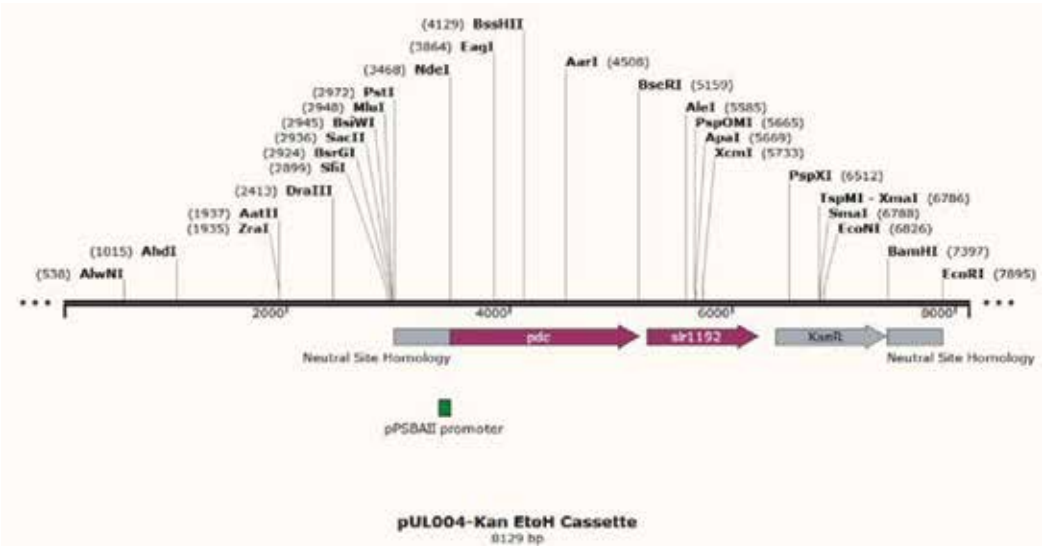

Figure 1. Structure of the ethanol cassette pUL004 [36]. The cassette contains the *Zymomonas pdc* gene (Zm*pdc*), the *Synechocystis sp.* PCC6803 *adh* gene (slr1192) and the kanamycin resistance determinant from the ICE R391 all under the control of the P_{psbA2} light inducible promoter. There is 500 bp at each end with homology to the neutral integration site and the construct is cloned into pUC18 for replication in *E. coli* prior to purification and transformation into *Synechocystis* PCC6803. Restriction sites within the cassette are also illustrated.

homology between the two 500 bp ends and the host chromosome leads to integration into the neutral site. In general, because of the polyploid nature of *Synechocystis*, selection for integration requires selection on increasing doses of kanamycin and PCR monitoring using primers across the neutral integration site. Initially many chromosomes will not contain an integrated cassette and this will show as a low molecular weight band (where no integration into the neutral site occurs). Those chromosomes that contain an integrated cassette will possess a higher molecular weight band where the cassette has integrated into the neutral site increasing the band size. At the initial stages, one would observe two bands one without and one with integration (one low and one high band). Following selection all chromosomes should contain a high molecular weight band (and no low molecular weight band) indicating that all chromosomes contain the cassette. This process illustrated in **Figure 2** (below) may take several weeks to segregate and stabilize. In the case of establishing an ethanol cassette, which provides no selective advantage on its host and in fact may be negative in selection terms as it causes diversion of pyruvate towards ethanol rather than biomass, selection and stabilization may take some time. Thus, strong selection

