Appl Microbiol Biotechnol (2006) 69: 627–642
DOI 10.1007/s00253-005-0229-x

MINI-REVIEW

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Ethanol fermentation from biomass resources: current state and prospects

Received: 18 July 2005 / Revised: 21 October 2005 / Accepted: 22 October 2005 / Published online: 6 December 2005

Abstract In recent years, growing attention has been devoted to the conversion of biomass into fuel ethanol, considered the cleanest liquid fuel alternative to fossil fuels. Significant advances have been made towards the technology of ethanol fermentation. This review provides practical examples and gives a broad overview of the current status of ethanol fermentation including biomass resources, microorganisms, and technology. Also, the promising prospects of ethanol fermentation are especially introduced. The prospects included are fermentation technology converting xylose to ethanol, cellulase enzyme utilized in the hydrolysis of lignocellulosic materials, immobilization of the microorganism in large systems, simultaneous saccharification and fermentation, and sugar conversion into ethanol.

Introduction

With the inevitable depletion of the world's energy supply, there has been an increasing worldwide interest in alternative sources of energy (Aristidou and Penttila 2000; Jeffries and Jin 2000; John 2004; Kerr 1998; Wheals et al. 1999; Zaldivar et al. 2001). It is now understood that it is important to use biomass energy as a means of providing modern energy to the billions who lack it. It would complement solar, wind, and other intermittent energy sources in the renewable energy mix of the future. One of the most immediate and important applications of biomass energy systems could be in the fermentation of ethanol from biomass.

Biomass is seen as an interesting energy source for several reasons. The main reason is that bioenergy can contribute to sustainable development (Van den Broek 2000; Monique et al. 2003). Resources are often locally available, and conversion into secondary energy carriers is feasible without high capital investments. Moreover, biomass energy can play an important role in reducing greenhouse gas emissions; since CO_2 that arises from biomass wastes would originally have been absorbed from the air, the use of biomass for energy offsets fossil fuel greenhouse gas emissions (Lynd 1996). Furthermore, since energy plantations may also create new employment opportunities in rural areas, it also contributes to the social aspect of sustainability. In addition, application of agro-industrial residues in bioprocesses not only provides alternative substrates but also helps solve their disposal problem. With the advent of biotechnological innovations, mainly in the area of enzyme and fermentation technology, many new avenues have opened for their utilization.

Nearly all fuel ethanol is produced by fermentation of corn glucose in the US or sucrose in Brazil (MacDonald et al. 2001; Rosillo-Calle and Cortez 1998), but any country with a significant agronomic-based economy can use current technology for fuel ethanol fermentation. This is possible because, during the last two decades, technology for ethanol production from nonfood-plant sources has been developed to the point at which large-scale production will be a reality in the next few years. Therefore, agronomic residues such as corn stover (corn cobs and stalks), sugarcane waste, wheat or rice straw, forestry, and paper mill discards, the paper portion of municipal waste and dedicated energy crops—collectively termed "biomass"—can be converted into fuel ethanol. In this field, although bioethanol production has been greatly improved by new technologies, there are still challenges that need further investigations. A further understanding of the ethanol fermentation needs to be reached.

This review will focus on the current status of ethanol fermentation including biomass resources, microorganisms, technology, the practical examples, and especially the promising prospects of ethanol fermentation.

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Biomass resources

There are various forms of biomass resources in the world, which can be grouped into four categories. Wood residues are by far the largest current source of biomass for energy production. It comes from the wood product industry which includes paper mills, sawmills, and furniture manufacturing. Municipal solid waste is the next largest, followed by agriculture residues and dedicated energy crops. Among these biomass resources including short-rotation woody crops and herbaceous crops, primarily tall grasses, dedicated energy crops seem to be the largest, most promising, future resource of biomass. This is because of the ability to obtain numerous harvests from a single planting, which significantly reduces average annual costs for establishing and managing energy crops, particularly in comparison to conventional crops (Monique et al. 2003).

Fermentation processes from any material that contains sugar could derive ethanol. The varied raw materials used in the manufacture of ethanol via fermentation are conveniently classified into three main types 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) must first be hydrolyzed to fermentable sugars by the action of enzymes from malt or molds. Cellulose (from wood, agricultural residues, waste sulfite liquor from pulp, and paper mills) must likewise be converted into sugars, generally by the action of mineral acids. Once simple sugars are formed, enzymes from microorganisms can readily ferment them to ethanol.

The most widely used sugar for ethanol fermentation is molasses which contains about 50 wt% of sugar and about 50 wt% of organic and inorganic compounds, including water. It is a thick, dark-colored syrup produced during refinement of sugar. Since molasses contains microorganisms which can disturb the fermentation, the molasses is taken first to the sterilizer and then to the fermentor. Then it is diluted with water to the mass fraction of 10±18% to reduce its viscosity in the pipeline. In addition, a very high concentration of sugar can give too much ethanol and results in a prolonged fermentation time and an incomplete sugar conversion. After the pH of the mash is adjusted to about 4–5 with mineral acid, it is inoculated with yeast or bacteria, and the fermentation is carried out nonaseptically at 20–32°C for about 1–3 days.

Most agricultural biomass containing starch can be used as a potential substrate for the ethanol fermentation by microbial processes. These substrates include corn (maize), wheat, oats, rice, potato, and cassava. On a dry basis, corn, wheat, sorghums (milo), and other grains contain around 60–75% (wt/wt) of starch, hydrolyzable to hexose with a significant weight increase (stoichiometrically the starch to hexose ratio is 9:10), and these offer a good resource in many fermentation processes (Jackman 1987).

Fermentation of starch is somewhat more complex than fermentation of sugars because starch must first be converted into sugar and then into ethanol. Starch is first hydrolyzed by adding α-amylase to avoid gelatinization, then cooked at high temperature (140–180°C). Next, the liquefied starch is hydrolyzed to glucose with glucoamylase. The resulting dextrose is fermented to ethanol with the aid of microorganisms producing CO_2 as a coproduct. During the process currently employed for industrial-scale ethanol fermentation from starchy materials, high-temperature cooking (140–180°C) is very effective for fermentation of starchy materials because it raises starch saccharification efficiency and achieves high levels of ethanol production under complete sterilization of harmful microorganisms. However, production costs are high due to the high energy consumption in the cooking process and the addition of large amounts of amylolytic enzymes. So processes to reduce the high production costs are required. To resolve these difficulties, noncooking and low-temperature cooking fermentation systems have been developed (Matsumoto et al. 1985).

Industrial ethanol production has been reported using various starchy materials such as corn, wheat, starch and potatoes, cassava root (Lindeman and Rocchiccioli 1979), corn stover (Kadam and McMillan 2003; Wilke et al. 1981), and starch (Maisch et al. 1979). Among many starchy materials, cassava starch is an inexpensive fermentable source. It is a tropical root crop produced in more than 80 countries (Sasson 1990). About 20% of the cassava starch was incorporated into animal feed. A similar amount was converted into starch for industrial use and another portion used for human food in some developing countries. The rest was lost since cassava is perishable after harvest. Harnessing the lost portion in addition to gains from new high-yielding varieties with outputs of 100 tons per hectare could provide the fermentation industry with an abundance of raw material (Anthony et al. 1996). Fresh cassava has a very high starch content, up to 30%. The content of sucrose is about 4%. Dried cassava has 80% fermentable substrate.

