

# Metabolic engineering applications to renewable resource utilization

Aristos Aristidou and Merja Penttilä

Lignocellulosic materials containing cellulose, hemicellulose, and lignin are the most abundant renewable organic resource on earth. The utilization of renewable resources for energy and chemicals is expected to increase in the near future. The conversion of both cellulose (glucose) and hemicellulose (hexose and pentose) for the production of fuel ethanol is being studied intensively, with a view to developing a technically and economically viable bioprocess. Whereas the fermentation of glucose can be carried out efficiently, the bioconversion of the pentose fraction (xylose and arabinose, the main pentose sugars obtained on hydrolysis of hemicellulose), presents a challenge. A lot of attention has therefore been focused on genetically engineering strains that can efficiently utilize both glucose and pentoses, and convert them to useful compounds, such as ethanol. Metabolic strategies seek to generate efficient biocatalysts (bacteria and yeast) for the bioconversion of most hemicellulosic sugars to products that can be derived from the primary metabolism, such as ethanol. The metabolic engineering objectives so far have focused on higher yields, productivities and expanding the substrate and product spectra.

## Addresses

VTT Biotechnology, PO Box 1500, 02044 VTT Espoo, Finland  
Correspondence: Aristos Aristidou; e-mail: Aristos.Aristidou@vtt.fi

*Current Opinion in Biotechnology* 2000, 11:187–198

0958-1669/00/\$ – see front matter

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## Abbreviations

<b>ADH</b>	alcohol dehydrogenase
<b>ED</b>	Entner–Doudoroff
<b>EMP</b>	Embden–Meyerhof–Parnas
<b>LDH</b>	lactate dehydrogenase
<b>PDC</b>	pyruvate decarboxylase
<b>PFL</b>	pyruvate formate lyase
<b>PGK</b>	phosphoglycerate kinase
<b>ScURA1</b>	<i>S. cerevisiae</i> URA1
<b>XDH</b>	xylitol dehydrogenase
<b>XI</b>	xylose isomerase
<b>XK</b>	xylokinase
<b>XR</b>	xylose reductase

## Introduction

In addition to the magnificent aesthetic value of the planet's flora, biomass represents a useful and valuable resource to man. The value of the biomass contents is related to the chemical and physical properties of the large molecules of which it is made. For millennia humans have exploited the solar energy stored in the chemical bonds of biomass by burning it as fuel and eating plants for the nutritional energy of their sugar and starch content. More recently, in the past few hundred years, humans have exploited fossilized biomass in the form of coal. This fossil fuel is the result of very slow chemical transformations that convert the sugar polymer fraction into a chemical composition that resembles

the lignin fraction. Thus, the additional chemical bonds in coal represent a more concentrated source of energy as fuel. After 1920, petroleum increasingly became the fossil fuel of choice, and after World War II petrochemicals began to dominate the synthetics market. Today, 65% of our clothing is made from oil, as are virtually all of our inks, paints, dyes, pharmaceuticals, plastics, and hundreds of intermediate chemicals. It is fair to characterize the 20<sup>th</sup> century as the hydrocarbon century. Because it takes millions of years to convert biomass into coal, fossil fuels are not renewable in the time frame over which we use them. Plant biomass is the only foreseeable sustainable source of organic fuels, chemicals, and materials, and at the end of the 20<sup>th</sup> century, there appears to be significant efforts to make the new century one that is based on renewable carbohydrates.