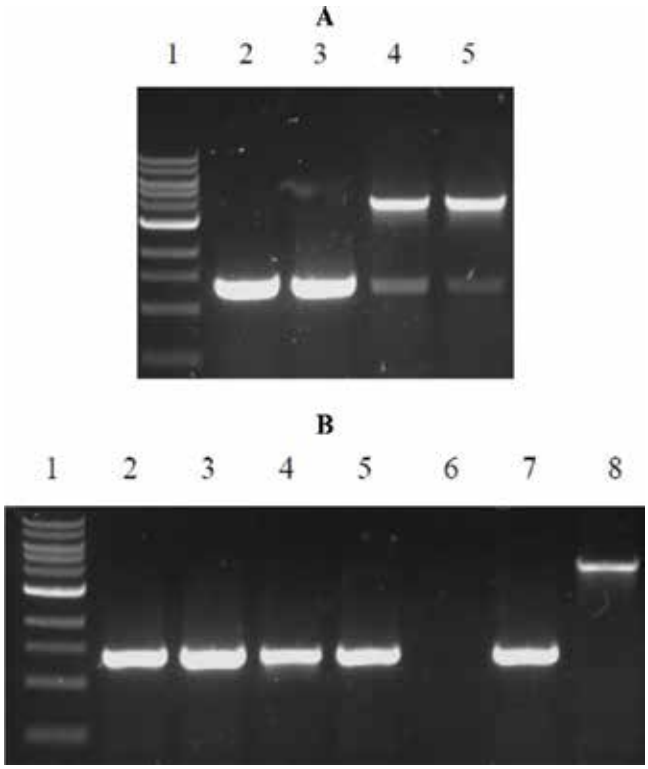

Figure 2. Agarose gel electrophoresis of PCR amplicons analyzing integration into the *psb*A2 neutral site of pUL004. A) Lanes 2 and 3 (and B lanes 2,3,4,5,7) illustrate amplicons using primers to amplify across the neutral integration site which in these cases are all of low molecular weight indicating no integration into the neutral site. A) Lanes 4 and 5 illustrate that two amplicons are detected the lower band with no cassette and the higher band with the cassette integrated. This pattern is detected in strains with partial segregation of the cassette. B) upon selection strains harboring integrated cassettes in all chromosomes should resolve (the cassette is present in all chromosomes of the polyploid genome) as illustrated in lane 8 panel B. This band (lane 8 panel B) can then be removed and sequenced to verify integration. Lanes 1 a and B illustrate a molecular weight ladder to determine amplicons size.

and monitoring is required to realize integration and maintenance of such cassettes. To insert a second cassette a different neutral integration site (and hence different homologous sequences within the cassette are required) and a different antibiotic resistance determinant such as zeocin [36] is needed as part of the cassette construction.

6. Issues and methodologies to enhance ethanol production levels

6.1. Ploidy as an issue in cloning in *Synechocystis*

Strains of *Synechocystis* are polyploid with the chromosome number varying as a function of growth phase [39]. This causes issues with metabolic engineering and stabilization of engineered genes into chromosomal locations as one must select for integration into all chromosomal copies. In addition, the high polyploid level causes problems with generation and selection of mutants, which again must be fixed into all chromosomal copies.

Griese et al. using a real time PCR method demonstrated that the motile 'Moscow strain' of *Synechocystis* contained 58 genome copies per cell at both the log and stationary phases, while the GT 'Vermass strain' contained somewhat less with 42 chromosome copies during the same time period [39]. The 'Kazuza' strain had been reported to possess 12 copies but may have altered its ploidy because of laboratory growth over extended periods [40]. High copy number during growth has also been seen in several archaeal and other bacterial species [41, 42] but the levels reported in *Synechocystis* are amongst the highest chromosome copy numbers recorded for any cyanobacterial species or indeed prokaryote. Given the high chromosome number, the energy expended on its maintenance is high and contributes significantly to the slow growth rate of many polyploid cyanobacteria. Equally, this high chromosome number poses added difficulties in genetic and metabolic engineering in requiring many rounds of selection and screening to achieve stable integration of cloned genes. One possibility of limiting the polyploidy may be to culture production strains in limited phosphate containing media as this may have the effect of limiting phosphate availability for DNA synthesis and can limit the chromosome number, making it easier to establish recombinants and ease stabilization of chromosomally inserted cassettes.

