# THE BIOREFYNERY OF SECOND GENERATION- NEW RESOURCES, TECHNOLOGIES AND IDEAS

P.Boiko c.t.s., as.prof.
M.Bondar c.t.s., as.prof.
A.Kuts c.t.s., as.prof.

*National University of food technologies,*
*Kyiv, Volodymyrska 68, 01601*

*Abstract. In this artical we describe the perspectives of bioethanol production by the refinery of second generation, including from cellulose as a raw material.*

*Key words: cellulose, fermentation, yeast, microorganisms*

## Introduction

The inevitable decline in petroleum reserves and the rise in demand for oil from rapidly growing economies have caused soaring oil prices, and coupled with climate change concerns have contributed to the current interest in renewable energy resources. In some parts of the world this interest has resulted in the introduction of legislations promoting the use of renewable energy resources and increasing government

incentives for commercialization of renewable energy technologies. Development of science and technologies for efficient conversion of lignocellulosic biomass to renewable liquid transportation fuels has become one of the high priority research areas of the day, and bioethanol is the most successful biofuel to date.

Corn- and sugarcane-derived first generation bioethanol is currently in wide use as a blendin fuel in gasoline sold in the United States, Brazil, and in a few other countries. However, there are a number of major drawbacks to these first generation fuels such as the effect on food prices as traditional food resources are utilized as raw materials, net energy balance, and poor greenhouse gas mitigation.

It is very interesting, that the bioethanol produsers are looking to the new type of raw materials, not typical for them before. E.g. UNICA, the Brazilian sugarcane industry association, has released data showing that Brazilian corn ethanol production has more than doubled over the past year and accounted for approximately two-thirds of the ethanol produced during the second half of December.

According to UNICA, mills in the south-central region of Brazil processed 733,860 tons of sugarcane during the second half of December. From the beginning of the current harvest, which began April 1, through the end of December, mills in the region processed 578.6 million tons of sugarcane, up 2.86 percent when compared to the same period of the previous harvest.

As of the end of 2019, UNICA said 253 units had ended harvest activities, compared to 267 that had completed the harvest by the end of 2018. During the final half of December, 31 units produced ethanol. This includes 21 that produced sugarcane ethanol, 5 that produced corn ethanol, and five that produced ethanol from both feedstocks.

Only 137.28 million liters (36.27 million gallons) of ethanol was produced during the second half of December, including 84.52 million liters of hydrous ethanol and 52.76 million liters of anhydrous ethanol. Approximately 90.38 million liters, or 66 percent, of the ethanol produced during the two week period was made from corn.

Since the beginning of the current season, mills in the region have produced 32.11 billion liters of ethanol, including 9.85 billion liters of anhydrous ethanol and 22.26 billion liters of hydrous ethanol. Of that total volume, corn ethanol accounted for 1.05 billion liters, up 104.03 percent when compared to the same period of 2018.

Ethanol sales by units in the south-central region reached 2.67 billion liters in December, including 120.64 million liters that were destined for export, and 2.55 billion liters that were sold domestically. The volume of anhydrous ethanol sold into the domestic market reached 681.86 million liters, with the volume of hydrous ethanol at 1.87 billion liters, up 4.91 percent when compared to December 2018 [1].

Cellulosic ethanol is a second generation biofuel produced from agricultural wastes, grasses, municipal wastes, and other feedstocks that do not double as food, so unlike traditional

corn-based ethanol, it promises to avoid encroaching upon and destabilizing the human food supply. In addition, cellulosic ethanol can be produced from a variety of abundant lignocellulosic biomass feedstocks, and should be able to be produced in substantial amounts to meet the growing global energy demand.

Cellulosic ethanol is the biofuel produced from many forms of lig-nocellulosic biomass such as grasses, wood, agricultural wastes, or inedible parts of plants. The use of lignocellulosic biomass for the production of biofuels, and particularly the cellulosic ethanol, has a number of advantages, as shown below.

1. Cellulosic biomass is the most abundant form of organic carbon on earth. Unlike corn and sugarcane now used to make most ethanol, cellulose is not used for food; therefore cellulosic ethanol will not have adverse effects on food supply and prices. As there is a very wide range of plant materials that can be used for cellulosic ethanol production, it can be grown in all parts of the world. Cellulosic ethanol can be made of many agricultural wastes like corn stover, wheat straw, rice straw, grasses, farm residues, industrial wastes, sawdust, forest thinnings, waste paper, and municipal wastes. Fast-growing woody crops such as poplar and willow are also attractive options for cel-lulosic ethanol production.
2. Cellulosic ethanol achieves a significant reduction in greenhouse gases compared to other forms of etha-nol. On a life-cycle basis, all biofuels produce lower GHG emissions com-pared to gasoline. Corn-based bioethanol offers rather limited benefits, as it reduces GHG emissions by only 18% compared to gasoline. In contrast, cellulosic

bioethanol results in almost 90% lower emissions. On a life-cycle basis, not all biofuels are equal in terms of environmental benefits. The net energy balance of biomass to bioethanol conversion is the key parameter that explains the interest in using bioethanol fuel instead of fossil gasoline. From a life-cycle assessment (LCA) viewpoint, the ratio of the energy content of bioethanol to the net non-renewable primary energy consumed in the whole production process must be taken into consideration. As the approach is LCA oriented, the energy input must be estimated in terms of primary energy. Studies have shown that corn-based bioethanol yields 20–30% more energy, typically fossil fuel energy, than is con-sumed in making it. On the other hand, sugarcane and cellulosic bioethanol yield renewable energy nine times worth the fossil energy used to produce them. The reductions in carbon dioxide emissions mean that bioethanol is better for the environment. Using renewable resources-based bioethanol or bioethanol-gasoline blends as transportation fuels can signifi-cantly reduce gasoline use and exhaust greenhouse gas emission.

3. Land-use change (LUC) is another parameter used in evaluating the biomass-based renewable fuels. Dunn and coworkers from Argonne National Laboratory, USA, have recently published their results on a land-use change and greenhouse gas emissions from corn and cellulosic ethanol. Land-use change occurs when land is converted to biofuel feedstock production from other uses or states, including forests, non-biofuel feedstock agricultural lands, and grasslands. This type of land-use change is at times called direct LUC. The resulting change in crop production levels like, for example, an increase in corn production, may cause a decrease in soybean production and in turn affect

corn exports in one country, shifting the land uses in other parts of the world through economic linkages.

There are two fundamental routes to produce cellulosic ethanol from renewable biomass:

the aqueous-phase biomass saccharification-fermentation route, and thermochemical gasifi cation route. The thermochemical route can be divided into two paths as syngas produced from biomass can be converted to ethanol by chemical or enzymatic methods.

## 1. Fermentation

Fermentation is the biochemical transformation by which sugars are converted to ethanol, and carbon dioxide is also formed as a byproduct in this reaction. This step can be applied in the cellulosic ethanol production process in two different ways.

1. Separate Hydrolysis and Fermentation (SHF)
   This classic configuration involves a sequential process where the hydrolysis of cellulose and fermentation are carried out in different units as shown in the scheme in Figure 1.1. In this route the first enzymatic or acid hydrolysis can be used to produce biomass hydrolyzate or C-5, C-6 sugar solution. Secondly this sugar solution is subjected to fermentation in a separate vessel and this configuration is known as separate hydrolysis and fermentation (SHF).

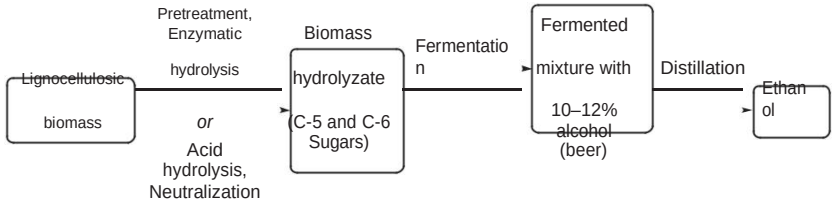


**Figure 1.1** Separate hydrolysis and fermentation (SHF).

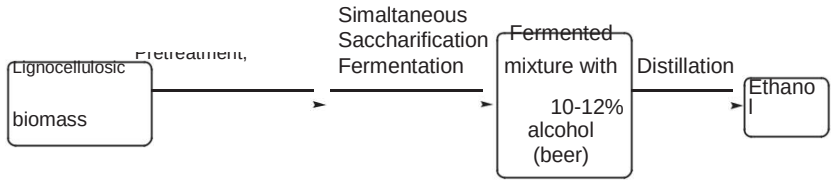


**Figure 1.2** Simultaneous saccharification and fermentation (SSF).

2. Simultaneous Saccharification and Fermentation (SSF)
   In this alternative route, pretreated biomass is subjected to hydrolysis and fermentation in a single unit as shown in the scheme in Figure 1.2. This method can be carried out in two different ways: (1) using a mixture of biomass saccharification microorganisms and fermentation microorganisms, or,
   (2)using genetically engineered microorganisms that can do both saccharification and fermentation.

## 1.1 Detoxification of Lignocellulosic Hydrolyzate

Detoxification of lignocellulosic hydrolyzate is an integral part of the cellulosic ethanol process, because during pretreatment and hydrolysis steps, a considerable amount of compounds that can inhibit the subsequent fermentation are formed in addition to the fermentable

sugars. The detoxification step is applied to remove or reduce the concentrations of these undesirable compounds to tolerable levels before biological processes. In the separate hydrolysis and fermentation (SHF) method, detoxification can be applied before exposure to cellulase enzymes, or in the case of direct acid hydrolysis type saccharification, detoxification is applied before the fermentation step. In the simultaneous saccharification and fermentation (SSF) technique detoxification is required before exposure to an enzyme cocktail affecting hydrolysis and fermentation. These inhibitory substances are produced in four different ways in pretreatments or exposure to acids. As a result of hydrolysis of the extractive components, organic or sugar acids, saponification of esters to acids like acetic, formic, glucuronic, galacturonic acids, and solubilization of phenolic derivatives.

1. Degradation of soluble sugars to furfural, 5-hydroxy-methylfurfural (HMF) and further degradation of these substances to levulinic acid, formic and acetic acid.

