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Fuel Ethanol Production from Corn Fiber

Current Status and Technical Prospects

BADAL C. SAHA,* BRUCE S. DIEN, AND RODNEY J. BOTHAST

*Fermentation Biochemistry Research Unit, National Center for Agricultural Utilization Research, Agricultural Research Service, US Department of Agriculture, Peoria, IL 61604***

ABSTRACT

Corn fiber, which consists of about 20% starch, 14% cellulose, and 35% hemicellulose, has the potential to serve as a low cost feedstock for production of fuel ethanol. Currently, the use of corn fiber to produce fuel ethanol faces significant technical and economic challenges. Its success depends largely on the development of environmentally friendly pretreatment procedures, highly effective enzyme systems for conversion of pretreated corn fiber to fermentable sugars, and efficient microorganisms to convert multiple sugars to ethanol. Several promising pretreatment and enzymatic processes for conversion of corn fiber cellulose, hemicellulose, and remaining starch to fermentable sugars were evaluated. These hydrolyzates were then examined for ethanol production in bioreactors, using genetically modified bacteria and yeast. Several novel enzymes were also developed for use in pretreated corn fiber saccharification.

Index Entries: Fuel ethanol; corn fiber; pretreatment; enzymatic saccharification; fermentation.

INTRODUCTION

In the United States, over 1.3 billion gallons of ethanol are produced annually, primarily from corn starch. Various agricultural residues, such as corn stover, straw, and bagasse, can also serve as low-value and abundant feedstocks for production of fuel ethanol. In general, these contain about 35–50% cellulose, 20–35% hemicellulose, and 5–25% lignin. Corn fiber

* Author to whom all correspondence and reprint requests should be addressed.

** Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

represents a renewable resource that is available in sufficient quantities from the corn wet-milling industries to serve as a low-cost feedstock for production of fuel ethanol. Currently, the utilization of corn fiber to produce fuel ethanol presents significant technical and economic challenges, and its success depends largely on the development of environmentally friendly pretreatment procedures, highly effective enzyme systems for conversion of pretreated corn-fiber substrate to fermentable sugars, and efficient microorganisms to ferment mixed sugars to ethanol. In this paper, a comprehensive progress report on this endeavor is presented.

Corn Fiber as Feedstock

In the United States, both wet- and dry-milling processes are currently used to produce ethanol from corn. Wet milling accounts for about 60% of total ethanol production. In a typical wet-milling process, cleaned corn is soaked in circulating water, slightly acidified with 0.1–0.2% SO_2, at 51–54°C for 24–48 h to soften the kernel, loosen the germ, and hull and swell the endosperm. Corn fiber is a mixture of corn hulls and residual starch not extracted during the milling process, making up 11% of the dry wt of the corn kernel *(1)*. Presently, corn fiber is marketed in corn-gluten feed. A typical composition of corn fiber is given in Table 1. It contains about 70% fermentable sugars, of which approx 20% comes from starch. Typically, 32 lb of starch is obtained from a bu of corn (56 lb), which yields about 2.5 gal of ethanol in industrial practice. From the same bu of corn, about 4.5 lb of corn fiber is obtained, which produces about 3.15 lb of fermentable sugars. These fermentable sugars will theoretically yield about 0.3 gal of ethanol. In practice, it may yield about 0.24 gal of ethanol per bu of corn. The low cost and high carbohydrate content of corn fiber makes it an attractive feedstock for conversion to fuel ethanol. Gulati et al. *(2)* estimated that a wet-milling facility that currently produces 100 million gal of ethanol per yr from starch could generate an additional $4–8 million of annual income, if the fiber components were processed into ethanol. The conversion of corn fiber to ethanol involves four basic steps: pretreatment, enzymatic saccharification, fermentation of hydrolyzate, and ethanol recovery. The enzymatic saccharification and fermentation can also be performed together in a process known as simultaneous saccharification and fermentation (SSF).