6.2. Gene dosage

The initial cloning strategies [4, 5] used one copy of the ethanol cassette inserted into a chromosomal neutral site. To enhance productivity two copies of the cassette where then utilized [9, 36]. This had the effect of increasing productivity all be it at the expense of biomass and indeed stability during production. Attempts in our laboratory to generate strains with three cassette copies per cell have thus far failed. This suggests there could be a limit to the gene dosage that can be utilized for ethanol cassettes at least. This limit may be due to several factors and many of these factors may combine to limit production. There is the ploidy issue such that during growth if the ploidy level is some 50 copies [39] then with one cassette the copy number is already 50, two copies would mean it would be approximately 100 and the cell may not be able to tolerate more. There may also be instability issues with recombination events between similar cassette sequences. In addition, there may be the issue of ability to supply

the co-factors ThDP, NADH and NADPH for this level of enzyme expression. There may be additional factors such as limitation of pyruvate for other essential cellular functions if high levels of enzyme activity are utilizing it to react to ethanol. This in turn may affect biomass production and synthesis of essential cell components and thus triggering a stress response. In addition, given the negative effect ethanol has on growth there may be the selective pressure to mutate the cassettes selecting for faster growing strains which do not have the burden of ethanol production. The nature of all these possibilities may need to be examined in more detail to generate optimal strains going forward.

That gene dosage can have an effect on production has been demonstrated by utilizing the small native *Synechocystis* plasmid pCA2.4 [43]. This plasmid has a copy number of seven per chromosome copy, thus potentially greater than 300 copies per cell. Cloning of the yellow fluorescent protein (YFP) into a neutral site on this highly stable plasmid resulted in >100 fold increase production levels of YFP relative to a chromosomal insert indicating the potential of gene dosage within *Synechocystis* [43] all be it in this case with a non-burdening or non-toxic product.

6.3. Promoter constructs

Most productivity studies for ethanol in *Synechocystis* have been carried out with the light inducible P_{psbA2} promoter [4, 5, 9, 36]. However, a number of other promoters have been examined specifically to improve yields (see **Table 1**). Recently heterologous strong promoters P_{trc} [44], P_{rnpB} [45] and P_{lac} [46] have been used for butanol, lactate and ethylene production respectively. Use of the super promoter P_{cpc560}, [47] was shown to produce functional proteins at a level of up to 15% of total soluble protein in *Synechocystis sp.* PCC6803, a level comparable to that produced in *E. coli*. This promoter appears to have 14 predicted transcription binding sites, which appear to be key to its high expression level [47]. Many of these promoters are always on and may not be optimal for controlled expression however.

A number of controllable promoters have also been analyzed [37] with the most useful being the Ni^{++} Co^{++} inducible, P_{nrsB}, which gives relatively silent expression in the un-induced state and can be induced some 40 fold to approximately the level of the P_{psbA2} promoter with inducer. Such promoters may allow tuneable promoter activity for ethanol production. Always on promoters, do not allow biomass to be generated as might happen in the yeast system where removal of aeration during production leads to the switch to anaerobic metabolism and ethanol productivity following adequate biomass production. This decoupling of growth from ethanol production could be achieved by tuneable promoters and has been reported [48] where by a riboswitch was incorporated in an ethanol cassette following the P_{psbA2} promoter. Such riboswitches can be induced by theophylline and has been used as a *proof of concept* to decouple biomass from ethanol production [48].

6.4. Knockout of competing pathways as an aid to greater production

Manipulation of carbon flux within the cell factory *Synechocystis* has been used to increase production of metabolically engineered products. Photoautotrophic growth in the light results in accumulation of a number of storage compounds in *Synechocystis* including the major storage polymers glycogen and polyhydroxyalkanoates (PHA), the best characterized

being polyhydroxybutyrate (PHB) [49]. Mutants deficient in accumulation of such storage compounds have been used to express metabolic engineered pathways such as in the production of lactate [50]. Here diverting flux away from storage has been demonstrated to have positive effects on production. In a similar way, inserting the ethanol cassette directly into the *pha* genes has also been shown to increase production of ethanol [9, 36].

Increasing levels of substrate, in this case pyruvate, have also been used to increase yield in metabolic engineered strains. Expressing the enzyme pyruvate kinase (PK), which transfers a phosphate group from PEP to ADP forming Pyruvate [51], has been shown to increase flux to product [52]. Thus, there appears to be some potential for manipulating the flux pathways to and from pyruvate as a means of increasing product yield, which may prove useful when coupled to ethanol production.

