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Biofuels Production Process: Microorganisms Utilizing Carbon to Produce Butanol and Ethanol

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Abstract

Microbial fermentation for bio-based products is quickly becoming an integral component of the world infrastructure, as the processes encompassing the synthesis of these natural products becomes more efficient, environmentally friendly and cost effective to compete with existing products. Rising energy costs and increased awareness of global warming have motivated production of biomass-derived fuels and polymers. Bio-butanol and bio-ethanol are currently the most desired fermentation products, as these compounds can be applied to multiple uses, including the foundation for green fuel sources. This review focuses on biofuel production; butanol and ethanol from yeast and bacteria, and how these products are efficient and can be suitable alternative for petrochemical products. It also focuses on utilizing waste to be used as the carbon source for microorganisms to produce bio-based products in an attempt to reduce the overall cost.

Keywords: *Fermentation, Butanol, Ethanol, Strain Development, Feedstock*

1. Introduction

Expected depletion of oil and fossil resources, and increasing concerns of the environment and climate change are urging researchers to develop alternative sources for fuels and energy beyond petroleum resources. Recently, there has been much interest and research in producing advanced biofuels possessing fuel characteristics similar to those of petroleum-derived fuels. Currently, ethanol is used as a major biofuel, since it can be easily produced by existing fermentation technology, but it is not the best biofuel due to its high vapour pressure, hygroscopy, and low energy density [1]. Higher alcohols, including butanol, which possess fuel properties more similar to those of petroleum-based fuel, have attracted interest as alternatives to ethanol. Metabolic engineering has been applied to microorganisms in order to improve butanol production, since the wild type strains do not allow production of these alcohols at high enough efficiencies.

Biofuels are being produced from sugars and starch-based feedstocks, such as corn and sugarcane, (molasses) for bio-ethanol production in the US and Brazil, respectively, and vegetable oil for biodiesel production in EU. Since these feedstocks are interfering with the food supply demand, non-grain based feedstocks such as lignocellulosic biomass resources, particularly agricultural residues, have been targeted for biofuels production [2].

Butanol has been targeted as an advanced biofuel. However, its high cost production through acetone–butanol–ethanol (ABE) fermentation by *Clostridia* is still an obstacle. Inexpensive and sustainable feedstocks such as lignocellulosic residues and dedicated energy crops are needed for butanol production at large scale to reduce the cost, but the process is more complicated, compared to ABE fermentation from sugar and starch-based feedstocks. Engineering heterologous species, such as *Saccharomyces cerevisiae* and *Escherichia coli* with butanol pathway is a potential solution to eliminate the formation of acetone and ethanol byproducts, so that butanol yield can be improved significantly [3].

Butanol and ethanol are produced by fermentation process utilizing the mentioned feedstocks by bacteria or yeast. The main objective of this review is to highlight the strains development for the microorganisms in order to improve biofuel production, and feedstock selection of sugars and starch-based feedstocks and lignocellulosic biomass, and to provide an update of the current commercial status of bio-butanol and bio-ethanol production.

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2. Bio-Butanol Production

Butanol is a four-carbon, straight chained alcohol, which is an important chemical precursor for plastics, paints, and polymers, and it has the potential to replace gasoline [4]. Butanol has traditionally been produced by anaerobic fermentation of sugar substrates using solventogenic *Clostridia* [5]. Meanwhile, non-solvent producing species such as *E. coli* and *S. cerevisiae* can be engineered with the butanol production pathway by metabolic engineering to produce high butanol yields since acetone and ethanol are not produced.

Treatment of biomass for the extraction of fermentable sugars results mainly in pentose, hexose, and disaccharide. *Clostridium* are capable of utilizing both pentose and hexose sugars [6]. However, in addition to utilization of inexpensive and sustainable feedstocks, there are other processes that must be considered for sustainable and economical production of butanol, such as high cost of fermentation substrate, substrate inhibition, and low solvent tolerance that results into low butanol concentration.