Many different biomass feedstocks can be used for the production of fuels and chemicals. These include various agricultural residues (corn stalks, wheat straws, potato or beet waste), wood residues (leftovers from harvested wood, and unharvested dead and diseased trees), specifically grown crops (hybrid poplar, black locust, willow, silver maple, sugarcane, sugarbeet, corn, and sweet sorghum), and waste streams (municipal solid waste, recycled paper, bagasse from sugar manufacture, corn fiber, and sulfite waste). The chemical composition of biomass varies among species, but biomass consists of ~25% lignin and ~75% carbohydrate polymers (cellulose and hemicellulose) [1]. Cellulose is a high-molecular weight linear glucose polysaccharide, with a degree of polymerization (DP) in the range of 200–2000 kDa (4000–8000 glucose molecules connected with  $\beta$ -1,4 links). Cellulose is very strong and its links are broken by cellulase enzymes. The cellulases can be separated into two classes, endoglucanases and cellobiohydrolases [2]. Cellobiohydrolases hydrolyze the cellulose chain from one end, whereas an endoglucanase hydrolyzes randomly along the cellulose chain. Hemicellulose, on the other hand, is a rather low-molecular weight heteropolysaccharide (DP < 200, typically  $\beta$ -1,3 links), with a wide variation in both structure and composition. Commonly occurring hemicelluloses are xylans, arabinoxylan, gluco-mannan, galacto-glucomann, and so on. In contrast to cellulose, which is crystalline, strong, and resistant to hydrolysis, hemicellulose has a random, amorphous structure with little strength. It is easily hydrolyzed by dilute acid or base, but nature provides an arsenal of hemicellulase enzymes for its hydrolysis [3,4]. The cellulose fraction of biomass is typically high (25–60%), whereas the hemicellulose fraction is typically in the range of 10–35%. The monomeric composition of lignocellulosic material can vary widely depending on the biomass source (see Table 1). In general, the carbohydrate fraction is made up primarily of the hexose sugar glucose (with small amounts

Table 1

## Composition of various biomass raw materials\*.

	Corn stover	Wheat straw	Rice straw	Rice hulls	Baggasse fiber	News-print	Cotton gin trash	Douglas fir
<b>Carbohydrate</b>								
Glucose (C6)	39.0	36.6	41.0	36.1	38.1	64.4	20.0	50.0
Mannose (C6)	0.3	0.8	1.8	3.0	–	16.6	2.1	12.0
Galactose (C6)	0.8	2.4	0.4	0.1	1.1	–	0.1	1.3
Xylose (C5)	14.8	19.2	14.8	14.0	23.3	4.6	4.6	3.4
Arabinose (C5)	3.2	2.4	4.5	2.6	2.5	0.5	2.3	1.1
Total C6	40.1	39.8	43.2	39.2	39.2	81	22.2	63.3
Total C5	18	21.6	19.3	16.6	25.8	5.1	6.9	4.5
<b>Non-carbohydrate</b>								
Lignin	15.1	14.5	9.9	19.4	18.4	21.0	17.6	28.3
Ash	4.3	9.6	12.4	20.1	2.8	0.4	14.8	0.2
Protein	4.0	3.0	–	–	3.0	–	3.0	–

\*Adapted from [5].

of the hexoses galactose and mannose); however, the pentose fraction is rather significant: xylose 5–20% and arabinose 1–5%. Xylose is second only to glucose in natural abundance and it is the most copious sugar in the hemicellulose of hardwoods and crop residues.

The conversion of both cellulose and hemicellulose for production of fuel ethanol is being studied intensively with a view to develop a technically and economically viable bioprocess. Ethanol is a versatile transportation fuel that offers high octane, high heat of vaporization, and other characteristics that allow it to achieve higher efficiency use in optimized engines than gasoline. Ethanol is low in toxicity, volatility, and photochemical reactivity, resulting in reduced ozone formation and smog compared to conventional fuels. Researchers at the National Renewable Energy Laboratory estimate that the United States potentially could convert 2.45 billion metric tons of biomass to 270 billion gallons of ethanol each year, which is approximately twice the annual gasoline consumption in the United States. Increased use of bioethanol, also used as a hydrogen fuel source for fuel cells, could become a vital part of the long-term solution to climate change.

The important key technologies required for the successful biological conversion of lignocellulosic biomass to ethanol have been extensively reviewed [5,6,7]. Microbial conversion of the sugar residues present in wastepaper and yard trash from US landfills alone could provide more than 400 billion liters of ethanol [8], 10 times the corn-derived ethanol burned annually as a 10% blend with gasoline [9]. The biological process of ethanol fuel production utilizing lignocellulose as substrate requires three steps (Figure 1): firstly, delignification to liberate cellulose and hemicellulose from their complex with lignin; secondly, depolymerization of the carbohydrate polymers (cellulose and hemicellulose) to produce free sugars; and finally, fermentation of mixed hexose and pentose sugars to produce ethanol (see Table 2). The development of a feasible biological delignification