However, cassava waste processing is difficult because it is high in toxic materials. The potential toxicity of cassava is due to the presence of cyanogenic glycosides, linamarin, and lotaustralin, which on hydrolysis yield hydrogen cyanide on its peel. Traditional methods of cooking like boiling and decanting remove cyanoglycosides to a certain extent, but even then a certain amount of residual toxicity remains in it (Westley 1980). Moreover, since starch particles in cassava are bigger and there are some branched structures, more glucoamylase has to be added into the reactor. Furthermore, the nitrogen content of the cassava is very low, so during the fermentation, nutrient has to be added into the reactor to maintain the normal growth of the microorganisms.

Among the three main types of raw materials, cellulose materials represent the most abundant global source of biomass and have been largely unutilized. The global production of plant biomass, of which over 90% is lignocellulose, amounts to about 200×10^9 tons per year, where about $8–20\times10^9$ tons of the primary biomass remains potentially accessible. However, the effective utilization of the lignocellulosic feedstock is not always practical because of its seasonal availability, scattered stations, and the high costs of transportation and storage of such large amounts of organic

material (Polman 1994). Recently, the enzymatic hydrolysis of biomass cellulose is considered to be the most promising technology available (Ogier et al. 1999; Yu and Zhang 2004). However, despite the work done, the industrial scale-up of this process appears to be still hindered by technological issues or by the lack of a biomass refinery approach in which ethanol is one of several products. In fact, because raw material cost comprises more than 20% of the production cost (Brown et al. 2001; Kaylen et al. 2000; Zhuang et al. 2001), the optimization of the cellulose conversion should be accomplished by correct management and utilization of all process streams. A consequence of this situation is that even limited government intervention is still crucial to maintaining ongoing research.

Furthermore, lignocellulose is a more complex substrate than starch. It is composed of a mixture of carbohydrate polymers (cellulose and hemicellulose) and lignin. The carbohydrate polymers are tightly bound to lignin mainly by hydrogen bonds but also by some covalent bonds. The biological process for converting the lignocellulose to fuel ethanol requires the following: delignification to liberate cellulose and hemicellulose from their complex with lignin, depolymerization of the carbohydrate polymers to produce free sugars, and fermentation of mixed hexose and pentose sugars to produce ethanol. Among the key processes described above, the delignification of lignocellulosic raw materials is the rate-limiting and most difficult task to be solved. Another problem is that the aqueous acid used to hydrolyze the cellulose in wood to glucose and other simple sugars destroys much of the sugars in the process. Extensive research has been carried out in this field for decades (Yu and Zhang 2004), and the first demonstration plant using lignocellulosic feedstocks has been in operation in Canada since April 2004 (Tampier et al. 2004). It is expected that the cost of lignocellulosic ethanol can undercut that of starch-based ethanol because low-value agricultural residues can be used.

General process

Besides the initial removal of large and unsuitable items, key components of an integrated residual waste treatment system based on ethanol fermentation include recyclable materials recovery and removal of contaminants via mechanical preprocessing, initial hydrolysis process (conversion to simpler compounds), fermentation of organics, postfermentation purification of ethanol (by distillation or filtration), gasification of solid residuals to provide process heat, and treatment and disposal of wastewater.

Nearly all of the ethanol fermentation technologies use an initial tipping floor removal of large or unsuitable materials, followed by mechanical preprocessing to remove recyclables and contaminants, and shredding of the material. Then the material is processed through vessels using various systems for the purpose of hydrolysis (breaking down to simpler compounds) of the material. Depending on the technology, this may include high temperature, acid treatment, and/or high pressure. Following the initial hydrolysis phase, the slurried material is then fermented to produce alcohol, which is then purified through distillation and/or filtration to produce the desired fuel-grade quality ethanol.

When cellulose was used as the raw material, the cellulase responsible for enzymatic hydrolysis of pretreated cellulosic biomass is strongly inhibited by hydrolysis products: glucose and short cellulose chains. One way to overcome cellulase inhibition is to ferment the glucose to ethanol as soon as it appears in solution. Simultaneous saccharification and fermentation (SSF) combines enzymatic hydrolysis with ethanol fermentation to keep the concentration of glucose low (as shown in Fig. 1). The accumulation of ethanol in the fermentor does not inhibit cellulase as much as high concentrations of glucose, so SSF is a good strategy for increasing the overall rate of cellulose to ethanol conversion. In comparison to the process where these two stages are sequential, the SSF method enables attainment of higher (up to 40%) yields of ethanol by removing end-product inhibition, as well as by eliminating the need for separate reactors for saccharification and fermentation (Bollók et al. 2000; Hari et al. 2001; Stenberg et al. 2000). Other advantages of this approach are a shorter fermentation time and a reduced risk of contamination with external microflora, due to the high temperature of the process, the presence of ethanol in the reaction medium, and the anaerobic conditions (Emert and Katzen 1980; Wyman 1994).

In spite of the obvious advantages presented by the SSF, it has some drawbacks. These lie mainly in different temperature optima for hydrolysis (45–50°) and fermentation (28–35°) (Ballesteros et al. 2004; Jeffries and Jin 2000; Jeffries and Shi 1999). Besides, ethanol itself and some toxic substances arising from pretreatment of the lignocellulose inhibit the action of fermenting microorganisms, as well as the cellulose activity (Targonski and Achremowicz 1986; Yu and Zhang 2004). Achieving microorganism–enzyme compatibility becomes a major issue in the SSF, since some compounds (e.g., proteolytic enzymes) that are released on cell lysis or are secreted by a particular strain can degrade the cellulases, alternately, components in the enzyme preparation, and reduce microbial viability leading to cell lysis. On the whole, several process parameters must be optimized: substrate concentration, enzyme to substrate ratio, dosage of the active components (α-glucosidase to glucanase ratio) in the enzymatic mixture, and yeast concentration.

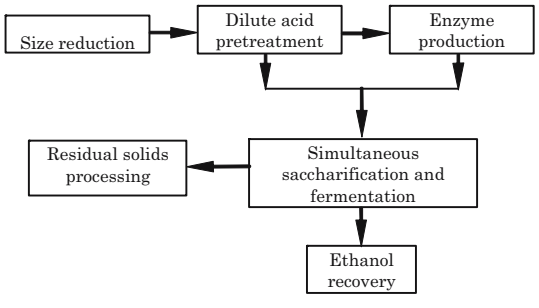

Fig. 1 Schematic diagram of the conversion of biomass feedstock to ethanol fuel

Microorganisms related to ethanol fermentation

Ethanol fermentation is a biological process in which organic material is converted by microorganisms to simpler compounds, such as sugars. These fermentable compounds are then fermented by microorganisms to produce ethanol and CO_2. During the whole process of ethanol fermentation, there are mainly two parts for microorganisms. One is for the microorganisms which convert fermentable substrates into ethanol, and the other is to produce the enzyme to catalyze chemical reactions that hydrolyze the complicate substrates into simpler compounds.