6.5. Mutagenesis strategies

Mutagenesis and mutant selection has been developed in *Synechocystis* however; the use of random mutagenesis is difficult. This stems from the polyploid nature of the organism and the need to establish the mutant genotype in all chromosomes before the phenotype is apparent. A novel microfluidics strategy has been developed as an aid to select mutants with higher ethanol production levels [53]. This micro-droplet technique can detect increased ethanol from single cells of engineered *Synechocystis* in micro-droplets. The technique is based on an enzymatic assay, which couples ethanol levels produced within the micro-droplet directly to resorufin, a fluorescent compound. The extra fluorescence apparent with a high ethanol producer can be detected and the droplet containing the higher producer collected [53]. Passage of large quantities of metabolic engineered ethanol producing *Synechocystis*, through the system coupled to laser detection and separation of high fluorescent strains facilitates separation of higher ethanol producers [53]. Such a technique could be used to rapidly screen a large library of transposon insertion mutants, a cloned library of genes potentially enhancing ethanol production (such as PK) or directed insertion libraries (such as PHA) to select higher producers.

6.6. Improving carbon capture

Several mechanisms of carbon accumulation have been described to operate in *Synechocystis*, which include both bicarbonate and CO_2 transporters [54, 55]. These systems include the high affinity bicarbonate transporter BCT1 (locus slr0040–44), the sodium dependent bicarbonate transporter *Sbt*A (slr1512), the medium affinity bicarbonate transporter *Bic*A (locus sll0834) and the multi component CO_2 transporters NDH [56]. Theoretically, manipulation of transporters could provide more carbon for fixation and conversion to products such as ethanol. While some of the transporters are multi subunit complexes and could be difficult to express to functional activities, some are single gene encoded activities and more easily amenable to metabolic engineering. The *Bic*A protein was expressed in *Synechocystis* [57] by engineering the strain to contain additional inducible copies. Studies revealed that this strain resulted in enhanced biomass yields. We confirmed that expressing *Bic*A did in fact increase biomass whereas expressing *sbt*A in our hands did not (O'Riordan, Armshaw and Pembroke, unpublished 2018). This offers a proof of concept that increasing carbon flux can affect productivity and may have applications in enhancement of product yield. Other

strategies also show potential in *Synechocystis*. Manipulating the Calvin-Benson-Bassham cycle, has been proposed as a strategy for improving cyanobacterial growth and product metabolites [58]. Four enzymes of the cycle, ribulose-1, 5-bisphosphate carboxylase/oxygenase (RuBisCO), fructose-1, 6/sedoheptulose-1, 7-bisphosphatase, transketolase and aldolase were co-expressed with an ethanol cassette (containing *pdc* and *adh*) expressed with the P_{nrsB} promoter in *Synechocystis*. In all cases there was a 55, 67, 37 and 69% increase respectively in ethanol production and also a relative increase in biomass [58], indicating the potential of increasing carbon flux within the cell factory as a means of improving product yield.

6.7. Neutral sites for integration

As integrative vectors, which utilize homologous recombination into the chromosome, are widespread when metabolic engineering *Synechocystis* [59] the characterization of integration sites, termed neutral sites, is important, particularly in terms of functionality and stability of insert. Pinto et al. carried out a systematic study of neutral sites using insertion and deletion at the site and expression of the green fluorescent protein module [38]. Although a large number and variety of sites were chosen based on genomic and sequence analysis further analysis revealed that many of these were unsuitable. Location of genuine neutral sites appears to be complicated by the possibility that non-coding regions may possess cis-acting sites thus a systematic assay using trials was needed [38]. Investigation indicated that not all neutral sites were the same and that insertion in some caused some growth defects relative to wild type. This systematic review points to a new and validated set of potential sites that can be utilized going forward which is important given the popularity of integration as the preferred tool for metabolic engineering in *Synechocystis*.

6.8. Replicative plasmids

Replicative plasmids have been utilized for genetic engineering in *Synechocystis* [60] and many are based on the broad host range chassis of the IncQ plasmid, RSF1010, which functions in *Synechocystis*. While this chassis allows ease of construction of inserts and can replicate in *E. coli* for generating transformation material [61] they are not widely utilized. Replicative plasmids in *Synechocystis* suffer from a number of drawbacks including recombination back into the chromosome, loss of the vector without selective pressure and unusual effects on transcription within the vector [38]. Also given the polyploid nature of the organism, segregation and maintenance of such vectors can be problematic.