2. Degradation of lignin to cinnamaldehyde, *p*-hydroxy-benzaldehyde, syringaldehyde, and related compounds.

3. Solubilization of metal ions from biomass.

Depending on the type of pretreatment and hydrolysis process employed, concentrations of these inhibitory substances can be varied in a wider range. Consequently, a variety of detoxification methods have been developed depending on the downstream requirements. As pointed out by Palmqvist and Hahn-Hägerdal, these methods cannot be directly compared because they vary in the

neutralization degree of the inhibitors. In addition, the fer-menting microorganisms have different tolerances to the inhibitors. The main features of the detoxification methods employed for lignocellulosic ethanol production and selected examples are summarized in Table 1. 1.

Detoxification methods can be divided into three groups: physical, chemical and biochemical methods, as shown in Table 1.1. In the model processes developed at National *Renewable Energy* Laboratory (NREL), ionic exchange and adding excess of $Ca(OH)_2$ or over-liming have been proposed as detoxification methods. The calcium hydroxide method is especially useful in the case of dilute-acid hydrolyzates, where furan aldehydes and phenolic compounds are formed and can be efficiently removed leading to great improvement in fermentability. Detoxification with calcium hydroxide (over-liming) has shown better results than treatment with sodium or potassium hydroxide, but this difference has not been understood. Martinez and coworkers have performed experimental optimization of the amount of lime needed in the detoxification, which depends on the content of acids in each hydrolyzate. In this study they developed a method for predicting the optimal addition dosage based on the titration of hydrolyzate with standardized NaOH. Persson *et al*. indicate that the positive effects of alkali

**Table 1.1** Common detoxification methods used on streams resulting pretreatment and hydrolysis of lignocellulosic biomass during bioethanol production.

| Methods | Procedure/ Remarks |
|---|---|
| **Physical methods:** | |
| Evaporation | Reduction of acetic acid and phenolic compounds in non-volatile fraction; roto-evaporation |
| Extraction | Solvent: diethyl ether<br>Removal of acetic, formic and levulinic acids, furfural, HMF |
| Adsorption | Activated carbon, Amberlite hydrophobic polymeric adsorbent<br>Reduction of furfural conc. from 1–5 to <0.01 g/L |
| **Chemical methods:** | |
| Neutralization | $Ca(OH)_2$ or CaO, pH = 6, then membrane filtration or adsorption |
| Alkaline detoxification (over liming) | $Ca(OH)_2$, pH = 9–10.5, then pH adjustment to 5.5–6.5 with $H_2SO_4$ or HCl |
| Ionic exchange | Weak base resins, Amberlyst A20, regenerated with ammonia<br>Poly(4-vinyl pyridine), Removal: >80% phenolic compounds, 100%, levulinic, acetic and formic acids, 70% furfural; considerable lost of fermentable sugars |

| **Biological methods:** | |
|---|---|
| Enzymatic detoxification | Laccase (phenol oxidase) and lignin peroxidase from *Trametes versicolor*, laccase selectively removes phenolic, low molecular weight compounds and phenolic acids |
| Microbial detoxification | Laccase (phenol oxidase) and lignin peroxidase from *Trametes versicolor*<br>*Pseudomonas putida* and *Streptomyces setonii* cells(biofilm reactor: PCS tubes attached to CSTR acetic acid, furfural and benzoic acid derivatives<br>Aerobic bacteria oxidize aromatic compounds |

treatment cannot be completely explained by the removal of inhibitors, and this method could have possible stimulatory effects on fermenting microorganisms as well.

In addition to the single-step methods, there are diverse and multistep detoxification methods which include: neutralization with lime followed by the addition of activated carbon, and filtration for acetic acid removal; partial removal of acetic acid, furfural and soluble lignin by molecular sieves; vapor stripping for removal of volatile inhibitors, and; adsorption using activated carbon, addition of diatomite, bentonite and zeolite after neutralization or over liming. Biological detoxification is an attractive alternative to physical and chemical methods. Khiyami *et al*. have proposed a

biofilm method, which uses a mixed culture of aerobic bacteria cells naturally immobilized on a plastic support. In this way, the biofilm-associated cells are more resistant to the toxic substances released during the biomass pretreatment. The use of extraction with supercritical fluids has also been tested as a detoxification method for wood hydrolyzates.

Most of the studies on the effects of toxic compounds on growth and ethanol production have been performed for common Baker's yeast *Saccharomyces cerevisiae* and xylose-fermenting yeast. Palmqvist *et al*. carried out extensive experiments for assessing the effect of acetic acid, furfural and *p*-hydroxybenzoic acid on growth and ethanol productivity of *S. cerevisiae* and *C. shehatae* through full factorial design. Oliva *et al*. performed a study of the effect of compounds released during the pretreatment of poplar biomass by steam explosion for the thermotolerant yeast *Kluyveromyces marxianus*, showing that growth is more affected than ethanol production, and this microorganism exhibits an important resistance to aromatic compounds. Additionally, Zaldivar *et al*. have investigated recombinant microorganisms regarding their tolerance capacity for fermenting lignocellulosic hydrolyzates containing common inhibitors.

## 1.2. Separate Hydrolysis and Fermentation (SHF)

In the separate hydrolysis and fermentation method aqueous sugar solution from the enzymatic or acid hydrolysis is taken for the fermentation. The main advantages of this method are that each step can be processed at its optimal operating conditions, and separate steps minimize interaction between the steps. The disadvantages of the SHF method are that the end

product inhibition minimizes the yield of ethanol, and there is a chance of contamination due to the long period processes.

The supernatant from enzymatic hydrolysis of lignocelluloses can contain both 6-carbon (hexoses) and 5-carbon (pentoses) sugars when both cellulose and hemicellulose are hydrolyzed. Depending on the lignocellulose source, the hydrolyzate typically consists of glucose, xylose, arabinose, galactose, mannose, fucose, and rham-nose. According to theoretical calculations one ton of 6-carbon polysaccharides, glucan, galactan, or mannan yields 1.11 tons of six-carbon sugars and could be fermented into 172.0 gallons of bioethanol, and fermentation of the representative 6-carbon sugars like glucose can be represented by the equation:

$$C_6H_{12}O_6 \rightarrow 2\ CH_3CH_2OH + 2\ CO_2 \qquad (1.1)$$

Similarly, one ton of 5-carbon polysaccharides, xylan or arabinan, yields 1.14 tons of 5-carbon sugars and could be fermented theoretically into 176.0 gallons of bioethanol. Fermentation of 5-carbon sugars like xylose can be represented by the equation:

$$3\ C_5H_{10}O_5 \rightarrow 5\ CH_3CH_2OH + 5\ CO_2 \qquad (1.2)$$

## 1.3. Microorganisms Used in the Fermentation

Biochemical conversion of sugars to ethanol or fermentation can be carried out by fungi or bacterial microorganisms.

**Table 1.2** Recombinant yeast strains capable of fermenting C-5 xylose sugars and co- fermenting mixtures of C-5, C-6 sugars and their references.

| Strain | Sugar/ sugar* mix (g/L) | Ethanol Production (g/L) | Ethanol Yield (g/g) |
|---|---|---|---|
| *S. cerevisiae* 1400 | G:X:A:Gal 31:15:10:2 | 22 | 90 |
| *S. cerevisiae* BH42 | G:X 50:50 | 28 | 56 |
| *S. cerevisiae* 1400 | 80 X | 27 | 66 |
| *S. cerevisiae* ZU-10 | 80 X | 30.2 | 75.6 |

* A – arabinose; G – glucose; Gal – galactose; X – xylose

The use of that cytosolic NADH kinase appeared to revert these effects during anaerobic metabolism of xylose by channeling carbon flow from ethanol to xylitol. Heterologous expression of a *xylose isomerase* (XI) can also be another approach to enable *S. cerevisiae* cells to metabolize xylose. In pursuing this approach, Brat and coworkers screened nucleic acid databases for sequences encoding putative *xylose isomerases* and cloned them to express a highly active *xylose isomerase* from the anaerobic bacterium *Clostridium phytofermentans* in *S. cerevisiae*, which resulted in an efficient metabolism of xylose as the only carbon and energy source by recombinant yeast cells [40].

In a real biomass application example, Zho and Xia utilized genetically modified yeast to ferment corn stover hydrolyzates [41]. In this study ethanol production from corn stover hemicellulosic hydrolyzate was investigated using immobilized recombinant *Saccharomyces cerevisiae* yeast cells. Detoxification of hemicellulosic hydrolyzate by roto-evaporation and lime neutralization was carried out to remove volatile fermentation

inhibitors. All furfural and more than 50% of the acetic acid in the hydrolyzate were removed, meanwhile the xylose concentration was enhanced to 71.8 g/L. The fermentability of the detoxified hydrolyzate was significantly improved using Ca-alginate immobilized cells of recombinant *S. cerevisiae* (ZU-10). An ethanol concentration of 31.1 g/L and the corresponding ethanol yield on fermentable sugars of 0.406 g/g were obtained within 72 h in batch fermentation of the detoxified hydrolyzate with immobilized cells; the concentration of ethanol in each batch maintained above 30.1 g/L with the ethanol yield on fermentable sugars over 0.393 g/g. With these experiments Zho and Xia demonstrated the viability of ethanol production from corn stover hydrolyzate using C-5 and C-6 co-fermenting recombinant *S. cerevisiae*, and the effect of immobilization of this yeast [36.

The time course of ethanol production from detoxified corn stover hemicellulose hydrolyzate by recombinant *S. cerevisiae* ZU-10 using free and immobilized cells is shown in Figure 1.3. In addition, repeated batch fermentation of immobilized recombinant *S.cerevisia* cells was attempted for ethanol production for five batches, demonstrating the reusability of the immobilized S. cerevisiae ZU-10. The results of the reusability experiment are shown in Figure 1.4.

In another example Carlos Martín *et al*. used recombinant xylose-utilizing *Saccharomyces cerevisiae* for ethanol production from enzymatic hydrolyzates of sugarcane bagasse. In their experiments, sugarcane bagasse was first pretreated by steam explosion at 205 and 215°C and hydrolyzed with cellulolytic enzymes.