Pretreatment and Enzymatic Saccharification of Corn Fiber

Native corn-fiber cellulose and hemicellulose are resistant to enzymatic hydrolysis. The pretreatment of corn fiber is thus crucial before enzymatic hydrolysis. Various pretreatment options can be used to solubilize, hydrolyze, and separate starch, cellulose, hemicellulose, and lignin components of corn fiber. A number of pretreatment procedures, such as dilute acid, alkali, ammonia, alkaline peroxide, and liquid hot-water treatments,

Table 1
Composition of Corn Fiber

Component[a,b]	%[c]
Carbohydrate	
Crude fiber	14.1 (0.97)
Starch	19.68 (0.91)
Glucose	37.19 (1.86)
Xylose	17.58 (1.76)
Arabinose	11.25 (1.46)
Galactose	3.59 (0.336)
Total sugars	69.6 (5.03)
Other components	
Protein	10.98 (0.52)
Klason lignin	7.78 (0.74)
Acetyl groups	1.71 (0.13)
Ash	0.6 (0.05)
Crude fat	2.53 (0.31)
Unknown	6.79

[a] Average concentration of each component is expressed in wt% of total dry solids.
[b] The sugar content is expressed on anhydrous basis.
[c] Numbers in parentheses refer to standard deviations.
From Ref. 1.

have been tried for pretreatment of corn fiber *(1,3–5)*. Each of them has distinct advantages and disadvantages. Osborn and Chen *(6)* reported that the starch fraction of the corn hull was completely hydrolyzed by glucoamylase after the corn hull was heated with steam for 5 min, and the destarched hemicellulose fraction of the corn hull was readily hydrolyzed with dilute sulfuric acid at 135°C. Grohmann and Bothast *(1)* investigated saccharification of corn fiber by treating first with dilute sulfuric acid (100–160°C), and then with enzymes (cellulase and glucoamylase, 45°C), after partial neutralization. The sequential treatment achieved a high (~85%) conversion of corn-fiber polysaccharides to monomeric sugars. The formation of compounds inhibitory to fermentative microorganisms was evident for all pretreatments tested at 140 and 160°C. Moniruzzaman et al. *(4)* optimized the conditions necessary for pretreatment of corn fiber with high moisture content (150% moisture, dry wt basis) by ammonia fiber explosion (AFEX). The best results were obtained at 90°C with an ammonia and dry corn fiber ratio of 1:1, and a residence time of 30 min (reactor pressure ~200 psig). More than 80% of the theoretical sugar yield was obtained during enzymatic hydrolysis of the AFEX-pretreated corn fiber with a combined mixture of commercial enzyme preparations (α-amylase, glucoamylase, cellulase, hemicellulase, and β-glucosidase).

However, xylooligosaccharides represented about 30–40% of xylan-degradation products, and very little xylose was produced *(7)*. Recently, Leathers and Gupta *(5)* reported that pretreatment of corn fiber with alkaline peroxide nearly doubled the susceptibility of corn-fiber hemicellulose to enzymatic digestion.

The hydrolysis of cellulose to glucose, which requires the synergistic actions of endoglucanase, cellobiohydrolase, and β-glucosidase, is very slow because of product and substrate inhibitions *(8)*. In addition, cellulases are expensive because of high production costs, and are difficult to reuse. Both endoglucanase and cellobiohydrolase are inhibited by cellobiose. Although β-glucosidase hydrolyzes cellobiose to glucose, most β-glucosidases are inhibited by glucose, as well as by cellobiose *(9)*. Freer *(10,11)* purified and characterized a unique β-glucosidase from *Candida wickerhamii*, and studied the kinetics of the enzyme in detail. The enzyme demonstrated optimal activity between pH 4.0 and 5.0, and was stable below 40°C. Cellodextrins were good substrates for the enzyme. No inhibition of *p*-nitrophenyl β-D-glucoside hydrolysis by the enzyme was detected with 50 m*M* glucose. Skory and Freer *(12)* have isolated and cloned the gene encoding this glucose-insensitive β-glucosidase in *Escherichia coli*. The introduction of this gene in *Saccharomyces cerevisiae* has the potential to yield a cellodextrins-fermenting yeast *(13)*. In a continuing search for a thermophilic β-glucosidase insensitive to glucose inhibition, Saha and Bothast *(14)* screened a variety of yeasts from the Agricultural Research Service (ARS) culture collection. Enzymes from 15 yeast strains showed very high glucose tolerance (<50% inhibition at 30% glucose). Optimal temperature and pH for these 15 β-glucosidase preparations varied from 30 to 65°C and pH 4.5 to 6.5. The β-glucosidase from *Debaryomyces yamadae* NRRL Y-11714 showed the highest optimal temperature at 65°C, followed by enzymes produced by *Candida chilensis* NRRL Y-17141 and *Kluyveromyces marxianus* NRRL Y-1195 at 60°C. The optimal pHs of these three enzyme preparations were 6.5, 6.0, and 6.5, respectively. The β-glucosidase from all these strains hydrolyzed cellobiose. The β-glucosidase from *Candida peltata* NRRL Y-6888 was purified and characterized *(15)*. Enzyme production was not repressed by glucose, making glucose a good carbon source for production of the enzyme. The optimal pH and temperature for the action of the purified enzyme were 5.0 and 50°C, respectively. The enzyme hydrolyzed cellobiose and cellooligosaccharides very well, and was highly tolerant to glucose, with a K_i of 1.4 *M* (252 mg/mL). The β-glucosidase was not inhibited by cellobiose (15%). Cellobiose (10%) was almost completely hydrolyzed to glucose by the purified β-glucosidase, in both the absence and presence of 6% glucose (Fig. 1). The β-glucosidase from a color-variant strain of *Aureobasidium pullulans* was purified, characterized, and found to be highly thermostable and optimally active at 75°C *(16)*. The half-life of the crude enzyme was 72 h at 75°C and 24 h at 80°C. The enzyme hydrolyzed both cellobiose and cellooligosaccharides very well, but was strongly inhibited by glucose with a K_i of 5.65 m*M*.

