



Progresses in the production of ethanol from lignocellulosic biomass using *Saccharomyces cerevisiae* by fermentation engineering

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Abstract

Ethanol fermentation is a biological procedure which converts sugars such as glucose, fructose, and sucrose into cellular energy, producing ethanol and carbon dioxide as by-products. Since yeasts perform this conversion in the absence of oxygen, alcoholic fermentation is generally considered to be an anaerobic process. Ethanol fermentation has many uses, including the production of alcoholic beverages, the production of ethanol fuel, and bread making. The increasing demand for biofuels around the globe has also prompted the necessity to seek other means to meet the demands. In this review, the general ideologies, methodologies, general chemistry and biochemistry and conditions of the production of ethanol by fermentation engineering using *Saccharomyces cerevisiae* are highlighted. The quest to reduce pressure on staple foods has necessitated the attention now given to the use of lignocellulose biomass, despite the complexity of the process. It concludes by suggesting ways to improve yield and commercialization of the use of lignocellulosic biomass for ethanol fermentation.

Keywords: Ethanol; Fermentation engineering; *Saccharomyces cerevisiae*; Lignocellulose biomass; Petrochemical

1. General Concepts of Ethanol Production

Fermentation engineering is the intentional use of engineering methods to induce fermentation by microorganisms such as bacteria and fungi as well as eukaryotic cells like CHO cells and insect cells, to make products useful to humans. Ethanol is produced from biomass predominantly through fermentation using glucose derived from sugars, starch or cellulose. In this process, the feedstock is converted first into glucose. In the case of sugars, the process is straightforward since the sugar is simply dissolved in water. Starch, however, requires pre-processing where the starch is transformed into glucose through a process call liquefaction and saccharification. This process through the addition of enzymes frees the glucose bound in the starch and makes it available for fermenting into alcohol. The microbial conversion of agricultural substrates into ethanol is, however, an ancient practice that certainly predates the science of microbiology, the chemistry of the distillation process, and the engineering of an ethanol fermentation plant [1].

Production of ethanol fuel from lignocellulosic biomass has been a topic of much interest from many researchers in recent times. This is in response to quest to find a more environmentally friendly, yet cost effective approach to the production of bioethanol and also to reduce the dependence on staple foods such as sugar cane, corn, among others. As compared to sugar and starch-based feedstocks, the bioconversion of lignocellulosic materials to fermentable sugars for bioethanol production is harder [2]. In the bioconversion of lignocellulosic materials to bioethanol, a pretreatment step to break the complex structure of lignocellulose is needed, after which the fermentable sugar is obtained by hydrolysis and then the obtained sugars are converted into bioethanol by microorganisms [2].

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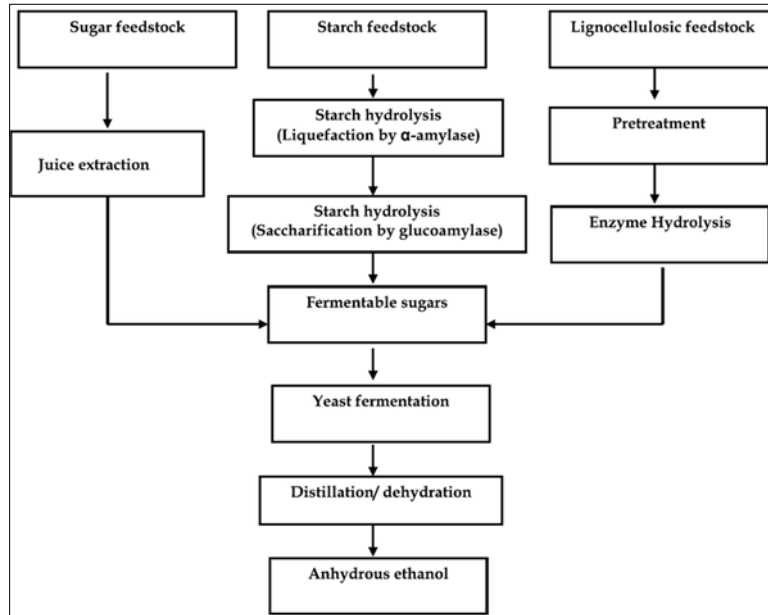


Figure 1 The schematic diagram of the general principle of ethanol production

1.1. The general chemistry and biochemistry of ethanol production

Alcoholic fermentation converts one mole of glucose into two moles of ethanol and two moles of carbon dioxide, producing two moles of ATP.