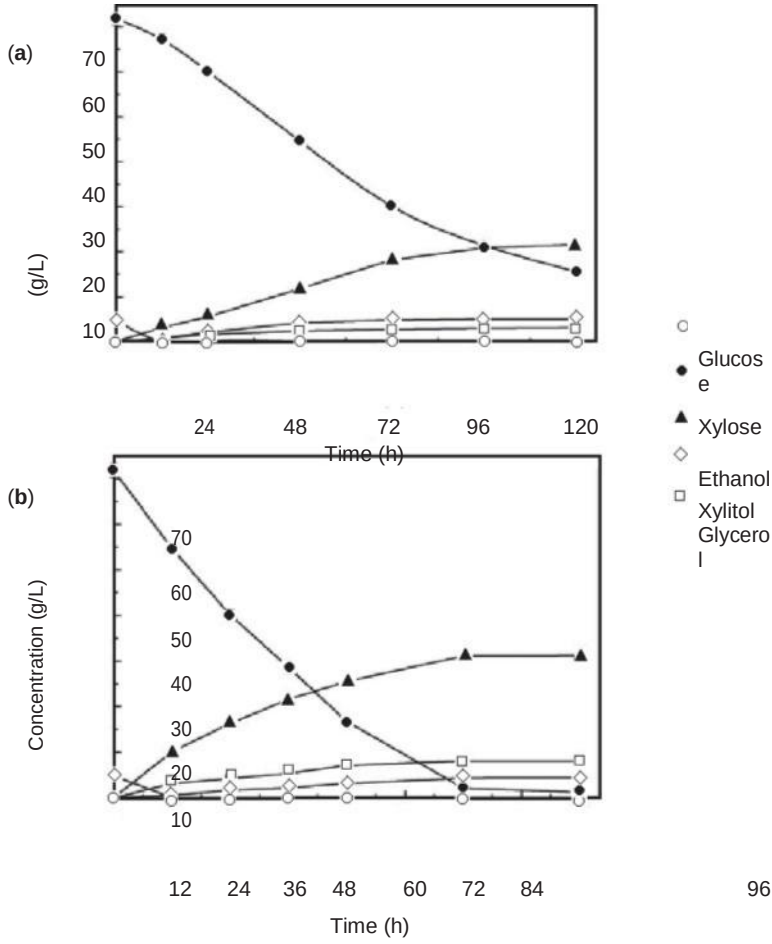


**Figure 1.3** Time course of ethanol production from detoxified corn stover hemicellulose hydrolyzate by recombinant *S. cerevisiae* ZU-10 (a) free cells; (b) immobilized cells.

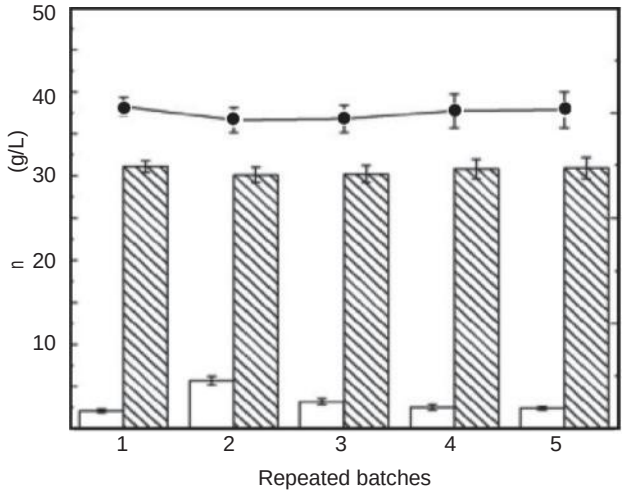


Figure 1.4 Repeated batches fermentation of hemicellulose hydrolyzate by immobilized recombinant S. cerevisiae ZU-10 cells: residual xylose (white); ethanol (dashed); ethanol yield (•). Error bars represent the standard deviation.

The hydrolyzates were then subjected to enzymatic detoxification by treatment with the phenoloxidase laccase and to chemical detoxification by over-liming. Approximately 80% of the phenolic compounds were specifically removed by the laccase treatment. Over-liming partially removed the phenolic compounds, but also other fermentation inhibitors such as acetic acid, furfural and 5-hydroxymethylfurfural. The resultant hydrolyzates were fermented with the recombi-nant xylose-utilizing *Saccharomyces cerevisiae* laboratory strain TMB 3001, a CEN.PK derivative with overexpressed xylulokinase activ-ity and expressing the *xylose reductase* and *xylitol dehydrogenase* of *Pichia stipitis*, and the *S. cerevisiae* strain ATCC 96581, isolated from spent sulphite liquor from a fermentation plant. They reported that the fermentative performance of the lab strain in undetoxi-fied hydrolyzate was better than the performance of the industrial strain. An almost two-fold increase of the specific productivity of the strain TMB 3001 in the detoxified hydrolyzates compared to the undetoxified hydrolyzates was observed. The ethanol yield in the fermentation of the hydrolyzate detoxified by over-liming was 0.18 g/g dry bagasse, whereas it reached only 0.13 g/g dry bagasse in the undetoxified hydrolyzate. Furthermore, a partial xylose utiliza-tion with low xylitol formation was observed with this recombi-nant yeast strain *Saccharomyces cerevisiae* TMB 300.

## 1.4.Fermentation Using Bacteria

Bacteria such as *Zymomonas mobilis*, *Escherichia coli* and *Klebsiella oxytoca* have attracted particular interest in ethanol fermentation given their rapid fermentation

which can be minutes compared to hours or days for yeasts. *Zymomonas mobilis* is a Gramnegative bacterium belonging to the genus *Zymomonas* and is notable for its bioethanol production capabilities, which surpass yeast in some aspects. *Z. mobilis* is well recognized for its ability to efficiently produce ethanol at high rates from glucose, fructose, and sucrose. It was originally isolated from alcoholic beverages like African palm wine and Mexican pulque, and was also a contaminant of cider and beer in European countries.

*Zymomonas mobilis* degrades sugars to pyruvate using the Entner-Doudoroff pathway. The Entner–Doudoroff pathway describes an alternate series of reactions that catabolize glucose to pyruvate using a set of enzymes different from those used in either glycoly-sis or the pentose phosphate pathway. The distinct features of this pathway are that it has a net yield of one ATP for every glucose molecule processed, as well as one NADH and one NADPH. By comparison, glycolysis has a net yield of two ATP and two NADH for every one glucose molecule processed. The pyruvate is then fermented to produce ethanol and carbon dioxide as the only products, similar to yeast.

The advantages of *Zymomonas mobilis* over *Saccharomyces cerevisiae* with respect to producing ethanol from lignocellulosic biomass can be summarized as follows:

1. Higher sugar uptake and higher ethanol yield
2. Higher ethanol tolerance up to 16% (v/v)
3. Does not require controlled addition of oxygen during the fermentation

Comparative performance trials on glucose have shown that *Zymomonas mobilis* can achieve 5% higher bioethanol yields and up to five-fold higher bioethanol volumetric productivity compared to wild type *Saccharomyces cerevisiae* yeast. *Zymomonas mobilis* can efficiently produce bioethanol from the hexose sugars glucose and fructose. However, wild-type *Z. mobilis* cannot ferment C-5 sugars like xylose and arabinose, which are important components of lignocellulosic hydrolyzates. Another disadvantage is that, unlike *E. coli* and yeast, *Zymomonas mobilis* cannot tolerate toxic inhibitors present in lignocellulosic hydrolyzates such as acetic acid and various phenolic compounds. Concentration of acetic acid in lignocellulosic hydrolyzates can be as high as 1.5% (w/v), which is well above the tolerance threshold of *Zymomonas mobilis*. Therefore efficient detoxification steps are essential in using this type of bacteria in the fermentation step.

### 1.4.1. Genetic Modifications of Bacteria

Metabolic engineering has allowed the development of recombinant microorganisms that will ferment glucose and xylose. Some of the most widely considered recombinant microorganisms as can-didates for industrial application include ethanologenic *Escherichia coli* xylose-fermenting Z. *mobilis*. The National *Renewable Energy* Laboratory (NREL), USA, has made significant contributions in recent years to engineer *Z. mobilis* to overcome its inherent defi-ciencies by expanding its substrate range to include C-5 sugars like xylose and arabinose. In one approach from NREL, two oper-ons encoding xylose assimilation and pentose

phosphate path-way enzymes were constructed and transformed into *Zymomonas mobilis* in order to generate a strain that grew on xylose, and efficiently fermented it to ethanol. Thus, anaerobic fermentation of a pentose sugar to ethanol was achieved through a combination of the pentose phosphate and Entner-Doudoroff pathways. Furthermore, this strain efficiently fermented both glucose and xylose, which is essential for economical conversion of lignocellulosic biomass to ethanol. The same group from NREL later developed an arabinose-fermenting *Zymomonas mobilis* strain by metabolic pathway engineering as well [48]. A number of research groups around the world have successfully engineered C-5, C-6 co-fermenting bacteria suitable for industrial applications. Some selected examples from these successes and their references are shown in Table 1.3.

**Table 1.3** Recombinant bacteria strains capable of fermenting C-5 xylose sugars and co-fermenting mixtures of C-5, C-6 sugars and their references.

| Strain | Sugar/sugar* mix (g/L) | Ethanol Production (g/L) | Ethanol Yield (g/L) |
|---|---|---|---|
| *Z. mobilis* ATCC 39767 | G:X:A 30:30:20 | 33.5 | 82–84 |
| *Z. mobilis* AX101 | A:G:X 20:40:40 | 42 | 84 |
| *Z. mobilis* ZM4 | G:X 65:65 | 62 | 90 |
| *Z. mobilis* CP4 | G:X 65:65 | 24.2 | 95 |
| *E. coli* FBR5 | A:X:G 15:30:30 | 34 | 90 |

| | | | |
|---|---|---|---|
| *E. coli* FBR5 | 95 X | 41.5 | 90 |

* A – arabinose; G – glucose; X – xylose

In a more recent example, Agrawal *et al*. reported the engineering of efficient xylose metabolism capabilities into an acetic acid-tolerant *Zymomonas mobilis* strain by introducing adaptation-induced mutations [53]. They reported that chromosomal mutation at the xylose reductase gene was critical to xylose metabolism by reducing xylitol formation, together with the plasmid-borne mutation impacting xylose isomerase activity, and these two mutations accounted for 80% of the improvement achieved by adaptation.

