

# Enzymatic Conversion of Biomass for Fuels Production

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Developed from a symposium sponsored  
by the Division of Cellulose, Paper and Textile  
at the 205th National Meeting  
of the American Chemical Society,  
Denver, Colorado,  
March 28–April 2, 1993



American Chemical Society, Washington, DC 1994

## Chapter 17

## Development of Genetically Engineered Microorganisms for Ethanol Production

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Although cellulosic biomass is a favorable feedstock for fuel ethanol production, substantial hurdles remain before a typical hydrolysate can be utilized efficiently as a fermentation substrate. Rapid and efficient conversion of pentose sugars, particularly xylose, remains one of the key economic bottlenecks in a biomass to ethanol process. Despite the development of recombinant strains with improved xylose fermentation performance, high ethanol yields from lignocellulosic hydrolysates, and increased product concentrations and ethanol tolerances are key targets that have yet to be achieved. The genetic modifications that can have the greatest impact on the economic feasibility of these fermentations include amplification and deregulation of rate-limiting reactions; metabolic engineering that redirects the normal carbon flow to ethanol, improves glycolytic efficiency, or reduces futile cycling; introduction of genes that broaden the substrate range; and manipulations that improve ethanol tolerance, osmotolerance, thermotolerance, and resistance to the inhibitory compounds normally present in lignocellulosic hydrolysates.

With the fuel alcohol industry producing a large-volume, low-value product, many of the raw materials used by the potable distiller are too expensive to consider as a feedstock. Currently, only sugarcane and corn are routinely used as feedstocks for the production of fuel ethanol. Brazil produces more than 3 billion gallons of ethanol per year from sugarcane, while most of the fuel ethanol produced in the United States is made from corn. In order to make this process competitive with the prevailing cost of gasoline, the U.S. government grants a credit of approximately \$0.50 per gallon to ethanol producers. Our challenge in the 21<sup>st</sup> century is to develop new, economically viable feedstocks as well as the microorganisms that can efficiently and rapidly convert these feedstocks to fuel ethanol.

Cellulosic biomass is a favorable feedstock because it is readily available and, because it has no food value, it is less expensive than corn or sugarcane. However, there remain substantial hurdles that must be overcome before a typical lignocellulosic hydrolysate can be efficiently utilized as a substrate by fermentative microorganisms. The typical feedstock contains approximately 30-60% glucose, 15-30% xylose, 10-20% lignin, and 5-30% of a mixture of arabinose, mannose, galactose, and a variety of other minor pentose and hexose sugars. Many microorganisms are capable of efficiently fermenting glucose, but the conversion of pentose sugars, particularly xylose, to ethanol remains one of the key economic bottlenecks in a biomass to ethanol conversion scheme. In addition, the feedstock will probably be presented to the selected microorganism at elevated temperature, low pH, and high salt concentration. The few organisms that can grow on all the sugars in lignocellulosic hydrolysates typically grow slowly and demonstrate marginal yields and productivities. The rapid and efficient utilization of all of these component sugars is an absolute requirement for a commercial process. In all likelihood, the ideal ethanol-producing microorganism will have the productivity of bacteria, the selectivity of yeast, and a broad substrate utilization range. It will also be a facultative anaerobe and it will tolerate the inhibitory compounds present in dilute-acid prehydrolysates.

Unfortunately, no one microorganism is known to possess all these traits. Recent advances in the application of recombinant DNA technology, while encouraging, have not yet yielded an organism with all of the desired features of an ideal ethanologen. Hence, processes based on their use have not been commercialized. However, the recombinant approach to add the ability to produce ethanol to a microorganism with an already broad substrate utilization range shows substantial promise. This paper reviews the recent advances in the development of recombinant microorganisms for ethanol production and highlights those strain development strategies that have demonstrated some measure of success and which appear to hold the most promise.

## Strain Development Strategies

*Saccharomyces cerevisiae*. The historical importance of *S. cerevisiae* in industrial fermentations cannot be overstated and, as one might expect, there is a long list of advantages to consider for its potential application in biomass fermentations. *S. cerevisiae* ferments glucose through the Embden-Meyerhoff-Parnas pathway (Figure 1) and demonstrates high fermentation selectivity, with ethanol as virtually the sole product (only small amounts of glycerol and acetate are formed in order to maintain intracellular cofactor balance). This yeast is known for its high ethanol tolerance and some strains can continue fermentation even at ethanol concentrations in excess of 30% w/v (1)! As a facultative anaerobe, *S. cerevisiae* is capable of anaerobic growth and, because it has a restricted respiratory system capable of a Crabtree effect, there is little yield loss to biomass during the fermentation. This effect is of fundamental importance in carbohydrate fermentations because it normally uncouples substrate utilization from respiration, even in the presence of oxygen, providing that some fermentable sugar is present (2,3). Its facilitated diffusion sugar transport system maximizes intracellular energy efficiency by eliminating the need for anaerobically-generated ATP. Also, because fermentation occurs under completely anaerobic conditions, there is no yield loss due to aerobic ethanol reassimilation.

