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Review

Ethanol as an alternative fuel from agricultural, industrial and urban residues

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Abstract

With world reserves of petroleum fast depleting, in recent years ethanol has emerged as most important alternative resource for liquid fuel and has generated a great deal of research interest in ethanol fermentation. Research on improving ethanol production has been accelerating for both ecological and economical reasons, primarily for its use as an alternative to petroleum based fuels. Field crops offer potential source of fuel, offering promise as large-scale energy and based on its genetic diversity, climatic adaptation, biomass and sugar production. Lignocellulosic biomass is the most abundant organic raw material in the world. Production of ethanol from renewable lignocellulosic resources may improve energy availability, decrease air pollution and diminish atmospheric CO_2 accumulation. The aim of the present review is to highlight on major agricultural, industrial and urban waste, which could be used for ethanol production in an ecofriendly and profitable manner. Primarily, the utilization of these wastes for ethanol production will reduce dependency on foreign oil and secondly, this will remove disposal problem of wastes and make environment safe from pollution.

Keywords: Ethanol; Alternate energy resource; Agriculture; Industry; Urban; Residue

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Contents

1. Introduction

In recent years, the consumption of petroleum products in India has been increasing at an annual growth rate of 5–6% (TERI, 2002). The total gasoline consumption increased from 3.6 Mt in 1990–1991 to 6.6 Mt during 2000/2001 (MoPNG, 2002). Due to diminishing fossil fuel reserves, alternative energy sources need to be renewable, sustainable, efficient, cost effective, convenient and safe (Chum and Overend, 2001). The transportation sector particularly depends upon about 77% imported oil, in addition to strategic vulnerability, such large dependence upon foreign oil cause significant economic difficulties. According to an estimation of Ministry of Petroleum and Natural gas, India has imported about 90 Mt of crude oil during the year 2003–2004, causing a heavy burden on foreign exchange (Balu, 2003). The demand of crude oil increases dramatically year by year. Every one dollar rise in crude oil prices inflates the import bill by about 620 million dollars per year (Badger, 2002). The country's production and import of crude oil is given in Table 1. The demand of fuel (gasoline and diesel) has been estimated about 80 Mt for the year 2011–2012, which is about 1.3 times higher than that of current demand (Planning Commission, GOI, 2003), while it is

Table 1
Production and import of crude oil in India

Year	Production (Mt)	Import (Mt)	Total (Mt)
1971	6.8	11.7	18.7
1981	10.5	16.2	26.7
1991	33.0	20.7	53.7
2000	32.0	57.9	89.9
2003–2004	33.4	90.4	123.8

Source: Ministry of Petroleum and Natural Gas, Government of India (2002).

Table 2
Demand of gasoline and diesel in India

Year	Gasoline (Mt)	Diesel (Mt)
2002–2003	7.62	42.15
2003–2004	8.20	44.51
2004–2005	8.81	46.97
2005–2006	9.42	49.56
2006–2007	10.07	52.33
2011–2012	12.85	66.90

Source: Planning Commission, Government of India (2003).

expected that crude oil production will start declining from 2010 (Campbell and Laherrere, 1998), therefore, alternative energy source is only option to fulfill the requirement in future (Table 2). The use of ethanol as an alternative motor fuel has been steadily increasing around the world for several reasons. Domestic production and use of ethanol for fuel can decrease dependence on foreign oil, reduce trade deficits, create jobs in rural area and reduce air pollution and carbon dioxide build-up (Badger, 2002).

Ethanol, being an excellent transportation fuel can be used as blend with gasoline, 10 and 22% blends are being used in the US and Brazil, respectively (Wyman, 1994). It is an oxygenated fuel that contains 35% oxygen, which reduces particulate and NOx emission from combustion. It may be used directly (95% ethanol and 5% water) as a fuel, such nearly pure ethanol fuel provides a number of environment benefits, due to their low pressure and reduced emission of ethanol in to the atmosphere along with their clean burning characteristics (Lynd et al., 1991). Ethanol-blended with gasoline oxygenates it thereby reducing the formation of carbon monoxide and ozone, which is desirable for the implementation of Clean Air Act Amendments (Wyman, 1994). With the increasing shortage of petroleum, urban air pollution and accumulation of carbon dioxide in the atmosphere, ethanol is expected to play a more significant role in the future. Government of India through a notification dated September 2002 made 5% ethanol-blending mandatory in petrol, in nine states and three Union Territories (The Gazette of India, 2002). In the next phase, supply of ethanol-blended petrol would be extended to the whole country and efforts would be made to increase the percentage of ethanol mixture in petrol to 10% (The Hindu, 2003).

According to a document published by Government of India, Ministry of Petroleum and Natural Gas for 5% ethanol-blend in gasoline required 500 M l ethanol per annum. Ethanol has about two-thirds the energy and heat value of petrol, but results in more efficient combustion. While more fuel is needed to run the same distance, the emissions are cleaner. When added in small quantities to unleaded petrol, as envisaged in India, it acts as an octane booster, replacing the conventional additive for this purpose (meta tertiary butyl ether, which can create adverse health impacts). When up to 15% of ethanol is blended with diesel and used in unmodified diesel engines, it is known to greatly reduce visible smoke. Smoke and smog are the greatest problems that diesel vehicles cause in congested cities (Warrier, 2002).

The increased realization of the finite nature of the world's oil supplies and vagaries in oil prices have rekindled interest in production of potable and industrial alcohol by fermentation of carbohydrate containing raw materials. Brazilian effort to reduce petroleum imports by adding ethanol to motor fuels is an interesting attempt in this direction. The rapid

increase in the price of crude oil and fear of the potential use of oil as a political weapon, since 1973, had led to the search of substitutes of liquid transport fuels particularly the renewable energy sources (Coombs, 1981; Perlman, 1978). Biomass has been shown to have considerable promise as a raw material for gaseous fuel, liquid fuel and certain petrochemical intermediates (Gutierrez-Correa et al., 1999; Yamada and Uno, 1999). Hence, it is possible to use agricultural, industrial and urban residues for energy production.

Agricultural, industrial and urban residues are abundant in most of the developing countries like India, which could be used for production of an important alternative fuel and cheap energy sources as ethanol. Therefore, knowing about the potentiality of these residues for ethanol production is very essential and beneficial for reducing pressure on energy sources and ecofriendly utilization of these residues.

2. Scope of ethanol production from alternative materials

Unlike fossil fuels, ethanol is a renewable energy source produced through fermentation of sugars. A dramatic increase in ethanol production using the current corn starch based technology may not be practical because corn production for ethanol will compete for the limited agricultural land needed for food and feed production. A potential source for low cost ethanol production is to utilize lignocellulosic materials such as crop residues, grasses, sawdust, wood chips, solid animal waste (Sun and Cheng, 2002) and industrial wastes.

2.1. Availability of agricultural residues and other wastes

Straw, a low-density residue, is the dominant residue. Rice husk, a byproduct of rice milling, accounts for 20% of paddy. Unlike the cereals, crops such as red gram, cotton, rapeseed, mustard, mulberry and plantation crops produce woody (ligneous) residues. Residue production for mulberry, coconut and sugarcane were estimated based on field studies (CES, 1995; Ravindranath and Hall, 1995). Industrial wheat bran usually accounts for 14–19% of the grain and comprises the outer coverings, the aleurone layer and the remnants of the starchy endosperm (Pomeranz, 1988). It consists mainly of starch, arabinoxylans, cellulose, β-glucan, protein and lignin (Maes and Delcour, 2001; Schooneveld-Bergmans et al., 1999) and has the potential to serve as low cost feedstock to increase the production of fuel ethanol (Palmarola-Adrados et al., 2005). The total crop residue production in India during 1996–1997 is estimated to be 626 Mt of air-dry weight (Table 3). The dominant residues are those of rice, wheat, sugarcane and cotton accounting for 66% of the total residue production. Sugarcane and cotton residue production is 110 and 50 Mt, respectively. Crop residues, which are used as fodder, will not be available as feedstock for energy. The total potential of non-fodder crop residues available for energy is estimated to be 325 and 450 Mt for 1996–1997 and 2010, respectively (Table 3). Only the woody (ligneous) crop residues, rice husk and bagasse are considered for energy production (Ravindranath et al., 2005).

Municipal solid waste (MSW) is normally collected, transported and dumped in the outskirts of towns and cities. Though sorting out for the recyclable materials by the rag pickers is common, other ways of handling, like composting, incineration, ethanol production, etc., also take place to some extent. The total quantity of solid wastes generated in larger towns

Table 3
Total crop residue production and their availability for ethanol production in India

Crop	Total residue production (Mt air-dry weight)		Non-fodder crop residues (Mt air-dry weight)	
	1996–1997	2010	1996–1997	2010
Rice	146.5	213.9	26.7	41.0
Wheat	110.6	157.6	8.8	21.4
Jowar	22.3	12.2	–	–
Bajra	15.8	13.6	0.0	1.4
Maize	26.3	32.5	5.3	–
Other cereals	9.4	2.8	–	6.2
Red gram	13.5	11.2	13.5	11.2
Gram	9.3	13.5	9.3	13.5
Other pulses	17.1	17.1	17.1	16.5
Ground nut	20.7	28.1	20.7	28.1
Rapeseed and mustard	13.8	24.1	13.8	24.1
Other oil seeds	18.2	27.1	18.2	27.1
Cotton	50.0	55.7	50.0	55.7
Jute	15.7	10.5	15.7	5.9
Sugar cane	110.8	185.4	99.7	163.5
Coconut + arecanut	20.0	28.2	20.0	28.2
Mulberry	3.0	3.3	3.0	3.3
Coffee + tea	3.42	3.9	3.4	3.9
Total	626.5	840.6	325.3	450.7

Source: CES (1995) and CMIE (1997).

and cities has been estimated at 20.7 Mt annually for an urban population of 217 million in 1991. This is expected to increase to 56 Mt by 2010 (Ahmed and Jamwal, 2000) as the urban population is increasing at a decadal growth rate of above 40%. The quantity of wastes generated per family in a week has also increased substantially from 7 kg during 1980s to 20–30 kg at present. In India, based on 1991 census data, the estimated quantity of MSW generated in 10 major cities is more than 10 Mt annually. The disposal of such huge quantities has become a major problem. Thus, the utilization of MSW for energy production would mean a solution of this problem. In addition to MSW, large quantity of wastewater is generated in certain industrial plants like breweries, sugar mills, distilleries, food-processing industries, tanneries, and paper and pulp industries. Out of this, food products and agro-based industries together account for 65–70% of the total industrial wastewater in terms of organic load (Pachauri and Sridharan, 1998). Ravindranath et al. (2005) estimated about 1056,730 Mm^3 wastewater generated in India by industries. Hence, agricultural, urban and industrial residue is present in huge amount and increasing day by day, which could be utilized for ethanol production to reduce its demand and it also be reduced disposal problem of wastes and will help to make clean environment.