Solutions for these problems have been found by researchers, including microorganism strain development for improved butanol titer and tolerance [6], development of *in situ* product recovery technologies to overcome the butanol toxicity to microorganism [7], and the application of several fermentation strategies to increase the cell density [8] as well as yield, productivity, and butanol titer [9].

The global market for bio-butanol was estimated in 2012 to be 3.8 million tons, with an estimated worth of approximately $7 billion, which is forecast to grow by 4.6% from 2013 to 2018 [10]. Butanol production cost by the petrochemical route is 1.52 $/kg, while its cost by fermentation using the first generation feedstock (grain-based) is 2.3 $/kg, and the cost using the second generation feedstock (lignocellulosic) is 1.87 $/kg [10].

2.1. Strains and Metabolic Engineering Development for Butanol Production

The disadvantage of ABE fermentation is its low butanol yield due to the significant production of the major byproducts acetone and ethanol, which makes the process expensive and the feedstock consumption very high [2]. Therefore, developing strains and applying metabolic engineering to the microorganisms will lead to an improved butanol yield and tolerance.

Since *Clostridia* have been the best known species for ABE fermentation, many efforts have been made on developing new strains. *C. acetobutylicum* strain EA2018 with a butanol–acetone–ethanol ratio of 7:2:1, instead of 6:3:1, was isolated in the 1980s from soil samples collected at a Shanghai suburb by Dr. Rui-Shen Jiao at Shanghai Institute of Plant Physiology and Ecology, Chinese Academy of Sciences [11]. A successful case of engineering solventogenic *Costridia* has been reported [12]. The double deletion of the genes *pta* and *buk*, along with the overexpression of a modified aldehyde/alcohol dehydrogenase, increased the butanol titer from 11.8 g/L to 18.9 g/L, and increased the butanol ratio from 65% to 88%.

Mutagenesis by physical or chemical methods has been studied. In the early 1990s, a hyperbutanologenic strain *C. beijerinckii* BA101 was produced by chemical mutagenesis using *N*-methyl-*N*-nitro-*N*-nitrosoguanidine. The most successful case of this approach resulted in a strain that could produce 19 g/L butanol and 29 g/L total solvents, compared to 9 g/L butanol and 13 g/L total solvents produced by the parent strain *C. beijerinckii* NCIMB 8052 [13].

Butanol at a much higher concentration of ~20 g/L [7] was produced using genome shuffling by the recursive genetic recombination [14]. A butanol tolerant strain *C. acetobutylicum* JB200 was bred in Prof. Shang-Tian Yang's lab at Ohio State University from *C. acetobutylicum* ATCC 55025 by long term adaptation.

Non-solvent producing species are being thoroughly investigated due to their biggest advantage, which is the elimination of the major byproducts acetone and ethanol, compared to *Clostridia*. *E. coli* and *S. cerevisiae* are two well established microorganisms for this purpose [7]. Other microorganisms such as *Synechococcus elongates*, *B. subtilis*, *C. tyrobutyricum*, and *Pseudomonas putida* are also under development [15].

Shen et al. (2011) [16] were successful in engineering a heterologous host, who introduced an integrated pathway using *ter* from *Treponema denticola*, and by blocking cellular NADH and acetyl-CoA consuming pathways in *E. coli*, which produced as a result a butanol titer of 15 g/L [10], and the yield reached up to 88% of the theoretical maximum. On the other hand, achieving homobutanol fermentation is challenging due to the complexity of the butanol synthesis pathway.

S. cerevisiae is more tolerant to butanol toxicity than *Clostridia* [17]. Its tolerance was supported by studies on the impact of butanol on its growth [18]. For more efficient butanol production, a deep understanding of the control of heterologous expression of genes and enzymes is needed in order to make the strategy economically competitive compared to the native pathway in *Clostridia* and the chemical synthesis.