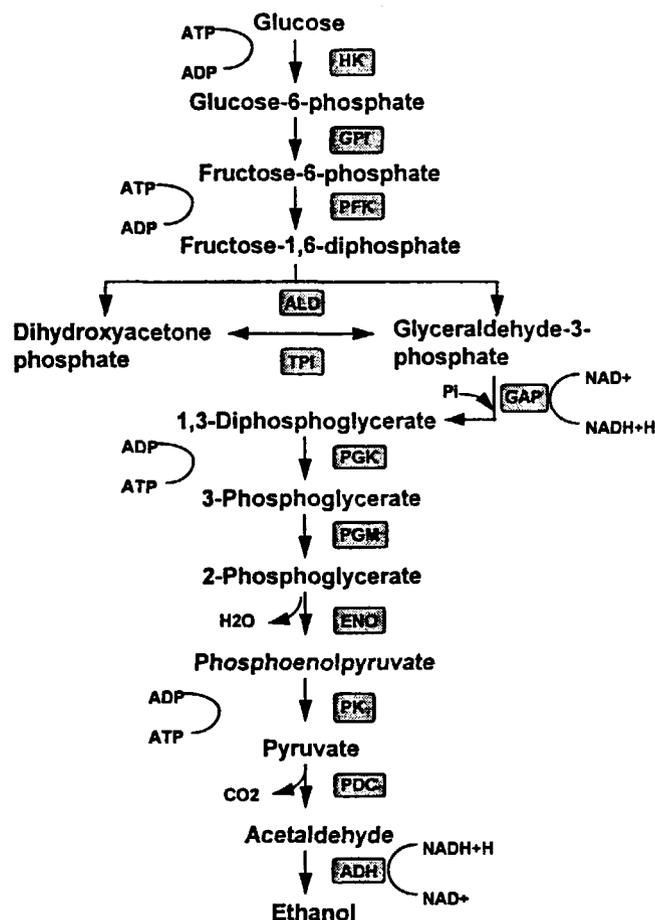


Figure 1. Embden-Meyerhoff-Parnas Pathway. HK, Hexokinase; GPI, Glucose-6-phosphate isomerase; PFK, Phosphofructokinase; ALD, Aldolase; TPI, Triose-phosphate isomerase; GAP, Glyceraldehyde-3-phosphate dehydrogenase; PGK, Phosphoglycerate kinase; PGM, Phosphoglycerate mutase; ENO, Enolase; PK, Pyruvate kinase; PDC, Pyruvate decarboxylase; ADH, Alcohol dehydrogenase.

This yeast's ability to ferment sugars at low pH provides protection against bacterial contamination during prolonged cultivation and precludes the need for the base addition required in bacterial fermentations. Other key advantages are its ability to grow and ferment sugars in the presence of lignocellulosic hydrolysates and its superior resistance to acetate at low pH under anaerobic conditions (4). The high indigenous levels of glucose-inducible pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) ensure rapid fermentation rates and high specific productivities and provide resistance to glucose catabolite repression during fermentation of mixed sugar hydrolysates. The availability of flocculent strains permit the development of processes based on cell-recycle and provide the opportunity to significantly reduce the costs associated with inoculum preparation. Certainly, the utility of *S. cerevisiae* in a simultaneous saccharification and fermentation process (SSF) has been well established (5-12) and its industrial scale-up is well understood. A well-developed gene transfer system is also available for metabolic engineering.

Clearly, the only real disadvantage with *S. cerevisiae* is its limited substrate range (although strains with higher thermotolerance would be more compatible in SSF processes utilizing cellulases with optimal enzymatic activities around 50°C). Unfortunately, this yeast lacks both a xylose-assimilation pathway and adequate levels of key pentose phosphate pathway enzymes. Xylose uptake by the facilitated transport system is relatively slow and occurs only in the presence of other metabolites, such as ribose (13). Like many other yeasts, *S. cerevisiae* ferments xylulose, but more slowly and not as efficiently as glucose (14-17). Senac and Hahn-Hagerdal (1990) found a significant accumulation of the intermediate sedoheptulose-7-phosphate in xylulose-grown cells compared to glucose-grown cells, suggesting transaldolase as a rate-limiting enzyme in the pentose phosphate pathway. The transaldolase specific activity, while essentially the same for glucose and xylulose-grown cells, was several orders of magnitude lower than that reported for xylose-assimilating yeasts such as *Candida utilis*. Similar accumulation in the presence of iodoacetate, a specific inhibitor of glyceraldehyde-3-phosphate dehydrogenase (GAP), suggested that slow xylulose fermentation could be the result of competition between transaldolase in the pentose phosphate pathway and GAP in glycolysis for the common intermediate, D-glyceraldehyde-3-phosphate (18). However, cell extracts in which up to 100-fold more transaldolase activity was added demonstrated the same sugar conversion rate and a decreased rate of ethanol formation (19).

Several attempts to genetically engineer xylose utilization into *S. cerevisiae* by introduction of bacterial xylose isomerase genes have been unsuccessful (20,21), apparently due to improper folding of the heterologous protein in the highly-reducing yeast cytoplasm. Greater success has been achieved in cloning and expressing the xylose reductase and xylitol dehydrogenase genes from xylose-assimilating yeasts. *S. cerevisiae* transformed with the *Pichia stipitis* xylose reductase gene was incapable of growth on xylose as the sole carbon source or of ethanol production (22), but converted xylose almost exclusively to xylitol (23). When transformed with the *P. stipitis* xylose reductase and xylitol dehydrogenase genes, recombinant *S. cerevisiae* fermented xylose as a sole carbon source, though incompletely and at a considerably slower rate than glucose (24). The low ethanol yields indicated that, contrary to aerobic glucose metabolism, xylose utilization was almost entirely oxidative. Further analysis of these recombinants indicated that the incomplete xylose conversion to

ethanol was the result of both cofactor imbalance and an insufficient capacity for xylulose conversion through the pentose phosphate pathway (25). No growth was observed in the absence of respiration and, under such conditions, maximal ethanol yields were 34% of theoretical with xylitol and ethanol as the major fermentation products. Inefficient ATP generation resulting from yield loss to xylitol coupled with slow xylose metabolism are believed to be the cause of growth arrest in the absence of respiration.