2.2. *Technologies involved in ethanol production from wastes*

Extensive research has been completed on the conversion of lignocellulosic materials to ethanol production in the last two decades (Azzam, 1989; Dale et al., 1984; Duff and

Table 4
Cellulose, hemicellulose and lignin content in common agricultural residues and wastes

Agricultural residue	Cellulose	Hemicellulose	Lignin
Hardwood stem	40–50	24–40	18–25
Softwood stem	45–50	25–35	25–35
Nut shells	25–30	25–30	30–40
Corn cobs	45	35	15
Grasses	25–40	35–50	10–30
Wheat straw	33–40	20–25	15–20
Rice straw	40	18	5.5
Leaves	15–20	80–85	0
Sorted refuse	60	20	20
Cotton seed hairs	80–90	5–20	0
Coastal Bermuda grass	25	35.7	6.4
Switch grass	30–50	10–40	5–20
Solid cattle manure	1.6–4.7	1.4–3.3	2.7–5.7
Swine waste	6.0	28	
Primary wastewater solids	8–15	NA	24–29
Paper	85–99	0	0–15
Newspaper	40–55	25–40	18–30
Waste papers from chemical pulps	60–70	10–20	5–10

Source: Boopathy (1998), Cheung and Anderson (1997), Dewes and Hunsche (1998), Kaur et al. (1998), McKendry (2002) and Reshamwala et al. (1995).

Murray, 1996; Martin et al., 2002; Reshamwala et al., 1995; Yanase et al., 2005). This conversion includes two processes: (i) hydrolysis of cellulose in the lignocellulosic materials to fermentable reducing sugars and (ii) fermentation of the sugars to ethanol. The hydrolysis is usually, catalyzed by cellulase enzymes and the fermentation is carried out by yeast or bacteria. The factors that have been identified to affect the hydrolysis of cellulose include porosity, i.e., accessible surface area of the waste materials, cellulose fiber crystallinity and lignin and hemicellulose content (McMillan, 1994). The presence of lignin and hemicellulose makes the access of cellulose enzymes to cellulose difficult, thus reducing the efficiency of the hydrolysis. The contents of cellulose, hemicellulose and lignin in common agricultural residues and wastes are presented in Table 4. Removal of lignin and hemicellulose, reduction of cellulose crystallinity and increase of porosity in pretreatment processes can significantly improve the hydrolysis (McMillan, 1994). Pretreatment must meet the following requirements: (1) improve the formation of sugars or the ability to subsequently form sugars by enzyme hydrolysis; (2) avoid the degradation or loss of carbohydrate; (3) avoid the formation of byproducts inhibitory to subsequent hydrolysis and fermentation processes; (4) be cost effective (Sun and Cheng, 2002).

2.3. Pretreatment of lignocellulosic materials

Waste materials can be comminuted by a combination of chipping, grinding and milling to reduce cellulose crystallinity (Sun and Cheng, 2002). Vibratory ball milling has been found to be more effective in breaking down the cellulose crystallinity of spruce and aspen chips and improving the digestibility of the biomass than ordinary ball milling (Millet et al.,

1976). Pyrolysis has also been used for pretreatment of lignocellulosic materials. When the materials are treated at temperatures greater than 300 °C, cellulose rapidly decomposes to produce gaseous products and residual char (Shafizadeh and Bradbury, 1979). The decomposition is much slower and less volatile products are formed at lower temperatures. Steam explosion is the most commonly used method for pretreatment of lignocellulosic materials (McMillan, 1994). In this method, chipped biomass is treated with high-pressure saturated steam and then the pressure is swiftly reduced, which makes the materials undergo an explosive decomposition. The process causes hemicellulosic degradation and lignin transformation due to high temperature, thus increasing the potential of cellulose hydrolysis (Sun and Cheng, 2002). Addition of H_2SO_4 or SO_2 or CO_2 in steam explosion can effectively improve enzymatic hydrolysis, decrease the production of inhibitory compounds and lead to more complete removal of hemicellulose (Morjanoff and Gray, 1987). The advantages of steam explosion pretreatment include the low energy requirement compared to mechanical comminution and no recycling or environmental costs. The conventional mechanical methods require 70% more energy than steam explosion to achieve the same size reduction (Holtzapple et al., 1989). Limitations of steam explosion include disruption of the lignin–carbohydrate matrix and generation of compounds that may be inhibitory to microorganisms used in downstream processes (Mackie et al., 1985). Because of the formation of degradation products that are inhibitory to microbial growth, enzymatic hydrolysis and fermentation, pretreated biomass needs to be washed by water to remove the inhibitory materials along with water soluble hemicellulose (McMillan, 1994).

Ammonia fiber explosion (AFEX) is another type of physico-chemical pretreatment in which lignocellulosic materials are exposed to liquid ammonia at high temperature and pressure for a period of time and then the pressure is swiftly reduced. AFEX pretreatment can significantly improve the saccharification rates of various herbaceous crops and grasses. It can be used for the pretreatment of many lignocellulosic materials including alfalfa, wheat straw, wheat chaff (Mes-Hartree et al., 1988), barley straw, rice straw, corn stover (Vlasenko et al., 1997), municipal solid waste, soft wood news paper, kenaf news paper (Holtzapple et al., 1992), coastal Burmuda grass, switch grass (Reshamwala et al., 1995), aspen chips (Tengerdy and Nagy, 1988) and bagasse (Holtzapple et al., 1991). The AFEX pretreatment does not significantly solubilize hemicellulose compared to acid pretreatment and acid catalyzed steam explosion (Mes-Hartree et al., 1988; Vlasenko et al., 1997). McMillan (1994) reported that AFEX process was not very effective for biomass with high lignin content such as newspaper (18–30% lignin) and aspen chips (25% lignin). The ammonia pretreatment does not produce inhibitors for the down stream biological processes, so water wash is not necessary (Dale et al., 1984; Mes-Hartree et al., 1988). AFEX pretreatment does not require small particle size for efficacy (Holtzapple et al., 1990). CO_2 explosion is also used for pretreatment of lignocellulosic materials. It was hypothesized that CO_2 would form carbonic acid and increase the hydrolysis rate. The yield was relatively low compared to steam or ammonia explosion pretreatment, but high compared to the enzymatic hydrolysis without pretreatment (Dale and Moreira, 1982). Zheng et al. (1998) compared CO_2 explosion with steam and ammonia explosion for pretreatment of recycled paper mix, sugarcane bagasse and repulping waste of recycled paper and found that CO_2 explosion was more cost effective than ammonia explosion and did not cause the formation of inhibitory compounds that could occur in steam explosion.

Ozone can be used to degrade lignin and hemicellulose in many lignocellulosic materials such as wheat straw (Ben-Ghedalia and Miron, 1981), bagasse, green hay, peanut, pine (Neely, 1984), cotton straw (Ben-Ghedalia and Shefet, 1983) and popular sawdust (Vidal and Molinier, 1988). The degradation was essentially limited to lignin and hemicellulose was slightly attacked but cellulose was hardly affected. Ozolysis pretreatment has few advantages, such as; it effectively removes lignin and does not produce toxic residues for the down stream process. The reactions are also carried out at room temperature and pressure (Vidal and Molinier, 1988). However, a large amount of ozone is required, making the process expensive (Sun and Cheng, 2002). In acid hydrolysis, concentrated sulfuric and hydrochloric acids have been used to treat lignocellulosic materials. Although they are powerful agent for cellulose hydrolysis but concentrated acids are toxic, corrosive and hazardous and requires reactors that are resistant to corrosion. In addition, concentrated acid must be recovered after hydrolysis to make the process economically feasible (Sivers and Zacchi, 1995). Thereafter, dilute acid hydrolysis has been successfully developed for pretreatment of lignocellulosic materials. The dilute sulfuric acid pretreatment can achieve high reaction rates and significantly improve cellulose hydrolysis (Esteghlalian et al., 1997). Recently in a study Lloyed and Wyman (2005) concludes that acid accelerated the rate of xylan solubilization relative to xylose degradation, resulting in higher maximum yields. Dilute acid hydrolysis process use less severe conditions and achieve high xylan to xylose conversion yields. Achieving high xylan to xylose conversion yields is necessary to achieve favorable overall process economics because xylan accounts for up to a third of the total carbohydrate in many lignocellulogic materials (Hinman et al., 1992). There are primarily two types of dilute acid pretreatment processes: high temperature (>160 °C), continuous flow process for low solid loading (5–10%) (Converse et al., 1989) and low temperature (<160 °C), batch process for high solid loading (10–40%) (Esteghlalian et al., 1997). Although dilute acid pretreatment can significantly improve the cellulose hydrolysis, its cost is usually higher than some physico-chemical pretreatment processes such as steam explosion or AFEX. A neutralization of pH is necessary for the down stream enzymatic hydrolysis or fermentation processes (Sun and Cheng, 2002). Studies of Liu and Wyman (2003, 2004) showed that increasing flow rate significantly enhanced removal of hemicellulose and lignin for pretreatment with compressed-hot water or very dilute sulfuric acid at elevated temperatures.

Alkaline hydrolysis can also be used for pretreatment of lignocellulosic materials and the effect of alkaline pretreatment depends on the lignin content of the materials (McMillan, 1994). The mechanism of alkaline hydrolysis is believed to be saponification of intermolecular ester bonds crosslinking xylan hemicellulose and other components. The porosity of lignocellulosic materials increases with the removal of crosslinks (Tarkow and Feist, 1969). Dilute NaOH treatment of lignocellulosic materials caused swelling, leading to an increase in internal surface area, a decrease in crystallinity, separation of structural linkage between lignin and carbohydrates, and disruption of the lignin structure (Fan et al., 1987). Using alkaline chemicals to remove lignin has been known to improve cellulose digestibility for years, but sodium hydroxide and other bases are too expensive and too difficult to recover and recycle to make them viable for producing fuels and chemicals (Hsu, 1996). However, pretreatment with ammonia has more recently been shown to be effective in improving cellulose digestion with the advantage of ammonia being recyclable due to its volatility (Wyman

et al., 2005a). Ammonia decrystallizes crystalline cellulose and deacetylates acetyl linkages (Gollapalli et al., 2002; Mitchell et al., 1990). Both of these effects increase the enzymatic hydrolysis of cellulose.

Lignin biodegradation could be catalyzed by the peroxidase enzyme with the presence of H_2O_2 (Azzam, 1989). The pretreatment of cane bagasse with H_2O_2 greatly enhanced its susceptibility to enzymatic hydrolysis. Bjerre et al. (1996) used wet oxidation and alkaline hydrolysis of wheat straw and achieved 85% conversion yield of cellulose to glucose. In the organosolvent process, an organic or aqueous organic solvent mixture with inorganic acid catalysts is used to break the internal lignin and hemicellulose bonds. Solvents used in the process need to be drained from the reactor, evaporated, condensed and recycled to reduce the cost. Removal of solvents from the system is necessary because the solvent may be inhibitory to the growth of organisms, enzyme hydrolysis and fermentation (Sun and Cheng, 2002).