In an attempt to produce new xylose fermenting strain in the presence of high acetic acid concentrations, they transferred the two mutations to an acetic acid-tolerant strain. The resulting strain fermented glucose + xylose (each at 5% w/v) with 1% (w/v) acetic acid at pH 5.8 to completion with an ethanol yield of 93.4%, outper-forming other reported strains [53]. Introduction of xylose metabo-lizing pathways from *E. coli* is an another example. Modified *Z. mobilis* has the advantages of requiring a minimum of nutrients, growing at low pH and high temperatures, and it is considered

**Table 1.4** Comparison of ethanol production using genetically engineered *Z. mobilis* by introducing xylose metabolizing pathways from *E. coli* and *E. coli*.

| | **Genetically engineered *Z. mobilis* by introducing xylose metabolizing pathways from *E. coli*** | **E. coli** |
|---|---|---|
| Ethanol (g $L^{-1}$) | 62 | 27 |
| Ethanol yield[a] (%) | 97 | 90 |
| Ethanol productivity | 1.29 | 0.92 |

| | | |
|---|---|---|
| ($g\ L^{-1}\ h^{-1}$) | | |

[a] Estimation from the theoretical yields.

generally recognized as safe (GRAS). A comparison between genetically engineered *Z. mobilis* by introducing xylose metabolizing pathways from *E. coli* and *E. coli* is shown in Table 1.4.

A genetic modification of wild-type *E. coli* to improve its fermentation profile is another approach. Saha and Cotta have reviewed the recent developments in recombinant *E. coli* strains in the fer-mentation of biomass hydrolyzate. Researchers at Bioenergy Research Unit, National Center for Agricultural Utilization Research, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Illinois, USA, have developed two recombinant *E. coli* strains (FBR4 and FBR5) that have been used for fermentation of corn fiber hydrolyzates. These strains carry the plasmid pLOI297, which contains the genes from *Zymomonas mobilis* neces-sary for efficiently converting pyruvate into ethanol. Both strains selectively maintained the plasmid when grown anaerobically. Each culture was serially transferred 10 times in anaerobic culture with sugar-limited medium containing xylose, but no selective antibiotic. An average of 93–95% of the FBR4 and FBR5 cells maintained pLOI297 in anaerobic culture. The fermentation performances of the repeatedly transferred cultures were compared with those of cultures freshly revived from stock in pH-controlled batch fermentations with 10% (w/v) xylose. Fermentation results were similar for all the cultures. Fermentations were completed within 60 h and ethanol yields were 86–92% of theoretical. Maximal ethanol concentrations were 3.9–4.2% (w/v). In order to test the applica-bility in real biomass situations, Dien and

coworkers tested these strains for their ability to ferment corn fiber hydrolyzate, which contained 8.5% (w/v) total sugars (2.0% arabinose, 2.8% glucose, and 3.7% xylose). *E. coli* FBR5 produced more ethanol than *E. coli* FBR4 from the corn fiber hydrolyzate. *E. coli* FBR5 fermented all but 0.4% (w/v) of the available sugar, whereas strain FBR4 left 1.6% unconsumed. The fermentation with FBR5 was completed within 55 h and yielded 0.46 g of ethanol/g of available sugar, which corresponds to 90% of the maximum obtainable.

Saha *et al*. from the same research laboratory also reported the use of these two recombinant *E. coli* strains (FBR 4 and 5), and in this case for the fermentation of wheat straw. In these experiments ethanol production by separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) have been compared using recombinant bacterium *E. coli* FBR 5, where high solid loading of wheat straw was used. The yield of total sugars from dilute acid pretreated wheat straw after enzymatic saccharification was 86.3 g/L. Then pretreated wheat straw was bioabated by growing a fungal strain aerobically in the liquid portion for 16 h. Ethanol yields and productivity under SHF and SSF conditions using recombinant bacterium *E. coli* FBR 5 on wheat straw are shown in Table 1.5.

A number of research groups have demonstrated that recombi-nant *E. coli* has the ability to ferment a wide spectrum of sugars, without the requirements for complex growth factors. However, the major disadvantages associated with using *E. coli* cultures are a narrow and neutral pH growth range (6.0–8.0), less hardy cultures compared to yeast, and public perceptions regarding the danger of *E. coli* strains. The lack of data

on the use of residual *E. coli* cell mass as an ingredient in animal feed is also an obstacle to its application.

A variety of ethanol-producing thermophilic microorganisms have been isolated and characterized due to their ability to degrade a broad variety of both hexoses and pentoses. These bacteria include *Thermoanaerobacter ethanolicus* [, *Thermoanaerobacter math-ranii*, *Clostridium thermohydrosulfuricum*, *Thermoanaerobium brockii*, *Clostridium thermosaccharolyticum*, etc. These types of thermophilic anaerobic bacteria have a distinct advantage over conventional yeasts for bioethanol production in their ability to use a variety of inexpensive biomass feedstocks and their ability to withstand temperature extremes. Nevertheless, the low bioethanol tolerance of thermophilic anaerobic bacteria (< 2%, v/v) is a major obstacle for their industrial exploitation for bioethanol production. *Thermoanaerobacterium saccharolyticum* is one of the thermophilic anaerobic bacteria which is able to directly ferment hemicellulosic oligomers as well as primary sugars found in cel-lulosic biomass, including cellobiose, glucose, xylose, mannose, galactose, and arabinose. The ability to ferment the full spectrum of sugars available in hydrolyzates promises to further enhance the overall fermentation of mixed solutions of hexoses and pentoses to ethanol, therefore this group of bacterium can be identified as a hopeful branch in fermentation microbiology.

**Table 1.5** Summary of fermentation activity of recombinant *Escherichia coli* FBR 5 from dilute acid pretreated wheat straw hydrolyzate at 35°C.

| Fermentation type | Fermentation time (h) | Total sugars (g/L) | EtOH produced (g/L) | EtOH productivity (g/L/h) | EtOH yield (g/g straw) |
|---|---|---|---|---|---|
| **SHF** | | | | | |
| Non-abated (pH 7.0) | 168 | 86.6 ± 0.3 | 41.1 ± 1.1 | 0.24 | 0.27 |
| Bioabated (pH 6.5) | 120 | 86.6 ± 0.3 | 41.8 ± 0.0 | 0.35 | 0.28 |
| **SSF** | | | | | |
| Non-abated (pH 7.0) | - | – | – | – | – |
| Bioabated (pH 6.0) | 104 | _ | 41.6 ± 0.7 | 0.40 | 0.28 |

The dilute acid (0.75% $H_2SO_4$, v/v) pretreatment of wheat straw (150 g/L) was performed at 160°C for 10 min. Enzymatic saccharification was carried out at pH 5.0 at 45°C for 72 h with a cocktail of three commercial enzyme (cellulase, *b*-glucosidase, and hemicellulase) preparations. Fed-batch SSF was performed by adding the substrate 4 times (0, 16, 21, and 24 h) in 4 equal portions. SHF – separate hydrolysis and fermentation; SSF – simultaneous saccharification and fermentation.

## 1.5. Simultaneous Saccharification and Fermentation (SSF)

In simultaneous saccharification and fermentation (SSF) pretreated biomass is subjected to hydrolysis and fermentation in a single unit. This method can be carried out in two different ways:

1. Using a mixture of biomass saccharification microor-ganisms and fermentation microorganisms.
2. Using genetically engineered microorganisms that can do both saccharification and fermentation.

Simultaneous saccharification and fermentation generally gives higher ethanol yields due to the removal of end product inhibition of the saccharification step, and another advantage is that the SSF method requires only one reactor for both steps. The disadvantage of the SSF method is that a difference in optimum temperature conditions of enzyme for hydrolysis and fermentation conditions can affect the yield when a mixture of biomass saccharification microorganisms and fermentation microorganisms are used. On the other hand, genetically engineered microorganisms that can do both sac-charification and fermentation are still a technology in the developing stages.

### 1.5.1. SSF Using a Mixture of Saccharification and Fermentation Microorganisms

In this type of simultaneous saccharification and fermentation (SSF) technique pretreated biomass is exposed to a cocktail of enzymes that can convert

cellulosic material to ethanol in one reactor. Cellulasesand xylanases in the cocktail first convert the carbohydrate poly-mers to fermentable sugars, and these enzymes are notoriously sus-ceptible to feedback inhibition by the products—glucose, xylose, cellobiose and other oligosaccharides. Therefore, this process has an enhanced rate of hydrolysis because sugars are concurrently removed by the fermentation step by yeast or bacteria in the solution. This SSF technique requires lower enzyme loading, results in higher bioethanol yields, and reduces the risk of contamination. Compatibility of enzymes and fermentation conditions is the major issue in this SSF technique, and it is essential to match enzymes and microorganisms that can operate under similar pH, temperature and substrate concentrations. In many cases, the low pH, e.g., lower than 5, and high temperature, e.g., > 40°C, may be favorable for enzymatic hydrolysis, whereas the low pH can surely inhabit the lactic acid production and the high temperature may adversely affect the fungal cell growth. For example, *Trichoderma reesei* cellulases, which constitute the most active preparations, have optimal activity at pH 4.5 and 55°C. On the other hand, *Saccharomyces* cultures are typically operated at pH 4.5 and 37°C.

Various forms of biomass such as corn stover, wheat straw, rice straw, barley straw, oat straw, switchgrass, sugarcane bagasse, sorghum bagasse, cogon grass , napier grass, guinea grass, Paja Brava straw, lespedeza stalks, eucalyptus wood, pine wood, aspen wood, algae, seaweed, paper sludge, and waste paper have been tested for bio-ethanol production using this type of

simultaneous saccharification and fermentation (SSF) technique. A selected sample of SSF on various biomass forms, saccharification enzymes used, operating conditions and ethanol yields are shown in Table 1.6.[2].