In another approach, the level of xylulokinase activity in *S. cerevisiae* was increased up to 230-fold following amplification of its xylulokinase gene on a high-copy number plasmid (26). The resulting strain could ferment xylulose up to 130% faster than the parental strain. Efforts to construct a xylose-fermenting *S. cerevisiae* continue and a genetically engineered yeast in which the genes encoding xylose reductase, xylitol dehydrogenase and xylulokinase were coordinately expressed can apparently ferment 5% xylose to ethanol within two days and with very little xylitol formation (N. Ho, personal communication). It is conceivable, however, that this approach will result in the development of a xylose-fermenting *S. cerevisiae* that is limited in the same fashion as the xylose-assimilating yeasts - notably in its oxygen requirement to maintain xylose transport and cofactor balance, its formation of xylitol byproduct and its aerobic ethanol reassimilation. Despite its numerous advantages, this yeast appears to have a fundamental deficiency in pentose fermentation that may preclude its use in commercial xylose fermentations for the time being.

Opportunities to improve this technology involve cloning a novel xylose isomerase into *S. cerevisiae* to provide a xylose-assimilation pathway. One such approach involves the cloning of a xylose isomerase from an acidophilic *Lactobacillus* (27). Longer-term opportunities to improve the metabolic efficiency of fermentation involve amplification and deregulation of the key rate-limiting pentose phosphate pathway enzymes, deregulation of the typical yeast diauxic response to the presence of mixed sugars, and elimination of CO<sub>2</sub> loss through futile cycling (16,19,28-30). In addition, introduction of a facilitated xylose transport system will probably be an essential element of an efficient recombinant yeast system.

**Xylose-Assimilating Yeasts.** A survey of over 400 yeasts (31) failed to identify any strains capable of fermenting xylose under strictly anaerobic conditions. This was somewhat surprising considering their historical importance in glucose fermentations and their ability to ferment xylulose (albeit not as efficiently as glucose). Upon further investigation it was found that 63% of the 466 species of yeasts examined were able to assimilate xylose under aerobic or microaerophilic conditions (32). It is now generally accepted that *Candida shehatae*, *Pachysolen tannophilus* and *Pichia stipitis* are the three wild-type yeasts best suited for xylose "fermentation". Unlike bacteria, which utilize xylose by direct isomerization to xylulose via xylose isomerase, these yeasts utilize a two-step pathway in which xylose is first reduced by an NAD(P)H-dependent xylose reductase to xylitol, which is then oxidized to xylulose by an NAD-dependent xylitol dehydrogenase (Figure 2). Xylulose is subsequently phosphorylated by xylulokinase to form xylulose-5-phosphate and then metabolized to ethanol through the pentose phosphate and Embden-Meyerhoff-Parnas pathways (15,33). The different cofactor specificities of these two enzymes; however, limits the efficiency by which these yeasts convert xylose to xylulose (25,34).

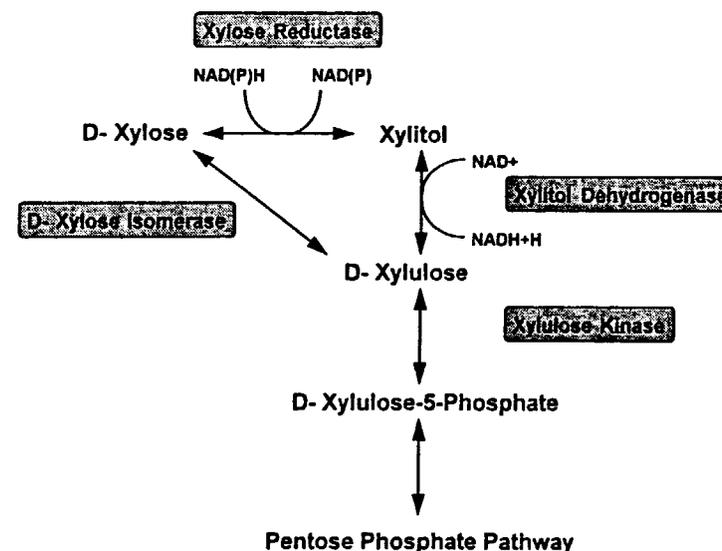


Figure 2. Xylose Assimilation Pathways in Bacteria and Yeast.

Generally, the xylose-assimilating yeasts are capable of achieving ethanol yields from 78-94% of theoretical and final ethanol concentrations of up to 5% (w/v), but demonstrate relatively low ethanol productivities (0.3 - 0.9 g/L/h), especially in the absence of oxygen (0.1 - 0.2 g/L/h). One advantage of their use in xylose fermentations is their low pH optima, ranging from pH 3.5 - 4.5, which precludes the need for the base addition typical in bacterial fermentations. *P. stipitis* demonstrates the best overall performance in terms of complete sugar utilization, minimal coproduct formation and insensitivity to temperature and substrate concentrations (35). Coproduct formation is negligible under ideal fermentation conditions and high selectivity is considered one of the key advantages to their use. Because the strains developed thus far are wild-type strains, no specialized containment equipment is required upon scale-up.