process should be possible if lignin-degrading microorganisms (e.g. *Phanerochaete chrysosporum* and *Phlebia radiata*), their ecophysiological requirements, and optimal bioreactor design are effectively coordinated. Some thermophilic anaerobes and recently developed recombinant bacteria have advantageous features for direct microbial conversion of cellulose to ethanol — that is, the simultaneous depolymerization of cellulosic carbohydrate polymers with ethanol production. New fermentation technology for converting xylose to ethanol also needs to be developed to make the overall conversion process more cost-effective. The fermentation of glucose, the main constituent of the cellulose hydrolysate, to ethanol can be carried out efficiently. On the other hand, although bioconversion of xylose (the main pentose sugar obtained on hydrolysis of hemicellulose) to ethanol presents a biochemical challenge, especially if it is present along with glucose, it needs to be achieved to make the biomass-to-ethanol process economical. A lot of attention has therefore been focussed on the utilization of both glucose and xylose to ethanol. The economics of the ethanol process is determined by the cost of sugar. The average biomass cost amounts to ~\$0.06 per kg of sugar, or a contribution to the feedstock costs for ethanol production of as low as \$0.10 per l.

Applied research in the area of biomass conversion to ethanol in the past 20 years has answered most of the major challenges on the road to commercialization but, as with any new technology, there is still room for performance improvement. Over the past decade, the total cost of ethanol has dropped from more than \$1.0 per l to ~\$0.3–0.5 per l, with a projected cost of less than \$0.25 per l in the near future. As a number of studies have indicated, efficient utilization of the hemicellulose component of lignocellulosic feedstocks offers an opportunity to reduce the cost of producing fuel ethanol by 25% [10]. This essentially requires the ability to convert all fermentable sugars (i.e. pentoses and hexoses) to product, which dictates the need to develop advanced hexose/pentose-fermenting

**Table 2****Comparison of fermentation products during aerobic and anaerobic growth of wild-type and recombinant *E. coli*\***

Growth	Plasmid	Fermentation product (mM)			
		Ethanol	Lactate	Acetate	Succinate
Aerobic	None	0	0.6	55	0.2
	PLO1308-10 (PET)	337	1.1	17	4.9
Anaerobic	None	0.4	22	7	0.9
	PLO1308-10 (PET)	482	10	1.2	5.0

\*Adapted from [66].

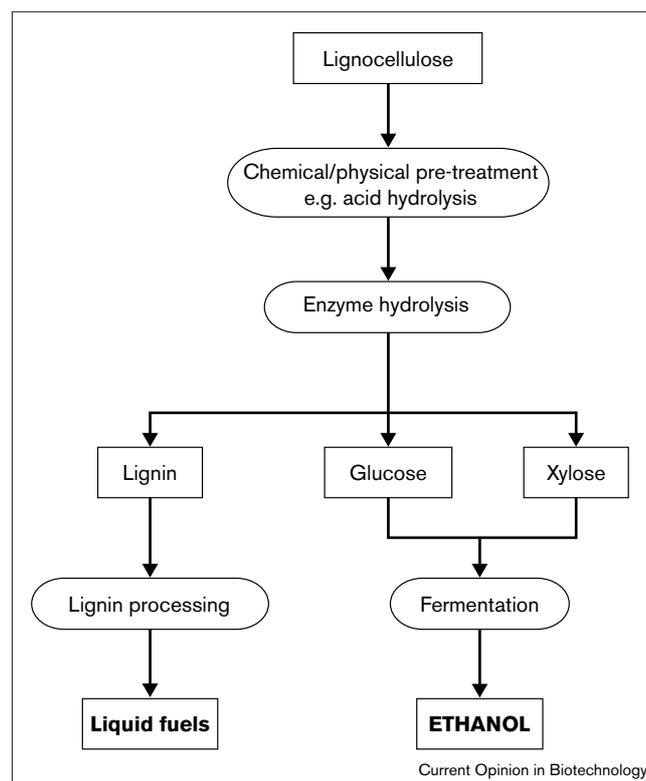
organisms. As no naturally occurring organism can satisfy all necessary specifications (e.g. high yield, high productivity, wide-substrate range, ethanol tolerance, tolerance to inhibitors present in hydrolysates, and biomass disposal cost), this has to come about by the utilization of modern genetic engineering techniques aimed at organisms that are endowed with most of the desirable properties for such bioprocesses. Along with the introduction of ethanol genes in enteric bacteria (*Escherichia coli*), parallel efforts were also undertaken to incorporate pentose-metabolizing pathways in natural ethanol producers such as *Saccharomyces cerevisiae* and *Zymomonas mobilis* (as discussed later).