In biological pretreatment processes, microorganisms such as brown, white and soft rot fungi are used to degrade lignin and hemicellulose in waste materials (Schurz, 1978). Brown rots mainly attack cellulose, while white and soft rots attack both cellulose and lignin. White rot fungi are the most effective basidiomycetes for biological pretreatment of lignocellulogic materials (Fan et al., 1987). The white rot fungus *Phanerochaete chrysosporium* produces lignin-degrading enzymes, lignin peroxidases and manganese-dependent peroxidases, during secondary metabolism in response to carbon or nitrogen limitation (Boominathan and Reddy, 1992). Both enzymes have been found in the extracellular filtrates of many white rot fungi for the degradation of wood cell walls (Waldner et al., 1988). Other enzymes including polyphenol oxidases, laccases, H_2O_2 producing enzymes and quinine-reducing enzymes can also degrade lignin (Blanchette, 1991). The advantages of biological pretreatment include low energy requirement and mild environmental conditions. However, the rate of hydrolysis in most biological process is very low (Sun and Cheng, 2002). Most of the sugars are released as oligomers that must still be hydrolyzed prior to fermentation by typical organisms (Heitz et al., 1991). Furthermore, sugar oligomers and lignin compounds solubilized during autohydrolysis precipitate when cooled and reattach to the pretreated fibers, impeding cellulose digestion. Low solids pretreatment or hot washing can reduce these compounds and enhance the digestion of the resulting fibers (Jacobsen and Wyman, 2002; Nagle et al., 2002).

As expected, dilute acid, neutral pH, and water only pretreatments solubilized mostly hemicellulose whereas addition of lime or percolation with ammonia removed mostly lignin. On the other hand, when ammonia was released at the end of the pretreatment process via AFEX, neither lignin nor hemicellulose was physically removed from cellulose and other components. When water was pushed through biomass in a flow through mode, virtually all of the hemicellulose and up to about 75% of the lignin were removed with or without addition of very dilute sulfuric acid (Wyman et al., 2005b). In a comparative study for pretreatment technologies to sugar recovery from corn stover Wyman et al. (2005b) suggested that each pretreatment made it possible to subsequently achieve high yields of glucose from cellulose by cellulase enzymes, and the cellulase formulations used were effective in solubilizing residual xylan left in the solids after each pretreatment. Thus, overall sugar yields from hemicellulose and cellulose in the coupled pretreatment and enzymatic hydrolysis operations were high for all of the pretreatments with corn stover.

Furfural is an important inhibitor of ethanol production from hemicellulose hydrolysate (Azhar et al., 1981). Even at low concentrations (3–15 mM), furfural can severely affect rate of ethanol production and final conversion, thus creating an unwanted limitation in ethanol production processes (Beall et al., 1991; Ranatunga et al., 1997; Taherzadeh et al., 1999a,b; Zaldivar et al., 1999). Various bacteria and yeast have been reported to partially transform furfural to either furfuryl alcohol or furoic acid, or a combination of both (Boopathy and Daniels, 1991; Boopathy et al., 1993; Gutierrez et al., 2002; Modig et al., 2002; Palmqvist et al., 1999; Schoberth et al., 1993; Wang et al., 1994). The enzyme, a furfural dehydrogenase from *Pseudomonas putida* Fu1, catalyzed the oxidation of furfural to 2-furoic acid, utilizing NAD^+ or dichlorophenolindophenol plus phenazine methosulfate as electron acceptors (Koenig and Andreesen, 1990).

The essentially irreversible reduction of furfural catalyzed by this enzyme supports its functional designation as an NADPH-linked furfural reductase. The only other bacterial enzyme activities that have been implicated in furfural metabolism are a furfuryl alcohol dehydrogenase and a furfural dehydrogenase from *P. putida* Fu1 (Koenig and Andreesen, 1990) that presumably contribute to an oxidative pathway not unexpected for such a versatile oxidative organism. These enzymes were shown to respectively catalyze the oxidation of furfuryl alcohol to furfural, and furfural to 2-furoic acid, with each reaction utilizing NAD^+ as natural co-enzyme. An enzyme activity that participates in NADH-dependent reduction of furfural in yeast has been reported but not characterized (Wahlbom and Hahn-Hagerdal, 2002). Furfural can also serve as a substrate for NADH-dependent alcohol dehydrogenase of *Saccharomyces cerevisiae* (Modig et al., 2002). This approach is under consideration for developing improved ethanologenic *Escherichia coli* strains, as it has been successful in selecting the *E. coli* LY01 strain with improved tolerance to relatively high concentrations of ethanol (Yomano et al., 1998). The identification, cloning and over-expression of a gene encoding the furfural reductase characterized in the study of Gutierrez et al. (2006), may allow a more direct and rapid approach to developing ethanologenic *E. coli* tolerant of the levels of furfural generated during dilute acid and steam pretreatments of lignocellulosic biomass for conversion to alternative fuels and biobased products.

2.4. Enzymatic hydrolysis of cellulose

Enzymatic hydrolysis of cellulose is carried out by cellulase enzymes, which are highly specific (Beguin and Aubert, 1994). Cellulases are usually a mixture of several enzymes. At least three major groups of cellulases are involved in the hydrolysis process: (1) endoglucanases (EG, endo-1,4-D-glucanohydrolase or EC 3.2.1.4.), which attacks regions of low crystallinity in the cellulose fiber, creating free chain ends; (2) exoglucanase or cellobiohydrolase (CBH, 1,4-β-D-glucan cellobiodehydrolase or EC 3.2.1.91.), which degrades the molecule further by removing cellobiose units from the free chain ends; (3) β-glucosidase (EC 3.2.1.21.), which hydrolyzes cellobiose to produce glucose (Coughlan and Ljungdahl, 1988). In addition to three major groups of cellulase enzymes, there are also a number of ancillary enzymes that attack hemicellulose, such as glucuronidase, acetylesterase, xylanase, β-xylosidase, galactomannanase and glucomannanase (Duff and Murray, 1996). During hydrolysis cellulose is degraded by the cellulases to reducing sugars, which can be fermented by yeasts or bacteria to ethanol (Sun and Cheng, 2002). Utility cost of enzymatic

hydrolysis is low compared to acid or alkaline hydrolysis because enzyme hydrolysis is usually conducted at mild conditions (pH 4.8 and temperature 45–50 °C) and does not have a corrosion problem (Duff and Murray, 1996). Both bacteria and fungi can produce cellulases for hydrolysis of lignocellulosic materials. These microorganisms can be aerobic/anaerobic, mesophilic/thermophilic.

The filamentous fungus *Fusarium oxysporum* is known for its ability to produce ethanol by simultaneous saccharification and fermentation (SSF) of cellulose. However, the conversion rate is low and significant amounts of acetic acid are produced as a byproduct (Panagiotou et al., 2005). A few microbial species such as *Neurospora, Monilia, Paecilomyces* and *Fusarium* have been reported to hold the ability to ferment cellulose directly to ethanol (Singh et al., 1992). *F. oxysporum* produces a broad range of cellulases and xylanases, which has been characterised earlier (Christakopoulos et al., 1995a,b, 1996a,b, 1997). Acetic acid was the major fermentation product of *Neocallimastix* sp., another ethanol producing fungus (Dijkerman et al., 1997).

Bacteria belonging to *Clostridium, Cellulomonas, Bacillus, Thermomonospora, Ruminococcus, Bacteriodes, Erwinia, Acetovibrio, Microbispora* and *Streptomyces* can produce cellulases (Bisaria, 1991). *Cellulomonas fimi* and *Thermomonospora fusca* have been extensively studied for cellulase production. Although many cellulytic bacteria, particularly the cellulytic anaerobes such as *Clostridium thermocellum* and *Bcteroides cellulosolvens* produce cellulases with high specific activity, they do not produce high enzyme titres. Because the anaerobes have a very low growth rate and require anaerobic growth conditions, most research for commercial cellulase production has focused on fungi (Duff and Murray, 1996). Fungi that have been reported to produce cellulases include *Sclerotium rolfsii, P. chrysosporium* and species of *Trichoderma, Aspergillus, Schizophyllum* and *Penicillium* (Duff and Murray, 1996; Fan et al., 1987; Sternberg, 1976).

The factors that affect the enzymatic hydrolysis of cellulose include substrates, cellulase activity and reaction conditions (Sun and Cheng, 2002). Substrate concentration is one of the main factors that affect the yield and initial rate of enzymatic hydrolysis of cellulose. At low substrate levels, an increase of substrate concentration normally results in an increase of the yield and reaction rate of the hydrolysis (Cheung and Anderson, 1997). However, high substrate concentration can cause substrate inhibition, which substantially lowers the rate of hydrolysis and the extent of substrate inhibition depends up on the ratio of total substrate to total enzyme (Huang and Penner, 1991; Penner and Liaw, 1994). Increasing the dosage of cellulases in the process, to a certain extent, can enhance the yield and rate of hydrolysis, but would significantly increase the cost of the process. Cellulase dosage of 10 FPU/g cellulose is often used in laboratory studies because it provides a hydrolysis profile with high levels of glucose yield in a reasonable time (48–72 h) at a reasonable enzyme cost (Gregg and Saddler, 1996). Enzymatic hydrolysis of cellulose consists of three steps: adsorption of cellulase enzymes onto the surface of cellulose, the biodegradation of cellulose to fermentable sugars and desorption of cellulase.

Cellulase activity decreases during the hydrolysis. The irreversible adsorption of cellulase on cellulose is partially responsible for this deactivation (Converse et al., 1988). Addition of surfactants such as non-ionic Tween 20, 80 (Wu and Ju, 1998), polyoxyethylene glycol (Park et al., 1992), Tween 81, Emulgen 147, amphoteric Anhitole 20BS, cationic Q-86W (Ooshima et al., 1986), sophorolipid, rhamnolipid and bacitracin (Helle et al., 1993), dur-

ing hydrolysis is capable of modifying the cellulose surface property and minimizing the irreversible binding of cellulase to cellulose. Cellulases can be recovered from the liquid supernatant or the solid residues and most recycled cellulases are from the liquid supernatant. Enzyme recycling can effectively increase the rate and yield of the hydrolysis and lower the enzyme cost (Mes-Hartree et al., 1987). Ramos et al. (1993) reported that the enzyme mixture of the commercial Celluclast and Novozym preparation was successfully recycled for five consecutive steps with an elapsed time of 48 h between each recycling step. The efficiency of cellulose hydrolysis decreased gradually with each recycling step. Cellulase activity is inhibited by the cellobiose and to a lesser extent by glucose. Several methods have been developed to reduce the inhibition, including the use of high concentrations of enzymes, the supplementation of β-glucosidases during hydrolysis and removal of sugars during hydrolysis by ultrafiltration or SSF. The SSF process has been extensively studied to reduce the inhibition of end products hydrolysis (Saxena et al., 1992; Zheng et al., 1998).