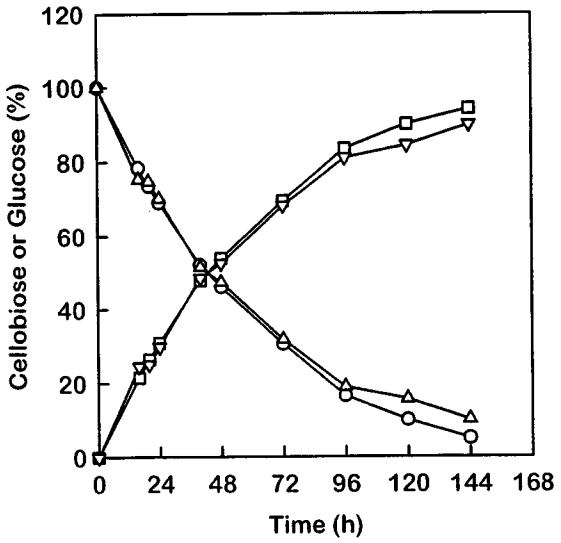

Fig. 1. Time-course of cellobiose (10%, w/v) hydrolysis by purified β-glucosidase (1.5 U/mL) from *C. peltata* Y-6888 in the absence and presence of glucose (6%, w/v) at pH 5.0 and 50°C *(15)*. Symbols: (○), cellobiose only; (□), glucose formed from cellobiose, (△), cellobiose with glucose; (▽), glucose formed from cellobiose in the presence of glucose (from Ref. 15).

The total hydrolysis of xylan requires endoxylanase, β-xylosidase, and several accessory enzymes, such as α-L-arabinofuranosidase, α-glucuronidase, acetyl xylan esterase, feruloyl esterase, and *p*-coumaroyl esterase, which are necessary for hydrolyzing various substituted xylans. Many xylanases do not cleave glycosidic bonds between xylose units that are substituted. Therefore, the side chains must be cleaved before the xylan backbone can be completely hydrolyzed *(17)*. On the other hand, several accessory enzymes only remove side chains from xylooligosaccharides. These enzymes require xylanases to partially hydrolyze hemicellulose before side chains can be cleaved *(18)*. The corn-fiber hemicellulose was resistant to hydrolysis to fermentable sugars by commercial hemicellulase preparations *(7)*. Structural analysis of the corn-fiber xylan suggests that over 70% of the xylose backbone residues have one or more arabinose, 4-O-methylglucuronic acid, or other side chains *(19)*. As a result, there are few regions in corn-fiber xylan where several contiguous xylose residues are unsubstituted, which makes enzymatic hydrolysis of xylan difficult.

Fermentation of Corn Fiber Hydrolyzates

Most of the xylose- and glucose-fermenting yeasts, such as *Pichia stipitis*, *Candida shehatae*, and *Pachysolen tannophilus*, do not have the ability to produce ethanol from L-arabinose *(20–22)*. Since corn fiber contains 12% L-arabinose, Dien et al. *(23)* screened 116 different L-arabinose-utilizing