While ethanol yields approaching theoretical have been reported for *P. stipitis* under ideal fermentation conditions on pure xylose (36), typical yields range from 80-90% of theoretical due to considerable formation and accumulation of xylitol (35). The accumulation of this intermediate is believed to result from an inhibition of xylitol dehydrogenase by excess NADH formed in the absence of sufficient respiration (37). Furthermore, unlike glucose fermentations with *S. cerevisiae*, there is also significant yield loss to biomass formation because of the absence of the Crabtree effect. Perhaps the greatest disadvantage with the use of these yeasts is their fundamental requirement for low levels of oxygen (2 mmol/L/h) to maintain cell viability, xylose transport and ethanol productivity. Whereas cells lose viability rapidly in the absence of sufficient oxygen, excess oxygen causes them to completely cease ethanol production and respire the substrate to form biomass (37,38-40). Clearly, the degree of control necessary to maintain this narrow operational margin on an industrial scale would be difficult and cost-prohibitive. Other disadvantages with these yeasts include: low to moderate ethanol tolerance (maximum ethanol concentrations ranging from 3-5% (w/v)); poor growth and fermentation performance on lignocellulosic hydrolysates (because of their sensitivity to inhibitory components); comparatively low volumetric productivity (0.3-0.9 g ethanol/L/h); low temperature optima (<30°C); low specific growth rates; and aerobic re-assimilation of ethanol.

The application of recombinant technology to improve the fermentation performance of these yeasts has been limited to the development of suitable gene transfer systems. Most efforts have been directed to a comparison of their performance characteristics in xylose fermentations. The gene transfer systems developed for *P. stipitis* are based on resistance to the antibiotic gentamicin (41) or on complementation of an *ura3* auxotroph with the native URA3 gene cloned on autonomously replicating or integrative shuttle vectors (42). In addition, many of the xylose assimilation genes have been cloned, including the xylose reductase and xylitol dehydrogenase genes from *P. stipitis* (2-24) and the xylulokinase gene from *P. tannophilus* (43). Unlike most xylose reductases that are linked solely to an NAD(P)H cofactor, the *P. stipitis* enzyme also has an NADH-dependent activity which is thought to allow reoxidation of the NADH generated by the NAD-dependent xylitol dehydrogenase reaction under anaerobic conditions. Attempts are now under way to modify the *P. stipitis* xylose reductase cofactor requirement by site-specific mutagenesis so that it requires only NADH (N. Ho, personal communication). This would eliminate the cofactor imbalance commonly encountered with these yeasts.

Opportunities to improve these yeasts involve metabolic engineering to increase their ethanol tolerance. It has been postulated that their low ethanol tolerance is, in part, due to the aerobic re-assimilation of ethanol leading to an intracellular accumulation of toxic levels of acetaldehyde (44). Genetic disruption of the ethanol assimilation genes (ADHII analogues) would have the beneficial effects of not only increasing ethanol tolerance but simultaneously preventing product re-assimilation. Another opportunity would be to deregulate expression of the *pdh* and *adh* genes to prevent their repression by the small amounts of oxygen necessary for xylose transport and cell viability. A third potential opportunity would be to develop a comparable system around *Candida tropicalis*, a xylose-assimilating yeast with inherently superior hydrolysate resistance and an available gene transfer system (45).

**Simultaneous Fermentation and Isomerization of Xylose (SFIX).** As noted above, the greatest single disadvantage to the use of *S. cerevisiae* in xylose fermentations is its lack of a xylose-assimilation pathway. While this is not a handicap with the xylose-assimilating yeasts, their comparatively low ethanol tolerances, oxygen requirements, volumetric productivities, temperature optima and specific growth rates, combined with their aerobic re-assimilation of ethanol, limit commercial application. An alternative approach couples the use of exogenously added xylose isomerase for conversion of xylose to the more readily fermentable xylulose with the simultaneous anaerobic fermentation by ethanol tolerant yeasts, such as *S. cerevisiae*, *Schizosaccharomyces pombe* or *C. tropicalis*. Because the xylulose:xylose ratio is low at equilibrium (1:5) (46) and the yeast ferments the xylulose as rapidly as it is formed, the SFIX process allows for complete xylose conversion in a single-step process (47, 48).

Providing these yeast with xylulose instead of xylose allows one to exploit many of the benefits associated with yeast glucose fermentations. The main disadvantage with this approach is the high cost for commercial xylose isomerase. This cost burden is compounded by the relatively poor stability of the enzyme (4), the incompatible pH optima between isomerization (pH 7.0) and fermentation (pH 4.0) (14,15), and the unfavorable equilibrium constant of xylose isomerase (17,46). Theoretical yields from pure xylulose of >90% have been reported and rival the efficiency of glucose fermentations (15). However, xylulose fermentations are typically up to ten-fold slower than glucose fermentations (15). While theoretical ethanol yields from xylose of up to 85% have been reported, there is significant yield loss to xylitol and to CO<sub>2</sub> through the oxidative portion of the pentose phosphate pathway (4,16,19,28-30).

The use of recombinant technology to improve the economics of the SFIX process has focussed on reducing the cost of xylose isomerase by overproduction in genetically engineered bacteria or, alternatively, on eliminating its cost altogether by attempting to obtain functional expression of cloned heterologous xylose isomerases in yeast. As an example of the former approach, the gene encoding the *Escherichia coli* xylose isomerase has been cloned under the control of the lambda P<sub>L</sub> promoter to obtain enzyme overexpression following induction of a pre-grown culture at 42°C (49). The overproduced enzyme, representing over 38% of the total cell protein, was found to be identical to the native enzyme by several biochemical and immunological criteria (50). Similar strategies to overexpress xylose isomerase in *E. coli* by cloning the gene under the control of its native (51), *lac* (52), *tac* (53) and *att-nutL-p-att-N* (thermal inverting) (54) promoters have also been reported.