The fermentation of ethanol generally follows the chemical equation:



Sucrose for instance, is a dimer of glucose and fructose molecules. Therefore, in order to produce ethanol through fermentation of sucrose, the microbial enzyme invertase first catalyzes the cleaving of the glycosidic linkage between the glucose and fructose molecules.

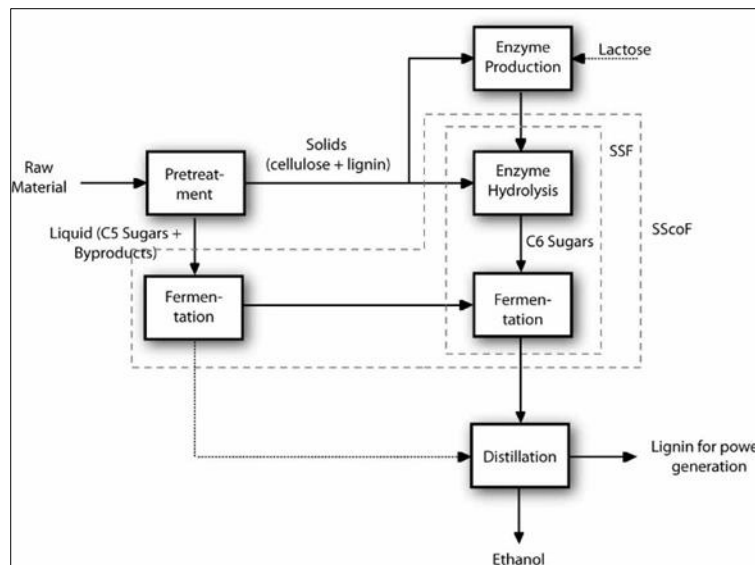
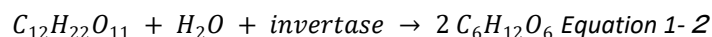
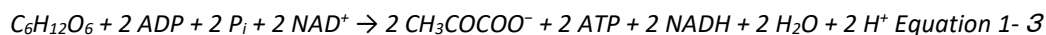


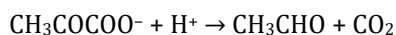
Figure 1 Biochemical route of enzymatic production of ethanol



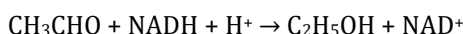
Each of the glucose molecules is then broken down into two pyruvate molecules via glycolysis [3]. Glycolysis follows the following equation:



CH₃COCOO⁻ is pyruvate, and P_i is inorganic phosphate. Conclusively, pyruvate is converted to ethanol and CO₂ in a two-step reaction, which in turn regenerate oxidized NAD⁺ require for glycolysis:



This reaction catalyzed by the enzyme pyruvate decarboxylase



This reaction is catalyzed by the enzyme alcohol dehydrogenase (ADH1 in baker's yeast) [4]

From the above equations, it can be deduced that glycolysis allows for the reduction of two molecules of NAD⁺ to NADH. Two ADP molecules are also converted to two ATP and two water molecules through substrate-level phosphorylation.

1.2. Sources of ethanol

As of 2003, about 5% of the world's ethanol was produced from the hydration of ethylene, catalyzed by sulfuric acid (H₂SO₄) [5]. The ethylene was sourced from petrochemicals such as coal, calcium carbide and others. Petroleum derived ethanol (synthetic ethanol) is chemically identical to bio-ethanol and can be differentiated only by radiocarbon dating [6]. This accounts for a non-fermentation source of ethanol production.

Using fermentation engineering methods, ethanol can be produced from various feedstocks such as sugar cane, miscanthus, sweet potatoes, sugar beet, bagasse, sorghum, grain, switchgrass, barley, hemp, potatoes, cassava, fruit, molasses, stover, grain, sunflower, corn, wheat, straw, kenaf, cotton, other biomass, as well as many types of cellulose waste.

An alternative process to produce bio-ethanol from algae is being analyzed by the company Algenol. Rather than grow algae and then harvest and ferment it, the algae grow in sunlight and produce ethanol directly, which is removed without killing the algae. It is claimed the process can produce 6,000 U.S. gallons per acre (5,000 imperial gallons per acre; 56,000 liters per hectare) per year compared with 400 US gallons per acre (330 imp gal/acre; 3,700 L/ha) for corn production [7].