In the process, reducing sugars produced in cellulose hydrolysis or saccharification is simultaneously fermented to ethanol, which greatly reduces the product inhibition to the hydrolysis. The microorganisms used in the SSF are usually the fungus *Trichoderma reesei* and *S. cerevisiae* (Sun and Cheng, 2002). Hydrolysis is usually the rate limiting process in SSF (Philippidis and Smith, 1995). Thermotolerant yeasts and bacteria have been used in the SSF to raise the temperature close to the optimal hydrolysis temperature. Ballesteros et al. (1991) have identified *Kluyveromyces marxianus* and *K. fragilis* that have the highest ethanol productivity at 42 °C from 27 yeast strains. *K. marxianus* has an ethanol yield of 0.5 g g^{-1} cellulose in 78 h using Solka Floc 200 as substrate at 42 °C (Ballesteros et al., 1991). Kadam and Schmidt (1997) found that thermotolerant yeast *Candida acidothermophilum*, produced 80% of the theoretical ethanol yield at 40 °C using dilute acid pretreated poplar as substrate. Compared to the two stage hydrolysis–fermentation process, SSF has some advantages like increase of hydrolysis rate by conversion of sugars that inhibit the cellulase activity, lower enzyme requirement, higher product yield, lower requirements for sterile conditions since glucose is removed immediately and ethanol is produced, shorter process time and less reactor volume because a single reactor is used. However, ethanol may also exhibit inhibition to the cellulase activity in the SSF process (Sun and Cheng, 2002). Wu and Lee (1997) found that cellulase lost 9, 36 and 64% of its original activity at ethanol concentrations of 9, 35 and 60 g/l, respectively at 38 °C during SSF process. According to Sun and Cheng (2002) this method has some disadvantages, which need to be considered for improvement of SSF includes incompatible temperature of hydrolysis and fermentation, ethanol tolerance of microbes and inhibition of enzymes by ethanol.

A reduction of the cost of ethanol production can be achieved by reducing the cost of either the raw materials or the cellulase enzymes. Reducing the cost of cellulase enzyme production is a key issue in the enzymatic hydrolysis of lignocellulosic materials.

3. Genetic engineering in microorganisms for ethanol production

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. Wood et al. (1997) reported the expression of recombinant endoglucanase genes from *Erwinia chrysanthemi* P86021 in *E. coli* KO11 and the recombinant system produced 3200 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).

Using genetically engineered microorganisms that can convert xylose and/or pentose to ethanol can greatly improve ethanol production efficiency and reduce the cost of production. The constructed operons encoding xylose assimilation and pentose phosphate pathway enzymes were transformed into the bacterium *Zymomonas mobilis* for the effective fermentation of xylose to produce ethanol (Zhang et al., 1995). The recombinant strain of *E. coli* with the genes from *Z. mobilis* for the conversion of pyruvate into ethanol has been reported by Dien et al. (2000). The recombinant plasmids with xylose reductase (XR) and xylitol dehydrogenase (XDH) genes from *Pichia stipitis* and xylulokinase (XK) gene from *S. cerevisiae* have been transformed into *Saccharomyces* spp. for the co-fermentation of glucose and xylose (Ho et al., 1998).

Efforts to metabolically engineer *S. cerevisiae* for xylose utilisation by insertion of the genes encoding XR and XDH from the xylose-metabolising yeast *P. stipitis* have resulted in poor ethanol production from xylose, with mainly xylitol formed under fermentative conditions (Kotter and Ciriacy, 1993; Kotter et al., 1990; Meinander et al., 1994; Tantirungkij et al., 1993, 1994). This has been attributed to a redox imbalance between the cofactor usage of XR and XDH (Bruinenberg et al., 1984) (Fig. 1) limitations in xylulose metabolism through the pentose phosphate pathway (Senac and Hahn-Hagerdal, 1990) and insufficient induction of glycolytic and ethanologenic genes (Boles et al., 1993, 1996; Hahn-Hagerdal

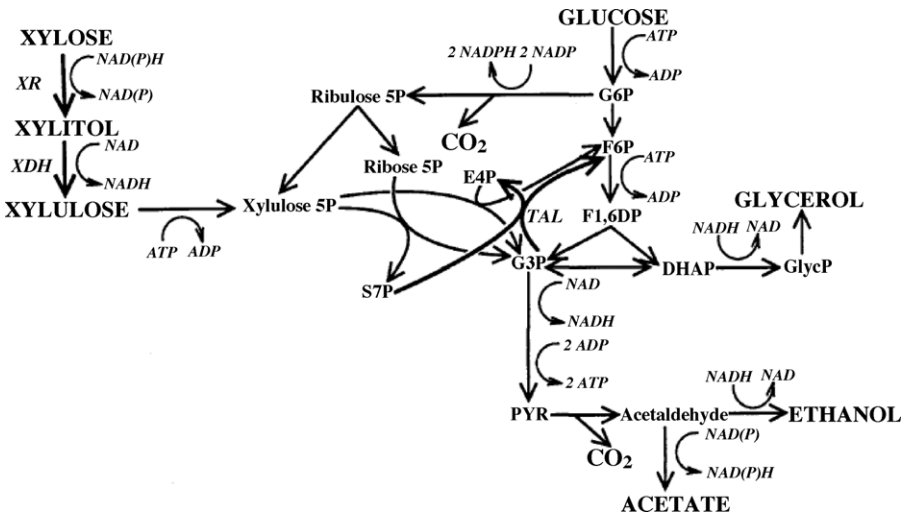

Fig. 1. Model of anaerobic xylose and glucose metabolism in metabolically engineered *S. cerevisiae* (*Source*: Meinander et al., 1999).

et al., 1996). Recently, the native transaldolase gene (*TAL1*) of *S. cerevisiae* was over-expressed in a XR- and XDH-containing strain, resulting in improved aerobic growth on xylose (Walfridsson et al., 1995). However, xylose consumption was severely reduced when aeration was decreased, indicating the presence of other metabolic limitations at low oxygen concentrations (Meinander et al., 1999). Previously, the *XYL1* + *XYL2* + *TAL1* strain has been reported to grow significantly better aerobically on xylose plates compared with the *XYLI* + *XYL2* strain, but in oxygen-limited batch cultures the enhanced TAL activity does not lead to ethanol formation from xylose (Walfridsson et al., 1995). Under anaerobic conditions investigated in the study conducted by Meinander et al. (1999), subtle differences in product formation between the strains with different transaldolase (TAL) activity were observed. The *XYLI* + *XYL2* + *TAL1* strain did not consume xylose significantly faster compared with the *XYL1* + *XYL2* strain, nor was the ethanol yield higher. However, on a xylose/glucose mixture the *XYLI* + *XYL2* + *TAL1* strain clearly gave a higher biomass yield on consumed sugar compared with the XYLI + XYL2 strain.

These observations show that over-expression of transaldolase, enhanced growth on xylose under anaerobic conditions, even though the effect was not as pronounced as on aerobic xylose plates (Walfridsson et al., 1995). This indicates that steps other than the TAL catalysed reaction exert a higher control over the rate of xylose consumption under anaerobic conditions. The simultaneous metabolism of glucose influenced the rate of xylose consumption more than the over-expression of *TAL1*. At high glucose concentrations xylose metabolism was slow because of competitive inhibition of xylose transport by glucose (Meinander and Hahn-Hagerdal, 1997). This is due to the orders of magnitude higher affinity of the common transport system for glucose than for xylose (Busturia and Lagunas, 1986; van Zyl et al., 1993).

The stimulation of xylose metabolism by glucose, indicated by the instantaneous decrease in xylose consumption rate after glucose exhaustion, can be explained by the generation of intermediary metabolites for the initial steps of xylose metabolism and the pentose phosphate pathway through glucose metabolism (Fig. 1). NADPH, the preferred co-substrate for xylose reduction is generated through the oxidative pentose phosphate pathway and acetate production (Meinander et al., 1996) and NAD^+ for oxidation of xylitol to xylulose is generated through glycerol production. Higher glyceraldehyde-3-phosphate levels are maintained through glucose metabolism (Kotter and Ciriacy, 1993), avoiding the limitation of the transaldolase reaction indicated by elevated sedoheptulose-7-phosphate levels in xylose-metabolising cells (Senac and Hahn-Hagerdal, 1990). A higher intracellular ATP concentration is maintained during glucose metabolism compared with xylose metabolism, as has been shown for *Candida tropicalis* (Lohmeier-Vogel et al., 1995), providing sufficient ATP for xylulose phosphorylation. The transient xylulose excretion after the depletion of glucose may thus be due to a limitation of xylulose phosphorylation caused by a decrease in ATP/ADP ratio (Meinander et al., 1999).

Glucose metabolism ensures sufficient concentrations of the glycolytic intermediates glucose-6-phosphate, fructose-6-phosphate and C3-metabolites, required for the induction of the ethanologenic enzymes pyruvate decarboxylase and alcohol dehydrogenase, and enolase II and pyruvate kinase in the lower part of the glycolytic pathway (Boles et al., 1996). Additionally, glucose represses the gluconeogenic enzyme fructose-1,6-bisphosphatase (Gancedo et al., 1967), which could reverse the direction of the upper part of the glycolysis,

channelling intermediates into the oxidative pentose phosphate pathway and causing carbon dioxide formation. After the transient excretion of xylulose, the only products of xylose metabolism observed after the exhaustion of glucose were xylitol and carbon dioxide.

3.1. Bacteria

Ethanol-producing bacteria have attracted much attention in recent years because their growth rate is substantially higher than that of the *Saccharomyces* presently used for practical production of fuel alcohol and, with the recent advances in biotechnology, they have the potential to play a key role in making production of ethanol more economical (Dien et al., 2003). Among such ethanol-producing bacteria, *Z. mobilis* is a well-known organism used historically in tropical areas to make alcoholic beverages from plant sap (Skotnicki et al., 1982). The advantages of *Z. mobilis* are its high growth rate and specific ethanol production; unfortunately, its fermentable carbohydrates are limited to glucose, fructose and sucrose. On the other hand, the Gram-negative strain *Zymobacter palmae*, which was isolated by Okamoto et al. (1993) using a broad range of carbohydrate substrates, is a facultative anaerobe that ferments hexoses, α-linked di- and tri-saccharides, and sugar alcohols (fructose, galactose, glucose, mannose, maltose, melibiose, sucrose, raffinose, mannitol and sorbitol). This strain produces approximately 2 mol of ethanol per mole of glucose without accumulation of byproducts and shows productivity similar to that of *Z. mobilis* (Okamoto et al., 1994).