yeasts for production of ethanol from L-arabinose, and found the following species able to ferment the sugar: *Ambrosiozyma monospora* (NRRL Y-1484), *Candida* sp. (NRRL YB-2248), *Candida auringiensis* (NRRL Y-11848), and *Candida succiphila* (NRRL Y-11998). These yeasts produced low levels (up to 4.1 g/L) of ethanol, and are potential candidates for mutational or other genetic enhancements for increased ethanol production. Most yeasts are inefficient in the regeneration of the co-factor required for the conversion of L-arabinose to ethanol. Saha and Bothast *(24)* studied the production of L-arabitol from L-arabinose by two superior L-arabitol producers *(Candida entomaea* NRRL Y-7785 and *Pichia guilliermondii* NRRL Y-2075). These two strains produced L-arabitol (0.70 g/g) from L-arabinose (50 g/L) at 34°C and pH 5.0 and 4.0, respectively. Both yeasts produced ethanol (0.32–0.33 g/g) from glucose (50 g/L), and only xylitol (0.43–0.51 g/g) from xylose (50 g/L). The yeasts co-utilized xylose (6.2–6.5 g/L) and L-arabinose (4.9–5.0 g/L) from a corn-fiber acid hydrolyzate simultaneously, and produced xylitol (0.10 g/g xylose) and L-arabitol (0.53–0.54 g/g L-arabinose). *Klebsiella oxytoca* strain P2 is a recombinant organism in which the pyruvate decarboxylase *(pdc)* and alcohol dehydrogenase *(adh B)* genes from *Zymomonas mobilis* have been integrated into the *pfl* gene within the chromosome of *K. oxytoca* M5A1, and expressed at high levels *(25)*. This strain diverts the metabolism of pyruvate to ethanol, and produces a lesser quantity of acetic acid. Bothast et al. *(26)* evaluated this organism for its ability to ferment L-arabinose, xylose, and glucose, alone and in mixtures, in pH-controlled batch fermentations. The recombinant organism produced 0.34–0.43 g ethanol/g sugar at pH 6.0 and 30°C on 8% sugar substrate, and utilized L-arabinose very well. With the mixture of sugars, *K. oxytoca* strain P2 showed a preference for glucose. Xylose utilization was slow, and approx 47 and 29% of supplied xylose were left unutilized in mixtures A (glucose:xylose:arabinose, 1:1:1) and B (glucose:xylose:arabinose, 1:2:1), respectively, even after 114 h fermentation. Sugar utilization was glucose > arabinose > xylose, and ethanol production was xylose > glucose > arabinose.

Grohmann and Bothast *(1)* investigated the fermentation of dilute acid hydrolyzates of 15% corn fiber slurries with recombinant *E. coli* K011. *E. coli* K011 is a recombinant derivative of *E. coli* B, in which the *Z. mobilis* genes encoding *pdc* and *adh B* genes have been integrated *(27)*. The fermentations were conducted in 2 L magnetically stirred bioreactors (Multigen™ 2000 fermenter, New Brunswick, Edison, NJ) or covered beakers (Fleaker,™ Corning, NY) equipped with pH, temperature, and agitation controls *(28)*. Final ethanol concentrations exceeded 3% w/v in 3 d, and yields ranged from 19–62% of theoretical. As mentioned earlier, the formation of inhibitory compounds became readily apparent for all pretreatments tested at 140 and 160°C. Asghari et al. *(29)* reported that hemicellulose hydrolyzates of the agricultural residues bagasse, corn stover, and corn hulls plus fibers were readily fermented to ethanol by recombinant *E. coli*

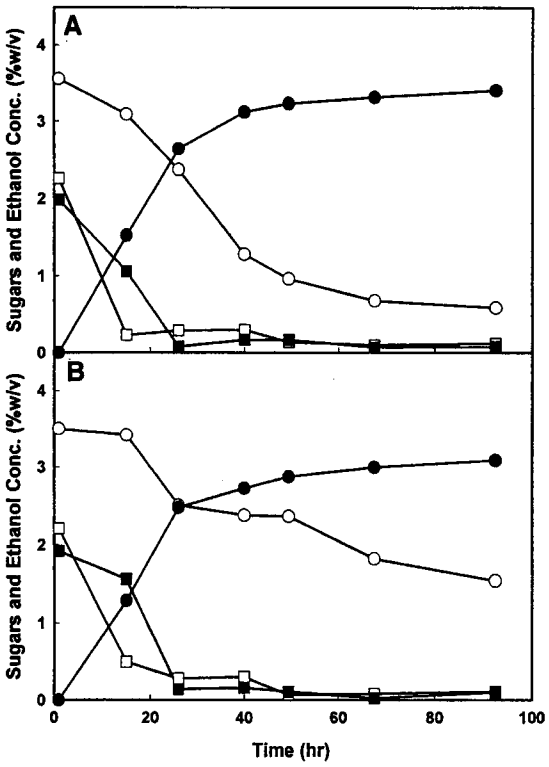

Fig. 2. Fermentation of corn fiber acid hydrolysate with recombinant *e. coli* **(A)** strain K011 and **(B)** strain SL40. Symbols: □, glucose; ■, L-arabinose; ○, xylose and galactose; ●, ethanol. Xylose and galactose comigrated during HPLC analysis (from Ref. 3).