6.9. Tolerance to ethanol

For high level, production of ethanol within *Synechocystis* it has been estimated that yields would need to be above 15 $g.L^{-1}$ but indeed as high as possible from a commercial perspective [62]. Currently levels of production are nowhere near these levels. However were such levels, approaching 15 $g.L^{-1}$, to be reached the tolerance of the organism to ethanol could become an issue, as this would stress the cell factory. In competitive yeast fermentation systems up to 20% ethanol (v/v) has been reported [63], which is far from the current production capacity of metabolically engineered *Synechocystis* (**Table 1**). However, in preparation for strains that

would have this capacity it is important to determine the level of tolerance to ethanol in engineered strains and examine toxic or stress related effects. Proteomic analysis has been used to determine the response of engineered strains [64] with current ethanol production levels and the response of strains with ethanol added up to projected or expected ethanol production levels [65]. In the case of added ethanol incubation with 1.5% (v/v) of ethanol for 24 h reduced growth of *Synechocystis* by 50% with cell aggregation visible [65]. Proteome analysis revealed some 32 unique proteins up-regulated and some 42 down-regulated after 24 h. This number of altered proteins increased after 48 h. incubation. Many of these proteins were demonstrated to be involved in the common stress response such as those associated with oxidative stress [65], transporters, cell-membrane modifying proteins and proteins associated with the photosystems. Many of the altered protein observed in the proteome response in *Synechocystis* were similar to those observed in the tolerance response of *Zymomonas mobilis* [66]. Proteomic analysis has also been observed on metabolic engineered *Synechocystis* producing levels as outlined in **Table 1** [64]. Here some 60–70% of the carbon fixed was converted to ethanol via a single ethanol cassette. At this rate of ethanol production there was no significant stress response observed rather there was a realignment of systems. Some upregulation of carbon concentrating mechanisms were observed, as were enzymes of the Calvin cycle and photosynthesis antennae proteins [64]. Interestingly the t*hi*C gene, encoding phosphomethylpyrimidine synthase involved in thDP synthesis (the PDC-cofactor), was also up-regulated suggesting that even at this low level of ethanol production that co-factor availability was limited [64].

Tolerance has also been examined via transcriptomic analysis following exogenous ethanol addition [67] with 1.2–3% ethanol addition to wild type *Synechocystis*. Addition of 1.5% caused a 50% reduction in growth rate with visible aggregation suggesting stress. Many of the genes up-regulated in the transcriptomic study were associated with energy metabolism particularly photosynthesis. The results observed were broadly in line those observed in the proteomic studies [64].

7. Linking metabolic engineering of *Synechocystis* to production

While progress is being made with metabolic engineering for ethanol production and establishing *Synechocystis* as a cell factory there needs also be to an understanding of the production landscape when developing the system at industrial scale. Generation of a viable ethanol producing photoautotroph will necessitate rolling out of a production system to commercial level. Thus, at one level, there are the limitations and possibilities of metabolic engineering which have been discussed above but there is a second level that also needs to be addressed, that of the production environment to realize the potential of metabolically engineered strains. Indeed a fuller understanding of the requirements at this stage can help inform the strategies used for optimal metabolic engineering of potential production candidates.

7.1. Overall process life cycle analysis

Implementation of an industrial process for ethanol production from cyanobacteria will be the next stage of development once the challenges of metabolic engineering have been addressed.