While several attempts to genetically engineer xylose utilization into *S. cerevisiae* by introduction of bacterial xylose isomerase genes have failed (20,21), similar efforts have apparently met with some success in *S. pombe* transformed with the *E. coli* xylose isomerase gene. Transformants demonstrated both low level xylose isomerase activity and the ability to grow very slowly on xylose as the sole carbon source (55,56). The xylose isomerase activity detected in transformants grown on xylose (6.2 nmol/h/mg) was very low compared to levels in induced wild-type *E. coli* (107 nmol/m/mg) (51), and only about 4-fold higher than that detected in the untransformed host grown on a mixture of xylose and xylulose. Subsequent studies have demonstrated enzymatic activity in non-denaturing PAGE zymograms, but the migration patterns from recombinant *S. pombe* were distinct from the native *E. coli* xylose isomerase (57). Xylose-inducible expression was apparently directed from either the native *E. coli xylA* promoter or from some fortuitous promoter on the shuttle vector. Nevertheless, the transformants demonstrated ethanol yields from xylose that were 80% of theoretical. Ethanol productivity was very low (0.15-0.19 g/L/h) and xylitol formation remained a significant problem. The detection of both xylose reductase and xylitol dehydrogenase activities in these transformants (57), coupled with their inability to ferment xylose under anaerobic conditions (58), may suggest the presence of a typical, albeit dysfunctional, yeast xylose assimilation pathway in *S. pombe*.

Despite its novel approach to reduce or eliminate many of the disadvantages associated with other yeast fermentations, the main limitation with the SFIX process is the high cost and poor performance characteristics of xylose isomerase. Absolute theoretical yields will be required to obtain the credit necessary to offset the cost of xylose isomerase. Potential opportunities to metabolically engineer SFIX yeasts involve eliminating the yield loss to xylitol by genetic disruption of the xylose reductase and xylitol dehydrogenase genes (48). Approaches to improve the metabolic efficiency of fermentation include amplification and deregulation of the key rate-limiting enzymes and elimination of CO<sub>2</sub> loss through the oxidative portion of the pentose phosphate pathway. As with *S. cerevisiae*, introduction of a facilitated xylose transport system will be a key element of an efficient recombinant yeast system. Reducing the cost of xylose isomerase by cloning and overproduction, or eliminating its cost altogether by successfully expressing the gene in an SFIX yeast, is an absolute prerequisite for a commercial process.

**Recombinant *Escherichia coli*.** Historically, the xylose-fermenting bacteria were viewed as being capable of high fermentation rates, but only at the expense of low ethanol yields due to the formation of numerous coproducts (i.e. acetate, succinate, lactate, formate, etc.). The construction of recombinant *E. coli* strains capable of directly fermenting xylose to ethanol represents the most significant recent advance in xylose fermentation research. By introducing the *pdc* and *adh* genes from *Zymomonas mobilis* cloned under the control of an *E. coli lac* promoter (PET operon), near-theoretical ethanol yields could be achieved with a microorganism not formerly known to produce ethanol (59). These results demonstrate the feasibility of improving bacterial ethanol production by introducing the highly efficient *Z. mobilis* PET operon into a host capable of utilizing a broad range of substrates. They also illustrate the

use of metabolic engineering to redirect intracellular carbon flow by introducing enzymes with kinetic properties that favor ethanol formation by out-competing other enzymes for the key metabolic intermediate pyruvate.

The key advantages to the use of these recombinant strains in xylose fermentation are their high ethanol yields and volumetric productivities, their broad substrate utilization range, and, by virtue of being a facultative anaerobe, their lack of an oxygen requirement to maintain fermentative capacity. *E. coli* ATCC 11303, containing plasmid pLOI297, has been shown to achieve 96% of theoretical ethanol yield from xylose with a maximum productivity of up to 0.7 g ethanol/L/h during anaerobic batch fermentations in a rich medium maintained at near-neutral pH (60). Generally, these fermentations last about 2 days and yield maximum final ethanol concentrations of 4.8% (w/v). Higher-than-theoretical ethanol yields have been reported and are believed to result from ethanol formation from medium buffers and components other than xylose (61). While the original strains required antibiotic selection for plasmid maintenance, strains with improved stability have since been developed by integration of the PET operon into the *E. coli* chromosome (62). The substrate utilization range for these strains has been reported to encompass glucose, lactose, mannose, galactose, arabinose and xylose (61). In addition, recombinant *Klebsiella oxytoca* strains have been developed along similar lines which are capable of fermenting cellobiose, xylobiose and xylotriose to ethanol (63).

Despite these advances, a number of significant disadvantages remain in the use of these strains for commercial xylose fermentations. Coproduct formation is still a problem despite expression of the kinetically superior *Z. mobilis* PDC and ADH enzymes to levels representing up to 17% of the total cellular protein (64). These acidic coproducts not only limit the conversion yield but adversely affect cell viability. Second-generation strains with reduced coproduct formation have since been developed, but exhibit lower productivity than the plasmid-bearing strain (62). Another disadvantage with an *E. coli*-based process is that its pH optimum requires the fermentations to be conducted at near-neutral pH, resulting in increased costs for base addition and the potential for contamination during prolonged large scale cultivation. Furthermore, a nutrient-rich and expensive growth medium is presently required to achieve the maximum reported yields and productivities. The low ethanol tolerance of *E. coli* and its sensitivity to the inhibitory compounds contained in real hydrolysates are perhaps the greatest fundamental limitations to commercial application in the conversion of lignocellulosic hydrolysates. *E. coli* is especially sensitive at low pH and under anaerobic conditions to the acetic acid generated during dilute-acid pretreatment of lignocellulosic biomass. In addition to its being an enteric microorganism lacking GRAS status, efforts to commercialize processes based on the use of *E. coli* could be hindered further by its recent implication in contaminated foodstuffs. Like any recombinant strain, scale-up will require specialized containment equipment.