In conclusion, there are advantages and disadvantages in the simultaneous saccharification and fermentation (SSF) using a mix-ture of biomass saccharification microorganisms which are outlined below.

The main advantages of SSF are:

1. Significantly reduces the enzyme inhibition by con-version of sugars that inhibit the cellulase activity compared to SHF, because immediate consumption of sugars by the microorganism produces low sugar concentrations in the fermenter, which results in increased saccharification rates.
2. Lower enzyme requirement.
3. Higher product yields.
4. Lower requirements for sterile conditions since glucose is removed immediately and bioethanol is produced.
5. Shorter process time compared to SHF.

**Table 1.6** Selected examples of simultaneous saccharification and fermentation (SSF) using a mixture of biomass saccharification microorganisms and fermentation microorganisms.

| Biomass | Pretreatment | Saccharification | Fermentation | Ethanol yield |
|---|---|---|---|---|
| Sweet sorghum (Sugar Drip Variety) | 0.5% $H_2SO_4$ solution, 180 °C for 5 min. | Novozymes North America (Franklinton, NC, USA), which included cellulase (NS50013), *b*-glucosidase (NS50010) and hemicellulase (NS22002) | *Saccharomyces cerevisiae* (ATCC 24858) | 89.4% yield, 38 g/L concentration and 1.28 g/L/h production rate |
| Sweet sorghum (*Sorghum bicolor* [L.] Moench) | liquid hot water (170–200 °C) | Celluclast 1.5 L in combination with Novozymes 188 | active dry yeast (ADY) Thermosacc® (Birkerød, Denmark) 2 g ADY/L | 53 g/L ethanol was achieved after 168 h |
| Lodgepole wood chips | dil. sulfuric acid, sodium bisulfite solutions, 180°C | cellulase cocktail, Cellic CTec 2, by Novozymes North America (Franklinton, NC). | *Saccharomyces cerevisiae* YRH400 | 47.4 g/L , calculated yield of 285 L/tonne of wood |

| Biomass | Pretreatment | Saccharification | Fermentation | Ethanol yield |
|---|---|---|---|---|
| Micro algae *Saccharina japonica* | 0.06% (w/w) sulfuric acid at 170 °C for 15 min. | Celluclast 1.5 L and Novozymes 188 (Novozymes A/S Bagsvaerd, Denmark) | Thermotolerant *S. cerevisiae* DK 410362 | 13.01 g/L, 67.41% based total available glucan |
| Corn stover | dilute acid-pretreated corn stover | cellulase cocktail | thermotolerant strain *Saccharomyces cerevisiae* DQ1 | ethanol titer of 48 g/L and yield of 65. 6 % |
| Rice straw | calcium hydroxide according to the alkali treatment method in CaCCO (calcium capturing by carbonation) process | Celluclast 1.5 L (Novozymes Japan Co. Ltd., Chiba, Japan), Novozymes 188 (Novozymes Japan Co. Ltd.) and UltrafloL (Novozymes Japan Co. Ltd.). | *Saccharomyces cerevisiae* cells were immobilized by entrapping in photocrosslinkable resin beads | ethanol production of approx. 38 g/L and an ethanol yield of 84.7% were obtained |
| Napier grass | 1% aqueous solution of NaOH at 95°C for 1 h. | cellulase from filamentous fungus *Acremonium cellulolyticum* (Acremozyme; Kyowa Kasei, Osaka, Japan) | *S. cerevisiae* NBRC 2044 and E. coli KO11 | 44.2% of the theoretical yield based on hexose (37.5 g) and pentose (26.5 g) derived from 100g napier grass |

**Table 1.6** (*Cont.*)

| Biomass | Pretreatment | Saccharification | Fermentation | Ethanol yield |
|---|---|---|---|---|
| Paper sludge | No pretreatment | A. cellulolyticus C-1 (Ferm P-18508 Tsukishima Kikai (Tokyo, Japan), | thermotolerant and high ethanol producing strain, *Saccharomyces cerevisiae* TJ14 | 11.34 kg/$m^3$ from 50 kg/$m^3$ untreated paper sludge |
| Kinnow mandarin (*Citrus reticulata*) peels | hydrothermally pretreated | strain of *Aspergillus oryzae* | thermotolerant strain of *Pichia kudriavzevii* | Ethanol production productivity of 33.87 g/L and 2.82 g/Lh, respectively, after 12 h. |
| Rice straw | 1% sodium hydroxide | 20 FPU $gds^{-1}$ cellulase, 50 IU gds-1 *b*-glucosidase, 15 IU $gds^{-1}$ pectinase | thermotolerant *Pichia kudriavzevii* HOP-1 strain | 24.25 g/L corresponding to 82% theoretical yield on glucan basis and ethanol productivity of 1.10 g/Lh |
| Corn stover | Ammonia Fiber Expansion (AFEX$^{TM}$) | Spezyme CP (Genencor Inc., USA) 22.4 mg protein/g glucan, Novozymes 188 (Sigma–Aldrich, USA) | xylose-fermenting strain *S. cerevisiae* 424A(LNH-ST) | 193.2 g ethanol per kg AFEX$^{TM}$- corn stover |

| Biomass | Pretreatment | Saccharification | Fermentation | Ethanol yield |
|---|---|---|---|---|
| Eucalyptus globulus Wood | 8 g of water/g of oven-dry wood, 210–230 °C | "Celluclast 1.5L" cellulases (from *Trichoderma reesei*) and NS50010 *b*-glucosidase (from *Aspergillus niger*), Novozymes (Madrid, Spain). | *Saccharomyces cerevisiae* CECT-1170, Spanish Collection of Type Cultures (Valencia, Spain | 67.4 g ethanol /L and 291 L ethanol /1000 kg oven-dry wood |
| Sugarcane bagasse | steam explosion at 200 °C for 7 min. | *Trichoderma reesei* cellulases (Celluclast 1.5L: 42.40 FPU/mL and 21.10 CBU/mL) and a *b*-glycosidase (1340 CBU/ mL) preparation (Novozym 188), both from Novozymes A/S (Bagsværd, Denmark) | Industrial strain *S. cerevisiae* UFPEDA 1238 | 150 L ethanol/t bagasse |
| Switchgrass (*Panicum virgatum*) | Hot water, 200 °C, 10 min. | cellulase enzyme (Fibrilase, Iogen, Ottawa, Canada) at a loading of 15 FPU $g^{-1}$ glucan | thermotolerant yeast strain *Kluyveromyces marxianus* IMB3 at 8% solid loading | 22.5 g/L after 168 h equivalent to 86% yield. |

**Table 1.6** (*Cont.*)

| Biomass | Pretreatment | Saccharification | Fermentation | Ethanol yield |
|---|---|---|---|---|
| Office paper, newspaper, handbills and cardboard | stream explosion | cellulase from *Aspergillus niger* and *Trichoderma viride* at the fixed loading rate of 20 FPU g -1 | *Saccharomyces cerevisiae* | 21.02 g/L after 36 h., production rate of 0.58 g ethanol /L h |
| Native aspen (*Populus tremuloides)* | sulfuric acid and sodium bisulfite. 170 °C for approximately 20–30 min. | Celluclast 1.5 L, Novozymes 188 (*b*-glucosidase), and Fibercare® were generously provided by Novozymes North America (Franklinton, NC) | *Saccharomyces cerevisiae* Y5 (Strain preserved No. CGMCC2660, China General Microbiological Culture Collection Center) Capital Normal University of Beijing, China | 211 L/ton wood at 59 g/L with SSF efficiency of 76% |
| Cogon grass | 10% (wt) NaOH at room temperature for 24 h. | Cellulase accellerase 1500 yeast | Ethanol red dry yeast | 76.2% yield |

The main disadvantages of SSF are:

1. Different temperature optima for saccharification and fermentation can make it difficult to optimize the process.

2. A typical fermentation will take 5–7 days; the long res-idence time may make contamination control difficult in a continuous process, but may be manageable in a batch process.

### 1.5.2. SSF Using Microorganisms that Can Do both Saccharification and Fermentation or Consolidated Bio-Processing (CBP)

A more recent development in simultaneous saccharification and fermentation (SSF) technique uses microorganisms that can do both saccharification and fermentation, thereby directly processing pretreated biomass to ethanol. This approach commonly involves four biochemical transformations:

1. Production of saccharolytic enzymes (cellulases and hemicellulases)
2. Hydrolysis of cellulose and hemicellulase present in pretreated biomass to sugars
3. Fermentation of hexose sugars (glucose, mannose and galactose)
4. Fermentation of pentose sugars (xylose and arabinose)

Since these four transformations occur in a single reactor and in one process configuration, this technique is known as consolidated bioprocessing (CBP). In recent years CBP has gained recognition as a promising

bioethanol production system since the costs of capital investment, substance and other raw materials, and utilities associated with the production of cellulase enzyme can be avoided or reduced as these enzymes are generated by the same microorganism. However, one of the major drawbacks in the SSF process and CBP is the optimum temperature required for the saccharification and fermentation stages. The optimum temperature for sacchari-fication with cellulolytic enzymes is around 50°C, while most fermenting microbes have a most favorable temperature for ethanol fermentation between 28°C and 37°C. In practice as well as following the current state of technology, it would be difficult to lower the optimum temperature of cellulases through genetic engineering. One possible answer to this problem could be the use of thermo-tolerant yeast strains that can ferment at higher temperatures as host for genetic manipulation of introducing saccharolytic enzyme producing genes.

There are two fundamental approaches for the construction of new microorganisms for consolidated bioprocessing (CBP) type simultaneous saccharification and fermentation process, which include:

1. Heterologous expression of cellulase genes in yeast.
2. Surface engineering of yeast strains to display cellulases on cell surface.