Numerous studies have addressed the challenges of breeding of alcohol-producing microorganisms that harbor a pet operon, including *E. coli* (Ingram et al., 1987), *E. chrysanthemi* (Tolan and Finn, 1987) and *Klebsiella oxitoca* (Ohta et al., 1991), which can produce ethanol from cellulosic materials. So far, however, the production of ethanol from cellulosic materials using these strains has not reached a level sufficient for commercial application. For that reason, Yanase et al. (2005) in their study focused on *Zymobacter palmae*, given its broad range carbohydrate substrates and ability to efficiently produce ethanol, but this organism could not ferment cellulose or its degradation products, cello-oligosaccharides and cellobiose. Therefore, as the first step, they breed a strain of *Zymobacter palmae* that can produce ethanol from cellulosic materials directly and investigated the possibility of conferring the ability to ferment cellobiose to the strain through metabolic engineering. To confer the ability to ferment cellobiose to the strain, they selected the β-glucosidase gene from *Ruminococcus albus* to be introduced into *Zymobacter palmae*. *R. albus* is an anaerobic cellulolytic rumen bacterium that produces highly active cellulolytic enzymes. In *R. albus*, β-glucosidase catalyzes the hydrolysis of cellobiose and cello-oligosaccharides during the final degradation of cellulosic materials (Ohmiya et al., 1985). The gene encoding β-glucosidase has been cloned and efficiently expressed in *E. coli*, from which the recombinant enzyme has been purified and characterized in detail by Ohmiya et al. (1990). To enable *Zymobacter palmae* to ferment cellobiose to ethanol Yanase et al. constructed a plasmid pMFY31-βg containing the β-glucosidase gene from *R. albus* and introduced into *E. coli* JM109 by electroporation. *Zymobacter palmae* carrying pMFY31-βg exhibited a higher level of β-glucosidase activity (1.05 U/ml) than *E. coli* (0.77 U/ml), confirming that the endogenous promoter from *R. albus* was functional in both *Zymobacter palmae* and *E. coli*. Yanase et al. (2005) found that 28.6% of the expressed activity in *Zymobacter palmae*

was localized in the cell-surface fraction, while 16.2% was in the periplasmic fraction, suggesting that *R. albus* β-glucosidase is translocated through the cytoplasmic membrane. By contrast, about 86% of the expressed β-glucosidase was present in the cytoplasm of *E. coli* cells. In any case, the level of β-glucosidase expression in *Zymobacter palmae* appeared to be sufficient to grow the organism on cellobiose (Yanase et al., 2005).

In the presence of a mixture of glucose and cellobiose, the recombinant *Zymobacter palmae* fermented both sugars to ethanol with growth of the organism, but glucose was preferentially utilized and fermented at a faster rate than cellobiose (Yanase et al., 2005). Notably, increased levels of cellobiose did not inhibit cell growth or ethanol production, suggesting that the level of β-glucosidase expression might limit the rate of cellobiose fermentation. In addition to cellobiose, β-glucosidase catalyzes the hydrolysis of cello-oligosaccharides, suggesting that the enzyme could be usefully placed within a series of cellulose-degrading enzymes. Although an amino acid sequence corresponding to a typical secretion signal-peptide has not been found in the N-terminal region of this enzyme, its activities are localized in both the periplasmic and cytoplasmic fractions in *R. albus* (Ohmiya et al., 1985). Interestingly, in *Zymobacter palmae* approximately 50% of the enzyme activity was found in the washing solution, which corresponds to the cell-surface fraction and osmotic-shock solution, which corresponds to the periplasmic fraction. This situation suited the fermentation of cellobiose by *Zymobacter palmae*, and the recombinant strain was able to assimilate cellobiose to produce ethanol (Yanase et al., 2005).

The recovery of ethanol from cellobiose by the recombinant strain reached approximately 95% of the theoretical yield from glucose; moreover, no glucose liberated from cellobiose or other common metabolites, such as pyruvate, lactate, acetate and glycerol, were not detected in the culture fluid (Yanase et al., 2005). There have been numerous investigations into the breeding sugar-utilizing bacteria (e.g., *E. coli* and *Klebsiella oxytoca*) carried out with the aim of conferring to them the ability to produce ethanol (de Carvalho Lima et al., 2002; Dien et al., 2000; Dumsday et al., 1999; Qian et al., 2003; Zaldivar et al., 2000; Zhou et al., 2001). Because these strains exhibit a broad range of utilizable carbohydrates, they are well suited for the production of ethanol from carbohydrates derived from lignocellulosic waste materials. On the other hand, these strains are susceptible to ethanol, and are thus not suitable for continuous production of ethanol at high concentrations. Attempts are in progress by Yanase et al. to introduce genes encoding endoglucanase and cellobiohydrolase into *Z. palmae*, thereby making it capable of producing ethanol directly from cellulose.

Anaerobic xylose conversion by two metabolically engineered *S. cerevisiae* strains, in the presence and absence of simultaneous glucose metabolism was investigated by Meinander et al. (1999). In this study one strain expressed *XYL1* encoding XR and *XYL2* encoding XDH from *P. stipitis*, whereas the other additionally over-expressed *TAL1* encoding TAL. Both strains formed xylitol as the main product of xylose metabolism. The *TAL1* over-expressing strain gave a higher biomass yield and produced less carbon dioxide and somewhat less xylitol compared with the *XYLI* + *XYL2* strain, indicating that TAL limited xylose metabolism in the latter. The ethanol yield was similar with both strains. The simultaneous metabolism of glucose enhanced xylose metabolism by causing a higher rate of xylose consumption and less xylitol and xylulose excretion, compared with xylose metabolism alone. Simultaneous xylose and glucose metabolism affected the growth rate negatively compared with growth on glucose alone.

3.2. Yeasts

Metabolic pathway engineering is constrained by the thermodynamic and stoichiometric feasibility of enzymatic activities of introduced genes. Engineering of xylose metabolism in *S. cerevisiae* has focused on introducing genes for the initial xylose assimilation steps from *P. stipitis*, a xylose-fermenting yeast, into *S. cerevisiae*, a yeast traditionally used in ethanol production from hexose. However, recombinant *S. cerevisiae* created in several laboratories have used xylose oxidatively rather than in the fermentative manner that this yeast metabolizes glucose (Jin and Jeffries, 2004). D-Xylose is a major component of the hydrolyzate of hemicellulose from biomass. Therefore, ethanol production from xylose is essential for successful utilization of lignocellulose (Jeffries, 1985). Many bacteria, yeast, and fungi assimilate xylose, but only a few metabolize it to ethanol (Skoog and Hahn-Hagerdahl, 1988).

Xylose-fermenting yeasts, such as *P. stipitis, Pachysolen tannophilus* and *Candida shehatae* require precisely regulated oxygenation for maximal ethanol production (Ligthelm et al., 1988; Skoog et al., 1992), and detoxification of the hydrolysate, because they withstand the inhibitory environment of lignocellulose hydrolysates poorly (Bjorling and Lindman, 1989; Hahn-Hagerdal et al., 1994; Sanchez and Bautista, 1988; van Zyl et al., 1991). These factors increase the cost of xylose fermentation. *S. cerevisiae* has an efficient anaerobic sugar metabolism, tolerates inhibitory industrial substrates better than other microorganisms (Olsson et al., 1992; Olsson and Hahn-Hagerdal, 1993) and ferments hexoses abundantly present in lignocellulosic hydrolysates, such as glucose, mannose and galactose with high yield and productivity. Due to lack of the XR and XDH enzymes, which catalyse the first two-steps in the xylose-metabolising pathway in yeasts, *S. cerevisiae* is unable to ferment xylose.

In yeast, xylose is reduced to xylitol by NADPH-linked XR and then xylitol is oxidized to xylulose by NAD-linked XDH. *S. cerevisiae*, traditionally used in ethanol production, is unable to utilize xylose. However, it can slowly metabolize xylulose, the ketoisomer of xylose. Thus, several research groups have tried to genetically engineer *S. cerevisiae* by introducing the genes (*XYL1* and *XYL2*) coding for XR and XDH from a xylose-fermenting yeast, *P. stipitis*. The resulting strains can grow on xylose aerobically and produce ethanol under oxygen-limited conditions (Jin et al., 2000; Kotter et al., 1990; Tantirungkij et al., 1994; Toivari et al., 2001; Walfridsson et al., 1997). Ho et al. (1998) reported that over-expression of endogenous xylulokinase (ScXKS1) under the background of *XYL1* and *XYL2* could enhance the xylose fermentation by recombinant *S. cerevisiae*. Recently, Jin et al. cloned the xylulokinase gene (*XYL3*) from *P. stipitis* (Jin et al., 2002) and were able to transfer a complete xylose pathway into *S. cerevisiae*. However, the resulting recombinant *S. cerevisiae* expressing *XYL1, XYL2* and *XYL3* still prefers oxidative utilization of xylose. The results of study conducted by Jin and Jeffries (2004) showed that recombinant *S. cerevisiae YSX3* expressing *XYL1, XYL2* and *XYL3* uses xylose in an oxidative manner. They found that the *YSX3* strain, like *P. stipitis* needed optimal aeration conditions for ethanol production from xylose. In silico phenotypes predicted by flux balance analysis (FBA) were consistent with experimental results, showing that aeration is critical to xylose fermentation by recombinant *S. cerevisiae*. Extreme pathways calculated from the metabolic network revealed that, unlike glucose metabolism, xylose metabolism requires oxygen due to the redox imbalance caused by cofactor difference between XR and XDH.

Most likely, however, there are other limitations, such as low intracellular metabolite levels and insufficient induction of glycolysis, which may be difficult to solve by genetic engineering techniques. With genetically stable strains, the application of a fermentation technique such as fed-batch or continuous fermentation that facilitates the simultaneous fermentation of xylose and other sugars may solve these problems.

4. Processes of ethanol production

Raw materials containing sugars, or materials which can be transformed into sugars, can be used as fermentation substrates. The fermentable raw materials can be grouped as directly fermentable sugary materials, starchy, lignocellulosic materials and urban/industrial wastes. Direct fermentation of sugarcane, sugar beet and sweet sorghum to produce ethanol has also been reported (Bryan, 1990; Ganesh et al., 1995; Ravi et al., 1997). Sugar containing materials require the least costly pretreatment, where starchy, lignocellulosic materials and urban/industrial wastes needed costly pretreatment, to convert into fermentable substrates (Sun and Cheng, 2002). Sugar containing materials which can be transformed into glucose, can be used as fermentation substrates under anaerobic conditions, glucose is converted to ethanol and carbon dioxide by glycolysis. The phosphorylation of carbohydrates is carried out through the metabolic pathway and the end products are two moles of ethanol and carbon dioxide (Ingram et al., 1998). The overall reactions to liberate energy for biosynthesis (Eq. (2)) produce two moles of ethanol and CO_2 for every mole of glucose consumed.

Although fungi, bacteria, and yeast microorganisms can be used for fermentation, specific yeast (*S. cerevisiae* also known as Bakers' yeast, since it is commonly used in the baking industry) is frequently used to ferment glucose to ethanol. Theoretically, 100 g of glucose will produce 51.4 g of ethanol and 48.8 g of carbon dioxide. However, in practice, the microorganisms use some of the glucose for growth and the actual yield is less than 100% (Badger, 2002).