Microorganisms producing ethanol

Several reports and reviews have been published on production of ethanol fermentation by microorganisms, and several bacteria, yeasts, and fungi have been reportedly used for the production of ethanol. Those microbes that are capable of yielding ethanol as the major product are shown in Tables 1 and 2.

As shown in Tables 1 and 2, there are some microorganisms which can accumulate high concentrations of ethanol. Historically, the most commonly used microbe has been yeast, among the yeasts, *Saccharomyces cerevisiae*, which can produce ethanol to give concentration as high as 18% of the fermentation broth, is the preferred one for most ethanol fermentation. This yeast can grow both on simple sugars, such as glucose, and on the disaccharide sucrose. *Saccharomyces* is also generally recognized as safe (GRAS) as a food additive for human consumption and is therefore ideal for producing alcoholic beverages and for leavening bread.

As with many microorganisms, *S. cerevisiae* metabolizes glucose by the Embden–Meyerhof (EM) pathway. Beside this, the Entner–Doudoroff (ED) pathway is an additional means of glucose consumption in many bacteria, such as *Zymomonas*. The high ethanol yield and productivity observed for *Zymomonas* are a consequence of its unique physiology. *Zymomonas* is the only microorganism that metabolizes glucose anaerobically using the ED pathway as opposed to the EM or glycolytic pathway (Matthew et al. 2005). The ED pathway yields only half as much ATP per mole of glucose as the EM pathway. As a consequence, *Zymomonas* produces less biomass than yeast, and more carbon is funneled to fermentation products. Also, as a consequence of the low ATP yield, *Zymomonas* maintains a high glucose flux through the ED pathway. All the enzymes involved in fermentation are expressed constitutively, and fermentation enzymes comprise as much as 50% of the cells' total protein (Sprenger 1996).

Zymomonas mobilis is an unusual Gram-negative microorganism that has several appealing properties as a biocatalyst for ethanol production. The microorganism has a homoethanol fermentation pathway and tolerates up to 120 g/l ethanol. It has a higher ethanol yield (5–10% more ethanol per fermented glucose) and has a much higher specific ethanol productivity (2.5×) than *Saccharomyces* sp. (Sprenger 1996). Furthermore, *Z. mobilis* is GRAS and has simple nutritional needs. It is so well suited for ethanol production that in the 1970s and 1980s, some researchers advocated it as superior to *S. cerevisiae*. Despite its advantages as an ethanologen, *Z. mobilis* is not well suited for all of the biomass resources conversion because it ferments only glucose, fructose, and sucrose. Moreover, for *Z. mobilis* on synthetic media containing either glucose, fructose or sucrose, the specific rates of sugar uptake and ethanol production are at a maximum when utilizing the glucose medium. In addition, *S. cerevisiae* is still preferred by the industry because of the yeast hardiness.

Engineering *Escherichia coli* is another valuable bacterial resource for ethanol production. The construction of *E. coli* strains to selectively produce ethanol (Millichip and Doelle 1989) was one of the first successful applications of metabolic engineering. *E. coli* has several advantages as a biocatalyst for ethanol production, including the ability to ferment a wide spectrum of sugars, no requirements for complex growth factors, and prior industrial use (e.g., for production of recombinant protein). 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.

Cellulose-to-ethanol biotransformation can be conducted by various anaerobic thermophilic bacteria, such as *Clostridium thermocellum* (Ingram et al. 1987), as well as by some filamentous fungi, including *Monilia* sp. (Saddler and Chan 1982), *Neurospora crassa* (Gong et al. 1981), *Neurospora* sp. (Yamauchi et al. 1989), *Zygosaccharomyces rouxii* (Pastore et al. 1994), *Aspergillus* sp. (Sugawara et al. 1994), *Trichoderma viride* (Ito et al. 1990), and *Paecilomyces* sp. (Gervais and Sarrette 1990). However, studies on the fermentation process utilizing these microorganisms have shown this process to be very slow (3–12 days) with a poor yield (0.8–60 g/l of ethanol), which most probably is due to the low resistance of microorganisms to higher concentrations of ethyl alcohol. Another disadvantage of this process (particularly in the case of bacterial fermentation) is the production of various by-products, primarily acetic and lactic acids (Herrero and Gomez 1980; Wu et al. 1986).

Hydrolysis enzymes and the related microorganisms

In addition to polymeric carbohydrates, raw material for ethanol fermentation contains varying amounts of polyphenolic lignin and other "extractables." These compounds are not directly fermentable by most yeasts, and they must be pretreated to hydrolyze the complicate compounds to simple sugars (Zertuche and Zall 1982). Development of an ideal pretreatment process is difficult, given that "biomass" includes such sources as hardwood and softwood trees, agricultural residues such as corn stover and nonrecyclable paper waste.