Opportunities to improve these strains involve the use of metabolic engineering to eliminate the acidic coproducts and, consequently, maximize fermentation yield, growth rate and cell density. However, some coproduct formation may be necessary to maintain cofactor balance during fermentation.

*Zymomonas mobilis*. *Z. mobilis* is a Gram-negative, facultative anaerobe that has been utilized for centuries in the tropical areas of the world as a natural fermentative agent for preparation of alcoholic beverages, such as pulque and palm wines produced from plant saps (65). Because of its potential value in industrial ethanol production, much attention has been paid to the genetics and biochemical engineering of this fermentative bacterium in the past 15 years (65-74). In comparison to ethanol production by *Saccharomyces carlsbergensis*, *Z. mobilis* has demonstrated several advantages, including two- to three-fold higher specific glucose uptake rates and productivities with ethanol yields of up to 97% of theoretical (66). The high specific productivity is the result of reduced yield loss to biomass formation during fermentation. Whereas yeasts produce 2 mol ATP/mol glucose through fermentation via the Embden-Meyerhoff-Parnas pathway (Figure 1), *Zymomonas* ferments glucose anaerobically by the Entner-Doudoroff pathway (Figure 3) and produces only 1 mol ATP/mol glucose (75-76). In addition, the existence of kinetically superior PDC and ADH enzymes results in high ethanol fermentation selectivity.

Because *Zymomonas* is acid tolerant and grows over a pH range from 3.5-7.5, industrial fermentations based on its use, like yeast fermentations, are resistant to bacterial contamination during prolonged large scale cultivation. *Zymomonas* is naturally resistant to many of the antibiotics (65) used for treatment of contaminated batch fermentations. *Zymomonas* also tolerates many of the inhibitors present in industrial feedstocks and demonstrates comparable performance to *S. cerevisiae* in fermentations of steam-pretreated salix (EH) and spent sulfite liquor (SSL) hydrolysates (27). While EH contains high concentrations of both glucose and acetic acid and low concentrations of microbial inhibitors, SSL contains low glucose and acetic acid concentrations but high levels of microbial inhibitors. *Zymomonas* naturally tolerates 1% NaCl (65), and industrially-useful mutants with improved salt tolerance (77), flocculence (78), ethanol tolerance (79, 80), and thermotolerance (81) have also been developed.

A key advantage to the use of *Zymomonas* is its ability to grow at high sugar concentrations (>25% glucose) and to produce and tolerate ethanol at concentrations up to 13% (w/v) (66). Unlike *S. cerevisiae*, *Zymomonas* does not require small amounts of oxygen for lipid synthesis (77). Although it can grow in the presence of oxygen, aerobic growth does not result in higher cell yields or growth rates compared to anaerobic conditions (73). In fact, aeration leads to a diversion of reducing power and a consequent decrease in ethanol production.

Several independent comparative performance trials have suggested that *Zymomonas* may become an important industrial ethanol-producing microorganism because of its 5-10% higher yield, up to 5-fold higher productivity and its lower biomass formation compared to traditional yeast fermentations. However, as is the case with yeast, success at the industrial scale appears to be strain dependent, with some strains being more susceptible to glucose or ethanol inhibition or to lactic acid bacterial contamination (82). Industrial practice with *Zymomonas* fermentations has been limited, although the Glucotech process developed at the University of Queensland, Australia, has been demonstrated on dry-milled milo at industrial scales of up to 586,000 L, apparently without contamination problems and with higher yields and productivities than comparable yeast fermentations (83). Distillers grain from this fermentation has received FDA approval for use as an animal feed. The *Zymomonas*-

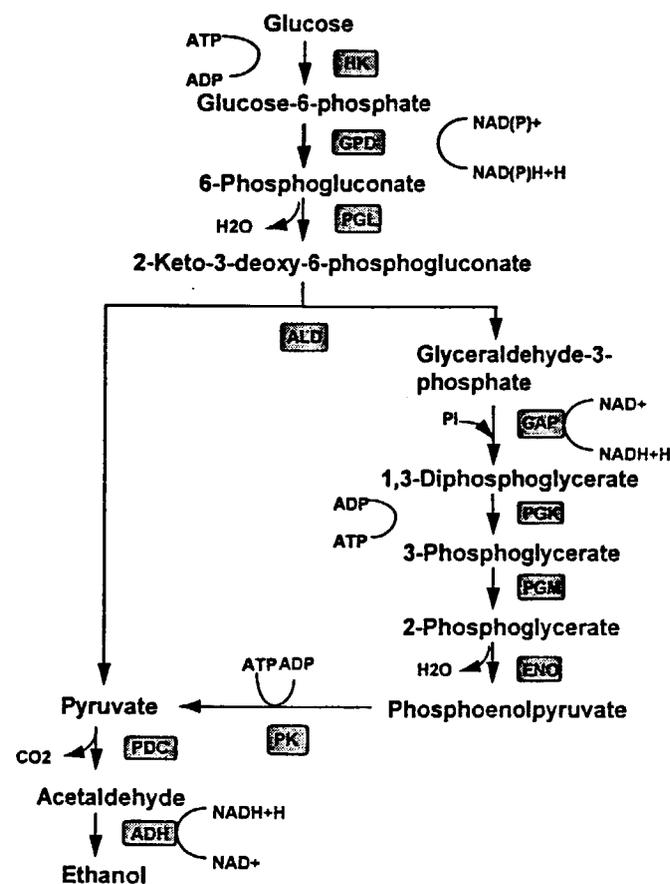


Figure 3. Entner-Doudoroff Pathway. HK, Hexokinase; GPD, Glucose-6-phosphate dehydrogenase; 6-Phosphogluconolactonase; ALD, 2-Keto-3-deoxy-6-phosphogluconate aldolase; GAP, Glyceraldehyde-3-phosphate dehydrogenase; PGK, Phosphoglycerate kinase; PGM, Phosphoglycerate mutase; ENO, Enolase; PK, Pyruvate kinase; PDC, Pyruvate decarboxylase; ADH, Alcohol dehydrogenase.