In recent times, the first-generation processes for the production of ethanol from corn makes use of only a small portion of the corn plant: the corn kernels are obtained from the corn plant and the starch alone, which accounts for about 50% of the dry kernel mass, is processed into ethanol. Two kinds of second-generation processes are still under analysis and development. The first type uses enzymes and yeast fermentation to convert the plant cellulose into ethanol while the second type proceed via pyrolysis to convert the whole plant to either a liquid bio-oil or a syngas. Second generation processes can also be used with plants such as grasses, wood or agricultural waste material such as straw [8].

2. Ethanol Production using *Saccharomyces cerevisiae*

2.1. Upstream Processing

Upstream processing is the first step of the production process, involving the formulation of fermentation medium, air sterilization, the fermentation medium and fermenter, inoculum preparation and medium inoculation. it begins with the isolation, improvement and production of microorganisms; followed by screening (primary and secondary screening).

2.1.1. Isolation of *Saccharomyces cerevisiae* and inoculum preparation

Saccharomyces cerevisiae is one of the oldest, most exploited and best studied microorganisms in both old and new biotechnologies and is known to be the world's premier industrial microorganisms which readily convert sugar into

alcohol and CO₂ in metabolic process called fermentation [9]. *Saccharomyces* strains were used widely and traditionally for industrial ethanol production because of its ability to produce high concentrations of ethanol from hexoses and its high tolerance to ethanol and other inhibitory compounds [10]. Tolerance to high temperatures and high ethanol concentrations are important properties of microorganisms of interest to industry [11]

Strains may be isolated from sugar cane molasses, dates and figs by a serial dilution procedure utilizing Yeast Malt Agar (YMA) medium containing yeast extract, malt extract, peptone, glucose, agar; the pH may be balanced to 5.5 within the nearness of an acid. Strains are filtered by streaking on YMA; an immaculate culture of each strain can be kept on strong culture medium YMA braces and stored at 4°C until required. A while later, the cells are collected by centrifugation, washed twice with sterile refined water, centrifuged and re-suspended on sterile water to get a suspension of high cell concentration.

Table 1 The Composition of The Medium

| Compound | Amount (g/l) in yeast malt agar |
|---------------|---------------------------------|
| Glucose | 10 |
| Yeast extract | 3 |
| Malt extract | 3 |
| Peptone | 5 |
| Agar | 15 |

2.1.2. Biomass preparation

Fermentation techniques from any material that includes sugar may be able to derive ethanol. The different raw materials used in the manufacture of ethanol by means of fermentation are easily labeled into three predominant sorts of raw materials: sugars, starches, and cellulose materials. Sugars (from sugarcane, sugar beets, molasses, and fruits) can be converted into ethanol directly. Starches (from corn, cassava, potatoes, and root crops) have to first be hydrolyzed to fermentable sugars through the activity of enzymes from malt or molds. Cellulose (from wood, agricultural residues, waste sulfite liquor from pulp, and paper mills) have to likewise be converted into sugars, generally, by the action of mineral acids. Once simple sugars are formed, enzymes from microorganisms can quite simply ferment them to ethanol. Most agricultural biomass containing starch can be used as a workable substrate for the ethanol fermentation via microbial processes. These substrates encompass corn (maize), wheat, oats, rice, potato, and cassava. On a dry premise, corn, wheat, sorghums (milo), and different grains contain around 60–75% (w/w) of starch, hydrolysable to hexose with a noteworthy weight increment (stoichiometrically the starch to hexose proportion is 9:10), and these offer a decent asset in numerous fermentation techniques [12]. Fermentation of starch is to some degree more intricate than of sugars since starch should initially be converted to sugar and afterward into ethanol. Starch is first hydrolyzed by adding α -amylase to prevent gelatinization, at that point cooked at high temperature (140–180°C). Next, the melted starch is hydrolyzed to glucose with glucoamylase. The subsequent dextrose is fermented to ethanol with the guide of microorganisms producing CO₂ as a by-product.

During the procedure as of now utilized for industrial-scale ethanol fermentation from starchy materials, high-temperature cooking (140-180°C) is extremely compelling for fermentation of starchy materials since it raises starch saccharification effectiveness and accomplishes significant levels of ethanol production under complete sterilization of harmful microorganisms.

