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Bacteria engineered for fuel ethanol production: current status

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Abstract The lack of industrially suitable microorganisms for converting biomass into fuel ethanol has traditionally been cited as a major technical roadblock to developing a bioethanol industry. In the last two decades, numerous microorganisms have been engineered to selectively produce ethanol. Lignocellulosic biomass contains complex carbohydrates that necessitate utilizing microorganisms capable of fermenting sugars not fermentable by brewers' yeast. The most significant of these is xylose. The greatest successes have been in the engineering of Gram-negative bacteria: *Escherichia coli*, *Klebsiella oxytoca*, and *Zymomonas mobilis*. *E. coli* and *K. oxytoca* are naturally able to use a wide spectrum of sugars, and work has concentrated on engineering these strains to selectively produce ethanol. *Z. mobilis* produces ethanol at high yields, but ferments only glucose and fructose. Work on this organism has concentrated on introducing pathways for the fermentation of arabinose and xylose. The history of constructing these strains and current progress in refining them are detailed in this review.

bioethanol industry that relies on lignocellulose as a feed source. The characteristics required for an industrially suitable microorganism have been cited previously (Bothast et al. 1999; Zaldivar et al. 2001) and are summarized in Table 1. Many of these traits have been suggested by experience from the current starch ethanol industry. The trait that has received the most attention is ethanol yield. Because feedstocks typically account for greater than one-third of the production costs, maximizing ethanol yield is imperative. Obtaining a high ethanol yield means using strains that produce ethanol with few side products, and metabolize all major sugars. Typically these include glucose, xylose, arabinose, galactose and mannose (Wiseloge et al. 1996).

In addition to these traits, the process may place additional constraints on the microorganism. The highest ethanol yields have been reported for processes using enzymatic digestion of cellulose. The biomass is pretreated for enzymatic digestion by heating in the presence of a mineral acid or base catalyst. Pretreating biomass completely or partially hydrolyzes the hemicellulose, removes the lignin and de-crystallizes the cellulose, thereby allowing the cellulase enzymes access to the cellulose fibers. Pretreated cellulose can be enzymatically hydrolyzed either prior to fermentation in sequential saccharification and fermentation or by adding the cellulase and inoculum together as in simultaneous saccharification and fermentation, (SSF). SSF gives higher reported ethanol yields and requires lower amounts of enzyme because endproduct inhibition from cellobiose and glucose formed during enzymatic hydrolysis is relieved by the yeast fermentation. However, SSF requires that enzyme and culture conditions be compatible with respect to pH and temperature. *Trichoderma reesei* cellulases, which constitute the most active preparations, have optimal activity at pH 4.5 and 55°C. For *Saccharomyces* cultures, SSF are typically controlled at pH 4.5 and 37°C.

While no one strain meets all of the requirements listed above, great strides have been made in the development of promising microorganisms for industrial ethanol produc-

Introduction

Improved biocatalysts and cellulase preparations are the major technical roadblocks to building a successful

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Table 1 Important traits for ethanol production

Trait	Requirement
Ethanol yield	>90% of theoretical
Ethanol tolerance	>40 g l ⁻¹
Ethanol productivity	>1 g l ⁻¹ h ⁻¹
Robust grower and simple growth requirements	Inexpensive medium formulation
Able to grow in undiluted hydrolysates	Resistance to inhibitors
Culture growth conditions retard contaminants	Acidic pH or higher temperatures

tion. The ethanologenic bacteria that currently show the most promise for industrial exploitation are *Escherichia coli*, *Klebsiella oxytoca* and *Zymomonas mobilis*. In the following sections, the development of Gram-negative bacteria for the production of ethanol is reviewed. Much good research has also been directed at developing Gram-positive and thermophilic bacteria for ethanol fermentation. However, metabolic engineering of these strains is still in its beginning stages and thus beyond the scope of this review. Recent research on yeast is also not included, but is reviewed in Jeffries and Jin (submitted).

While the specifics of how each of these microorganisms was engineered for ethanol fermentation vary, the reader will notice many similarities. Following the successful expression of genes needed for either xylose metabolism or ethanol production, vastly improved strains were developed by screening hosts with a wide variety of genetic backgrounds. Ethanol yields were subsequently improved by eliminating competing reactions. These early constructed strains relied on plasmids for gene expression, which can be lost from cultures growing in non-selective medium. An important step in strain development, therefore, was integrating the recombinant genes while still maintaining proper gene expression for optimal ethanol production. Furthermore, the eventual success of each depended upon proper medium optimization and, for some, on long-term adaptation strategies. Finally, all of the researchers paid careful attention to maintaining a balanced or healthy microbial physiology to ensure hardy cultures and high productivities.

Engineering *E. coli* for ethanol production

The construction of *E. coli* strains to selectively produce ethanol (Ingram et al. 1987) was one of the first successful applications of metabolic engineering. *E. coli* has several advantages as a biocatalyst for ethanol production, including the ability to ferment a wide spectrum of sugars, no requirements for complex growth factors, and prior industrial use (e.g., for production of recombinant protein). The major disadvantages associated with using *E. coli* cultures are a narrow and neutral pH growth range (ca. pH 6.0–8.0), less hardy cultures compared to yeast, and public perceptions regarding the danger of *E. coli* strains.