Table 1 Yeast species which produce ethanol as the main fermentation product

Strain-species	Temperature (°C)	pH value	Carbon source and concentration (g/l)	Nitrogen source and concentration (g/l)	Incubation time (h)	Concentration of ethanol produced (g/l)	References
27817-*Saccharomyces cerevisiae*	30	5.5	Glucose (50–200)	Peptone (2) and ammonium sulfate (4)	18–94	5.1–91.8	Vallet et al. 1996
L-041-*S. cerevisiae*	30 or 35	–	Sucrose (100)	Urea (1) or ammonium sulfate (1–2)	24	25–50	Leticia et al. 1997
181-*S. cerevisiae* (aerobic)	27	6.0	Glucose (10)	Peptone (5.0)	40–160	–	Todor and Tsonka 2002
UO-1-*S. cerevisiae* (aerobic)	30	5.0	Sucrose (20)	Ammonium sulfate (1)	60–96	–	Camacho-Ruiz et al. 2003
V5-*S. cerevisiae*	24	–	Glucose (250)	–	36	–	Virginie et al. 2001
ATCC 24860-*S. cerevisiae*	30	4.5	Molasses (1.6–5.0)	Ammonium sulfate (0.72–2.0)	24	5–18.4	Ergun and Mutlu 2000
Bakers' yeast-*S. cerevisiae*	30	4.5	Sugar (150–300)	–	192	53 (max)	Roukas 1996
Bakers' yeast-*S. cerevisiae*	28	5.0	Sucrose (220)	Peptone(5) and ammonium dihydrogen phosphate (1.5)	96	96.71	Caylak and Vardar 1996
Fiso-*S. cerevisiae*	30	5.0	Galactose (20–150)	Peptone, ammonium sulfate and casamino acid (10)	60	4.8–40	da Cruz et al. 2003
A3-*S. cerevisiae*	30	5.0	Galactose (20–150)	Peptone, ammonium sulfate and casamino acid (10)	60	4.8–36.8	da Cruz et al. 2003
L52-*S. cerevisiae*	30	5.0	Galactose (20–150)	Peptone, ammonium sulfate and casamino acid (10)	60	2.4–32.0	da Cruz et al. 2003
GCB-K5-*S. cerevisiae*	30	6.0	Sucrose (30)	Peptone (5)	72	27	Kiran et al. 2003
GCA-II-*S. cerevisiae*	30	6.0	Sucrose (30)	Peptone (5)	72	42	Kiran et al. 2003
KR_{18}-*S. cerevisiae*	30	6.0	Sucrose (30)	Peptone (5)	72	22.5	Kiran et al. 2003
CMI237-*S. cerevisiae*	30	4.5	Sugar (160)	Ammonium sulfate (0.5)	30	70 (max)	Navarro et al. 2000
2.399-*S. cerevisiae*	30	5.5	Glucose (31.6)	Urea (6.4)	30	13.7 (max)	Yu and Zhang 2004
24860-*S. cerevisiae*	–	–	Glucose (150)	Ammonium dihydrogen phosphate (2.25)	27	48 (max)	Ghasem et al. 2004
27774-*Kluyveromyces fragilis*	30	5.5	Glucose (20–120)	Peptone (2) and ammonium sulfate (4)	18–94	48.96 (max)	Vallet et al. 1996
30017-*K.fragilis*	30	5.5	Glucose (20–120)	Peptone (2) and ammonium sulfate (4)	18–94	48.96 (max)	Vallet et al. 1996
30016-*Kluyveromyces marxianus*	30	5.5	Glucose (100)	Peptone (2) and ammonium sulfate (4)	18–94	44.4 (max)	Vallet et al. 1996
30091-*Candida utilis*	30	5.5	Glucose (100)	Peptone (2) and ammonium sulfate (4)	18–94	44.4 (max)	Vallet et al. 1996
ATCC-32691 *Pachysolen tannophilus*	30	4.5	Glucose (0–25) and xylose (0–25)	Peptone (3.6) and ammonium sulfate (3)	100	7.8 (max)	Sanchez et al. 1999

Table 2 Bacterial species which produce ethanol as the main fermentation product

Mesophilic organisms	Mmol ethanol produced per mmol glucose metabolized	References
Clostridium sporogenes	up to 4.15[a]	Miyamoto 1997
Clostridium indoli (pathogenic)	1.96[a]	Miyamoto 1997
Clostridium sphenoides	1.8[a] (1.8)[b]	Miyamoto 1997
Clostridium sordelli (pathogenic)	1.7	Miyamoto 1997
Zymomonas mobilis (syn. Anaerobica)	1.9	Miyamoto 1997
Zymomonas mobilis subsp. *pomaceas*	1.7	Miyamoto 1997
Spirochaeta aurantia	1.5 (0.8)	Miyamoto 1997
Spirochaeta stenostrepta	0.84 (1.46)	Miyamoto 1997
Spirochaeta litoralis	1.1 (1.4)	Miyamoto 1997
Erwinia amylovora	1.2	Miyamoto 1997
Escherichia coli KO11	0.7–0.1	Dien et al. 2003; Matthew et al. 2005
Escherichia coli LY01	40–50 g ethanol produced/l	Dien et al. 2003
Leuconostoc mesenteroides	1.1	Miyamoto 1997
Streptococcus lactis	1.0	Miyamoto 1997
Klebsiella oxytoca	0.94–0.98	Matthew et al. 2005
Klebsiella aerogenes	24 g ethanol produced/l	Ingram et al. 1998
Mucor sp. M105	–	Ingram et al. 1998
Fusarium sp. F5	–	Ingram et al. 1998

These diverse feedstocks have caused researchers to test numerous pretreatment processes ranging from hot water and steam explosion treatments, to alkaline and solvent pretreatments, to many useful versions of acid pretreatment (Kaar and Holtzapple 2000; Maiorella 1985; Sun and Cheng 2002). However, they acknowledge that detoxification of acid-hydrolyzed lignin and other "extractables" in the sugar hydrolysate will present additional costs for the total hydrolysis process, costs that could be avoided entirely if a fully enzymatic process (yet to be developed) is implemented instead.

Traditionally, starch was, and still is, hydrolyzed to low molecular weight dextrins and glucose using acid, but enzymes have several advantages. First, the specificity of enzymes allows the production of sugar syrups with well-defined physical and chemical properties. Second, the milder enzymatic hydrolysis results in few side reactions and less "browning." Indeed, for the production of glucose syrups from starch, enzymic hydrolysis is essential. A summary of starch degrading enzymes is shown in Fig. 2 (Hsu 1996).

There have been several reports about yeasts that could produce extracellular α-amylase and glucoamylase. These include *Candida tsukubaensis* CBS 6389 (Aktinson and Mavituna 1991), *Filobasisium capsuligenum* (Aktinson and Mavituna 1991), *Lipomyces kononenkoae* (de Mot and Verachtert 1985), *Lipomyces starkeyi* (Spencer-Martins and Van Uden 1979), *Saccharomycopsis bispora* (formerly *Endomycopsis bispora*) (Kelly et al. 1985), *Saccharomycopsis capsularis*, *Saccharomycopsis fibuligera* (Ebertova 1966; Stepanov et al. 1975), *Schwanniomyces alluvius* (Gasperik et al. 1985), *Schwanniomyces castelli* (Simoes-Mendes 1984), and *Trichosporon pullulans* (Silla et al. 1984).

In addition, for the production of cellulolytic enzymes to be used in the hydrolysis, the lignocellulose-degrading fungus *Trichoderma reesei* can be used (Sharma 2000). This fungus is able to metabolize pentose and hexose sugars and also oligomers, and it is insensitive to inhibitors generated from the lignocellulosic material, because these are normally present in its natural environment.

In this field, it was investigated whether the cellulolytic fungus *T. reesei* could degrade inhibitory compounds present in a hemicellulose hydrolysate obtained after steam pretreatment of willow and thereby decrease its inhibitory effect on the ethanolic fermentation by *S. cerevisiae*. It was also investigated whether the inhibitor containing fraction could be used as a carbon source for the production of high-quality cellulolytic enzymes to be used in the hydrolysis.

Kinetic models

Generally, economic restrictions force industrial processes to work in a very small range of operating conditions. For some batch processes which have long operating times in each cycle and depend strongly on the operating variables, it is very important to define the optimum conditions to achieve sufficient profitability. Kinetic models describing the behavior of microbiological systems can be a highly appreciated tool and can reduce tests to eliminate extreme possibilities.