Be that as it may, production costs are high because of the high energy utilization in the cooking procedure and the addition of a lot of amylolytic proteins. Along these lines, procedures to decrease the high production costs are required. To resolve these challenges, noncooking and low temperature cooking fermentation frameworks have been created [13].

Among the three important sorts of raw materials, cellulose materials characterize the amplest world supply of biomass and have been largely unutilized. The global production of plant biomass, of which over 90% is lignocellulose, amounts to about 200×10⁹ heaps per year, where about 8–20×10⁹ heaps of the most important biomass remain potentially accessible. However, the high-quality utilization of the lignocellulosic feedstock is no longer continually realistic due to the fact of its seasonal availability, scattered stations, and the high expenses of transportation and storage of such giant amounts of natural fabric [14]. Recently, the enzymatic hydrolysis of biomass cellulose is regarded to be the most

promising technology accessible [15] However, no matter the work done, the industrial scaleup of this manner seems to be nevertheless hindered via technological issues or by way of the lack of a biomass refinery approach in which ethanol is one of countless products. In fact, because raw material value comprises greater than 20% of the production cost [15], the optimization of the cellulose conversion should be carried out by correct administration and utilization of all manner streams. An end result of this situation is that even constrained authorities' intervention is still crucial to preserving ongoing research. To make lignocellulosic biomass an appropriate raw material for ethanol fermentation, new engineering techniques and techno-economical modelling ought to be devised to genetically enhance plants/algae and acquire the desired structural and chemical properties.

Pretreatment methods can be generally classified into 4 groups – physical, chemical, physio-chemical and biological. Physical pretreatment approaches employ the mechanical comminution or irradiation approaches to change only the physical traits of biomass. The physio-chemical process makes use of steam or steam and gases, like SO₂ and CO₂.

The chemical methods employ acids (H₂SO₄, HCl, organic acids etc.) or alkalis (NaOH, Na₂CO₃, Ca (OH)₂, NH₃ etc.). The acid treatment commonly indicates the selectivity toward hydrolyzing the hemicelluloses components, whereas alkalis have better selectivity for the lignin. The fractionation of biomass aspects after such processes assists in improving the enzymes accessibility which is additionally important to the efficient utilization of enzymes.

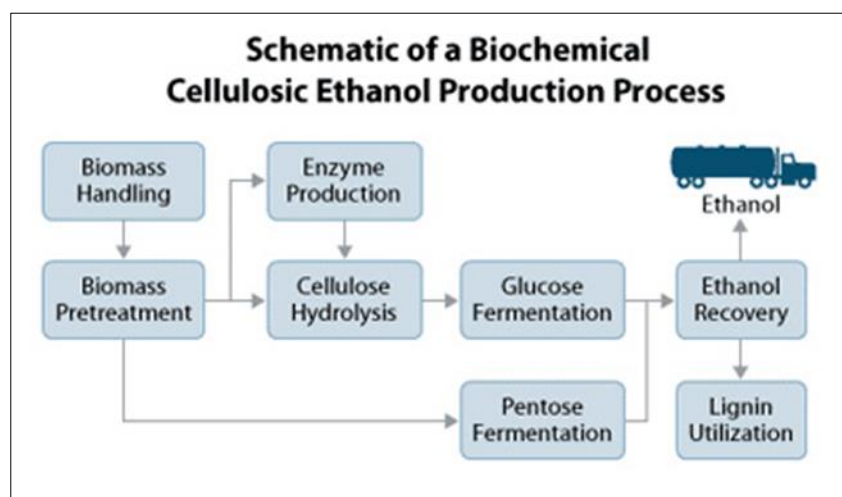


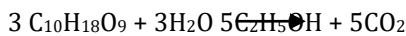
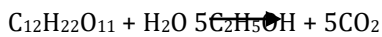
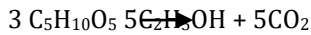
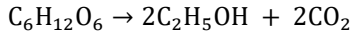
Figure 3 Schematic flow diagram of the biochemical production process of cellulosic ethanol