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

E. coli ferments sugars to a mixture of ethanol and organic acids (Fig. 1A). Ethanol is produced from pyruvate using pyruvate formate lyase (PFL). This fermentation pathway is unbalanced because one NADH, H⁺ is generated for each pyruvate made from sugars, and two NADH, H⁺ are required for converting pyruvate into ethanol. *E. coli* balances its fermentation by also producing acetic and succinic acids. Yeasts and *Z. mobilis* are homo-ethanol fermentative because they convert pyruvate to ethanol utilizing pyruvate decarboxylase (PDC), which only consumes one NADH, H⁺ for each ethanol produced. Therefore, it was expected that expressing *pdh* in *E. coli* would cause it to produce only ethanol. Early attempts to introduce a yeast-like pathway for converting pyruvate to ethanol were unsuccessful because the native alcohol dehydrogenase (ADH) activity of *E. coli* was not sufficient to achieve high ethanol yields. Ingram et al. (1987) isolated *adh II* from a genomic library of *Z. mobilis* using a novel indicator medium for ADH activity. *E. coli* transformed with a plasmid expressing *pdh* and *adh II* produced ethanol almost exclusively (Fig. 1B) (Ingram et al. 1987). The *adhII* and *pdh* genes were co-expressed under the control of the native *lac* promoter, and the construct was named the PET (production of ethanol) operon (Ingram et al. 1987).

Ingram and colleagues proceeded to evaluate several *E. coli* strains (Alterthum and Ingram 1989) as hosts. The major criteria for the screening included tolerance to harsh growing conditions (e.g., ethanol tolerance), plasmid stability in non-selective medium, and superior ethanol yield on xylose. *E. coli* ATCC11303 (pLOI297) was determined to be the most promising strain. Xylose fermentations with ATCC11303 (pLOI297) were run under a wide-variety of culture conditions to fully characterize the strain (Beall et al. 1991). Optimal culture conditions were determined to be below 42°C and above pH 6.0. Under these growth conditions, xylose-fermenting cultures had a maximum ethanol tolerance of 53–56 g/l and a final average productivity (for 80 g/l xylose) of 0.72 g l⁻¹ h⁻¹.

The initial *E. coli* strains each depended upon a plasmid for expression of the *adh* and *pdh* genes. While plasmids carrying the *adh* and *pdh* genes were not readily lost in the absence of antibiotics, these strains were still too genetically unstable for use in industrial fermentations. Therefore, Ingrams research group sought to stabilize ethanol production by integrating the genes into the chromosome of strain ATCC11303 (Ohta et al. 1991a). The PET operon was inserted into the *pyruvate formate lyase (pfl)* gene, in an attempt to eliminate an enzyme competing for pyruvate. However, the resulting construct yielded much lower amounts of ethanol than the plasmid-bearing strains. This was attributed to reduced gene dosage. A strategy was developed for selecting mutants with increased *adh* and *pdh* expression. The integrated DNA contained a chloramphenicol resistance marker.

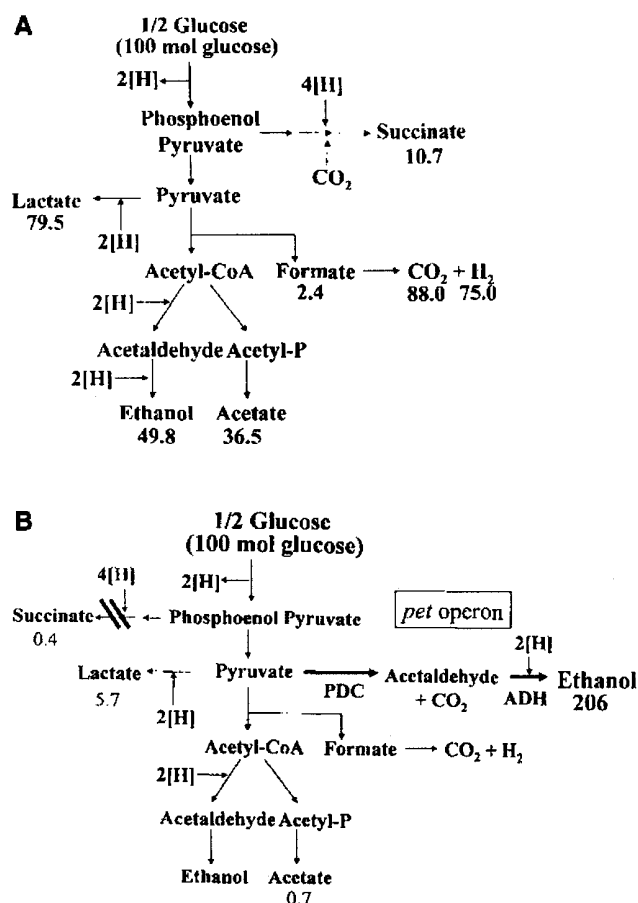


Fig. 1. A Typical fermentation products made by a K12 *Escherichia coli* fermenting glucose. Products are in moles produced per 100 mol fermented glucose (Gottschalk 1986) with 91% of the carbon accounted for as fermentation products. B Transforming *E. coli* with *pet* operon diverts almost all glucose to ethanol. This strain (KO11) also carries a mutation that blocks succinate production. Amount of each fermentation product is shown per 100 mol glucose. (Ohta et al. 1991a). Moles of CO₂ produced was not measured, but should be 206 mol based on ethanol production.