Development of the downstream aspects of production will require optimization of several parameters and a more favorable economic outlook. Capital expenditure (CapEx) will be a key driving force with many components needing to be considered. Chief amongst these is the nature of the producing organisms being a recombinant strain. This poses potential safety and containment considerations, which would add to the economics of plant construction and operation. The need for sunlight (which may limit location of production facilities) or continuous LCD exposure again adds costs with either cyclic day exposure in high light climates or continuous growth with added light, which would come with an added energy cost. Equally, calculations of volumes that would be needed suggest large CapEx expenditure on plant, large water requirements and effluent processing costs. Many geographical areas that have high sunlight with marginal land, such as desert areas, at first sight might seem suitable but will suffer from water limitations. Other issues that are related to CapEx relate to the growth of the production strains themselves and the provision of optimal conditions for growth and production. Currently as one diverts photosynthetic intermediates to ethanol, one is affecting the flux to biomass. The more ethanol that is produced the slower the growth and the less biomass that can be produced. This impinges significantly on the growth rate and hence competitiveness of production strains. Given that, growth under sterile conditions in photobioreactors would be economically unsustainable (due to cost and the low value of the product ethanol); competitor contamination would need to be built into the growth cycle. Thus, slow growth of producers would have two major potential consequences that could affect the process. Firstly, there may be mutational selection for faster growers, which have lost the engineered ethanol cassette reducing the yield during production, and secondly given that axenic conditions could not be maintained during aseptic but non-sterile culturing, contaminants could easily outgrow the engineered strains. Strategies that might mitigate this could be the addition of mutualistic consortia, which might stimulate the production strains by providing vitamins or co-factors while limiting the growth of contaminants [68]. Thus, strategies that would aid production at large scale would need to be factored in at the initial stages of metabolic engineering.

7.2. Reactor design for large-scale economic production

The need for significant scale up of photoautotrophic ethanol production in a high light environment can add significantly to initial CapEx. Within the reactor system itself, several components may need significant attention. It is impractical for low value ethanol products, at least in comparison to current fuel costs, for growth and production to be carried out in sterile photobioreactors (PBRs) with full control over light, and key physiological conditions. Although the technologies for such photobioreactors are well developed their practicality can reasonably only be considered suitable for high value products [69]. In addition to containment issues, there are issues with inoculum development for non-axenic culturing to insure that initial inoculum is stable, productive and clonal. Depending on the plant size, this may require significant CapEx.

The most frequent types of PBRs proposed are non-sterile horizontal tubular or vertical flat panel PBRs, which have several limitations including: (a) cost, which have been estimated at €2400 m^2 for small scale, reducing in cost slightly with scale [70]. This would result in a cost

of some €12.6 kg^{-1} [71], (b) High energy consumption [72] from mixing, CO_2 supply, pumping, separations, cleaning, and (c) Maintenance, cleaning and labor costs [70], (d) The reactor design must be able to withstand photo-oxidation, prevent evaporative loss of product, while maintaining axenic conditions as long as possible. Given the generally slow growth, rates of cyanobacterial species, largely because of the photoautotrophic lifestyle, need to manufacture most of their metabolites, maintain a polyploid genome because of the high sunlight and UV exposure the design of PBRs suitable for low value ethanol production from cyanobacteria is a challenge.

In production terms once one moves away from a controlled PBR design one halves the production cycle and level of photosynthetic production due to the night-day diurnal cycle and in addition there is less process control over the operation. Many approaches have been taken in an attempt to reduce cost; this has included use of bicarbonate-based systems for supply of carbon following carbon capture [73]. This may have significant cost savings in terms of CO_2 sparging, transport costs and CO_2 loss due to outgassing. Bag type culturing [70] which can be once off or be reusable can offer another potential solution. This may mitigate against some of the limitations of more traditional PBRs. Controlling contaminants in non-axenic culture might be carried out by use of pH as a control mechanism for limiting contamination, however this may necessitate use of more alkaliphilic cyanobacterial species [73]. Indeed adapting the production strain to the process or vice versa may offer a way forward in developing optimal reactor configurations with reduced CapEx. Thus incorporating knowledge of the production cycle, the types of conditions required for growth into a metabolic engineering strategy can be important during initial development of strains and strategies.

7.3. Temperature control, energy and evaporative loss

By virtue of the fact that ethanol-producing cyanobacteria will be recombinant strains, the current experimental systems tend to be enclosed due to regulatory constraints with GMO's. In geographical locations which are suitable for maximal sunlight and hence photosynthesis, enclosing a facility may raise issues with temperature control unless this is designed into the build. Direct exposure to air circulation or venting may also not be feasible due to safety issues while heat buildup beyond optimal growth temperatures, such as 30°C for *Synechocystis*, may easily occur. In such cases, utilizing a thermophilic or thermoduric strain as a cell factory may be more feasible although this is currently not being done. In addition evaporative loss of the product ethanol may occur given the rather long growth and production rates, thus strategies to constantly remove and collect product during production may be essential, which might mitigate against needing ethanol tolerant strains. These issues illustrate the potential interplay between knowledge of the production system and the metabolic engineering needs and strategies.