### 1.5.3 Heterologous Expression of Cellulase Genes in Yeast *S. cerevisiae* for the Development of CBP

As we discussed earlier, common yeast *S. cerevisiae* has many advantages as a producer of lignocellulosic ethanol, such as faster sugar consumption, higher ethanol yield, and higher resistance to ethanol and fermentation inhibitors present in pretreated lignocellulosic materials. In addition to this, *S. cerevisiae* is ame-nable to genetic manipulation and is generally regarded as safe (GRAS) due to its long association with the food and beverage industries. Therefore, a number of researchers have focused their efforts in the heterologous expression of cellulase genes with yeast hosts in order to produce genetically engineered yeasts for CBP. A genetically engineered yeast cell acts as a host cell for

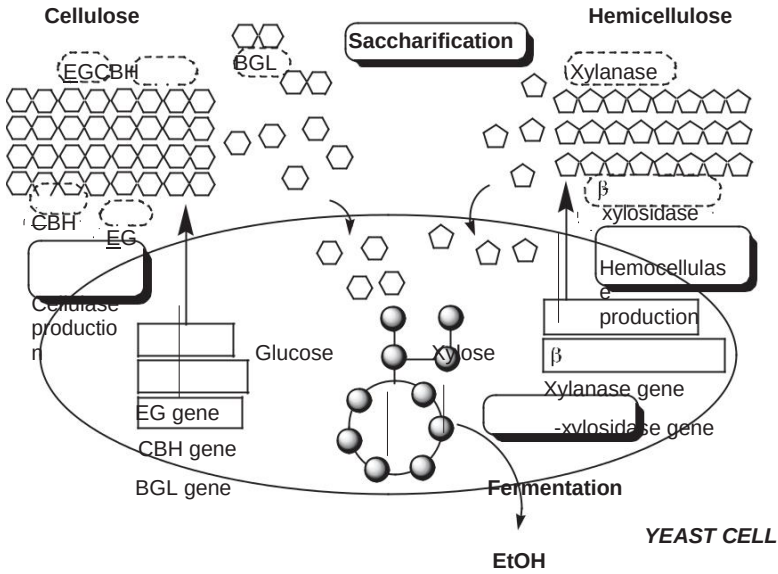


**Figure 1.5** Genetically engineered yeast cell acting as a host cell for cellulase genes with promoters which secretes the cellulases endoglucanase (EG), exoglucanases including cellobiohydrolase (CBH) and cellodextrinase, and *b*-glucosidase (BGL), and its operation in consolidated bioprocessing. (Reprinted with permission from reference [110]; copyright 2012 Elsevier).

cellulase genes with promoters which secretes the cellulases and its operation in consolidated bioprocessing is illustrated in Figure 1.5. There are several examples of expression of cellulases and hemi-cellulases by *Saccharomyces cerevisiae* in recent literature, especially after 2008, and some of the selected examples and their references are shown in Table 1.7.

Multiple enzymatic activities are required to hydrolyze cellulose into soluble sugars. These include endo-glucanase (EG), exoglucanases including cellobiohydrolase (CBH) and cellodextrinase, and *b*-glucosidase (BGL). Endoglucanase produces nicks in the cellulose polymer, exposing reducing and non-reducing ends for cellobiohydrolase, which liberates cellooligo-saccharides, cellobiose and glucose. In the last step of saccharification, *b*-glucosidase cleaves the cellooligosaccharides and cellobiose to liberate glucose. Given that cellobiose and cellooligosaccharide are potent inhibitors of cellulose hydrolysis, *b*-glucosidase action has been shown to be one of the major rate-limiting steps in the hydrolysis of cellulose. Therefore, *b*-glucosidase genes with a fun-gal origin such as BGL1 from *Saccharomycopsis fibuligera*, BGL1 from *A. aculeatus*, bglA from *Aspergillus kawachii*, bglB from *Candida wick-erhamii*, bgl from *Trichoderma reesei*, and BGL1 from *Endomyces fibu-liger*, have been heterologously expressed in *S. cerevisiae*. Cellobiose fermentation ability of industrial *Saccharomyces* strains carrying *S. fibuligera* BGL1 depends on their ability to accumulate BGL1 but also on their genetic background.

**Table 1.7** Cellulase and hemicellulase expression by *Saccharomyces cerevisiae.*

| **Organism source Cellulase and hemicellulase (gene)** | **Yeast strain** |
|---|---|
| Endoxylanase from *Trichoderma reesei*, *b*-xylosidase from *Aspergillus oryzae*, and *b*-glucosidase from *Aspergillus aculeatus* but that also assimilated xylose through the expression of xylose reductase and xylitol dehydrogenase from *Pichia stipitis* and xylulokinase from *S. cerevisiae* | *Saccharomyces cerevisiae* |
| *b*-glucosidase (BGL) and *b*-xylosidase (XYL) genes, and a gene cassette for xylose assimilation | *Saccharomyces cerevisiae* OC-2HUT |
| Cellulases and exoglucanase encoded by cel6A of *Neocallimastix patriciarum*, a *b*-glucosidase encoded by cel3A of *Saccharomycopsis fibuligera* and an endoglucanase encoded by cel7B of *Trichoderma reesei* | *Saccharomyces* cerevisiae |
| Saccharolytic enzymes (bglA, cel48A, celpin, xylA, and xylB) from a cDNA library prepared from the anaerobic fungus *Piromyces sp E2* | *Saccharomyces* cerevisiae Y294 |
| Genes encoding cellulases (endoglucanase, exoglucanase and *b*-glucosidase) | *S. cerevisiae* K1-V1116 |
| Endoglucanase E (Clostridium thermocellum) and *b*-glucosidase 1 (*Saccharomycopsis fibuligera*) | *Saccharomyces* cerevisiae |

In one of the early examples, Den Haan *et al.* demonstrated the construction of a yeast strain capable of growing on and one-step conversion of amorphous cellulose to ethanol. This report represents a significant progress towards realization of one-step processing of cellulosic biomass in a consolidated bioprocessing configuration. In 2007, Den Haan *et al*. claimed this was

the first report of a recombinant strain of *S. cerevisiae* growing on pure cellulose. In this study, they expressed two cellulase encoding genes, an endoglucanase of *Trichoderma reesei* (EGI) and the *b*-glucosidase of *Saccharomycopsis fibuligera* (BGL1) combination in *Saccharomyces cerevisiae*. The resulting strain was able to grow on phosphoric acid-swollen cellulose (PASC) through simultaneous production of sufficient extracellular endoglucanase and *b*-glucosidase activ-ity. Anaerobic growth (0.03 $h^{-1}$) up to 0.27 g $l^{-1}$ DCW was observed on medium containing 10 g $l^{-1}$ phosphoric acid-swollen cellulose as the sole carbohydrate source with concomitant ethanol production of up to 1.0 g $l^{-1}$.

In another example, Jeon and coworkers reported the direct cellulosic alcohol fermentation using recombinant *Saccharomyces cerevisiae* engineered for the production of *Clostridium cellulovo-rans* endoglucanase and *Saccharomycopsis fibuligera b*-glucosidase. In this study, *Saccharomyces cerevisiae* was engineered for simultaneous saccharification and fermentation of cellulose by the overexpression of the endoglucanase D (EngD) from *Clostridium cellulovorans* and the *b*-glucosidase (Bgl1) from *Saccharomycopsis fibuligera*. To promote secretion of the two enzymes, the genes were fused to the secretion signal of the *S. cerevisiae a* mating factor gene. The recombinant yeast developed could produce ethanol through simultaneous production of sufficient extracellular endoglucanase and *b*-glucosidase. When direct ethanol fermentation from 20 g $L^{-1}$ *b*-glucan as a substrate was performed with these recombinant strains, the ethanol concentration reached 9.15 g $L^{-1}$ after 50 h of fermentation. Furthermore, Jeon and coworkers reported the con-version ratio of ethanol from *b*-glucan as 80.3% of the theoretical ethanol concentration produced from 20 g $L^{-1}$ *b*-glucan.

Recently, genes encoding *T. reesei* endoglucanase II (EGII) and cellobiohydrolyase II (CBHII) and *A. aculeatus* BGL1 were integrated into the chromosome of a wine yeast strain with a single vector car-rying a gene responsible for resistance to the antibiotic G418. The resultant *S. cerevisiae* strain produced ethanol from pretreated corn stover cellulose without addition of exogenously-produced enzymes. When ethanol fermentation was performed with 10% dry weight of pretreated corn stover, the recombinant strain fermented 63% of the cellulose in 96 h and the ethanol titer reached 2.6% v/v.

Yamada and coworkers constructed a diploid *Saccharomyces cere-visiae* strain optimized for expression of cellulolytic enzymes, and attempted to improve the cellulose-degradation activity and enable direct ethanol production from rice straw. In this study they found that the engineered diploid strain, which contained multiple copies of three cellulase genes integrated into its genome, was pre-cultured in molasses medium (381.4 mU/g wet cell) and displayed approximately six-fold higher phosphoric acid-swollen cellulose (PASC) degradation activity than the parent haploid strain (63.5 mU/g wet cell). When used to ferment PASC, the diploid strain produced 7.6 g/l ethanol in 72 hours, with an ethanol yield that achieved 75% of the theoretical value, and also produced 7.5 g/l ethanol from pretreated rice straw in 72 hours.

### 1.5.4 Surface-Engineered Yeast Strains for the CBP

Another approach for producing cellulolytic yeast strain is displaying various types of functional proteins on

microbial cell surfaces without loss of their activity. This can be achieved by yeast cell sur-face engineering, where functional proteins are genetically fused to an anchor protein such as *a*-agglutinin, a-agglutinin and Flo1p, and expressed on the yeast cell surface. Display of *S. fibulig-era* BGL1 on the *S. cerevisiae* cell surface by fusing the mature pro-tein and *a*-agglutinin anchoring moieties enabled the recombinant strain to grow on cellobiose at almost the same rate as on glucose under anaerobic conditions. The application of surface-engineered yeast cell with endoglucanase (EG), exoglucanases including cellobiohydrolase (CBH), cellodextrinase, and *b*-glucosidase (BGL) fused on to yeast cell surface in consolidated bioprocessing is shown in Figure 1.6.

A number of examples of using cell surface engineering to express cellulases and their applications in direct conversion of cellulose and lignocellulosic materials have appeared in recent literature. Guo and coworkers reported a recombinant *S. cerevisiae* expressing cell-wall associated BGL1 from *S. fibuligera* utilized 5.2 g/L cellobiose and produced 2.3 g/L ethanol in 48 h, while a comparable *S. cerevisiae* secreting BGL1 into the culture broth used 3.6 g/L cellobiose and produced 1.5 g/L ethanol over the same period. In another example, Fujita and coworkers produced ethanol from pure cellulose such as phosphoric acid-swollen cellulose as well as from biomass such as barley straw without the addition of cellulases using recombinant S. cerevisiae strains displaying T. reesei EGII and CBHII and A. aculeatus BGL1 on the cell surface.