These transformants were further screened for increased chloramphenicol (Cm) resistance (e.g., 40–600 mg/l) because it was reasoned that hyper-Cm tolerance might correlate with increased expression of the PET operon gene products. The hyper-resistant mutants were finally screened on acetaldehyde selection plates, and hyper-ethanol producing strains were successfully recovered. Disrupting the terminal gene in the succinate pathway, fumarate reductase (*frd*), eliminated succinate production and further increased ethanol yield. This final strain (KO11) was able to convert glucose and xylose to ethanol at yields 103 to 106% of theoretical. The extra ethanol was thought to arise from fermentation of carbohydrates present in the rich medium that were not accounted for in the sugar balance. Recently, researchers noticed that KO11 grows faster on xylose-containing medium than its parent strain ATCC 11303. Comparison of global gene expression by microarray technology, demonstrated that KO11 over-expresses xylose metabolism genes (Tao et al.

2001). Strain KO11 has been evaluated for fermentation of hemicellulose hydrolysates from *Pinus* wood, sugarcane bagasse and corn stover (Asghari et al. 1996; Barbosa et al. 1992). The strain has not been widely used for SSF, probably because the optimal culture pH (6.5) is not compatible with *T. reesei* cellulase complex, which has a pH optimum of 4.6.

The literature is inconsistent regarding the phenotypic stability of KO11 for high ethanol yields. While researchers in several laboratories have been able to replicate Ingrams results (Dien et al. 1997; Hahn-Hagerdal et al. 1994), Lawford and Rousseau reported lower than expected ethanol yields, which they attributed to genetic instability (Lawford and Rousseau 1995, 1996). Dumsday et al. (1999) carried out the most comprehensive culture studies. When KO11 was grown in continuous cultures, high ethanol yields were maintained in glucose-fed cultures, but not in xylose or glucose/xylose mixtures. When xylose alone was used as the carbohydrate source (xylose feed, 20 g/l; dilution rate, 0.06/h), ethanol yields declined after 5 days and cells screened from the continuous culture began to lose Cm hyper-resistance (600 mg/l) after 30 days. However, as Dumsday et al. (1999) suggest, the instability they observed might be a hindrance only for utilizing KO11 in continuous fermentation processes. The same laboratory also evaluated the ethanol-producing *E. coli* strain FBR5 in continuous cultures and determined that this strain maintained consistently high ethanol yields when fed either glucose or xylose (N.B. Pamment, personal communication).

The FBR strains were constructed using parental strains that had been mutated so they could not grow fermentatively. These mutants carry *pfl* and *ldh* mutations that block the ability of the strains to reduce pyruvate and recycle the NADH, H⁺ generated from glycolysis. Transforming the strains with plasmid pLOI297, which encodes the PET operon, restored fermentative growth. The transformed strains selectively produced ethanol from arabinose, glucose or xylose. Furthermore, the maintenance of pLOI297 is positively selected for in anaerobic culture (Dien et al. 1998; Hespell et al. 1996) because cells that lose the plasmid cannot grow. These strains have been used to ferment hydrolysates prepared from corn hulls and germ meal (Dien et al. 1999, 2000). Ethanol production yields for the best strain, FBR5, were 0.46–0.51 g/g, and fermentations were complete within 36–60 h (Dien et al. 2000, and unpublished data). More recently, variants of these strains have been constructed—mutants that are not repressed by glucose because they all carry a mutation in their phosphoenolpyruvate-glucose phosphotransferase system (*ptsG*-) (Nichols et al. 2001). These strains have the ability to utilize arabinose, glucose and xylose simultaneously. However, *ptsG*- also disables active glucose transport in *E. coli*. As a result, mutants grow slower on glucose and are more sensitive than FBR5 to inhibitors present in corn hull hydrolysates (data not shown).

Recently, Yomano et al. (1998) used long-term adaptation on medium supplemented with ethanol to increase the

ethanol tolerance of KO11 by approximately 10%. Strain KO11 was serially cultured over 3 months in Luria-Bertani (LB) medium supplemented with 50 g/l glucose and increasingly elevated ethanol concentrations (35–50 g/l ethanol). Every 3–4 transfers the culture was re-selected on solid medium supplemented with 600 mg/l Cm. A number of isolates from the adapted culture were tested for ethanol tolerance and one (strain LY01) was chosen for further study. Adaptation successfully reduced the time required to ferment 140 g/l xylose from 120 h (for strain KO11) to 96 h (Yomano et al. 1998). Of added benefit, LY01 also tolerated hydrolysate-associated inhibitors, including aldehydes (Zaldivar et al. 1999), alcohols (Zaldivar et al. 2000) and organic acids (Zaldivar and Ingram 1999), better than KO11. Cultures of LY01 were able to tolerate up to 25 g/l acetic acid, 3.5 g/l HMF (hydroxymethylfurfural) and 3.5 g/l furfural. However, not surprisingly, when mixed these chemicals were observed to inhibit growth of LY01 at lower concentrations.