2.1.3. Fermentation process

After pretreatment and hydrolysis of lignocellulosic biomass, simple sugars are produced as a result of depolymerization of cellulose and hemicellulose that are then fermented by means of the applicable microorganisms and converted into ethanol. The normal method is referred to as fermentation. Ethanol fermentation can be completed both by submerged or solid-state fermentation. In submerged fermentation, water is a vital liquid that is used to make fermentation mash through mixing a pre-defined solid with water. On the other hand, solid state fermentation is the bioconversion of the lignocellulosic biomass in its natural state. In a solid-state fermentation, lignocellulosic biomass is moistened with a thin layer of water on the surface of the biomass, using weight ratios of water to lignocellulose are usually between 1:1 and 10:1. The anticipated benefits of solid-state fermentation over submerged fermentation are: (a) smaller fermenter volume, because there is no excess water in the fermenter; (b) decrease sterilization strength costs, because less water needs to be heated; (c) less complicated aeration, because air can flow into freely between the substrate particles, and due to the fact, the liquid film covering the substrate has a massive surface area compared to its volume; (d) reduced or eliminated capital and operating costs for stirring and for effluent treatment; (e) lower costs for product recovery and drying; (f) a more natural environment for lignin-degrading fungi: many of these fungi grow and operate better under SSF than underneath submerged conditions; and (g) a less favorable environment for many bacteria, decreasing the hazard of contamination. However, exploitation of solid-state fermentation on commercial ethanol production from lignocellulosic biomass is far away due to technoeconomic limitations of this process.

Fermentation can be performed by way of three modes such as batch, fed-batch and continuous fermentation. The choice of most suitable process relies upon on the kinetic properties of fermenting microorganisms and type of feedstocks, in addition to aspects of process economics. Batch subculture is commonly achieved in a closed culture

system through inoculating microorganism into a preliminary fermentation media containing defined amount of nutrients and allowed to ferment until the finishing of the nutrients. It is the simplest mode of fermentation where nothing is added after inoculation besides maybe acid or alkali for pH control. In batch fermentation, microorganism works in high substrate concentration initially and an excessive product concentration finally. During fermentation, microbial conversion of pentoses, hexoses and disaccharides derived from cellulose and hemicellulose occurred based on the following reactions. The theoretical ethanol yield can additionally be calculated from these equations. In general, ethanol yield, productivity and percentage theoretical ethanol yield can be calculated for any substrate in the fermentation system by the use of the Equations.



Ethanol yield is calculated as the amount of ethanol produced per unit of substrate utilized as

$$Y_{EtOH} = CV/m \text{ Equation 2- 1}$$

where, Y_{EtOH} is ethanol yield (kg^{-1}), C is ethanol concentration (g/L), V is initial volume of liquid medium (L), and m is the mass of the substrate (kg). Ethanol productivity is estimated as the amount of ethanol produced per unit of substrate utilized per unit of time. It is typically determined when ethanol concentration is maximum.

Ethanol productivity is calculated as

$$Q_{EtOH} = CV/mt = 1000 Y_{EtOH} /t \text{ Equation 2- 2}$$

where, Q_{EtOH} is ethanol productivity ($mg/kg h$), and t is the time at which the ethanol concentration produced on substrates is maximum (h).

The maximum theoretical ethanol yield was calculated as follows:

$$Y_{max} (\%) = [Ethanol produced in reactor (g) / Initial sugar in reactor (g) \times 0.511] \times 100 \text{ Equation 2- 3}$$

2.2. Downstream processing

In the distillation step, the ethanol is separated from the other components and leaves the head of the column as an azeotropic mixture. This processing step is very energy intensive and requires a smart internal use of the heat. Subsequently the ethanol-water mixture is dehydrated to a desired ethanol content of 99.5 wt%. The residue gained at the bottom of the distillation column is called stillage. This is a suspension comprising water, lignin, and other organic components that are not utilized during the simultaneous saccharification and fermentation (SSF). The stillage is subsequently subjected to solid-liquid separation, where it is split into insoluble solids and a liquid fraction. The solids, mainly containing lignin, are used after a drying step in the co-generation plant for process steam and power generation.

A common method in treating the liquid fraction of the distillation stillage is evaporation. It is used to separate the main constituents of the distillation stillage, water and soluble organic compounds, to make them accessible for reutilization in the process or as by-products [16]. From an energetic point of view this stillage treatment step is disadvantageous, since a multistage evaporation utilizes a high amount of primary steam to evaporate water and to produce a concentrate. It is therefore not unreasonable to use the liquid fraction rich in organic matter to produce biogas. With biogas as an additional main product, a more variable process control can be maintained, and the unused sugar from the SSF, proteins, and extracts can be thus exploited in the production of biogas, which results in a cascading use of sugar. The biogas production consists of an anaerobic fermentation, and for wastewater treatment, a subsequent aerobic step could be implemented.