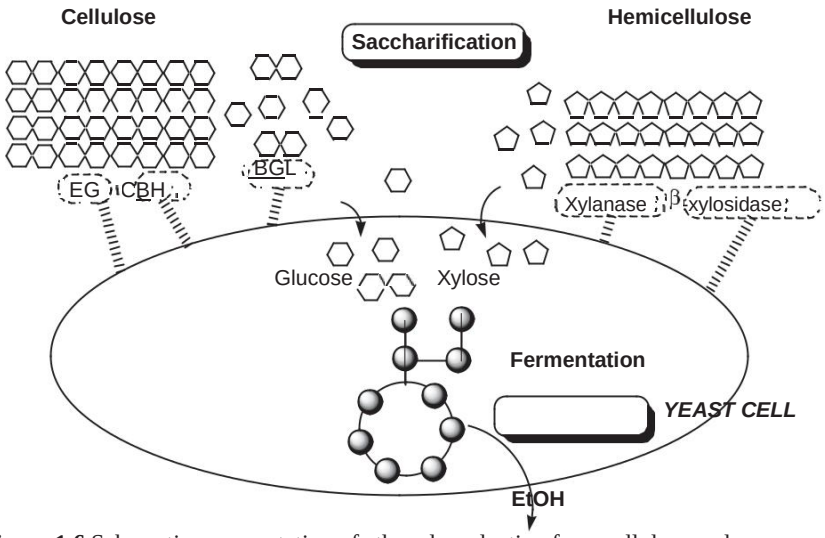


**Figure 1.6** Schematic representation of ethanol production from cellulose and hemicellulose by a recombinant yeast cell displaying cellulases and hemicellulases on the cell surface. (Reprinted with permission from reference [110]; copyright 2012 Elsevier).

A yeast strain codisplaying endoglucanase II and cellobiohydrolase II showed significantly higher hydrolytic activity with amorphous phosphoric acid-swollen cellulose than one displaying only endoglucanase II, and its main product was cellobiose; codisplay of *b*-glucosidase 1, endoglucanase II, and cellobiohydrolase II enabled the yeast strain to directly produce ethanol from the amorphous cellulose. The yield of ethanol produced from the PASC con-sumed was 0.45 g/g, which corresponds to 88.5% of the theoretical yield.

Additionally, Matano and coworkers reported the enhancement of cellulase activities on a recombinant yeast cell surface displaying *T. reesei* EGII, CBHII and *A. aculeatus* BGL1by additionally inte-grating EGII and CBHII genes into the recombinant strain. As a result, high-liter ethanol (43.1 g/L) was produced from high-solid (200 g-dry weight/L) rice straw by performing 2 h

liquefaction and subsequent 72 h fermentation in the presence of 10 FPU/g biomass added cellulase. The yield of ethanol produced from the cellulosic material by the recombinant strain reached 89% of the theoretical yield, which was 1.4-fold higher than the wild-type strain. Consequently, cell surface engineering successfully reduced the amount of commercial enzyme required for the fermentation of cellulose. Notably, the recombinant strain was able to hydrolyze a portion of the cellulosic material that was not hydrolyzed by commercial cellulase [2].

Displaying the cellulases on the yeast cell surface has certain advantages as well as disadvantages, some of the main advantages include:

1. Close proximity of multiple cellulases on the cell surface enables synergistic hydrolysis of cellulose, which leads to increased sugar availability for ethanol production.
2. Glucose liberated from cellulose is concurrently taken up on the yeast cell surface so that the glucose concentration is maintained at low levels, which reduces both the risk of contamination by other glucose-dependent organisms and product inhibition by cellulases.
3. Since the steady-state concentration of glucose in the medium can be maintained near zero, glucose repression, which prevents the uptake, catabolism or both of non-glucose sugar, is alleviated to facilitate consumption of xylose.
4. Reutilization of the yeast cells enables reuse of the enzymes displayed on their cell surface without repro-duction of the yeast cells, which would reduce the cost of yeast propagation as well as enzyme addition.

5. Cellulolytic enzymes are genetically self-immobilized on the yeast cell surface so that the activities of the enzymes are retained as long as the yeast continues to grow, while the activity of enzymes secreted into the medium is poorly maintained over a long reaction period.

However, despite all these advantages, surface immobilization of cellulases on yeasts is still an immature technology and the main disadvantage is that

ethanol yields are still very low and most of the studies so far have been done on simple model compounds or pure cellulose or xylan. Significant biotechnological advances are needed in engineering recombinant yeast that can display a vast array of cellulases required to handle very complex lignocellulosic biomass forms.

### 1.5.5 Cell Recycle Batch Fermentation (CRBF)

Cell recycle batch fermentation (CBRF) is a recently developed batch fermentation method where yeast cells are separated and reused in the next batch. The cell recycling techniques can significantly reduce time and costs associated with inoculum preparation and are also an interesting strategy for improving ethanol production in the simultaneous saccharification fermentation (SSF). However, in the case of fermentation of lignocellulosic materials, it is very difficult, or in many cases impossible, to collect only microbial cells by conventional cell collection procedures. This is because lignocellulosic residue, which is not utilized by microbes, is retained in the fermentation medium with yeast cells in the solid fraction after fermentation. Lignin left as solid residue in the fermentation medium also inhibits cellulase activity. Therefore, the removal of lignin is required for the efficient saccharification of cellulose in the subsequent batch.

Matano and coworkers have recently developed a new approach of cell recycle batch fermentation of high-solid lignocellulose using a recombinant cellulase-displaying yeast strain for consolidated bioprocessing. In this method a two-phase separation consisting of rough removal of lignocellulosic residues by low-speed centrifugation and solid-liquid separation enabled effective collection of *Saccharomyces cerevisiae* cells

with decreased lignin and ash. A schematic diagram of Matano and coworkers CRBF method for fer-mentation of lignocellulosic materials is shown in Figure 1.7.

After the fermentation, C-5 and C-6 sugars in the fermentation medium and the broth are separated by centrifugation at low gravity (20, 50, or 300 g) for 2 min to obtain a supernatant and a pellet; this pellet contains the lignocellulosic residue. Then the supernatant is separated by decantation, and then centrifuged for 5 min at 4000 g to precipitate yeast cells. The pellet obtained at the second stage was used as the yeast cell fraction for the next batch fermentation.

According to Matano and coworkers, five consecutive batch fermentations of 200 g/L hydrothermally pretreated rice straw led to an average ethanol titer of 34.5 g/L. Moreover, they claimed that the display of cellulases on the recombinant yeast cell surface increased ethanol titer to 42.2 g/L. After five-cycle fermentation only 3.3 g/L sugar was retained in the fermentation medium because cellulase displayed on the cell surface hydrolyzed cellulose that was not hydrolyzed by commercial cellulases or free secreted cellulases. They reported that fermentation ability of the recombinant strain was successfully kept during five-cycle repeated batch fermenta-tion with 86.3% of theoretical yield based on starting biomass.

### 1.5.6 Comparison of Different Fermentation Configurations

As discussed earlier, there are many fermentation configurations, and the efficiencies of these configurations can depend on several factors such as microorganism used, operating conditions, pretreatment, and the type of biomass used. A few studies reported

comparisons of various fermentation configurations. In one example of a comparison of different fermentation configurations, María López-Abelairas and coworkers reported the ethanol yields from corn stover under various pretreatments and fermentation configurations; some of the data and references are shown in Table 1.8.

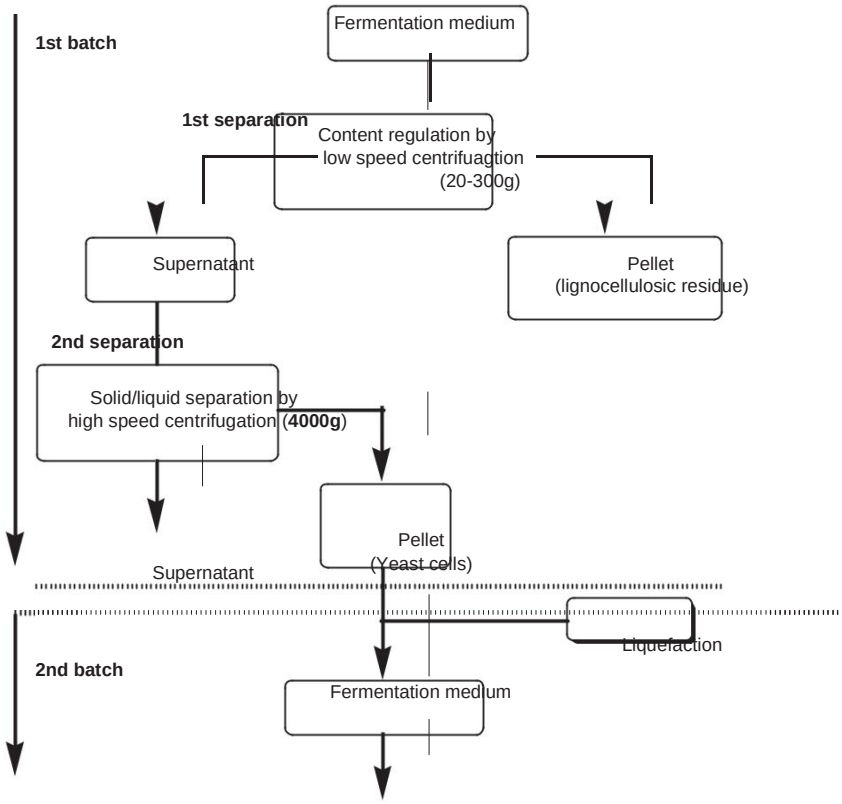


**Figure 1.7** A schematic diagram of Matano and coworkers cell recycle batch fermentation (CRBF) method.

## 1.6 Immobilization of Yeast

Immobilization of yeast is another valuable technique applicable to industrial ethanol fermentation due to its high cell density, greater volumetric productivity, tolerance to higher concentrations of sub-strate and products, relative ease of downstream processing, and most importantly, easy reusability. Many forms of solid support materials have been tested for cell immobilization, varying from natural materials like wood chips to synthesized polymers like polyacrylamide, polyurethane and polyethylene. A representative list of

some solid supports used in the immobilization of yeast is given in Table 1.9. There are advantages and disadvantages in all these solid supports; for example, synthesized polymers are non-biodegradable and could cause toxic effects on cell growth. For natural polymers, although they demonstrate non-toxic, biocom-patible, biodegradable and antimicrobial properties, they have the problems of unsatisfactory mechanical strength and insufficient space for live cells, which could lead to cell breakdown and leakage to the medium. Then there is some interest in immobilization of yeast cells on membranes, which will further enhance the recyclability. Additionally, the entrapment type of immobilization could cause physical constraints for cell growth and the natural adsorption cannot satisfy the stability requirements. Therefore, one of the promising aspects for immobilization is self-flocculation on supporting materials, which seems to have superior attributes among the yeast cell immobilization technologies in terms of the simple process and natural environment for cell growth.