Various kinetic models have been proposed in the literature for freely suspended cells in either batch or continuous operation (Ramon-Portugal et al. 1997; Reynders et al. 1996; Tan et al. 1996). Unstructured models give the most fundamental observations concerning microbial metabolic processes and can be considered a good approxi-

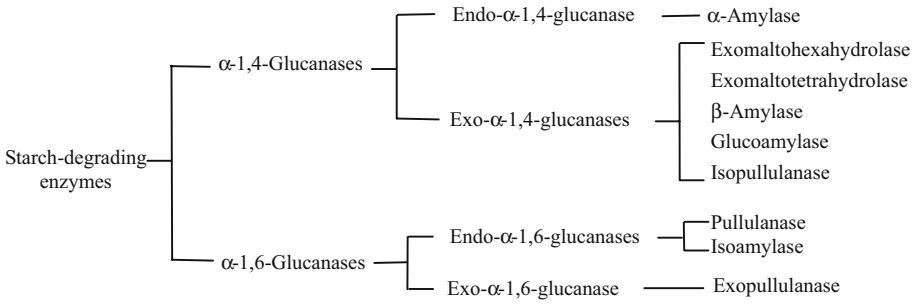

Fig. 2 A summary of starch degrading enzymes

mation when the cell composition is time dependent or when the substrate concentration is high compared to the saturation constant (Sonnleitner et al. 1997). Control models for routine operation of industrial fermentations are often based on simple, unstructured models since the process computer will adjust the model parameters based on the response of the system to disturbances.

When cultured in glucose media, unstructured models have been found effective for describing the exponential phase of the batch fermentation kinetics of cell growth and ethanol production for strains of *Z. mobilis* ZM4 and ATCC 10988 (Moser 1985). These models, incorporated with the bottleneck model approach, provide a base for establishing a structured model that can describe the transient behavior of a batch fermentation. An additional parameter, reflecting the quality of the inoculum, is adjusted to match the model prediction with the corresponding experimental result. In continuous culture, the experimental findings suggest that the specific substrate uptake rate is not linearly dependent upon the specific growth rate, μ. A structured two-compartment model was introduced by Jobses et al. (1985) to describe the fermentation of *Z. mobilis*. According to this model, the specific substrate (glucose) uptake rate in steady-state continuous culture is a nonlinear second-order function of μ.

Gulnur et al. (1998) investigated the mathematical description concerned with the basic metabolic processes of *S. cerevisiae* in immobilized form. Glucose utilization, ethanol production, and growth pattern of yeast cells immobilized in calcium alginate gel beads were determined in a stirred batch system using four different initial substrate concentrations. Eleven different mathematical models taking into account the possibility of glucose or ethanol inhibition on both yeast cell growth and ethanol production were studied. The batch performance curves predicted by the models were compared with the experimental data, and the results were analyzed in terms of the possible effects of initial condition (Doruker et al. 1995).

During the simulation of batch alcoholic fermentation with the different initial conditions employed 11 different models: the models of Monod, Moser, and Teissier were used to represent inhibition-free substrate limitation kinetics; the models of Andrews and Noack, Aiba and Luong include substrate inhibition effects, whereas the models of Levenspiel, Aiba, Jerusalimsky, Ghose and Tyagi, and Hinshelwood include product inhibition effects. The models proposed by Monod and Hinshelwood were found to be more appropriate for describing the batch growth and ethanol production of immobilized *S. cerevisiae* at low and high initial glucose concentrations, respectively (Gulnur et al. 1998).

Structured models describing culture kinetics are important in the control of bioreactors, as they provide a mathematical description of the mechanism of the process which are required for optimization and control. The objective of structured modeling is to obtain expressions that quantitatively describe the behavior of the process under consideration. A wide variety of models have been proposed for the kinetics of the process; these range from very simple models (Mori et al. 1970; Namba et al. 1984) to more complex global models (Park and Toda 1990; Park et al. 1990, 1991), which take into account the activating and inhibiting effects of the substrate (glucose and oxygen) and the product (ethanol and acetic acid; Oh et al. 2000). However, none of these studies have put forward a general model sufficiently well developed to permit the design of a good simulator which is capable of performing simulations with batch processes.

Moreover, structured models have been used to predict the influence of operating parameters on cell concentration, substrate utilization rate, and ethanol production rate. These models may lead to the development of better strategies for the optimization of the fermentation process to ensure its economic viability. Although four factors (substrate limitation, substrate inhibition, product inhibition, and cell death) are known to affect ethanol fermentation, none of these models accounts for these kinetic factors simultaneously. Monod's (1950) equation accounts only for substrate limitation. The models of Hinshelwood (1946), Holzberg et al. (1967), Egamberdiev and Jerusalimsky (1968), Nagatani et al. (1968), Ghose and Tyagi (1979), Hoppe and Hansford (1982), and Lee (1988) account only for ethanol inhibition. The models of Aiba et al. (1968), Aiba and Shoda (1969), and Luong (1985) include only substrate limitation and substrate inhibition terms. An appropriate ethanol fermentation model should therefore account for the four kinetic factors.

A developed mathematical model capable of predicting the cell, substrate, and ethanol concentrations during the continuous anaerobic fermentation is necessary. However,

it cannot be expected that any kinetic model will be directly applicable to a real process situation. Therefore, mathematical modeling should start with the simplest type, but it must be reiterated, modified, and extended until it eventually leads to an adequate process kinetic model.

Pilot plants producing ethanol

Approximately 80% of the ethanol produced in the world is still obtained from fermentations; the remainder comes largely by synthesis from the petroleum product, ethylene. The alcohol produced in the US is primarily used in alcoholic beverages, but this is not always the case elsewhere in the world. Brazil has embarked on a major program to produce ethanol for fuel and thereby diminish petroleum imports. As of 1984, approximately 7.9 million tons of ethanol was produced by fermentation in Brazil, with sucrose from sugarcane as the carbon source. The US is also substantially increasing its fuel alcohol production, originally because of the rapid increase in petroleum costs during the 1970s, and the subsequent need for developing alternative energy sources.

In spite of extensive research on fuel ethanol production from biomass (shown in Table 3), until 1995, not a single plant capable of converting cellulosic feedstock to ethanol, via biological processing on the industrial scale, has been put into operation anywhere in the world, although some pilot scale plants have been commissioned (Szczodrak and Fiedurek 1996).

During World War II, when wartime conditions changed economic conditions and priorities, several ethanol-from-cellulose (EFC) plants were built and operated in various countries to provide an alternative fuel source. These countries include Germany, Russia, China, Korea, Switzerland, and the US among others. Since the end of the war, competition from synthetically produced ethanol has forced many of these plants to close (Badger 2002). Since April 2004, the first demonstration plant using lignocellulosic feedstocks in Canada has been in operation (Tampier et al. 2004). The target volume of 100 million liters of ethanol, anticipated by 2006, will likely be met or exceeded by 2007. There is also progress on pretreatment of softwood residues and pentose fermentation (Natural Resources Canada's management team 2005).