based Bio-Hol process developed at the University of Toronto has also been scaled-up to 3000 L with cell recycle, demonstrating 97% theoretical yield and producing 12% ethanol at 14 g/L/h with a residence time of less than 7 h (84). With feedstock accounting for about 70% of all production costs, the 5-10% improvement in conversion yield afforded by *Zymomonas* resulted in an extra 40 L of ethanol per ton of corn. *Z. mobilis* appears to offer a number of advantages for industrial ethanol production, including high ethanol yield and tolerance, high specific productivity, low pH optimum, considerable tolerance to inhibitors in feedstocks and GRAS status. In addition, *Z. mobilis* has been used as a host for heterologous cellulase gene expression (85) and consequently could be compatible in an SSF process.

Despite these apparent advantages, *Z. mobilis* has a narrow substrate utilization range, which is limited to only glucose, fructose and sucrose. Thus, opportunities exist to genetically engineer this organism for the fermentation of other hexose and pentose sugars. Gene transfer systems based on conjugation, transformation and electroporation using native or broad-host range plasmids already have been developed (86-90). Expression vectors have been constructed to maximally express heterologous genes (91-93). Considerable research has been directed towards the development of lactose-fermenting strains which can be used to produce ethanol from whey. Both the lactose operon (91,94) and the *lac* transposon, Tn951 (95,96), have been introduced into *Z. mobilis* and shown to express beta-galactosidase activity. Although the recombinant strains were unable to grow on lactose, they were able to ferment it to ethanol. The primary reasons for the lack of growth on lactose appear to be insufficient expression of lactose permease and product inhibition by galactose. Introduction of a *gal* operon appears essential to permit effective lactose utilization. Attempts to introduce a xylose catabolic pathway from either *Xanthomonas* or *Klebsiella* into *Zymomonas* have only met with limited success (97,98). Although the genes were functionally expressed in *Z. mobilis*, none of the recombinant strains were capable of growth on xylose as the sole carbon source. Recent studies have shown that *Zymomonas* also lacks one of the key enzymes in the pentose-phosphate pathway (transaldolase) (98), and linkage between the pentose-phosphate and Entner-Doudoroff pathways will be essential for effective pentose utilization. Thus, introduction of both xylose operon and key pentose pathway genes will be necessary to enable *Zymomonas* to effectively ferment xylose.

**Lactobacillus.** *Lactobacillus* is used commercially in the preparation of a variety of food and feed products (99) and provides several potential advantages in biomass fermentations. These include high ethanol tolerance, resistance to the inhibitors present in lignocellulosic hydrolysates, fermentation at low pH, thermotolerance and GRAS status. These gram-positive, non-spore-forming bacteria ferment many of the carbohydrates found in biomass, such as glucose, starch, cellobiose, lactose, xylose, arabinose and ribose, and produce high concentrations of lactate (100-103). One of their most desirable properties is the ability of some strains to tolerate ethanol concentrations as high as 20% (103-105)! Anaerobic fermentations are typically conducted under conditions which are resistant to contamination during prolonged large scale cultivation (pH 5.4-6.2 and temperatures of 30-45°C). Since the lactobacilli are facultative aerobes, the strict exclusion of oxygen from the fermentor is unnecessary. In addition, lactobacilli show considerable resistance to the inhibitory agents found in lignocellulosic hydrolysates (27).

Some lactobacilli ferment glucose to lactate as the sole end product with yields of over 95% (102-103). Catabolism occurs via glycolysis, converting 1 mol of hexose to 2 mol of lactic acid. These obligate homofermentative strains are typically thermophilic and resistant to glucose catabolite repression. A xylose-fermenting homofermentative strain would be an ideal host for ethanol production. Unfortunately, these strains are incapable of both pentose fermentation and ethanol production. While it is not yet clear if these strains lack the genes necessary for xylose utilization, their inability to ferment pentose sugars is thought to result from the absence of a phosphoketolase pathway, which metabolizes xylulose-5-phosphate to acetyl-phosphate and the glyceraldehyde-3-phosphate precursor to ethanol. However, since glucose fermentation occurs via the key intermediate pyruvate, successful introduction and expression of the xylose and PET operon genes could result in high yields of ethanol from lignocellulosic hydrolysates. A survey of 31 *Lactobacillus* strains identified several potential hosts for the PET operon that were at once capable of growth at ethanol concentrations up to 16% (v/v) and at least 90% conversion of glucose, cellobiose, lactose or starch to lactic acid, ethanol and acetic acid (103). Unfortunately, no strains were found that were able to convert xylose at similar efficiencies. Thus far the xylose operon from *Lactobacillus pentosus* has been cloned and expressed in other heterofermentative lactobacilli (106). Since a gene transfer system already has been developed (107), similar experiments can now be conducted with obligate homofermentative lactobacilli. Cloning and amplification of genes encoding key pentose-phosphate pathway enzymes may also be necessary to optimize ethanol production in homofermentative strains.