Ethanol production from grain involves milling of grain, hydrolysis of starch to release fermentable sugar, followed by inoculation with yeast. Chemically starch is a polymer of glucose (Peterson, 1995). Yeast cannot use starch directly for ethanol production. Therefore, grain starch has to be wholly broken down to glucose by combination of two enzymes, viz., amylase and amyloglucosidase, before it is fermented by yeast to produce ethanol. The biochemical reactions and processes involved in starch hydrolysis and fermentation shown in Figs. 2 and 3 and given in Eqs. (1) and (2). Alcohol produced from fermented broth and remaining spillages is processed to produce Distiller's Dried Grain and Soluble (DDGS), which is an excellent ingredient for animal feed (Sheorain et al., 2000).

$$(C_6H_{10}O_5)n + nH_2O \rightarrow nC_6H_{12}O_6 \quad (1)$$

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2 + \text{energy} \quad \text{(stored as ATP)} \quad (2)$$

Ethanol production from biomass by fermentation is possible by using free or immobilized cells. Both have advantages and disadvantages. 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

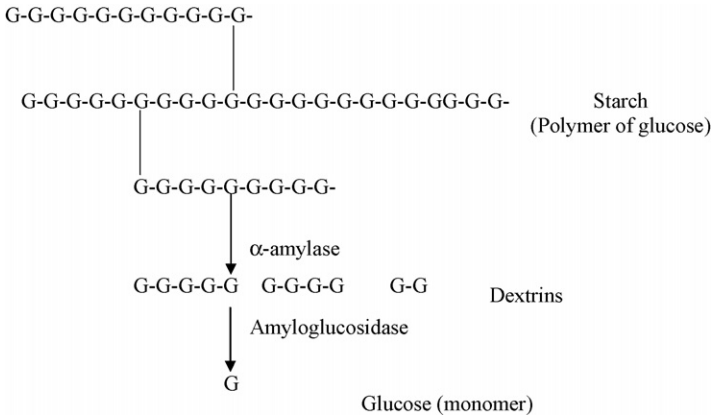

Fig. 2. Enzymatic hydrolysis of starch to glucose.

alcohol. The use of immobilized whole cells in industrial processes has attracted considerable attention during the past few years due to advantages over traditional processes. Immobilization is the restriction of cell mobility within a defined space. Immobilization provides high cell concentrations and cell reuse. It also eliminates washout problems at high dilution rates and the costly processes of cell recovery and cell recycle. High volu-

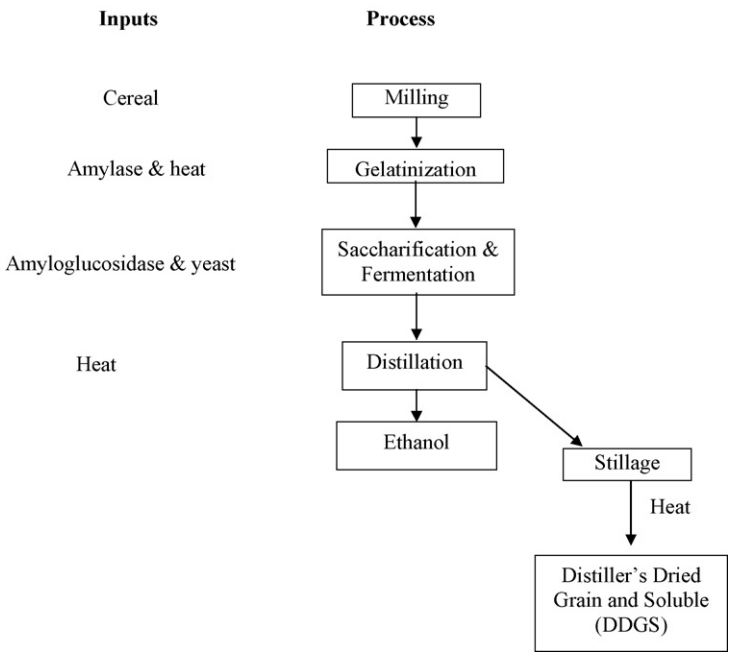

Fig. 3. Flow chart of ethanol production from cereal grains.

metric productivities can also be obtained with the combination of high cell concentrations and high flow rates. Immobilization may also improve genetic stability (Caylak and Vardar Sukan, 1998).

Ethanol can be produced by four main types of industrial operations: batch, continuous, fed-batch and semi-continuous. In batch fermentation, substrate and yeast culture are charged into the bioreactor together with nutrients. Most of the ethanol produced today is done by the batch operation since the investment costs are low, do not require much control and can be accomplished with unskilled labour (Caylak and Vardar Sukan, 1998). Complete sterilization and management of feedstocks are easier than in the other processes. The other advantage of batch operation is the greater flexibility that can be achieved by using a bioreactor for various product specifications. In the continuous process, feed, which contains substrate, culture medium and other required nutrients, is pumped continuously into an agitated vessel where the microorganisms are active. The product, which is taken from the top of the bioreactor, contains ethanol, cells, and residual sugar (Maiorella et al., 1981).

The fed-batch operation, which may be regarded as a combination of the batch and continuous operations, is very popular in the ethanol industry. In this operation, the feed solution, which contains substrate, yeast culture and the required minerals and vitamins, are fed at constant intervals while effluent is removed discontinuously. The main advantage of the fed-batch system is that intermittent feeding of the substrate prevents inhibition and catabolite repression. If the substrate has an inhibitory effect, intermittent addition improves the productivity of the fermentation by maintaining a low substrate concentration. It is essential to keep the culture volume constant in continuous operation, whereas there is volume variation in the fed-batch processes. In semi-continuous processes, a portion of the culture is withdrawn at intervals and fresh medium is added to the system. In the continuous processes it is essential to maintain a constant culture volume, whereas there is volume variation in semi-continuous processes. This method has some of the advantages of the continuous and batch operations. There is no need for a separate inoculum vessel, except at the initial startup. Time is also not wasted in non-productive idle time for cleaning and resterilization. Another advantage of this operation is that not much control is required. However, there is a high risk of contamination and mutation due to long cultivation periods and periodic handling. Furthermore, since larger reactor volumes are needed, slightly higher investment costs are required (Caylak and Vardar Sukan, 1998).

5. Ethanol from cane molasses and other directly fermentable feedstocks

In India, sugar cane molasses is the only major raw material for alcohol production. Molasses, which is byproduct of the sugar industry, contains about 45–50% fermentable sugars. Because of the ease with which this can be fermented into ethanol and its low price have made this raw material ideal for ethanol production. As a consequence, no other raw material at present in India can match the economy of molasses for ethanol production. In recent years because of decontrol, a jump in molasses price and limitation on molasses availability, ethanol production has been greatly affected in molasses-based distilleries. In particular, the Indian-made foreign liquor industries are concerned about competition in

the international liquor market. All India Distillers Association (AIDA) has emphasized on alcohol from substrate other than molasses on account of shortage of molasses, as well as the extremely high rate at which it is being sold to distillers, worse still, even after payment of high rates the quantity of available molasses is not up to mark (AIDA, 1996). Presently, in India ethanol is mainly produced from sugarcane molasses and its total production is not sufficient for meeting the demands of alcohol for fuel. Molasses-based distilleries are already causing pollution hazard due to the disposal problem of its effluent, which is generated at the rate of 40 billion liters per year (Joshi et al., 1996).

Other than sugarcane, sugar beet and sweet sorghum can be utilized as sources of sugar for ethanol production (Hill et al., 1990; Gibbons et al., 1986; Phowchinda et al., 1997; Rudolph et al., 1979). In Brazil, the production of bioethanol from cane juice is practiced on a large-scale (Borrero et al., 2003; Jackson, 1976; Pimentel, 1980). The potential of fodder beets juice for fermentation to ethanol was examined by Kosaric et al. (1983) with two strains of yeast *S. cerevisiae, S. diastaticus* and *K. marxianus* and found that 119.77 l absolute alcohol/metric tonnes of fodder beet can be produced. Sweet sorghum (*Sorghum bicolor* (L.) Moench) is a high biomass and sugar yielding crops (Almodares and Sepahi, 1997; Bryan, 1990). Smith and Buxton (1993) reported that average ethanol yields for two years study were above 3100 l ha^{-1} and ranged up to 5235 l ha^{-1} from different sweet sorghum varieties. Whereas Reddy and Reddy (2003) reported alcohol is produced at 6106 l ha^{-1} from sweet sorghum while only 4680 l ha^{-1} from sugarcane is produced. This crop is ideally suited for semi-arid agro climatic region of our country and it gives reasonably good yield with minimum requirement of irrigation and fertilizer (Maiti, 1996). Lengthy growing period and high water requirement are the disadvantage in sugarcane (*Saccharum officinarum*) and sugerbeet (*Beta vulgaris*), in those areas where irrigation facilities are not available. These factors along with the comparative disadvantage of molasses (higher price and water and air pollution) are expected to increase the interest in sweet sorghum (Reddy and Reddy, 2003). Sorghum stalks are ideal for ethanol production, as the ethanol from sorghum is significantly cleaner than that from sugarcane (Ravi et al., 1997).

6. Ethanol from starchy material

Starch is very important and abundant natural solid substrate. Many microorganisms are capable to hydrolyse starch, but generally its efficient hydrolysis requires previous gelatinization. Some recent works concern the hydrolysis of the raw (crude or native) starch as it occurs naturally. Essentially starch is composed of two related polymers in different proportions according to its source: amylose (16–30%) and amylopectin (65–85%). Amylose is a polymer of glucose linked by α-1,4 bonds, mainly in linear chains. Amylopectin is a large highly branched polymer of glucose including also α-1,6 bonds at the branch points. Within the plant, cell starch is stored in the form of granules located in amyloplasts, intracellular organelles surrounded by a lipoprotein membrane. Starch granules are highly variable in size and shape depending on the plant material. Granules contain both amorphous and crystalline internal regions in respective proportions of about 30/70. During the process of gelatinization, starch granules swell when heated in the presence of water, which involves the breaking of hydrogen bonds, especially in the crystalline regions

(Raimbault, 1998). Many microorganisms can hydrolyse starch, especially fungi which are then suitable for SSF application involving starchy substrates. Glucoamylase, α-amylase, β-amylase, pullulanase and isoamylase are involved in the processes of starch degradation. Mainly α-amylase and glucoamylase are of importance for SSF. α-Amylase is an endo-amylase attacking α-1,4 bonds in random fashion, which rapidly reduce molecular size of starch and consequently its viscosity producing liquefaction. Glucoamylase occurs almost exclusively in fungi including *Aspergillus* and *Rhizopus* groups. This exo amylase produces glucose units from amylose and amylopectin chains. Microorganisms generally prefer gelatinised starch. But large quantity of energy is required for gelatinization so it would be attractive to use organisms growing well on raw (ungelatinised) starch. Different works are dedicated to isolate fungi producing enzymes able to degrade raw starch, as has been done by Abe et al. (1988), Bergmann et al. (1988) and Soccol et al. (1994).

Ethanol production by a co-culture of *S. diastaticus* and *S. cerevisiae* 21 was found 24.8 g l^{-1} using raw unhydrolyzed starch in single step fermentation. This was 48% higher than the yield obtained with the monoculture of *S. diastaticus* (16.8 g l^{-1}). The maximum ethanol fermentation efficiency was achieved (93% of the theoretical value) using 60 g l^{-1} starch concentration in co-culture fermentation with *Endomycopsis capsularis* and *S. cerevisiae* 21, maximum ethanol yield was 16.0 g l^{-1}, higher than the yield with the monoculture of *E. capsularis*. In batch fermentations using monocultures maximum ethanol production occurred in 48 h of fermentation at 30 °C using 60 g l^{-1} starch. Fermentation efficiency was found lower in a two-step process using α-amylase and glucoamylase treated starch (Verma et al., 2000). Horn et al. (1992) reported that increasing the grain sorghum concentration from 8 to 28% (w/v) did not affect the final yield of 0.45 g ethanol/g glucose equivalent, although the fermentation rate decreased considerably at the higher slurry concentration, requiring 8 days for complete of the fermentation. 28% grain sorghum slurry yielded 12.5% (w/v) ethanol indicating that nearly 390 l could be produced per tonne of grain sorghum. In another study, a bushel of maize grain (25.3 kg or 56 lb at 15% moisture) can produce from 9.4 to 10.9 l (2.5–2.9 gallons) of pure ethanol (Badger, 2002). About 5% of corn in the world is wasted, if this wasted corn could be fully utilized to produce bioethanol, then 9.3 Gl of bioethanol could be produced, thereby replacing 6.7 Gl of gasoline, if ethanol is used as an alternative fuels for motor vehicles (Kim and Dale, 2004).