Current work by Ingrams laboratory on strain KO11 has focused on formulating an inexpensive culture medium and improving inhibitor tolerance. Initial fermentations used standard laboratory formulated medium (LB) containing yeast extract and peptone. Medium containing 15 g/l corn steep liquor (CSL) and 4 g/l yeast autolysate gave higher ethanol yields than LB (0.43 vs 0.48 g/g), albeit with a decline in productivity (from 0.80 to 0.62 g l⁻¹ h⁻¹). They subsequently observed a correlation between ADH and PDC activities and nutrient requirements. For example, the KO11-related strain LY01 transformed with plasmids for over-expressing either *adh* or *pdh*, especially the latter, required lower levels of supplemental nutrients than the untransformed strain (Martinez et al. 1999). Results were based upon 16 h fermentations of 50 g/l xylose. In more recently published reports Underwood et al. (2002a, 2002b) showed that strain KO11 was nutritionally starved for biosynthetic precursors. Two successful strategies were pursued to correct this metabolic imbalance. In the first, a citrate synthase (*citZ*) from *Bacillus subtilis* was expressed in strain KO11. Unlike that of *E. coli*, the Gram-positive citrate synthase is not repressed by elevated NADH₂ concentrations. Expression of *citZ* in KO11 increased growth and ethanol yield of this strain by 75% in CSL (10 g/l) medium. Alternately, increasing the acetyl-CoA pool (e.g., supplementing with acetic acid, acetaldehyde, etc.) also led to increased ethanol yields and productivity on similar CSL medium.

Engineering *Z. mobilis* for arabinose and xylose metabolism

Z. mobilis is an unusual Gram-negative microorganism that has several appealing properties as a biocatalyst for ethanol production. The microorganism has a homo-ethanol fermentation pathway and tolerates up to 120 g/l ethanol. It has a higher ethanol yield (5–10% more ethanol per fermented glucose) and has a much higher specific

ethanol productivity (2.5×) than *Saccharomyces* sp. (Rogers 1982). Furthermore, *Z. mobilis* is generally regarded as safe (GRAS). *Z. mobilis* is so well suited for ethanol production that in the 1970s and 1980s some researchers advocated it as superior to *S. cerevisiae* for conversion of starch to ethanol. While *S. cerevisiae* is still preferred by the industry because of the yeasts hardiness, industrial scaled trials were successfully carried out using *Z. mobilis* (Doelle et al. 1989; Millichip and Doelle 1989).

The high ethanol yield and productivity observed for *Zymomonas* are a consequence of its unique physiology. *Zymomonas* is the only microorganism that metabolizes glucose anaerobically using the Entner-Doudoroff (ED) pathway as opposed to the Embden-Meyerhoff-Parnas (EMP) or glycolytic pathway. The ED pathway yields only half as much ATP per mole of glucose as the EMP pathway. As a consequence, *Zymomonas* produces less biomass than yeast and more carbon is funneled to fermentation products. Also, as a consequence of the low ATP yield, *Zymomonas* maintains a high glucose flux through the ED pathway. All the enzymes involved in fermentation are expressed constitutively, and fermentation enzymes comprise as much as 50% of the cells' total protein (Sprenger 1996). *Zymomonas* also has simple nutritional needs. Some strains require only pantothenate and biotin for growth (Rogers et al. 1982).

Despite its advantages as an ethanologen, *Z. mobilis* is not well suited for biomass conversion because it ferments only glucose, fructose and sucrose. However, over the last decade, researchers at the National Renewable Resources Laboratory (Department of Energy, United States) have successfully engineered strains capable of fermenting xylose and arabinose. The first recombinant strain was engineered to ferment xylose (Fig. 2) (Zhang et al. 1995a). This required introduction and expression of four *E. coli* genes: xylose isomerase (*xylA*), xylulose kinase (*xylB*), transketolase (*tktA*), and transaldolase (*talB*) (Zhang et al. 1995a). Xylose isomerase and xylulose kinase convert xylose into xylulose-5-phosphate, an important intermediate in the pentose phosphate pathway. Xylulose-5-phosphate is next converted to intermediates of the ED pathway by transketolase and transaldolase. The genes were expressed on a plasmid using either the *enl* or *glyceraldehyde-3-phosphate dehydrogenase* promoters from *Z. mobilis*; both of which are strong constitutive promoters. The transformed strain CP4 (pZB5) grew on xylose, and the ethanol yield was 86%. The strain also simultaneously fermented glucose and xylose. Xylose uptake depends upon the native glucose permease; *Z. mobilis* does not have active sugar transport systems (Parker et al. 1995).