Thus to ensure maximal production and recovery of ethanol, systems may need to be engineered to trap and recover ethanol during production which again may add considerably to CapEx. Jorquera et al. estimated, in a comparative analysis of power consumption of different photosynthetic reactors that horizontal tubular PBRs consumed 2500 $W.m^3$, which reduced to

54 W.m^3 for flat panel PBRs and to 3.7 W.m^3 for raceway systems [74]. However mixing rates are quite different in the different systems such that in raceway systems there is little mixing, which effects movement of producing cells into light and poor mass transfer limiting overall productivity. Thus, power consumption unless linked to wind or solar in an integrated way may be a key hurdle to overall process efficiency and economy.

7.4. Ethanol recovery from production media

Lignocellulose based fermentations tend to be more dilute than starch based systems due to the presence of hemicellulose which increases viscosity and the presence of fermentation based inhibitors [75, 76]. This is currently similar in terms of cyanobacterial production of ethanol, which is also dilute and low in terms of yield. Recovery of ethanol from dilute production streams in an energy efficient and economical manner poses significant technical difficulties. Traditionally ethanol is recovered via distillation, however in the case of biofuel ethanol from cyanobacterial production the energy costs of distillation would be far too high particularly from dilute streams. It has been estimated that in a well-integrated lignocellulose to ethanol plant the process would require 4350 MJ.m^{-3} equivalent to approximately 20% of the energy content of the ethanol produced [76]. Thus, alternatives to distillation are needed to drive economy from cyanobacterial production systems. A number of techniques are available which may be suitable for the recovery of ethanol from cyanobacterial production such as membrane permeation or pervaporation, vacuum stripping, gas stripping, solvent extraction, adsorption and various hybrid processes [76]. However, the efficiency is dependent on the initial ethanol concentration (which is currently low for metabolically engineered *Synechocystis*) and often multiple cycles of processing would be required to achieve optimal yield. Thus, there are currently significant challenges to optimal recovery from dilute streams such as cyanobacterial systems although the potential for energy savings over distillation are possible. This thus implies that the higher the yield initially the better in terms of downstream processing, hence the current focus on optimizing metabolic engineering for yield.

8. Conclusions and perspectives

The basic proof of concept for photoautotrophic ethanol production from model cyanobacteria such as *Synechocystis* has been carried out. Strategies for increasing ethanol yields are currently being investigated but there are challenges going forward. These include the effect of ethanol synthesis on the metabolism of the producing strain, which include understanding and optimizing carbon flux, the tolerance of the organism to ethanol, growth and production rates and the challenges of integrating a production strategy that can inform the metabolic engineering strategy. Indeed the lessons learned from model organisms such as *Synechocystis* may need to be applied to different candidate strains which grow faster, can be genetically modified, are more robust in non-axenic culture, are more competitive or are more tolerant to the product once yields are increased. It is very much a case of much work done but significant challenges to future implementation of a viable production system.

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Edited by Thalita Peixoto Basso and Luiz Carlos Basso

This book offers a broad understanding of bioethanol production from sugarcane, although a few other substrates, except corn, will also be mentioned. The 10 chapters are grouped in five sections. The Fuel Ethanol Production from Sugarcane in Brazil section consists of two chapters dealing with the first-generation ethanol Brazilian industrial process. The Strategies for Sugarcane Bagasse Pretreatment section deals with emerging physicochemical methods for biomass pretreatment, and the non-conventional biomass source for lignocellulosic ethanol production addresses the potential of weed biomass as alternative feedstock. In the Recent Approaches for Increasing Fermentation Efficiency of Lignocellulosic Ethanol section, potential and research progress using thermophile bacteria and yeasts is presented, taking advantage of microorganisms involved in consolidating or simultaneous hydrolysis and fermentation processes. Finally, the Recent Advances in Ethanol Fermentation section presents the use of cold plasma and hydrostatic pressure to increase ethanol production efficiency. Also in this section the use of metabolic-engineered autotrophic cyanobacteria to produce ethanol from carbon dioxide is mentioned.

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