**Table 1.8** Ethanol yields for corn stover under different pretreatment and fermentation configurations and references.

| **Pretreatment** | **Saccharification and fermentation configuration** | **Fermentative microorganism** | **EtOH yield** | | |
|---|---|---|---|---|---|
| | | | **mg/g consumed sugars** | **mg/g untreated wheat straw** | |
| $H_2SO_4$ | SSF | *K. marxianus* | – | 45 | |
| $H_2SO_4$ | SHF | *P. tannophilus* | 380 | 98 | |
| Steam explosion | SSF | *S. cerevisiae* | – | 120 | |
| Steam explosion | SSF | *S. cerevisiae* | – | 132 | |
| Biological (*I. lacteus*) | SHF | *P. tannophilus* | 430±11 | 128±2 | |
| Biological (*I. lacteus*) | SSF | *P. tannophilus* | 440±14 | 143±2 | |
| Biological (*I. lacteus*) | C6/C5 | *P. tannophilus*/ *S. cerevisiae* | 421±12 | 99±2 | |
| Biological (*I. lacteus*) | H5–SSF | *P. tannophilus* | 452±10 | 163±4 | |
| Biological (*I. lacteus*) SHF | SHF | *S. cerevisiae* | 484±13 | 97±4 | |
| Conditioning+biological (*I. lacteus*) | SHF | *S. cerevisiae* | 481±11 | 161±3 | |
| Biological (*I. lacteus*) | SHF | *S. cerevisiae* | – | 123±5 | |
| Biological (*I. lacteus*) | SSF | S. cerevisiae | – | 144 | |
| Biological (*P. chrysosporium*) | SSF | S. cerevisiae | | 62 | |
| Biological (*C. subvermispora*) | SSF | *S. cerevisiae* | – | 120 | |

Sing and coworkers recently reported a comparative study on etha-nol production from pretreated sugarcane bagasse using immobilized *Saccharomyces cerevisiae* on various matrices. In this experiment, first alkali pretreated sugarcane bagasse was enzymatically hydrolyzed by crude unprocessed enzymes: cellulase (filter paper activ-ity 9.4 FPU/g), endoglucanase (carboxymethylcellulase, 148 IU/g), *b*-glucosidase (116 IU/g) and xylanase (201 IU/g) produced by *Aspergillus flavus*. Then the resulting sugar solution was exposed to *Saccharomyces cerevisiae* immobilized on sugarcane bagasse, calcium alginate and agar-agar for the production of ethanol. Fermentation parameters used in batch fermentation of sugarcane bagasse enzy-matic hydrolyzate with immobilized cells of *S. cerevisiae* and the eth-anol yields for different solid supports are shown in Table 1.10.

The yield of ethanol was 0.44 g ethanol/g bagasse in the case of yeast immobilized sugarcane bagasse, 0.38 gp/gs using Ca-alginate and 0.33 g ethanol/g bagasse using agar-agar as immobilization matrices. The immobilized yeast was used up to 10 cycles in the case of immobilized sugarcane bagasse and up to 4 cycles in the case of agar-agar and calcium alginate, for ethanol production under repeated batch fermentation conditions. With all three solid supports, studied ethanol yield reached their maximum values after about 36 hr, as shown in the Figure 8.8 time course of ethanol production by *S. cerevisiae* from microwave alkali pretreated sugarcane bagasse hydrolyzate (ISB: immobilized on sugarcane bagasse; ICA: immobilized on calcium alginate; IAA: immobilized on agar-agar).

**Table 1.9** Some solid materials used in immobilization of yeast and their references.

| **Solid support** | **Reference** |
|---|---|
| Wood shavings | [3], [4] |
| Corn cobs | [3], [5] |
| Cane bagasse | [3], [6], [7] |
| Pseudo-boehmite (*a*-AlOOH) mixed with Na alginate | [8] |
| Microporous divinyl benzene copolymer (MDBP) | [9] |
| Corn stalks | [10] |
| Microporous and mesoporous zeolites, including ZSM-5, H-*b*, H-Y, and MCM-41, modified with 3-aminopropyl-triethoxysilane (APTES), mixed with alginate | [11] |
| A thin-shell silk cocoon (TSC), a residual from the silk industry | [12] |
| Porous cellulose carriers | [13] |
| Calcium alginate | [14], [7] |
| A gel containing 2% sodium alginate, 15, 30 or 50% iron powder (or Ba-ferrite) in $CaCl_2$ solution | [15] |
| Polymer carriers, poly(hydroxyethyl acrylate (HEA)-methoxy polyethylene glycol methylacrylate (M-23G)) and poly(hydroxyethyl acrylate (HEA)-glycidyl methylacrylate (GMA) prepared by radiation polymerization at low temperature | [16] |
| Bacterial cellulose membrane | [3156] |
| Agar-agar | [7] |
| Polystyrene | [17] |
| Carbon-nanotubes | [18] |
| Hydroxy apatite ceramics | [19] |
| Divynyl benzene co-polymer | [9] |
| Organic polymer supports ( natural and synthetic) - Review | [20] |

**Table 1.10** Fermentation parameters obtained in batch fermentation of sugarcane bagasse enzymatic hydrolyzate with *S. cerevisiae* cells immobi-lized on sugarcane bagasse, calcium alginate and agar-agar [163].

| Parameters | Sugarcane bagasse immobilized | Calcium alginate immobilized | Agar-agar immobilized |
|---|---|---|---|
| Initial sugar concentration (gs/L) | 50 | 50 | 50 |
| Residual sugar (gs/L) | 15 | 19 | 22 |
| Ethanol (gp/L) | 15.4 | 11.8 | 9.4 |
| Ethanol yield (gp/gs) | 0.44 | 0.38 | 0.33 |
| Volumetric ethanol productivity (gp/L/h) | 0.42 | 0.32 | 0.26 |
| Efficiency of sugar conversion to ethanol (%) | 86.2 | 74.5 | 64.7 |

Total incubation time 72 h; maximum ethanol was produced within 36 h of incubation.

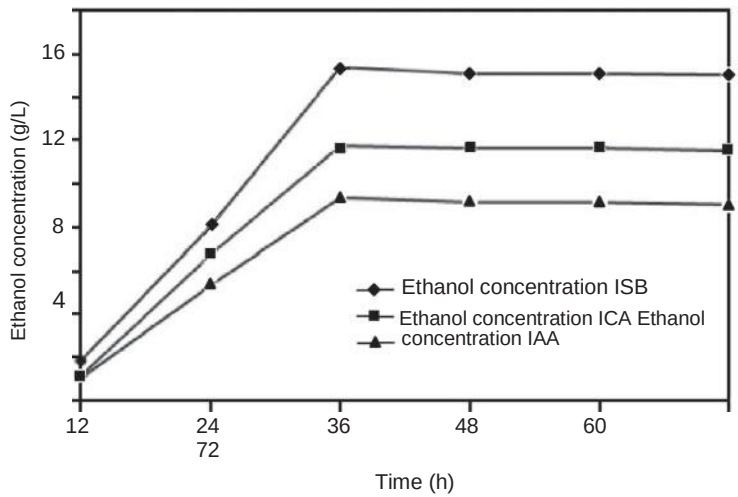


**Figure 1.8** Time course of ethanol production by *S. cerevisiae* from alkali pretreated sugarcane bagasse hydrolyzate (ISB: immobilized on sugarcane bagasse; ICA: immobilized on calcium alginate; IAA: immobilized on agar-agar).

**Summary.** As of mid 2013, several indicators have shown a steady progress in the cellulosic ethanol industry, even though the earlier targets set in the United States have not been met, as expected. Technoeconomic analysis plays an important role in the realization of cellulosic ethanol. The overreaching goal for the DOE's office of the biomass program is to demonstrate the cost-competitiveness of cellulosic ethanol with petroleum fuels.

The cellulosic biofuel industry 2013–2018 progress report is a more realistic and a vital indicator, which summarizes the global perspective of the industry. This report gives a detailed snapshot of advancements made towards the commercial deployment of cellulosic ethanol. However, entering into a commercial operation phase is an encouraging sign and a testimony for investor confi- dence on cellulosic ethanol technology. The future of cellulosic ethanol looks promising and the goal of large-scale production of fuel ethanol from abundant lignocellulosic biomass to meet the global energy demand is realizable in the near future.

The fuel producers are looking to the ethanol more interested than a past.

The utilization of biofuels in Low Temperature Combustion has shown great potential to decrease emissions and improve overall lifecycle energy efficiency. In particular, wet ethanol (a mixture of ethanol and water) as a domestically sourced biofuel has shown such potential. This study aims to determine what blend of wet ethanol would optimize combustion properties under HCCI operating conditions, both naturally aspirated and boosted. Four different blends are tested, and it is determined that WE80 (80% ethanol and 20% water by mass) exhibits optimal combustion characteristics when examining upstream intake temperature, combustion efficiency and thermal efficiency, regardless of intake boost level [21].

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## CONTENT