The same general strategy used to engineer *Z. mobilis* for xylose metabolism was also used to construct a strain that ferments arabinose (Deanda et al. 1996). In this case, a plasmid was constructed with five genes isolated from *E. coli*: L-arabinose isomerase (*araA*), L-ribulose kinase (*araB*), L-ribulose-5-phosphate-4-epimerase (*araD*), transketolase (*tktA*) and transaldolase (*talB*). The first three enzymes are responsible for converting arabinose to xylulose-5-phosphate. And, as before, xylulose-5-phos-

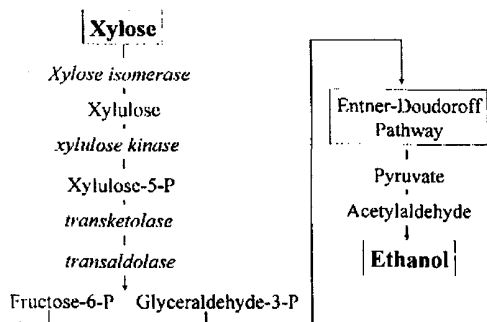


Fig. 2 *Zymomonas mobilis* engineered for metabolism of xylose (Zhang et al. 1995a). See text for details

phate was converted to ED pathway intermediates via transketolase and transaldolase. The resulting transformed strain, ATCC39676 (pZB206), successfully fermented arabinose (25 g/l) to ethanol and displayed a very high yield (98% of theoretical). But the rate of arabinose fermentation was much lower compared to that observed for the xylose-fermenting strain, which the authors ascribed to the low affinity of the glucose permease transporter for arabinose (Parker et al. 1995).

Since this initial work, Zhang and collaborators have continued to construct improved strains with higher ethanol productivities and yields. Rogers laboratory, which had previously evaluated numerous *Z. mobilis* strains for ethanol production from glucose, transformed pZR5 into their best *Z. mobilis* ethanol producing strain ZM4; ATCC 31821 (Joachimsthal and Rogers 2000). The resulting strain ZM4(pZB5) demonstrated much higher ethanol tolerance than the CP4 derivatives on glucose/xylose mixtures. For example, when both strains were used to ferment an equal mixture of glucose (65 g/l) and xylose (65 g/l), strain Zm4(pZB5) produced 62 g/l ethanol in 48 h, while CP4(pZB5) produced only 52 g/l after 60 h. However, when the sugar concentration was further increased to 75 g/l of each, the Zm4(pZB5) fermentation stalled after the ethanol concentration rose to 67 g/l (Joachimsthal and Rogers 2000). Therefore, the maximum ethanol tolerance for strain Zm4(pZB5) when fermenting a glucose/xylose mixture appears to be approximately half that of the wild-type strain fermenting glucose.

In other work, Zhang and collaborators transformed *Z. mobilis* strain ATCC39767 with a plasmid encoding the genes conferring the ability to ferment xylose. Strain ATCC39767 was identified as a good candidate for biomass conversion based on its growth in hydrolysate prepared from yellow poplar wood (Zhang et al. 1995b). The strain has previously been used for industrial scaled trails (up to 586,000 l) for fermenting ground milo (sorghum), corn or wheat (Doelle et al. 1989; Millichip and Doelle 1989). Furthermore, the fermentation residue from one of these industrial trials was analyzed for composition for use as animal feed and determined to have a nutrient profile comparable to residues from *S. cerevisiae* fermentations (Millichip and Doelle 1989). However, strain 39767 is highly sensitive to microbial inhibitors commonly associated with hydrolysates, especially acetic acid (Lawford et al. 1999). Furthermore, growth on xylose exacerbates the strains sensitivity to acetic acid (Joa-

chimsthal et al. 1999). Lawford et al. (1999) successfully adapted xylose-fermenting strain 39767(pZBL4) to tolerate higher concentrations of acetic acid, as well as other inhibitors, by culturing the strain in continuously higher levels of hydrolysate. The hydrolysate used was clarified dilute-acid-pretreated Yellow Poplar wood. The continuous culture was run for 149 days and the level of hydrolysate in the medium increased from 10 to 50% (v/v). Isolates recovered at the end of the fermentation demonstrated significantly improved ethanol productivity in the presence of acetic acid compared to the unadapted strain.

The adapted strain was subsequently evaluated for converting poplar wood hydrolysate to ethanol in SSF. The hydrolysate was prepared by steam exploding the poplar wood chips followed by over-liming to reduce inhibitor levels. The fermentations were conducted at 34°C and pH 5.5, which represents a compromise between the optimal conditions for the cellulase enzymes and typical culture conditions for *Z. mobilis*. After 7 days, the fermentation reached an ethanol concentration of 30 g/l, a yield of 54% based upon total initial carbohydrates (McMillan et al. 1999). While these results compare favorably to *S. cerevisiae* SSF, the process is still uneconomical.

Recently, Zhang and collaborators have made a number of improvements to their *Z. mobilis* strains. The newest strain (AX101, parental strain ATCC39676) ferments both arabinose and xylose and carries the seven necessary recombinant genes as part of its chromosomal DNA. Integrating the genes dispenses with the need for antibiotic for plasmid maintenance. Cultures of AX101, transferred for 160 generations on glucose, retained the ability to ferment both arabinose and xylose, demonstrating the genetic stability of the inserted genes. As observed for previous strains, AX101 ferments arabinose much slower than xylose, and arabinose fermentations are often incomplete. For example, when AX101 was evaluated on arabinose (20 g/l), it only fermented one-half of the initial arabinose within 48 h (Lawford and Rousseau 2002). In contrast to this result, parallel fermentations conducted with glucose or xylose were completed within 10 and 30 h, respectively. However, the yield and rate of arabinose fermentation was improved in the presence of other sugars. When AX101 was used to ferment a mixture of sugars (40 g/l glucose, 40 g/l xylose and 20 g/l arabinose) it fermented all of the glucose and xylose, and 75% of the arabinose in 50 h (Lawford and Rousseau 2002; Mohagheghi et al. 2002). Overall, final ethanol yields were a respectable 0.43–0.46 g/g with only minor side-production of xylitol (3.35 g/l), lactic acid (0.21 g/l) and acetic acid (0.84 g/l). Lactic acid production was reduced in this strain compared to previous pentose-utilizing strains, possibly because the arabinose fermentation genes were integrated at the site of the putative *ldh* gene (Lawford and Rousseau 2002). Whereas most reported fermentations using AX101 have employed laboratory media formulations that include expensive yeast extract (5–10 g/l) as a nitrogen source, the strain requires only the addition of 0.5% v/v CSL to meet its nutritional needs (Lawford and Rousseau 2002).