3. Advantages and challenges of using *S. cerevisiae* in ethanol fermentation

3.1. Advantages

It keeps the distillation cost low as it gives a high ethanol yield, a high productivity and can withstand high ethanol concentration. It as it tolerates a wide range of pH thus making the process less susceptible to infection. They are also used during biological fermentation for ethanol production as it facilitates downstream processing, allows operation at high cell density and gives higher overall productivity [17], [18]. It reduces the cost of cells recovery as it separates easily from the fermentation medium without centrifugation.

3.2. Challenges

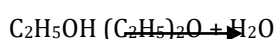
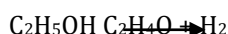
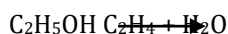
S. cerevisiae strains are unable to compete with wild-type yeast which cause contamination during industrial processes. Stressful conditions like an increase in ethanol concentration, temperature, osmotic stress and bacterial contamination are the reasons why the yeast cannot survive during the fermentation [17]. Increase in ethanol concentration during fermentation can cause inhibition to microorganism growth and viability [19], [20], [17]. Inability of *S. cerevisiae* to grow in media containing high level of alcohol leads to the inhibition of ethanol production [21].

One of the major hindrances in using *S. cerevisiae* for ethanol fermentation from lignocellulosic biomass is the incapability of fermenting xylose, which are produced together with glucose as a result of hydrolysis of hemicelluloses and cellulose.

4. Catalytic dehydration of ethanol Ethylene: petrochemical precursor

Catalytic dehydration of ethanol is an alternative route for production of ethylene. Ethylene or ethene (CH₂=CH₂) is the first member of the alkenes. It is a colorless gas with a normal boiling point of -103.7 °C and is slightly soluble in water and alcohol. This compound is highly active and reacts easily when added to many chemical reagents. For instance, addition of water to ethylene produces ethyl alcohol or ethanol. Ethylene is a good starter molecule to build on due to its simple chemical formula combined with the double bond. It is also the raw material for the production of different grades of polyethylene and other bulk and base chemicals [22], [17].

Straight chain olefins can be produced by the oligomerization of ethylene through a process called the Shell higher olefin process (SHOP). These higher chain hydrocarbons (α -olefins) are used primarily as comonomers to produce linear low-density polyethylene and to manufacture detergents and synthetic lubricants as well as gasoline, diesel, and jet blend stocks [22], [23], [24], [13]. For example, ethylene oxide, produced by oxidation of ethylene, is a key raw material in the production of surfactants and detergents. Another major chemical intermediate obtained by alkylation of ethylene is ethylbenzene which is precursor for styrene production. Styrene is utilized for manufacturing of polystyrene for insulation, packaging, rubber for tires, and footwear [22], [23]. In the industry, the alcohol dehydration mainly occurs in the vapor phase of two-catalyst systems, i.e., supported phosphoric acid and activated alumina. The ethanol dehydration is an endothermic reaction. Therefore, the reaction temperature affects the yield of ethylene. The highest selectivity towards ethylene is obtained at 300–500 °C. Higher temperatures shift the reaction towards acetaldehyde production, while lower temperatures result in production of diethyl ether. Isothermal and adiabatic modes of operations have been suggested for the dehydration of ethanol to ethylene, while the latter is more economically feasible [25], [26], [27], [28].



5. Progresses in ethanol fermentation from lignocellulosic biomass technology

Although generation of ethanol from lignocellulosic biomass is considered advantageous and promising, still there are some technical and economic challenges, despite the fact that substantial research efforts have been made to improve the conversion process. Requirement of different technological steps, such as pretreatment, detoxification, hydrolysis and fermentation has made the conversion process more complicated and economically non-competitive, thereby confining it into pilot or demonstration plants.

5.1. Process integration

In recent years, integration of processing steps at different configuration has been analyzed as attempts to reduce steps as well as capital investments, energy consumption and process time. The most promising process integration in ethanol production is the simultaneous saccharification and fermentation (SSF), which is widely used both in industrial and laboratory scales. SSF is referred to as the performance of both cellulose hydrolysis and fermentation of hexose sugars in a single reactor. When SSF includes co-fermentation of hexose and pentose sugars, it is called simultaneous saccharification and co-fermentation (SSCF).