strain K011. Corn-steep liquor and crude yeast autolyzate served as excellent nutrients. Fermentations were essentially complete within 48 h, often achieving over 40 g ethanol/L.

Dien et al. *(3)* treated corn fiber with dilute sulfuric acid (1%, v/v H_2SO_4) at 121°C for 1 h. Following hydrolysis, insoluble materials were removed by filtration, and the hydrolyzates were neutralized to pH 6.5 with $Ca(OH)_2$. Insoluble $CaSO_4$ was then removed by filtration. The total reducing sugar present in a typical hydrolyzate preparation was 89.8 g/L with glucose (26.1 g/L), xylose and galactose (40.9 g/L), and L-arabinose (22.8 g/L). These hydrolyzate preparations were then fermented to ethanol using recombinant *E. coli* strains K011 and SL40. *E. coli* SL 40 is a phosphomycin-resistant mutant of strain K011 that has been reported to produce 60 g/L ethanol from 120 g/L xylose in 60 h, 20% more than K011 under identical conditions *(30)*. Ethanol yields were 0.38–0.41 g/g of sugar consumed, and fermentations were complete within 60 h. Both strains fermented glucose and L-arabinose well, but the fermentation of xylose was slow and incomplete (Fig. 2). Both strains produced minor amounts of organic acids such as lactic acid (2.1–2.2 g/L), acetic acid (0.5–0.8 g/L),

Table 2
Fermentation of AFEX-Pretreated Corn Fiber Enzymatic Hydrolyzate (EH) and Simulated Sugar Mixtures (SM) by Recombinant Bacteria[a]

Strain	Substrate	Cell mass (g/L)	Base consumed[b] (mmol/L)	Maximum ethanol[c] (g/L)	Ethanol yield[d] (g/g)
E. coli strain K011	EH	3.2	65	27.1	0.47
	SM	3.2	69	27.1	0.47
E. coli strain SL40	EH	2.9	61	26.6	0.46
	SM	3.2	69	27.2	0.47
K. oxytoca strain P2	EH	2.1	57	20.0	0.35
	SM	2.9	62	20.3	0.35

[a] Values reported are from duplicate experiments.
[b] Base (KOH) refers to that added automatically to maintain pH at 6.0 during fermentation.
[c] Ethanol yields are corrected for dilution by base addition.
[d] Ethanol yields in g/g of substrate available for fermentation.
From Ref. 31.

and succinic acid (0.4–0.5 g/L). The amount of base (2 *M* KOH) added to the cultures to maintain at pH 6.5 was 163 mmol/L in the case of strain K011 and 120 mmol/L in the case of strain SL40. Overall, strain K011 tended to use sugars better than strain SL40. The presence of glucose in the fermentation medium inhibited xylose utilization by *E. coli* strain K011 *(28)*.

Moniruzzaman et al. *(31)* studied ethanol production from AFEX-pretreated corn fiber by recombinant *E. coli* strains K011 and SL40, and by *K. oxytoca* strain P2, under pH-controlled conditions. Enzymatic digestion of AFEX-pretreated corn fiber produced a hydrolyzate containing (g/L): glucose, 30.2; xylose, 3.1; L-arabinose, 4.0; and galactose, 2.0. The hydrolyzate was supplemented with additional xylose and arabinose, so that theoretical concentrations of xylose (15.2 g/L) and L-arabinose (10.5 g/L) were realized. Both *E. coli* strains (K011 and SL40) efficiently utilized most of the sugars contained in the hydrolyzate and produced a maximum of 27.1 and 26.6 g/L ethanol, respectively, equivalent to 92 and 90% of the theoretical yield. Very little difference was observed in cell growth and ethanol production between the fermentation of the enzymatic hydrolyzate and mixtures of pure sugars used to simulate the hydrolyzate (Table 2). These results indicate good compatibility of AFEX-pretreatment, and subsequent fermentation with recombinant bacteria.