The major shortcoming of AX101 cultures is their low tolerance to acetic acid, especially in the presence of ethanol. Acetic acid is commonly found in hydrolysates and originates from acetyl side-chain groups of the hemicellulose. Adding only 2.5 g/l acetic acid (pH 5.5) was sufficient to slow ethanol productivity in xylose fermentation cultures by 50%. Acetic acid inhibition was exacerbated in the presence of ethanol. Only 50% of the xylose (initial concentration 30 g/l) was consumed when both acetic acid (2.5 g/l, pH 5.5) and ethanol (30 g/l) were added to the medium (Lawford and Rousseau 2002). Acetic acid tolerance is also a problem when fermenting sugar mixtures. Mohagheghi et al. (2002) examined acetic tolerance for AX101 growing in a continuous culture on a sugar mixture (40 g/l glucose, 40 g/l xylose and 20 g/l arabinose) at pH 5.5. The culture was challenged with increasing levels of acetic acid. Residual xylose began to accumulate once the acetic acid concentration exceeded 4.5 g/l (Mohagheghi et al. 2002). There are two ways to circumvent this problem: (1) adapting the strain to acetic acid or (2) removing acetic acid from the hydrolysate prior to the fermentation. Improving acetic acid tolerance for xylose-fermenting cultures of recombinant *Z. mobilis* strains to match those found in hydrolysates may be challenging because cells growing on xylose have a lower energetic state than those growing on glucose (Kim et al. 2000).

Engineering *K. oxytoca* and *Erwinia chrysanthemifor* ethanol production

In addition to *E. coli*, Ingram and colleagues have also transformed the related Gram negative bacteria, *K. oxytoca* and *E. chrysanthemi* with the *pet* operon. Though the resulting strains have lower ethanol yields than *E. coli*, significant progress has since been made in developing improved *K. oxytoca* strains especially well suited for converting cellulose to ethanol.

K. oxytoca is an enteric bacterium found growing in paper and pulp streams as well as around other sources of wood. The microorganism is capable of growing at a pH at least as low as 5.0 and temperatures as warm as 35°C. *K. oxytoca* will grow on a wide variety of sugars including hexoses and pentoses, as well as on cellobiose and cellotriose. This latter characteristic makes the strain especially appealing for cellulose fermentations. Cellulase preparations are inhibited by cellobiose and consequently SSF cultures with cellobiose-fermenting organisms require less cellulase addition than those with non-cellobiose fermenters (Freer and Detroy 1983). Also, using cellobiose-fermenting strains would reduce growth of some contaminants by eliminating glucose from the fermentation broth.

K. oxytoca ferments glucose to a variety of organic acids and neutral products. Ethanol is formed through the PFL pathway. After *K. oxytoca* strain M5A1 was transformed with plasmids containing the *pet* operon, ethanol increased to greater than 90% of the fermentation products (on a molar basis; Ohta et al. 1991b). Expressing the *pet* operon on a lower copy number plasmid (pLOI555) gave higher ethanol productivity than for the

higher copy number plasmid (pLOI297). This was the opposite of what was observed for *E. coli* strains (Ohta et al. 1991b). Ethanol yields for M5A1 carrying either plasmid was 94–98% of theoretical based upon initial glucose. Strain M5A1 (pLOI555) appears to be particularly well suited for fermenting xylose. It fermented xylose as rapidly as glucose (2 g/l/h during first 24 h) and twice as fast as *E. coli* strain KO11.

The PET operon was genetically stabilized in *K. oxytoca* M5A1 by integrating the operon along with a chloramphenicol acetyl transferase (*cat*) marker at the site of the chromosomal *pfl* gene (Wood and Ingram 1992). While the initial M5A1 transformants recovered demonstrated poor ethanol yield and productivity, screening for strains hyper-resistant to Cm (600 mg/l), as done earlier for ethanologenic *E. coli* strains, yielded mutants with enhanced ethanol production characteristics (Wood and Ingram 1992). The best mutant isolated (P2) readily fermented either glucose (100 g/l) or cellobiose (100 g/l) to ethanol with yields of 44–45 g/l within 48 h. Results for P2 were similar to control fermentations inoculated with M5A1 (pLOI555), indicating the integrated strain was producing sufficient ADH and PDC.