Currently, some countries in locations with higher ethanol and fuel prices are producing ethanol from cellulosic feedstocks. It is only recently that cost-effective technologies for producing EFC in the US have started to emerge (Badger 2002). In Canada, Iogen Corporation built a small commercial-scale cellulose–ethanol plant using proprietary enzymatic hydrolysis technology. In 1997, they partnered with Petro-Canada to produce cellulose–ethanol beginning with a 1-million-gallon-per-year ethanol demonstration facility, located at Iogen's headquarters in Ottawa, using corn stover and switchgrass (Energy & Environmental Research Center, 2001). In summer 2005, a Swedish plant in Örnsköldsvik started to produce ethanol from sawdust. The production is still in a start-up phase, but the optimism is high. In a not so distant future, Sweden could become self-sufficient of ethanol from wood and wood residues, which would be a much more sustainable way of supplying ethanol to the Swedish market (Advanced course in LCA 2005).

Nowadays, in the field of sugar and starch utilization, the large-scale application of modern bioenergy conversion technologies has already occurred in a number of countries, both in the industrialized and developing worlds. In the US, the Minnesota Pollution Control Agency (MPCA) has scheduled a public information meeting in early 2005 to discuss the proposed Heron Lake BioEnergy ethanol project. The proposed plant would cover 37 acres at a site about 1 mile northeast of the city of Heron Lake in Jackson County. It would process 21.7 million bushels of corn annually to produce 55 million gallons of ethanol and 193,300 tons of distiller dried grains (Sullivan 2005). Another example is that of Brazil, a country that has committed itself to the development of its modern bioenergy

Table 3 The lists of pilot plants for ethanol production from biomass

Year	Place	Substrates	Capacity (ton/day)	Production (l ethanol/day)	References
1976	US	–	1	–	Emert and Katzen 1980; Emert et al. 1980
1981	Canada	Grain	960	27,400–220,000	Robert 2004
1983	–	Cellulose	2,000	57,750	Emert et al. 1983
1983	Japan	–	720	150–200	Morikawa et al. 1985a,b; Morikawa and Tadokoro 1987
1984	Canada	–	1	–	Bente 1984
1988	France	Cellulose	96	160–190 kg/1,000 kg wood	Ballerini et al. 1994; Nativel et al. 1992
1993	US	Concentrated sweet whey	7.5	5,178	National Renewable Energy Laboratory 1996
2001	US	Corn	–	155,000	Gary 2002
2002	US	MSW	–	10,360,000	Badger 2002
2003	Canada	Lignocellulosic	–	41,500	Tembec 2003
2005	US	–	–	570,000	MN Pollution Control Agency 2005

potential. Its sugarcane-based ethanol industry annually produces around 15 billion liters from about 350 distilleries and satisfies over 33% of the country's gasoline needs (Agama Energy 2003).

For the Global ethanol market, Brazil has more than 300 plants, producing 15 billion liters per year and supplying 3 million cars with pure ethanol. In the US, there are more than 80 plants producing 10 billion liters per year, which it intends to increase to 19 billion liters by 2010. China could create 3 billion liters of ethanol per year. India's annual production of ethanol is 2.7 billion liters, and Eastern Europe's 2.5 billion liters. Western Europe's production ability is 2 billion liters and in Canada, 0.24 billion liters could be achieved and possibly expanded to 1.4 billion liters (Klein 2005).

Moreover, a fuel tax exemption is necessary for ethanol to compete with gasoline. Biodiesel from waste vegetable oil is already nearly competitive with conventional diesel, which cannot be said of biodiesel made from far more expensive virgin oils. It is foreseen that within the next 5–10 years, renewable, alternative transportation fuels from biomass and wastes will be competitive with fuels derived from petroleum at about US $ 0.2 per liter.

Generally, economic restrictions force industrial processes to work in a very small range of operating conditions. For some batch processes which have long operating times in each cycle and depend strongly on the operating variables, it is very important to define the optimum conditions to achieve sufficient profitability. Kinetic models describing the behavior of microbiological systems can be a highly appreciated tool and can reduce tests to eliminate extreme possibilities.

Most promising prospects

Ethanol fermentation involves significantly greater challenges, owing to the necessity of converting xylose as well as glucose to ethanol in the process, the microorganism–enzyme compatibility in SSF, and the low rates of cellulose hydrolysis. Recently, research has concentrated on the development of improved processes; however, there are still challenges that need further investigations.

Fermentation technology converting xylose to ethanol

Major fermentable sugars in hydrolyzate from cellulose and hemicellulose are glucose and xylose. Glucose fermentation to ethanol can be carried out efficiently by *S. cerevisiae*. In contrast, xylose fermentation is challenging because only a few traditional ethanol-producing microorganisms can readily ferment xylose, though many microorganisms utilize xylose as a carbon source. Efforts were made to improve ethanol fermentation from xylose (Jeffries and Shi 1999; Ho et al. 1999; Ingram et al. 1987; Zhang et al. 1995).

However, low ethanol yields, by-product formation, neutral pH requirement for growth, and intolerance to high ethanol concentration are disadvantages in using bacteria in large-scale fermentation (Bothast et al. 1999). Currently, the bacterial conversion of xylose to ethanol has been studied mostly with utilizing the recombinant microorganisms.

The recombinant *E. coli* was used for ethanol production from xylose, and this ethanologenic strain (KO11) was able to convert glucose and xylose to ethanol at yields of 103–106% of theoretical value (Gonzalez et al. 2003; Tao et al. 2001). The extra ethanol was thought to arise from fermentation of carbohydrates present in the rich medium that was not accounted for in the sugar balance. Moreover, KO11 grows faster on xylose-containing medium than its parent strain ATCC11303. Comparison of global gene expression by microarray technology demonstrated that KO11 overexpresses xylose metabolism genes (Tao et al. 2001). During the combination, two genes are needed, one for pyruvate decarboxylase and another for alcohol dehydrogenase. These enzymes working together in the cell will divert pyruvate away from other fermentation products to ethanol. This would convert *E. coli* into an ethanologenic microorganism. The steps by initial *E. coli* and ethanologenic *E. coli* in alcoholic fermentation are shown in Fig. 3 (Gottschalk 1986; Matthew et al. 2005).

Similarly, the ethanol-producing bacterium *Z. mobilis* was metabolically engineered to broaden its range of fermentable substrates to include the pentose sugar xylose. Two operons encoding xylose assimilation and pentose phosphate pathway enzymes were constructed and transformed into *Z. mobilis*. The recombinant efficiently fermented both glucose and xylose, which is essential for economical conversion of lignocellulosic biomass to ethanol (Ingram and Doran 1995; Lynd et al. 2002; McMillan et al. 1999; Sun and Cheng 2002; Zhang et al. 1995). Currently, bacteria modified by this approach must operate at neutral pH where control of invasion by other organisms is more difficult than at the more acidic pH levels typical of most yeasts.