2.2. Feedstock Selection

Since the cost of feedstocks is approximately 3/4 of the total cost of butanol production [10], access to cheap fermentation raw materials is an important issue to consider. In the butanol fermentation industry, sugar and starch-based feedstocks, which are known as first-generation feedstock, are currently used for butanol production, as *Clostridia* possess strong amylase activities that enable it to effectively utilize starchy substrates without the need for hydrolysis pretreatment [19]. This sparked the debate food versus fuel, since it is not sustainable for butanol production at a large scale for fuel use. Second-generation biofuel production that utilizes lignocellulosic biomass, particularly residues from agriculture and forest industries that are abundantly available at low cost, is under development. Non-grain crops, such as wheat B-starch, cassava, Jerusalem artichoke, and sweet sorghum, could also be used for butanol fermentation [20]. However, these feedstocks also present challenges for butanol production due to their recalcitrance to degradation [21].

Starch based packing peanuts have been used for butanol production by ABE fermentation using *C. beijerinckii* BA101. Over a 110h fermentation, butanol of 18.9 g/l was produced from 80.0 g/l packing peanuts [22]. Madihah et al. (2001) [23] reported the application of gelatinized sago starch as fermentation feedstock for butanol production by *C. acetobutylicum*. The fermentation resulted in 16.0 g/l butanol production with a yield of 0.24 g/g glucose.

Cassava starch and chips have been used as fermentation feedstocks for butanol production [24]. *C. saccharoperbutylacetonicum* N1–4 possess hyper amylolytic activity, which gives it the ability to produce butanol directly from starch without the addition of a hydrolysis pretreatment step. Fermentations of cassava starch and cassava chip hydrolysate resulted in 16.4–16.9 g/L butanol with a yield of 0.26–0.35 g/g substrate [24]. Although sucrose and starch-based substrates are good for butanol fermentation, these crops will get more expensive due to high demand and potentially

compete with food supply, thus resulting in the food versus fuel debate.

The main components of lignocellulosic biomass are cellulose, hemicellulose and lignin; they are the most abundant polysaccharides, and are the major fraction of agricultural, industrial, forest, and wood waste residues [25]. These polysaccharides are being considered due to the low cost and availability of the feedstocks, and because these substrates do not compete with food supply. Another advantage can be found in low greenhouse gas emissions from biofuels produced from lignocellulosic biomass as compared to those biofuels obtained from corn [26]. However, it is difficult to obtain fermentable sugars from these lignocellulosic materials [27]. It requires physical, chemical, or biological pre-treatment. Solventogenic *Clostridia* can utilize pentose and hexose [28]. Therefore, significant research effort has been made towards utilization of lignocellulosic hydrolysate for butanol fermentation [29]. Saccharides obtained from domestic organic waste (DOW) were used as substrate for butanol production by *C. acetobutylicum* ATCC 824 [30]. Wheat straw hydrolysate has been used as a fermentation substrate for butanol production *by C. beijerinckii* P260 [30]. Experiment was started with 60.2 g/L total sugar obtained by hydrolysis of 86.0 g/L wheat straw, which resulted in the production of 12.0 g/L butanol with a yield of 0.20 g/g total sugar [30]. Another substrate was used as a fermentation feedstock for butanol production using *C. beijerinckii* ATCC 55025 [31] called wheat bran, a byproduct of wheat milling industry. Using the wheat bran hydrolysate containing 53.1 g/L total sugar, 8.8 g/L of butanol was produced in 72 h [31]. These results suggest that lignocellulosic biomass can be efficiently utilized as a fermentation substrate for butanol production.

Glycerol is a renewable carbon source and has widely been used as a fermentation feedstock for the production of ethanol, polyhydroxyalkanoates, and biosurfactants [32]. There are some reports on using glycerol as fermentation substrate for butanol production using *C. pasteurianum* as the biocatalyst [33]. This microorganism has been reported as the only native producer of butanol by glycerol fermentation. A maximum of 17.0 g/L butanol production has been produced by glycerol fermentation using *C. pasteurianum* [33]. The product has similar tolerance and a better yield compared to sugar and starch-based butanol fermentation. The study suggested that the major disadvantage of using glycerol as a feedstock is the high cost [33]. This disadvantage could be avoided, as studies have shown that glycerol is produced as a major co-product of biodiesel industry. Since the feedstock cost for sugar-based fermentation processes is usually 60–80% of the total production cost [34], it is suggested that the butanol fermentation process can be integrated with a biodiesel production facility, and there would be little to no cost for the feedstock. As a result, a significant decrease in the production cost for butanol.