Strain P2 has been tested successfully on various feedstocks including mixed office paper (Brooks and Ingram 1995), sugarcane bagasse (Doran et al. 1994), corn fiber (Moniruzzaman et al. 1996) and sugar beet pulp (Doran et al. 2000). The results with sugarcane bagasse provide an example of this strains performance (Doran et al. 1994). The hydrolysate was prepared by pretreating with dilute sulfuric acid at 140°C and then treating with cellulase (20 filter paper units/g biomass) for 24 h, which partially hydrolyzed the cellulose and lowered the viscosity of the hydrolysate. The fermentation using *K. oxytoca* P2 lasted 7 days and produced 38.6 g/l ethanol; a yield of 70% based upon total beginning carbohydrates. Because *K. oxytoca* ferments cellobiose, the cellulase was not supplemented with additional beta-glucosidase activity. A two-stage saccharification and fermentation scheme was also developed using un-ground sugarcane that increased the final ethanol yield to 40 g/l while halving the enzyme loading. However, this process took a total of 13 days. Golias et al. (2002) compared strain P2 and cellobiose-fermenting yeasts for fermenting micro-crystalline cellulose (Sigmacell 50). They determined that P2 fermentations were 25–50% faster than those inoculated with the yeasts, but the final ethanol Concentration was limited to 37 g/l (e.g., maximum ethanol tolerance), which is significantly lower than that reported earlier by Wood and Ingram (1992). Golias et al. (2002) finally relied on co-cultures of P2 and yeasts to achieve higher ethanol concentrations.

More recently, variants of strains P2 have been constructed that express endoglucanase, a component of cellulase mixtures. Engineering strains to produce their own cellulase is one strategy for reducing ethanol production costs. Zhou and Ingram (2000) integrated two extracellular endoglucanase genes (CelZ and CelY) from *E. chrysanthemi*, a plant pathogen, into the chromosomal DNA of strain P2 and introduced the required auxiliary transporter gene (*out*) on a plasmid (pCPP2006). The cellulolytic strain was named SZ21 (Zhou et al. 2001).

Endoglucanase production measured in glucose-grown cultures of strain SZ21 (pCPP2006) were 20 U/ml, about 1% of the activity in commercial cellulase preparations (Zhou et al. 2001). The strain fermented cellulose (Sigmacell 50) poorly without supplementing with additional cellulase activity. Encouragingly, when commercial cellulase was added, SZ21(pCPP2006) produced a 7–16% higher ethanol yield than the parental strain.

Doran et al. (2000) compared strains *K. oxytoca* P2, *E. coli* KO11 and *E. chrysanthemi* EC 16 (pLOI555) for production of ethanol from sugar beet pulp (106 g/l) with simultaneous enzymatic hydrolysis of pectin and cellulose. The *E. coli* KO11 fermentations produced 40% more ethanol than the others. A possible, or partial, explanation for the higher yield obtained from strain KO11 compared to the other strains is that sugar beet pulp is rich in galacturonic acid, and *E. coli* KO11 had a significantly higher ethanol yield on this carbohydrate.

Conclusions

Culture characteristics of each of the strains discussed are compared in Table 2. *E. coli* and *K. oxytoca* have wider substrate ranges than *Z. mobilis* (Table 2). However, with the notable exception of softwoods, the substrate range of recombinant *Z. mobilis* is sufficient for most sources of biomass. Strains of *Saccharomyces* sp. are capable of fermenting galactose, glucose, and mannose, and recombinant strains have been constructed that metabolize either xylose or just recently arabinose (Jeffries and Jin, submitted). The yeast *Pichia stipitis* has the natural ability to ferment galactose, glucose, mannose, and xylose (Jeffries and Jin, submitted). Therefore, the major qualitative advantage of recombinant bacteria compared to yeasts is the ability of the former to ferment arabinose. In terms of temperature, *E. coli* has a higher optimal fermentation temperature than the other two bacteria, but is also limited to a fairly neutral pH range. Recombinant strains of both *K. oxytoca* and *Z. mobilis* have been

Table 2 Culture characteristics of host strains used for ethanol production. *Ara* Arabinose, *Gal* galactose, *Glc* glucose, *Man* mannose, *Xyl* xylose

Host	Ara	Gal	Glc	Man	Xyl	T (°C) ^a	pH ^a
<i>Escherichia coli</i>	+	+	+	+	+	35	6.5
<i>Klebsiella oxytoca</i>	+	+	+	+	+	30	5.5
<i>Zymomonas mobilis</i>	+	–	+	–	+	30	5.5

^aTypical culture conditions for single-sugar fermenting cultures; conditions are varied for simultaneous saccharification and fermentation

cultured at higher temperatures (35 and 34°C) for SSF experiments.