Moreover, Tolan and Finn (1987) transformed *Klebsiella planticola* ATCC 33531 with multicopy plasmids containing the *pdc* gene inserted from *Z. mobilis*, and expression of the gene markedly increased the yield of ethanol to 1.3 mol per mole of xylose, or 25.1 g/l. Concurrently, there was significant decrease in the yield of other organic by-products (i.e., formate, acetate, lactate and butanediol).

There have also been several yeast strains which were capable of fermenting xylose to produce ethanol in batch culture. Fein et al. (1984) isolated seven strains which were capable of fermenting xylose to produce ethanol from crude wood hydrolyzate in batch culture. Xylitol was found to be one of the major by-products, and the amount of xylitol varied depending on the strain used. *Candida tropicalis* showed the greatest potential for ethanol production from xylose. The crude acid hydrolyzate was inhibitory to all strains of yeast, even at dilute hydrolyzate concentrations. Strain acclimatization and chemical pretreatment resulted in a marked increase in utilization of substrates in acidic crude hydrolyzate. In an attempt to develop a xylose-fermenting yeast for industrial ethanol production, UV light-induced mutants of *Pachysolen*

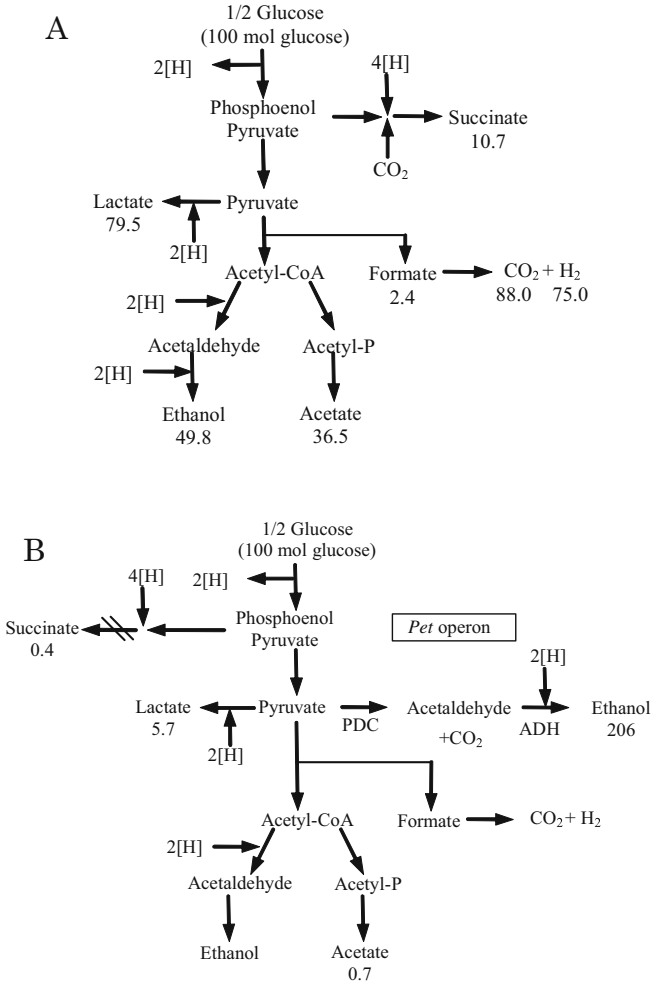

Fig. 3 **a** Typical fermentation products made by a K12 *Escherichia coli* fermenting glucose. Products are in moles produced per 100 mol fermented glucose (Dien et al. 2003; Gottschalk 1986) with 91% of the carbon accounted for as fermentation products. **b** Transforming *E. coli* with pet operon diverts almost all glucose to ethanol. This strain (KO11) also carries a mutation that blocks succinate production. Amount of each fermentation product is shown per 100 mol glucose (Dien et al. 2003; Ohta et al. 1991). Moles of CO_2 produced was not measured, but should be 206 mol based on ethanol production

tannophilus have been isolated, which can grow faster on xylose. Several other yeast strains for xylose utilization have been reported (Jeewon 1997).

On the other hand, *S. cerevisiae* traditionally has been used for ethanol production, such as beer and wine fermentation. This yeast does not exhibit many of the limitations encountered with bacteria. However, *S. cerevisiae* is not able to ferment xylose. Therefore, metabolic engineering of xylose fermentation in *S. cerevisiae* is an attractive approach (Sonderegger and Sauer 2003).

Although some significant progress can be noted in this field, there are still some problems which exist. One of them is ethanol inhibition. Ethanol inhibition of yeasts and other microorganisms has received much attention in microbial conversion of xylose to ethanol (Ghasem et al. 2004; Jeewon 1997; Palmqvist and Hahn-Hagerdal 2000). Xylose-fermenting yeasts do not grow under anoxic conditions and do not ferment when fully aerobic. Therefore, development of fermentation glucose and xylose efficiently is required for large-scale industrial application.

Cellulase enzyme

Using lignocellulosic materials such as agricultural residues, grasses, forestry wastes, and other low-cost biomass can significantly reduce the cost of raw materials (compared to corn) for ethanol production. A reduction of the cost of ethanol production can be achieved by reducing the cost of either the raw materials or the cellulase enzymes. It was predicted that the use of genetically engineered raw materials with higher carbohydrate content combined with the improvement of conversion technology could reduce the cost of ethanol by US $0.11 per liter over the next 10 years (Wooley et al. 1999).

Xylose metabolism employs pathways distinctly different from those involved in the utilization of glucose. With most yeast, xylose metabolism requires aerobic conditions at which cellular respiration is promoted; however, xylose is fermented to ethanol in poor yields and at low rates. To get around this problem, it has been proposed that the xylose fraction first be converted to readily fermentable xylulose, i.e., enzyme-mediated fermentation of xylose to ethanol using the bacterial enzyme, xylose isomerase.

Reducing the cost of cellulase enzyme production is a key issue in the enzymatic hydrolysis of lignocellulosic materials. Genetic techniques have been used to clone the cellulase coding sequences into bacteria, yeasts, fungi, and plants to create new cellulase production systems with possible improvement of enzyme production and activity. Riley et al. (1996) and Wood et al. (1997) reported the expression of recombinant endoglucanase genes from *Erwinia chrysanthemi* P86021 in *E. coli* KO11, and the recombinant system produced 3,200 IU endoglucanase/l fermentation broth (IU, international unit, defined as a micromole of reducing sugar as glucose released per minute using carboxymethyl cellulose as substrate). The thermostable endoglucanase E1 from *Acidothermus cellulolyticus* was expressed in *Arabidopsis thaliana* leaves (Ziegler et al. 2000), potato (Dai et al. 2000), and tobacco (Hooker et al. 2001).

Immobilization

As in the case of microalgae culture in open ponds, microecological engineering techniques will need to be developed to maintain such strains in large systems which could be subject to invasion and contamination by potentially much faster growing wild microbes. Such microecological techniques would relieve the constraints of having to maximize the amounts and activities of the enzymes used in this process and/or maintain strictly aseptic conditions which are not economical. If intact mi-

crobial cells are directly immobilized, the removal of microorganisms from downstream product can be omitted, and the loss of intracellular enzyme activity can be kept to a minimum level (Najafpour 1990).