Genetic improvements in the microorganism cultures have been made either to enlarge the range of substrate utilization or to channel metabolic intermediates specifically toward ethanol. These contributions represent real significant advances in the field and have also been adequately dealt with from the point of view of their impact on utilization of both cellulose and hemicellulose sugars to ethanol. The bioconversion process of lignocellulosics to ethanol could be successfully developed and optimized by aggressively applying the related novel science and technologies to solve the known key problems of conversion process; for example, simultaneous saccharification and fermentation (SSF), continuous ethanol processes based on flocculent yeast, and continuous end-product removal. The efficient fermentation of xylose and other hemicellulose constituents may prove essential for the development of an economically viable process to produce ethanol from biomass [11].

This review deals with the metabolic strategies to generate efficient biocatalysts (bacteria and yeast) for the bioconversion of most hemicellulose sugars to products that can be derived from the primary metabolism, such as ethanol.

### Pentose fermentation

The fermentation organism must be able to ferment all monosaccharides present and, in addition, withstand potential inhibitors in the hydrolysate. Pentose-fermenting microorganisms are found among bacteria, yeasts, and fungi, with the yeasts *Pichia stipitis*, *Candida shehatae*, and

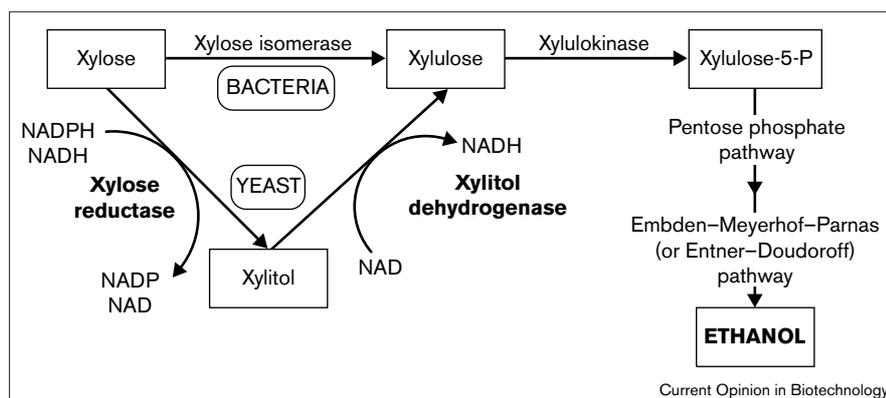
**Figure 1**

Conversion of lignocellulose to ethanol. Crystalline cellulose, the largest (50%) and most difficult fraction, is hydrolyzed by a combination of acid and enzymatic processes. During these steps 95–98% of the xylose and glucose are recovered. These monosaccharides are subsequently converted to ethanol by appropriate microorganisms.

*Pachysolen tannophilus* being the most promising naturally occurring microorganisms. Yeasts produce ethanol efficiently from hexoses by the pyruvate decarboxylase (PDC)–alcohol dehydrogenase (ADH) system. During xylose fermentation, however, the byproduct xylitol accumulates, thereby reducing the yield of ethanol. Furthermore, yeasts are reported to ferment L-arabinose only very weakly. Only a handful of bacterial species are known to possess the important PDC–ADH pathway to ethanol. Among these, *Z. mobilis* has the most active PDC–ADH system; however, it is incapable of dissimilating pentose sugars.

Microorganisms, in general, metabolize xylose to xylulose through two separate routes (Figure 2). The one-step pathway catalyzed by xylose isomerase (XI; EC 5.3.1.5) is typical in bacteria, whereas the two-step reaction involving xylose reductase (XR) and xylitol dehydrogenase (XDH) is usually found in yeast. Xylulose is subsequently phosphorylated with xylulokinase (XK) to xylulose-5-phosphate, which can be further catabolized via the pentose phosphate pathway and the Embden–Meyerhof–Parnas (EMP) pathway (or the Entner–Doudoroff [ED] pathway in organisms such as *Z. mobilis*).