Fermentation results for the various recombinant bacteria cannot be precisely compared because the studies all used different medium formulations, sugar mixtures, and inoculation protocols. Table 3 lists results for batch fermentations of xylose and sugar mixtures, which were formulated to mimic corn-fiber hydrolysate. Ethanol yields are listed as a percentage of the maximum theoretical (0.51 g/g) based on the starting sugar(s) concentration. Average ethanol productivity is calculated based on the final ethanol concentration and fermentation time. The results listed were either taken directly from the reference cited or (when not possible) calculated from the relevant reported results. Ethanol yields range from 84 to 95% of theoretical, which are comparable to current industrial fermentation yields from corn (ca. 90%). *Z. mobilis* appears to have higher ethanol yields and productivities on sugar mixtures containing glucose than when solely fermenting xylose. However, the only available results for xylose fermentation by *Z. mobilis* were for an earlier constructed strain. With two exceptions, ethanol productivities listed ranged from 0.59 to 0.96 g l⁻¹ h⁻¹. The exceptions are for *Z. mobilis* fermenting xylose and *K. oxytoca* P2 fermenting a sugar mixture, where productivities were 0.32 and 0.35 g l⁻¹ h⁻¹, respectively. Bothast et al. (1994) attributed the low ethanol yield and productivity for *K. oxytoca* P2 to a lower than expected ethanol

Table 3 Comparison of batch fermentations with xylose and sugar mixtures for ethanologenic bacterial strains

Strain	Host	Sugars ^a	Maximum ethanol (g l ⁻¹)	Ethanol yield (%)	Ethanol production (g l ⁻¹ h ⁻¹)	Citation
<i>E. coli</i>	KO11 ^b	Xyl 90	41.0	89	0.85	Yomano et al. 1998
		Ara:Gal:Glc:Xyl, 23:11:27:39	41.7	90	0.62	Asghari et al. 1996
	FBR5	Xyl95	41.5	90	0.59	Dien et al. 2000
		Ara:Xyl:Glc 15:30:30	34.0	90	0.92	Dien et al. 2000
	LY01	Xyl 140	63.2	88	0.66	Yomano et al. 1998
<i>K. oxytoca</i>	M5A1(pLOI555)	Xyl 100	46.0	95	0.96	Ohta et al. 1991b
	P2	Ara:Xyl:Glc 20:40:20	34.2	84 ^c	0.35	Bothast et al. 1994
<i>Z. mobilis</i>	AX101	Ara:Glc:Xyl 20:40:40	42 ^d	84	0.61	Mohagheghi et al. 2002
	CP4:pZB5	Xyl 60	23.0	94	0.32	Lawford and Rousseau 1999

^aSugars were analytical grade. Numbers refer to grams per liter

^bOn 140 g/l xylose, strain KO11 produced 59.5 g/l ethanol in 120 h (Yomano et al. 1998)

^cApproximately one-third of added xylose not fermented

^dEstimated from Fig. 5 (p 892) in Mohagheghi et al. 2002

tolerance (in this regard also see results by Golias et al. 2002). Therefore, the reported yields and productivity would probably have been much improved if less sugar had been added to the medium. The lower productivity for KO11 fermenting a sugar mixture compared to xylose probably reflects differences in basal medium: xylose was supplemented with LB and the sugar mixture with CSL and yeast autolysate.

Important characteristics not presented in the table include growth requirements and sensitivity to hydrolysate-associated microbial inhibitors. *Z. mobilis* AX101 and *E. coli* strain KO11 have both been used in fermentations using inexpensive CSL as a nutrient source, and wild-type strains of both can grow in very simple medium. The *Z. mobilis* and *E. coli* strains have been most thoroughly characterized for their sensitivity to inhibitors. *Z. mobilis* is extremely sensitive to acetic acid; however, work is underway to increase its tolerance. Inhibitor tolerance remains a concern for all of these strains, and efforts in this area will probably need to be coordinated with research on pretreatment strategies. Future development for these Gram-negative bacteria will continue to emphasize increasing inhibitor tolerance, reducing growth factors and improving ethanol productivity. Further progress is also expected in engineering other types of bacteria for ethanol production. Both Gram-positive and thermophilic bacteria have unique advantages compared to Gram-negative bacteria. Several reported attempts to express the PET operon in Gram-positive bacteria have given disappointing results including very low ethanol yields. The authors expect further attempts to engineer these bacteria either by expressing the *pdv* from *Sarcina ventricola* or a synthetic *pdv* gene. The utility of thermopiles is limited by the absence of a genetic system, which can be used to improve ethanol yields. Once a system is developed, it is expected that strains with industrial potential will be developed.

Representative strains of recombinant *E. coli*, *K. oxytoca*, and *Z. mobilis* strains either have been, or are currently being, considered for commercial scale-up. The commercial ethanol industry, however, has shown a bias, not without justification, for relying on *Saccharomyces* strains. For example, even though *Z. mobilis* has a higher ethanol yield on starch, all commercial producers continue to ferment starch using yeast. The willingness of ethanol producers to consider using bacterial strains as opposed to *Saccharomyces* will depend on demonstrating that bacterial strains are capable of producing ethanol reliably in larger bioreactors, fermentations need not be fully aseptic to avoid contamination, and that strains can be developed that have qualitative advantages compared to yeast, such as greatly reduced needs for saccharification enzymes (e.g., cellulases). Just as significantly, industrial acceptance of recombinant bacteria will also depend upon the relative success of yeast microbiologists in developing industrially relevant pentose-fermenting *Saccharomyces* strains. Specifically, their success in developing strains capable of fermenting pentoses in anaerobic culture with high yield and productivity, and possessing excellent tolerance to inhibitors. While these combined goals have been largely elusive for *Saccharomyces*, future progress should be greatly aided by utilizing micro-array and robotic-aided directed evolution technologies.

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