Use of biofilm reactors for ethanol production has been investigated to improve economics and the performance of fermentation processes (Vega et al. 1988). Immobilization of microbial cells for fermentation has been developed to eliminate inhibition caused by high concentration of substrate and product and also to enhance productivity and yield of ethanol. The work on ethanol production in an immobilized cell reactor (ICR) showed that production of ethanol using *Z. mobilis* was doubled (Ghasem et al. 2004; Takamitsu et al. 1993). The immobilized recombinant *Z. mobilis* was also successfully used with high concentrations of 12–15% sugar (Yamada et al. 2002).

Recently, immobilized biomass activity has been given more attention since it has been acknowledged to play a significant role in bioreactor performance (Gikas and Livingston 1997; Yamada et al. 2002). Frequently, immobilized cells are subjected to limitations in the supply of nutrients to the cells. Thus, because of the presence of heterogeneous materials such as immobilized cells, there is no convective flow inside the beads and the cells can receive nutrients only by diffusion (Riley et al. 1996). Immobilization of cells to a solid matrix is an alternative means of high biomass retention. The cells divide within and on the core of the matrix (Senthuran et al. 1997).

Simultaneous saccharification and fermentation

Simultaneous saccharification and fermentation (SSF) gives higher reported ethanol yields and requires lower amounts of enzyme because end-product inhibition from cellobiose and glucose formed during enzymatic hydrolysis is relieved by the yeast fermentation (Banat et al. 1998; McMillan et al. 1999). However, it is not easy to meet all the requirements of industry due to their low rates of cellulose hydrolysis, which is the stage limiting the rate of alcohol production. Another problem arises from the fact that most microorganisms used for converting cellulosic feedstock cannot utilize xylose, a hemicellulose hydrolysis product. Moreover, SSF requires that enzyme and culture conditions be compatible with respect to pH and temperature. *T. reesei* cellulases, which constitute the most active preparations, have optimal activity at pH 4.5 and 55°C. For *Saccharomyces* cultures, SSF are typically controlled at pH 4.5 and 37°C.

To overcome the problems related to SSF, many species of yeasts, as well as the bacterium *Z. mobilis*, have been tested with cellulases produced by *T. reesei* mutants (Chaudhuri and Sahai 1993; Haltrich et al. 1994; Spindler et al. 1992). The currently most promising ethanologenic bacteria for industrial exploitation are *E. coli*, *Klebsiella oxytoca*, and *Z.mobilis* (Matthew et al. 2005). Genetic engineering made it possible to transfer cellulose genes from *Trichoderma* to *S. cerevisiae* (Shoemaker 1984). However, the cellulases were produced at a concentration too low to be useful. There is a group of microorganisms (*Clostridium*, *Cellulomonas*, *Trichoderma*, *Penicillium*, *Neurospora*, *Fusarium*, *Aspergillus*, etc.) showing a high cellulolytic and hemicellulolytic activity, which are also highly capable of fermenting monosaccharides to ethanol. It may be possible, within this group of microorganisms, to produce "superstrains" via genetic engineering capable of hydrolyzing cellulose and xylan along with fermentation of glucose and xylose to ethanol.

Moreover, to make the SSF process more effective, it has also been found necessary to search for thermostable strains capable of producing substantial amounts of ethyl alcohol at temperatures optimal for saccharification and suitably resistant to ethanol (Szczodrak and Targonski 1988).

Roychoudhury et al. (1992) have developed a notable way of eliminating the negative effects which excessive concentrations of ethanol have on yeast activity and cellulased within the SSF system. They used a vacuum cycling reactor where the concentration of ethanol was kept at a relatively low level by its removal from the flash chamber.

However, more efforts have to be made in the development of microorganisms for industrial ethanol production. In addition, it is important to keep the rate-limiting step in mind. In SSF, the ethanol production rate is controlled by the cellulase hydrolysis rate and not the glucose fermentation, and hence, steps to increase the rate of hydrolysis will lower the cost of ethanol production via SSF.

Sugar conversion

Since sugars are already available in a degradable form and yeast cells can metabolize sugars directly, these substrates require the least costly preparation. The other carbohydrates must be hydrolyzed to sugars before they can be metabolized. Several studies have dealt with the economic assessment of using cellulose hydrolysate, either from waste (Cysewski and Wilke 1976; Green et al. 1989; Maiorella et al. 1984; Wilke et al. 1976) or from wood (Hinman et al. 1992; Marco et al. 2002). One disadvantage with the application of these materials is their low sugar content resulting in low cell and ethanol concentrations. Hence, although starchy or cellulosic materials are cheaper than sugar-containing raw materials, the requirement for converting the starchy or cellulosic materials to fermentable sugars is a disadvantage of these substrates (Lynd et al. 2001).

Moreover, microorganisms used in industry are selected to provide the best possible combination of characteristics for the process and equipment being used. The selected strains should have tolerance to high concentrations of sugar and ethanol (Keim 1983; Oh et al. 2000).

Ethanol inhibition of yeasts and other microorganisms has received much attention (Casey and Ingledew 1986) in microbial production of ethanol. Lucas and van Uden (1985) investigated the effects of temperature on ethanol tolerance and thermal death of *Candida shehatae* and

determined that it was more tolerant of ethanol at lower temperatures. Du Preez et al. (1987) quantitatively evaluated the effects of ethanol on the growth of the xylose-fermenting yeasts *C. shehatae* and *Pichia stipitis* using Luong kinetics. The effect of ethanol on metabolic rate has been examined with ethanol added exogenously. Both Lucas and van Uden (1985) and du Preez et al. (1987) placed cells into media containing different concentrations of ethanol and measured the specific growth rate which ensued. Unfortunately, less inhibition is observed with exogenous ethanol than with the same concentration of ethanol produced endogenously (Hoppe and Hansford 1982; Novak et al. 1981; du Preez et al. 1987). Some have claimed that the apparently greater inhibition by endogenously produced ethanol reflects the tendency of actively fermenting cells to accumulate ethanol intracellularly (Casey and Ingledew 1986; Ghasem et al. 2004); however, the yeast plasma membrane is known to be very permeable to ethanol, which casts doubt on this hypothesis. Whatever the reason for the different effects of externally added and internally generated ethanol, realistic assessments of ethanol inhibition ought to involve ethanol generated in situ.

On the other hand, since the distillation cost per unit amount of ethanol produced is substantially higher at low ethanol concentrations (Zacchi and Axelsson 1989), several investigators have dealt with the idea of concentrating sugar solutions prior to fermentation (Cysewski et al. 1976; Iraj et al. 2002; Oh et al. 2000; Zacchi and Axelsson 1989). Clearly, it is necessary to solve the problem between the concentration of ethanol produced and sugar added if an economically sustainable system is to be created using this method.

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