Figure 2



Comparison of xylose-utilizing pathways in bacteria and yeast. P, phosphate.

Empowered with the modern tools of genetic engineering, a number of groups have been pursuing for the past decade or so the construction of organisms (yeasts or bacteria) that can efficiently convert most of the sugars present in biomass-derived hydrolysates to useful products. The approaches can be divided in two groups: firstly, to engineer organisms with an expanded substrate spectrum; and secondly, to engineer organisms with enhanced abilities of converting key intermediates of central carbon metabolism (e.g. pyruvate, the ultimate product of glycolysis) to useful compounds (mainly ethanol). The former approach has focused on good ethanologenic organisms (mostly yeast and the bacterium *Z. mobilis*), with the aim of introducing the pathways for xylose or arabinose metabolism (e.g. [12,13]). The latter approach is to start with organisms that have a wide substrate range, including hexoses and pentoses (e.g. *E. coli*), and introducing pathways for converting, for example, pyruvate to ethanol [14–20].

### Genetically engineered bacteria

Initial studies were only partially successful in redirecting fermentative metabolism in *Erwinia chrysanthemi* [21], *Klebsiella planticola* [22], and *E. coli* [23]. The first generation of recombinant organisms amplified the PDC activity only and depended on endogenous levels of ADH activity to couple the further reduction of acetaldehyde to the oxidation of NADH (Figure 2). As ethanol is just one of a number of fermentation products normally produced by these enteric bacteria, a deficiency in ADH activity together with NADH accumulation contributed to the formation of various unwanted byproducts.

#### *Escherichia coli*

Most recent work in the engineering of bacteria has focused on *E. coli*. This is an attractive host organism for the conversion of renewable resources to ethanol and other useful products for a number of reasons. Firstly, it can grow efficiently on a wide range of carbon substrates that includes five-carbon sugars (i.e. it has the ability to ferment — besides glucose — all other sugar constituents of lignocellulosic material: xylose, mannose, arabinose, and

galactose). Secondly, it can sustain high glycolytic fluxes (both aerobically and anaerobically). Finally, it has a reasonable ethanol tolerance (at least up to 50 g per l).

Redirection of glycolytic fluxes to ethanol in *E. coli* was accomplished by transforming this organism with a plasmid containing the *Z. mobilis* genes coding for PDC and ADH (*pdc* and *adhB*) in an artificial operon (the so-called PET operon). In the recombinant *E. coli*, both enzymes (PDC and ADH), required to divert pyruvate metabolism to ethanol, were overexpressed to high levels. The combined effect of high PDC overexpression and the low apparent  $K_m$  value of this enzyme for pyruvate (i.e. higher affinity for pyruvate; see [Table 3]) is to effectively divert carbon flow to ethanol, even in the presence of native fermentation enzymes, such as lactate dehydrogenase (LDH). When the recombinant strain was grown on mixtures of sugars typically present in hemicellulose hydrolysates, sequential utilization was observed with glucose consumed first, followed by arabinose and xylose, to produce near-maximum theoretical yields of ethanol [24].

Under aerobic conditions, wild-type *E. coli* metabolizes pyruvate through pyruvate dehydrogenase (PDH) and