Starchy materials require a reaction of starch with water (hydrolysis) to break down the starch into fermentable sugars (saccharification). Typically, mixing the starch with water to form slurry, this is then stirred and heated to rupture the cell walls. Specific enzymes that will break the chemical bonds are added at various times during the heating cycle (Badger, 2002). Starchy grains and effluent generated from starch generating unit are the cheap substrates and could be used as potential raw materials for ethanol fermentation (Verma et al., 2000).

7. Ethanol from lignocellulosic biomass

Lignocellulose occurs within plant cell walls, which consist of cellulose microfibrils embedded in lignin, hemicellulose and pectin. Each category of plant material contains variable proportion of each chemical compound. Two major problems can limit lignocellu-

lose breakdown: (i) cellulose exists in four recognised crystal structures known as celluloses I–IV. Various chemical or thermal treatments can change the structure from crystalline to amorphous, and (ii) different enzymes are necessary in order degrade cellulose, e.g., endo- and exo-cellulases plus cellobiase (Raimbault, 1998).

Lignocellulosic materials are such an abundant and inexpensive resource that existing supplies could support the sustainable production of liquid transportation fuels (Pitkanen et al., 2003). Xylose is the most abundant pentose sugar in the hemicellulose (25% of dry weight) of hardwoods and crop residues and is second only to glucose in natural abundance. Thus, the efficient utilization of the xylose component of hemicellulose in addition to hexoses offers the opportunity to significantly reduce the cost of bioethanol production (Olsson and Hahn-Hagerdal, 1996). *S. cerevisiae*, which is one of the most prominent ethanol production organisms using hexose sugars, has the drawback that it is unable to utilize xylose. The initial steps of xylose utilization are as follows: xylose uptake through the cell membrane, xylose isomerization to xylulose and conversion of xylulose to glyceraldehyde-3-phosphate and fructose-6-phosphate in pentose phosphate pathway (Prior and Kotter, 1997). *S. cerevisiae* can uptake xylose with the same systems it uses for glucose. However, xylose uptake is very inefficient compared to that of glucose. Reported K_m values for xylose transport vary between 130 mM and 1.5 M (Kotter and Ciriacy, 1993; Singh and Mishra, 1995), which are at least 5–200-fold higher than that for glucose. *S. cerevisiae* can catabolize xylulose (Jeffries, 1981), however, it cannot utilize xylose due to the absence of an active isomerization system (single or two-step) to convert xylose to xylulose (Jeffries, 1990). Attempts to introduce bacterial xylose isomerases in *S. cerevisiae* have met with some success so far (Walfridsson et al., 1996), but despite good efforts they have not yet provided a breakthrough. In yeast D-xylose is first reduced to xylitol by D-xylose reductase that uses either NADH or NADPH, but with a preference towards NADPH. Xylitol is then oxidized to D-xylulose with NAD^+ by XDH. The different cofactor specificities would lead to serious cofactor imbalance, if the cell would not be able to compensate it elsewhere in the metabolism. Before entering the pentose phosphate pathway xylulose is phosphorylated to xylulose-5-phosphate by XK. In the pentose phosphate pathway non-oxidative reactions convert xylulose-5-phosphate to glyceraldehyde-3-phosphate and fructose-6-phosphate, which link the pentose phosphate pathway to glycolysis. The non-oxidative pentose phosphate pathway is a sequence of many reversible reactions, which operate near equilibrium. Thus, this step lacks irreversible reactions such as kinases with large differences in Gibbs free energies, which would drive the reactions efficiently forward (Jeffries, 1990). The most common approach to construct xylose utilizing recombinant *S. cerevisiae* strains has been the expression of XR- and XDH-encoding genes *XYL1* and *XYL2* from *P. stipitis* (Kotter et al., 1990; Walfridsson et al., 1997). Furthermore, over-expression of the endogenous XK improves xylose utilization, as has been demonstrated in recent studies (Eliasson et al., 2000; Toivari et al., 2001). Despite the successful expression of the three-enzyme set, XR/XDH/XK, the rate of xylose utilization is still very low, and conversion yields poor due to xylitol accumulation. This has been attributed to limitations in all steps mentioned above.

D-Xylose is one of the major components of lignocellulosic biomass. Alcoholic fermentation of this renewable carbon source is considered to be of potential economic value. Since the discovery of the pentose fermenting ability of some naturally occurring yeasts, considerable interest in the study of the xylose catabolic pathway has arisen (Mishra and

Singh, 1993; Schneider, 1989). Yeasts convert xylose to xylulose through sequential reduction and oxidation. The subsequent phosphorylation of xylulose allows entry of the sugar phosphate into the pentose phosphate pathway. However, at present overall regulation of xylose catabolism is largely obscure. Currently, three species of xylose-fermenting yeasts, *Pachysolen tannophilus, P. stipitis* and *Candida shehatae*, have been extensively characterized. Xylose reductases have been isolated from all three species (Bolen et al., 1985; Ho et al., 1990; Verduyn et al., 1985a,b) but the genes encoding XR and XDH, *XYL1* and *XYL2*, respectively, have been cloned and characterized only from *P. stipitis* (Amore et al., 1991; Kotter et al., 1990).

The different cofactor specificities of the two-step oxidoreductase reaction of xylose create a futile cycle between pentose phosphates, fructose-6-phosphate and glucose-6-phosphate with serious consequences. Let us envision the metabolic network as two sections, upstream and downstream, the upstream consisting of the hexose and pentose phosphates and the downstream of triose phosphates and organic acids. At the upstream, there is a route for NADPH generation, oxidative pentose phosphate pathway, but it consumes carbon in the form of evolved carbon dioxide reducing the flow of carbon downstream to pyruvate. Hence, NADPH generation is not a problem for the cell but it causes problems further downstream since the main NAD^+ generating routes start from pyruvate. The flow of carbon is preferentially used for the generation of NADPH, leaving a lesser amount for the generation of NAD^+. Thus, at the end, generation of NAD^+ becomes a bottleneck for xylose metabolism. Furthermore, under aerobic conditions the shuttling of NADH into the mitochondrion is likely to play a limiting role in xylose metabolism when no glucose is provided. Metabolic engineering of xylose metabolism would benefit on finding a mechanism for production of NADPH upstream in metabolism through a route, which would not result in loss of carbon. For example, simultaneous deletion of glucose-6-phosphate dehydrogenase encoding gene (Jeppsson et al., 2002) and expression of a partly NADPH-dependent glyceraldehyde-3-phosphate dehydrogenase encoding gene could direct the flow of carbon directly towards pyruvate and ethanol without the loss of carbon (Verho et al., 2002). By using solely metabolic flux analysis it was not possible to study the effects of xylose uptake or pentose phosphate reactions, since their role as a limiting step originates from their kinetic properties such as K_m values for uptake and Gibbs free energies for pentose phosphate pathway. However, application of the metabolic flux analysis to data from a series of cultivations where a factor, i.e., glucose level in the feed was changed proved useful in providing correlations against the changing glucose level. The metabolic flux analysis helped to concretize and visualize the tentative thoughts about the metabolic fluxes in xylose metabolism.

Various crop residues rich in lignocellulosics like wheat straw, rice straw, corn stalk and cobs, ground nut shell, sunflower stalks and hulls and alfalfa fiber has been exploited for ethanol production (Austin et al., 1994; Bothast and Saha, 1997; Hatfield, 1990; Kadam and McMillan, 2003; Koegel and Straub, 1996; Olsson and Hahn-Hagerdal, 1996; Sharma et al., 2002). The importance of some lignocellulosic biomass is given in Table 5. Cellulosic biomass contains ~40–50% cellulose, a glucose polymer; ~25–35% hemicellulose, a sugar heteropolymer; ~15–20% lignin, a non-fermentable phenylpropene unit plus lesser amounts of minerals, oils, soluble sugars, and other components; a vast resource that could be used for production of fuel (Holtzapple, 1993; Kaur et al., 1998; Wyman, 1994). In lignocelluloses,

Table 5
Importance of some agricultural waste for production of ethanol

Agricultural residue	Importance	References
Corn fiber	Rich in hemicellulose, produced from corn hull during the wet milling of corn	Gulati et al. (1996)
Sunflower hulls	Low commercial value, disposal problem due to their low bulk density, contained 53% cellulose, 17.5% hemicellulose and 11.4% lignin	Sharma et al. (2004)
Rice straw	Easily available agricultural waste in bulk, contained 40% cellulose, 18% hemicellulose and 5.5% lignin	Kaur et al. (1998)
Alfalfa fiber	Consist of cellulose, hemicellulose, lignin, small amount of pectin and proteins. Potent in the production of juice derived co-products	Austin et al. (1994), Hatfield (1990), Koegel and Straub (1996)

the cellulose and hemicelluloses are associated with lignin (Cowling and Kirk, 1976; Okeke and Obi, 1994). The lignin component acts as a physical barrier and must be removed to make the carbohydrates available for further transformation. Because lignin is believed to be a major hindrance to enzymatic hydrolysis (Chang and Holtzapple, 2000; Mooney et al., 1998; Yoon et al., 1995), its removal enhances cellulose digestion and also reduces non-productive binding of cellulose to lignin. For example, the digestibility of ARP (ammonia recycle percolation) treated corn stover is in the vicinity of 90% with 10 FPU/g glucan of enzyme loading, substantially higher than yields with α-cellulose under similar reaction conditions. Lignin and its derivatives are known to inhibit microbes (Palmqvist and Hahn-Hagerdal, 2000a,b), and lignin removal improves microbial activity. Thus, to utilize lignocellulosic biomass, it must be first pretreated to increase surface area, bulk density and decrease the crystallinity of the cellulose, so as to make it accessible for hydrolysis (Nikolov et al., 2000; Viesturs et al., 1996; Wood and Saddler, 1988). Biological routes are built around using enzymes to break down cellulose (cellulase) and perhaps hemicellulose (hemicellulase) to sugars. These sugars are then fermented to ethanol or other products, which are recovered and purified to meet market requirements (Wyman et al., 2005a).