In contrast, pentose sugars are readily fermented by facultative heterofermentative strains to equimolar amounts of lactate and acetate via the phosphoketolase pathway. While glucose and xylose are fermented at similar rates, sugar consumption rates are relatively low compared to *S. cerevisiae* and *Zymomonas* (66,106). Another approach to the development of ethanologenic lactobacilli involves the introduction of a PET operon into these strains. Since the  $K_m$  of most lactate dehydrogenases (LDH) for pyruvate (0.37 to 10 mM) (108) are higher than that of PDC (0.4 mM), the latter has the potential to out-compete LDH for the key intermediate pyruvate. However, targeted inactivation of the genes that encode enzymes which compete for key intermediates may also be necessary to eliminate coproduct formation.

**Clostridium.** The clostridia have been of commercial interest for the fermentative production of chemicals and fuels for many years. There has been renewed interest in their use for ethanol production from renewable biomass substrates because of the ability of some species to grow at high temperature and to ferment a variety of low-cost substrates to solvents, such as acetone, butanol and ethanol. Despite this interest, however, there remained a number of significant drawbacks associated with the commercial use of these obligate anaerobes dating from the 1950's.

Acetone and butanol production by *Clostridium acetobutylicum* in particular has received considerable attention. Although certain strains have been reported to produce industrial solvents at concentrations of up to 10-12%, ethanol tolerance is poor and final ethanol concentrations are too low (<4%) for economic recovery (109). While saccharides are converted via glycolysis to the common intermediate pyruvate

further catabolism occurs through an interconnected series of electrochemical transformations leading to the formation of various coproducts, such as H<sub>2</sub>, acetate, butyrate, and isopropanol. As a result, product yields are typically quite low. The high pH optimum of these gram-positive bacteria makes prolonged large scale cultivation susceptible to contamination. Low growth rates, cell densities and productivities also limit commercial fermentation with these organisms. Special equipment would be required to maintain anoxic conditions and to compensate for the high gas pressures generated during the fermentation.

The presence of restriction enzymes in these bacteria has slowed the use of genetic engineering to eliminate many of these undesirable properties. Although a breakthrough has recently been made to circumvent this problem in one species (110), serious reservations remain concerning the use of any spore-forming recombinants in industrial fermentation processes due to their ability to survive many of the treatments designed for their containment. Despite such limitations, these organisms contain several enzymes with properties suited for the metabolic engineering of other ethanologenic hosts, and thus provide a source of novel genes.

### Future Prospects

A recent economic analysis of xylose fermentation has identified high ethanol yields and product concentrations as the most important factors influencing production costs, with volumetric productivity being of secondary importance (111). Accordingly, the microbial characteristics that appear to be indispensable or, at the very least, desirable in a commercial biomass-to-ethanol process are presented in Table I. Despite the development of recombinant strains with improved xylose fermentation performance, many important issues remain unresolved concerning their use in industrial processes. For example, high ethanol yields from lignocellulosic hydrolysates, increased ethanol product concentrations and ethanol tolerances are key targets that have yet to be achieved. Aspects such as these drive the need to develop novel recombinant microorganisms capable of rapid, high yield fermentation of xylose and other sugars to ethanol.

A review by Stokes et al. (1983) on recombinant genetic approaches for efficient ethanol production identified several economic bottlenecks in biomass conversion that potentially could be relieved through genetic improvement of microbial strains. Ten years later, while some of these bottlenecks have been eliminated through recombinant technology, many others remain. The types of genetic modifications that can have the greatest impact on the economic feasibility of these fermentations include: amplification and deregulation of rate-limiting enzymatic reactions in fermentative pathways; metabolic engineering that redirects the normal carbon flow to ethanol as the sole fermentation product, improves glycolytic efficiency and reduces futile cycling; introduction of genes encoding pathways that broaden the substrate utilization range of ethanologenic hosts; and genetic manipulations that improve ethanol tolerance, osmotolerance, thermotolerance, and resistance to the inhibitory compounds normally present in lignocellulosic hydrolysates.<sup>2</sup> These latter traits may best be achieved (at least in the near term) by selection for superior mutants or adaptation of production strains to increasingly hostile fermentation conditions. Generally, the strategy that has so far proven to be most fruitful involves the introduction of the

Table I  
Microbial Performance Characteristics in a Biomass-to-Ethanol Process

Essential Traits	Desirable Traits
• High conversion yield	• High sugar consumption rate
• High ethanol tolerance	• High specific productivity
• Resistance to hydrolysates	• High specific growth rate
• No oxygen requirement	• High volumetric productivity
• Low fermentation pH	• Hexose/penitose co-fermentation
• High fermentation selectivity	• Minimal nutrient requirements
• Broad substrate utilization range	• High salt tolerance (acetate)
	• Capable of Crabtree effect
	• Embur-Douderoff pathway
	• Facilitated sugar transport
	• GRAS status
	• Non-sporulating
	• Non-conjugative
	• Amenable to scale-up
	• Availability of "industrial" strains
	• Compatibility with SSF
	• Thermotolerance
	• High shear tolerance
	• Cellulase producer
	• Availability of a gene transfer system

"Ethanol Production Operon" (PET operon) into microbial hosts with an inherently broad substrate utilization range. The complementary approach of introducing sugar assimilation pathways into ethanologenic hosts has also received considerable attention, but has not yet achieved a similar measure of success.

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RECEIVED January 19, 1994

Reprinted from ACS Symposium Series No. 566  
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Michael E. Himmel, John O. Baker, and Ralph P. Overend, Editors  
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