SSF is considered as an economic solution for its reduced process time and less equipment requirement, even though still there are some bottlenecks with this technique. Recently, a novel technique has been developed for process integration, namely simultaneous saccharification, filtration and fermentation (SSFF) that combines both SHF and SSF. In SSFF, pretreated LCB is hydrolyzed in a reactor, while the suspension is continuously pumped through a cross-flow membrane. The retentate goes back to the hydrolysis vessel, while a clear sugar-rich filtrate continuously perfuses through the fermentation vessel before it is pumped back to the hydrolysis vessel. Another emerging and promising technique is the consolidated bioprocessing (CBP), which is an alternative to technique to SSF.

5.2. Co-fermentation of glucose and xylose

Compared to starch-based ethanol production that use hydrolysate of starch as the fermentation media containing mostly glucose, the hydrolysate of lignocellulosic biomass often contains mixers of sugars, pentoses and hexoses, particularly xylose and glucose, respectively. Glucose is readily fermented to ethanol by many naturally occurring microorganisms with high yield. Although natural microorganisms, especially *S. cerevisiae* have been widely used in fermentation over many years, there are some limitations with this yeast in lignocellulosic ethanol production. Substantial research efforts have been made in recent years to ferment both glucose and xylose that focus on the development of novel microorganisms altering the genetic pattern of the natural microorganisms with desired traits through either recombinant DNA technology [29], or evolution and adaptation techniques [30].

Although the concept of developing recombinant strains brings some advantages in lignocellulosic ethanol production, several challenges exist with this technological approach, in addition to the fact that commercial exploitation of recombinant strains is still difficult due to some technical barriers.

5.3. Immobilization of enzymes and cells

An important yet cost increasing factor for lignocellulosic ethanol is the requirement of enzymes and fermenting microorganisms. Typically, enzyme can be immobilized through the attachment on the solid particles and enzyme cross-linking techniques. Immobilized cells have prolonged cellular stability and increased tolerance to high substrate concentration. The major advantages of immobilization are increased ethanol yield, greater volumetric productivity, reduced end-product inhibition, reduction of risk of microbial contamination due to high cell densities, decreased energy demands and process expenses due to easier product recovery, regeneration and reuse for extended periods, recycling in repeated batch fermentations, and protection of cells against toxic substances [31]. Self-flocculation of yeast cells is a nonsexual and reversible cell aggregation where yeast cells adhere to each other and form a floc [32].

This technique is more promising than the immobilization using the supporting materials as it is technically simple and economically competitive, completely eliminates the contamination, offers the cells to grow without being affected by environmental factors, and gives the feasibility to control desired concentration in the reactors and recovery of yeast cells through purging the reactors [33].

5.4. Use of high solid load

The initial solid content in the slurry is a factor for overall conversion efficiency and outcomes. In most cases, utilization of high biomass load is considered one of the essential approaches, as it enhances fermentable sugar and ethanol yields that meet the techno-economic requirements of large-scale ethanol production [34]. Nevertheless, excessively high solid load in the slurry poses some fundamental challenges that affect efficiency of the overall conversion process. These challenges include increase in the viscosity and inhibitors of the medium, poor mass transfer, inhibition of the yeast cells, reduced yield and productivity [35]. On the other hand, major challenges for fermenting microorganisms are the tolerance to high sugar and ethanol concentrations. In the current practice of commercial ethanol production from sugars and starch, strategies for overcoming these challenges have been achieved successfully.

6. Conclusion and Future Perspectives

This work along with other works prove lignocellulosic biomass to be one of the main renewable feedstocks for sustainable ethanol production in near future, since these are mostly waste materials, available with low and stable price, rich in carbohydrates, versatile and non-competitive to staple food and feed. Although good research efforts have been made in this area, ethanol fermentation from lignocellulosic materials remain the most complex. This limits its exploitation into the commercial production. Another challenge for commercial ethanol production using lignocellulosic biomass is the low ethanol yield, which is due the low fermentable sugars obtained as a result of nature of biomass and technical difficulties of the process. Lignocellulose contents of biomass greatly differ with regard to the source and type. A further research effort should be encouraged to overcome these technical barriers towards implementation on commercial scale. Improvement in enzymatic hydrolysis of LCBs through the development of potential enzyme systems with decreased production costs and novel technologies for high solid handling would be another promising option.

Compliance with ethical standards

Disclosure of conflict of interest

The authors declare that there is no conflict of interest.

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