2.3. Current Commercial Status of Bio-Butanol Production in China

As mentioned above, lignocellulosic biomass is a great sustainable feedstock, even though it is difficult to process. The only commercialized bio-butanol factory in the world is that of Lignicell Refining Biotechnologies Ltd in China [10]. The overall cost of the *n*-butanol was estimated as 1.32 $/kg in 2014, which is competing with that of the petrochemical cost 1.52 $/kg [10]. Further improvements can be focused on multiple targets, such as enzymes and pre-treatment that is 35% of butanol cost, utilities and waste water treatment (20%), as well as strain improvement. As data have shown in 2014 [10], 11 ABE fermentation factories based on corn-fermentation in China were newly established or restored to production. The total annual solvent production capacity of ABE fermentation was estimated to be 1 million tons when all these factories are put into full production.

3. Bio-ethanol Production

Ethanol is the most common renewable fuel today produced from sugar and starch-based substrates by *S. cerevisiae* fermentation not requiring saccharolytic and enzymatic hydrolysis of starch as compared to *Clostridial* strains. Currently, ethanol is being produced on a large scale in Brazil, the US and some European countries, and it is produced from sugarcane in Brazil, and starch in the USA at competitive prices, where the conversion of starch to ethanol includes a liquefaction step and a hydrolysis step to produce glucose that is then fermented [35]. As a final product, the ethanol is ready to be used as a fuel in dedicated engines, either pure or blended with petrol, taking advantage of the higher octane number and higher heat of vaporization.

Bio-ethanol production has been categorized into different generations based on the feedstock used. First generation is produced from either corn or sugarcane. Since the US Renewable Fuel Standards mandate (RFS; US Energy Policy Act (EPAct) 2005) requires that 44% of renewable fuel to be blended into gasoline by 2022 should be derived from non-food cellulosic biomass, and the European Council aim to 10% target for renewable energy in the transport sector by 2020 (EU Renewable Energy Directive (RED;2009/28/EC) [36], second generation fuels were introduced.

Second generation biofuels use more complex, non-food based biomass as feedstocks, such as wood residues, industrial and municipal solid waste, and agricultural waste. The most abundant renewable form of biomass is lignocellulose [35]. A part of the lignin, the principal solid part of the biomass remaining, can be burned to provide heat and electricity for the process.

Biomass is not readily fermentable and expensive pre-treatments, both physical (milling and steam explosion) and chemical (acid and alkaline hydrolysis) treatments are required to extract the sugars within the biomass [36]. Lignocellulosic biomass can contain 5–20% of the pentose sugar xylose, which is by far the most abundant pentose sugar. Since wild type *S. cerevisiae* cannot ferment xylose [35], the research on yeast conversion of xylose to ethanol has been intensively studied [37][38].

Bioethanol production from xylose is important for utilization of lignocellulosic biomass as raw materials. Despite the variety of approaches, the levels of ethanol produced are ranging from 4 to 25 g/L [36], which is lower than what is achievable by native yeasts using glucose or sucrose.

In order to reach maximum efficiency and economic viability of bioethanol production, third generation biofuels or development of consolidated bioprocessing (CBP) was studied [36]. In CBP, a single organism performs all steps in the same reaction vessel, and is capable of both producing biomass-hydrolyzing enzymes and fermenting the sugar product to ethanol.