Moniruzzaman et al. *(32)* also studied ethanol production from AFEX-pretreated corn fiber by recombinant *Saccharomyces* strain 1400 (pLNH32). *Saccharomyces* strain 1400 (pLNH32) was genetically engineered to ferment

xylose by expressing genes encoding a xylose reductase, a xylitol dehydrogenase, and a xylulose kinase *(33)*. The fermentation experiment with the recombinant yeast was performed in a pH 5.0-controlled flask under anaerobic conditions with an inoculum level of 2.0 g/L (dry wt). The recombinant yeast fermented the AFEX-pretreated corn-fiber enzymatic hydrolyzate containing glucose (33.5 g/L), xylose (7.5 g/L), arabinose (5.0 g/L), and galactose (1.0 g/L), and produced ethanol.

Although genetically engineered bacteria and yeast hold tremendous potential for the fermentative conversion of multiple substrates to ethanol, questions still remain concerning the stability or hardiness of these organisms and their ability to perform in a large-scale industrial process. Ingram et al. *(34)* were the first to report a metabolically engineered *E. coli* for high alcohol production. The resultant recombinant strain produced more than 4% w/v ethanol from glucose in media containing ampicillin, with positive selection pressure for the plasmid. A considerably more stable strain was developed by Ohta et al. *(27)*, by integrating the PET operon (gene cluster producing ethanol) and chloramphenicol (cm) resistance gene into the *E. coli* chromosome. The resultant *E. coli* K011 strain did not require cm in the growth media for retention of the PET operon, but, in the absence of cm, ethanol production was lower, presumably because of reduced PET gene copy number. When mutants were selected for resistance to high levels (600 μg/mL) of cm, high ethanol production was restored. Hespell et al. *(35)* have undertaken an alternative approach to eliminate the requirement for antibiotics. A lactate dehydrogenase *(ldh)*–pyruvate formate lyase *(pfl)* double mutant of *E. coli* was used as the cloning host *(36)*. Although capable of aerobic growth, this mutant strain FMJ39 is incapable of anaerobic growth, because of its inability to regenerate oxidized pyridine nucleotides by reduction of pyruvate to lactate. Clones having recombinant plasmids containing a *ldh* gene can be isolated by complementing for anaerobic growth by this strain. It was reasoned that an alternative complementation for anaerobic growth would be the PET-operon-containing plasmids, because expression of the *pdc* and *adh* genes would convert pyruvate to ethanol and regenerate oxidized pyridine nucleotides. If so, the resultant strains should be quite anaerobically stable for ethanol production. The strains should also not require antibiotics (ampicillin or tetracycline) in the growth media to maintain positive selective pressure for cells containing the PET operon plasmid, because loss of the plasmid would be a conditionally lethal event under anaerobic growth conditions. *E. coli* strains FBR1 and FBR2 were created by transforming *E. coli* FMJ39 with the PET operon plasmids pL01295 and pL01297, respectively. Both strains were capable of anaerobic growth and displayed no apparent PET plasmid losses after 60 generations in serially transferred (9×) anaerobic batch cultures. In contrast, similar aerobic cultures rapidly lost the PET operon plasmids. In high-cell-density batch fermentations, up to 4.4% w/v ethanol was produced from 10% glucose. In anaerobic, glucose-limited continuous

culture, one strain grown for 20 d (51 generations, 23 with tetracycline, and then 28 after tetracycline was removed) showed no loss of antibiotic resistance. Anaerobic, serially transferred batch cultures and high-density fermentations were inoculated with cells taken at 57 generations from the previous continuous culture. Both cultures continued high ethanol production in the absence of tetracycline. The genetic stability conferred by selective pressure for PET-containing cells, without requirement for antibiotics, suggests potential commercial suitability for these *E. coli* strains.

CONCLUSION

It is now possible to convert corn fiber hydrolyzates containing mixed sugars (glucose, xylose, L-arabinose, and galactose) to ethanol by using recombinant microorganisms. However, many fundamental problems need to be overcome before this becomes a commercial reality. They include:

1. High cost of cellulase enzymes.
2. Ineffectiveness of commercial hemicellulases to degrade corn fiber xylans.
3. Production of inhibitory substances to microbial fermentation during dilute acid pretreatment.
4. Stability and ethanol tolerance of the recombinant organisms.
5. Recovery of dilute ethanol from fermentation broth.
6. Disposal of waste containing recombinant organisms.

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