Lignocellulosic biomass such as crop residues and sugar cane bagasse are included in feedstock for producing bioethanol (Kim and Dale, 2004). There are about 73.9 Tg dry wasted crop in the world that could potentially produce 49.1 Gl year^{-1} of bioethanol. About 1.5 Pg years^{-1} of dry lignocellulosic biomass from these seven crops is also available for conversion to bioethanol. Lignocellulosic biomass could produce up to 442 Gl year^{-1} of bioethanol. Thus, the total potentiality of bioethanol production from crop residues and wasted crops is 491 Gl year^{-1}, about 16 times higher than the current world ethanol production. The potential bioethanol production could replace 353 Gl of gasoline (32% of the global gasoline consumption). Pretreated sunflower hulls hydrolyzed with *T. reesei* Rut C30 cellulose showed 59.8% saccharification. Enzymatic hydrolysate concentrated to 40 g l^{-1} reducing sugars was fermented with *S. cerevisiae* var. ellipsoideus under optimum conditions of time (24 h), pH (5.0), temperature (30 °C) and inoculum size 3% (v/v) with 2.1×10^8, maximum ethanol yield of 0.454 g g^{-1}. Ethanol production scaled up in 1 and 15 l fermentors under optimum conditions revealed maximum ethanol yields of 0.449 and 0.446 g g^{-1}, respectively (Sharma et al., 2002). Generally, acid hydrolysis of lignocellu-

lose is conducted with mineral acids such as dilute H_2SO_4 or HCl (in range of 2–5%), at temperature of about 160 °C and pressure of about 10 atm (Sun and Cheng, 2002). In this process, acid concentration and temperature are crucial factors for forming toxic compounds. Moderate temperature (<160 °C) has proven adequate for hemicellulose hydrolysis, promoting little sugar composition. On the other hand, temperature above 160 °C favors cellulose hydrolysis, generating a high quality of sugars and lignin decomposition products (McMillan, 1994). Concentrated acids including H_2SO_4 or HCL are toxic, corrosive and hazardous, requiring corrosion-resistant reactors. Besides, such acids must be recovered after hydrolysis to make the process economically feasible, ecofriendly and safe. Several inhibitory compounds are also formed during hydrolysis of the raw material; the hydrolytic process has to be optimized so that inhibitors formation can be minimized. When low concentrations of inhibitory compounds are present in the hydrolyzate, detoxification is easier and fermentation is cheaper (Mussatto and Roberto, 2004). The cost of ethanol production from lignocellulosic material is relatively high based on current technologies and the main challenges are to low yield and high cost of hydrolysis process (Sun and Cheng, 2002).

Because cellulosics are competitive in price with oil, a key challenge to commercializing production of fuels and chemicals from cellulosic biomass is to reduce processing costs enough to achieve attractive investor returns (Lynd et al., 1999; Wyman, 1999). Biological conversion promises such low costs because it has the potential to achieve nearly theoretical yields and the modern tools of biotechnology can improve key process steps. Cellulosic biomass must be pretreated to realize high yields vital to commercial success in biological conversion (Mosier et al., 2005). Pretreatment is among the most costly steps and has a major influence on the cost of both prior (e.g., size reduction) and subsequent (e.g., enzymatic hydrolysis and fermentation) operations (Lynd et al., 1996; Wooley et al., 1999). Better pretreatment can reduce use of expensive enzymes. Thus, more attention must be given to gaining insight into interactions among these operations and applying that insight to advance biomass conversion technologies that reduce costs. In addition, although several pretreatments are promising, their relative attributes differ, but comparisons have been difficult due to differences in research methodology and substrate use. Improving the understanding of differences among pretreatment technologies and the effect of each pretreatment on other operations can facilitate selection, reduce commercialization risk and suggest opportunities for stepchange improvements.

8. Ethanol from industrial and urban wastes

The utilisation of renewable lignocellulosic agro-industrial residues has been attracting recent interest with high petrol prices, depletion of fossil fuel reserves and more recently, increasing environmental and political pressures. There are a number of different urban and industrial wastes such as cotton linters, spent sulfite liquor, cheese whey, wastes from vegetable and fruit industries, coffee waste, etc. These waste materials are presented in the form of solids and liquids and have to be processed for avoiding pollution of the environment. As these wastes can be used for ethanol production, their processing can become profitable (Kosaric et al., 1981). Currently, the Tennessee Valley Authority (TVA) is developing technology for dilute acid conversion of municipal solid waste (MSW) in

to ethanol (Lambert et al., 1990). MSW is the polymer of glucose (cellulose 45%,) hemicellulose 9% (polymer of sugar) could be used for ethanol production (Wyman, 1994; Wyman and Goodman, 1993). Spent sulfite-pulping liquor (SSL is a high organic byproduct of acid bisulfite pulp manufacture), which is fermented to make industrial ethanol. SSL typically concentrated to 240 g l^{-1} (22%, w/w) total solid to prior to fermentation and contains up to 24 g l^{-1} xylose sugar depending upon wood species used (Steve et al., 2004). Xylose in SSL and breakdown products acetic acid, lignosulphonates, and sulfite and ammonia can also be present if the pulping process is ammonia based (Rydholm, 1985). These compounds are potentially inhibitory towards sugar fermentation to ethanol (Batt et al., 1986; Rydholm, 1985). By using *S. cereviseae* 295ST for SSL fermentation up to 130% more ethanol can be produced to fermentations using non-xylose-fermenting yeast (Steve et al., 2004).

The production of ethanol from corn or wheat starch results in the concurrent production of stillage from distillation in quantities of up to 20 l of stillage for each litre of ethanol. Stillage can result in a polluting stream that can exceed a chemical oxygen demand (COD) of 10,000 mg/l (Wilke et al., 2000). The fermentation of sugars derived from the cellulose and hemicellulose fractions of concentrated stillage is an important way to improve the yield and productivity of the starch-to-ethanol processes. The concentrated byproduct, from this process, contained approximately 50% carbohydrates (including 32%, w/w hemicellulose), which has the potential to improve the yield of ethanol with little additional cost. The low lignin content (approximately 1%, w/w) of stillage makes it particularly attractive because phenol monomers obtained from lignin degradation are potent inhibitors of ethanol fermentation (Delgenes et al., 1996; Ranatunga et al., 1997). By comparison, typical lignocellulosic material such as wheat straw contains 8–15%, w/w lignin (Radomir, 1999). Since D-xylose is a major component of hemicellulose, its fermentation together with hexose sugars (mainly glucose from cellulose) represents an opportunity to improve the economics of fuel ethanol production (Wyman, 1995). The efficient hydrolysis of stillage to fermentable sugars is a critical step in this fermentation.

Stillage hydrolysates require a microorganism that can co-ferment xylose and glucose and also arabinose if possible. A number of potential ethanologens have been studied to determine their metabolic characteristics, with key fermentation criteria including high ethanol yield, ethanol tolerance, high specific productivity and resistance to inhibitory compounds typically present in lignocellulosic hydrolysates (Picataggio and Zhang, 1996; Zaldivar et al., 2001). For fermentation of molasses and starch hydrolysates, *S. cerevisiae* has been the microorganism of choice and currently enjoys a monopoly in the fuel ethanol industry. However, *Z. mobilis* is now attracting increasing attention for fuel ethanol production by virtue of its high specific rates of sugar uptake and ethanol production, ethanol tolerance and high ethanol yields when compared to other fermentation organisms (Rogers et al., 1997). However, both *Z. mobilis* and *S. cerevisiae* lack the complete pentose metabolism pathway necessary for fermentation of lignocellulosic hydrolysates. Metabolic engineering of *Z. mobilis* to ferment pentoses to ethanol offers an excellent opportunity for process improvement. Recombinant strains of *Z. mobilis* capable of metabolising a broad range of sugars including xylose and arabinose have been developed (Lawford and Rousseau, 2002; Zhang et al., 1995). Laboratory and pilot scale trials indicate that recombinant *Z. mobilis* can generate near theoretical yields from lignocellulosic feedstocks (Rogers et al., 1997).

Kinetic studies have been reported for both wild-type and recombinant *Z. mobilis* (Lawford and Rousseau, 2001).

Furfural was the main inhibitor present at a concentration of approximately $3.2\,g\,l^{-1}$ in the 2% acid hydrolysate. Higher concentrations of $5.6\,g\,l^{-1}$ were detected with 4% acid hydrolysate, which corresponded to a decrease in the recovery of sugar at this concentration. The presence of furfural has been reported to create a lag-phase but not to reduce the final yield of ethanol in yeast fermentation (Chung and Lee, 1985). Growth and final ethanol concentration of *Z. mobilis* have also been reported to be inhibited by 64 and 44%, respectively in the presence of $2\,g\,l^{-1}$ of furfural (Delgenes et al., 1996). It was therefore necessary to include an overliming detoxification step in the hydrolysate pretreatment. The procedure which involved raising the pH to 9–10, followed by neutralisation to pH 5.5 using 90% sulphuric acid has been reported by several researchers (Millati et al., 2002; Ranatunga et al., 2000). Davis et al. (2005) reported that the concentration of furfural was reduced by up to 55% after 'liming' to pH 5, the optimum pH for *Z. mobilis* fermentation.

Concentrated stillage from the fermentation of wheat starch hydrolysates is a readily available, high carbohydrate substrate that provides an opportunity for additional production of fuel ethanol. Dilute acid hydrolysis was found to be an effective method of hydrolysing the hemicellulosic fraction of stillage prior to fermentation. Further research is required to develop an enzyme complex that is effective in hydrolysing cellulose in stillage following dilute acid pretreatment cost effectively. Recombinant *Z. mobilis* ZM4 (pZB5) demonstrated its potential suitability for fermentation of the concentrated stillage acid hydrolysates (Davis et al., 2005).

9. Conclusion

An increased use of biofuels would contribute to sustainable development by reducing greenhouse-gas emissions and the use of non-renewable resources (Henke et al., 2005). In recent years it has been suggested that, instead of traditional feedstocks (starch crops), cellulosic biomass (cellulose and hemicellulose), including agricultural and forestry residues, waste paper, and industrial wastes, could be used as an ideally inexpensive and abundantly available source of sugar for fermentation into transportation fuel ethanol. Agricultural biomass is composed of three fractions, viz., cellulose, hemicellulose and lignin. The efficiency of biomass conversion to ethanol depends upon the ability of the microorganism used in the process to utilize these diverse carbon sources and amount of fraction present in biomass. All low quality cereal grains (wheat, rice, corn and barley), potatoes, beans field peas, cassava roots, etc., might be also good substrate for ethanol production.

This review indicates the potentiality of sugar crops, agro and urban/industrial residues feedstocks for production of ethanol as an alternative fuel and energy sources, which is renewable, sustainable, efficient, and safe for environment. The cost of ethanol production from lignocellulosic material is relatively high based on current technologies, and the main challenges are to low yield and high cost of hydrolysis. There is need of process optimization for detoxification and maximize conversion of agro and urban/industrial residues feedstocks for production of ethanol as a cheaper substrate like molasses and other directly fermentable materials.

Although bioethanol production has been greatly improved by new technologies, there are still challenges that need further investigations. These challenges include maintaining a stable performance of the genetically engineered microorganisms in commercial scale fermentation operations and developing more efficient pretreatment technologies for the lignocellulosic biomass and integrating the optimal components into economic ethanol production systems.

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