3.1. Strains and Metabolic Engineering Development for Bio-ethanol Production

S. cerevisiae has several advantages due to its high ethanol productivity, as well as its high ethanol and inhibitor tolerance. To date, only a limited number of *S. cerevisiae* strains that ferment xylose have been generated. Several researchers were

able to engineer *S. cerevisiae* in order to produce ethanol [37]. Hector et al., 2013 [39] produced 13.6 g/L of ethanol from xylose by recombinant *S. cerevisiae* yeast strains (YRH1114) expressing xylose utilizing enzymes. Also, Matsushika et al., 2009 [40] produced 15.7 g/L from xylose by using *S. cerevisiae* (MA-T4). And Fujitomi et al., 2012 [41] achieved 25.4 g/L ethanol biosynthesis by *S. cerevisiae* (BY4741X).

Researchers were successful in the engineering of the Gram-negative bacteria: *E. coli*, *Klebsiella oxytoca*, and *Zymomonas mobilis*. *E. coli* and *K. oxytoca* are naturally able to ferment a wide range of sugars, and work was performed on engineering these strains to produce ethanol [37]. *Z. mobilis* ferments only glucose and fructose, and produces ethanol at high yields [37].

3.2. Current Commercial Status of Bio-Ethanol Production in Brazil

Until 2010, Brazil is the world's largest exporter and the world's second largest producer of ethanol fuel. Together, the United States and Brazil lead the industrial production of ethanol fuel [42] [43]. The successful Brazilian ethanol model is sustainable due to the abundant availability of low cost sugarcane and its advanced agro-industry [44]. The residual cane-waste (bagasse) is used to produce heat and power, which results in a very economic cost and in a high energy balance.

There are no vehicles in Brazil running on pure gasoline. The mandatory blend is 25% of anhydrous ethanol and 75% gasoline or E25 blend [45]. The Brazilian car manufacturing industry developed flexible-fuel vehicles that can run on any proportion of gasoline (E20-E25 blend) and hydrous ethanol (E100) [46].

Sucrose extracted from sugarcane is about 30% of the chemical energy stored in the plant; 35% is in the leaves and stem tips, and 35% are in the fibrous material left over from pressing[47]. These substances are used as leftover chemical energy to generate electricity and process ethanol [48]. Utilizing the most out of this plant resulted in a low cost ethanol product.

As of 2008, the average price of E25 gasoline was $4.39 per gallon [49], while the average price for ethanol was 2.69 $/gallon [50]. The price ratio between gasoline and ethanol fuel has been above 30% in Brazil. According to Brazilian producers, ethanol can remain competitive if the price of oil does not fall below $30 a barrel [51].

Brazilian fermenters are able to produce ethanol for 22 cents per litre, compared with the 30 cents per litre for corn-based ethanol in the US [52]. It costs 30% more because the corn starch must first be converted to sugar before being fermented into alcohol [53].

4. Future Pathways

In Jordan, we can utilize the olive's flesh, which is rich with fatty acids after being pressed and squeezed for olive oil production, and these waste streams can be used as feedstocks for the fermentation process, just like Brazil has utilized sugarcane and converted its waste to a value-added product. In the US, waste utilization is growing fast. Researchers are studying chitin extraction from lobster and shrimp shells to use it as feedstocks in order to produce polyhydroxyalkanoate, a bio-plastic that is an alternative for petroleum-based plastics.

5. Conclusion

Butanol is a superior biofuel compared to ethanol. Its production cost must be reduced in order to become competitive to be used as a biofuel. It is important to use inexpensive, non-food carbon substrates obtained from renewable biomass and develop efficient fermentation and processing strategies. Lignocellulosic biomass should be used to lower the feedstock cost and to ensure the sustainable production of butanol. The used strain also influences the method of butanol production and the methods used for pre-treatment and hydrolysis of biomass. The selected strain can be improved by applying metabolic engineering.

Development of strains and processes in order to increase butanol titer, productivity and yield, increase tolerance to butanol, and improvement of cost effective purification method, as well as preparation of fermentable sugars from the least expensive and most abundant biomass, will be continuously pursued and studied.

The ultimate goal of research into third generation bio-ethanol is to engineer an organism capable of CBP, which will decrease the cost dramatically.

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