Table 3

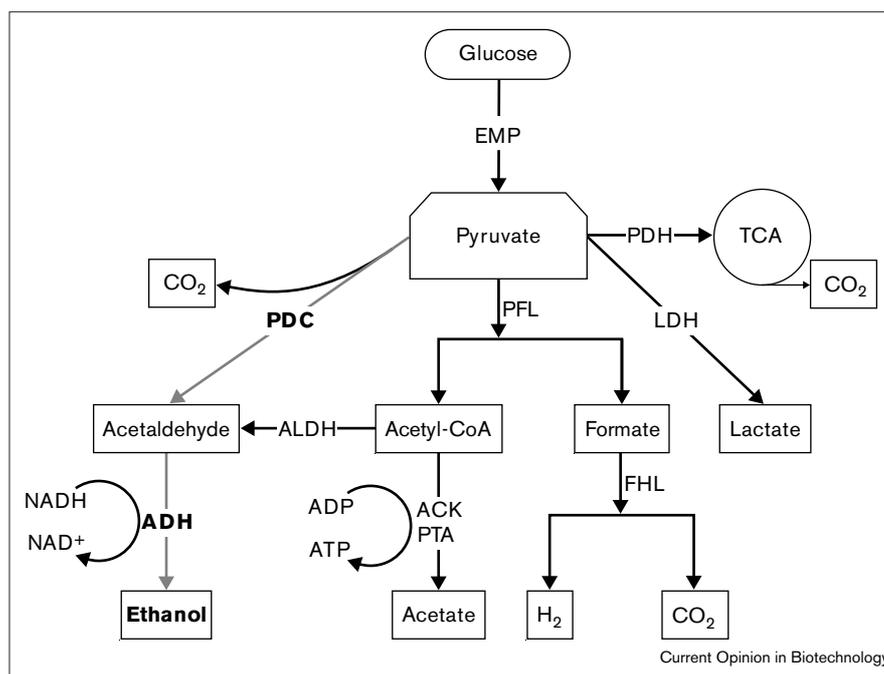
Comparison of apparent  $K_m$  values for pyruvate for selected *E. coli* and *Z. mobilis* pyruvate utilizing enzymes.

Organism	Enzyme	$K_m$	
		Pyruvate	NADH
<i>E. coli</i>	PDH	0.4 mM	0.18 mM
	LDH	7.2 mM	> 0.5 mM
	PFL	2.0 mM	
	ALDH		50 $\mu$ M
	NADH-OX		50 $\mu$ M
<i>Z. mobilis</i>	PDC	0.4 mM	
	ADH II		12 $\mu$ M

ALDH, aldehyde dehydrogenase; ADH II, alcohol dehydrogenase II; NADH-OX, NADH oxidase; PDH, pyruvate dehydrogenase.

**Figure 3**

Competing pathways at the pyruvate branchpoint. ACK/PTA, acetate kinase and phosphotransacetylase; ALDH, acetaldehyde dehydrogenase; EMP, Embden–Meyerhof–Parnas (enzymes and intermediates); FHL, formate hydrogen lyase; PDH, pyruvate dehydrogenase; TCA, tricarboxylic acid cycle.



pyruvate formate lyase (PFL) ( $K_m$  0.4 and 2.0 mM, respectively; Table 3), with the main products being  $\text{CO}_2$  and acetate (formed by the conversion of excess acetyl-CoA). The apparent  $K_m$  for the *Z. mobilis* PDC is similar to that of PDH and lower than those of PFL and LDH, thereby facilitating acetaldehyde production.  $\text{NAD}^+$  regeneration under aerobic conditions primarily results from biosynthesis and from the  $\text{NADH}$  oxidase (coupled to the electron transport system). Again, because the apparent  $K_m$  for *Z. mobilis* ADH II is over fourfold lower than that for *E. coli*  $\text{NADH}$  oxidase, the heterologous ADH II effectively competes for endogenous pools of  $\text{NADH}$ , allowing the reduction of acetaldehyde to ethanol. Under anaerobic conditions, wild-type *E. coli* metabolizes pyruvate primarily via LDH and PFL. As indicated again in Table 3, the apparent  $K_m$  values for these two enzymes are 18-fold and fivefold higher, respectively, than that for *Z. mobilis* PDC. Furthermore, the apparent  $K_m$  values for primary native enzymes involved in  $\text{NAD}^+$  regeneration are also considerably higher in *E. coli* than those of *Z. mobilis* ADH. Thus, overexpressed ethanologenic *Z. mobilis* enzymes in *E. coli* can favorably compete with the native enzymes for pyruvate and redox co-substrates channeling carbon (pyruvate) into ethanol.

The University of Florida was awarded US Patent No. 5,000,000 [P1] for the ingenious microbe created at its Institute of Food and Agricultural Sciences. Significant amounts of ethanol were produced in recombinant *E. coli* containing the above mentioned PET operon under both aerobic and anaerobic conditions (Table 2). Typical final

ethanol concentrations are in excess of 50 g per l (e.g. 54.4 and 41.6 g per l were obtained from 10% glucose and 8% xylose, respectively) [16] at near-maximum theoretical yields of 0.5 g of ethanol per g of sugar (sugar  $\rightarrow$  2 ethanol + 2  $\text{CO}_2$ ). Published volumetric and specific ethanol productivities with xylose in simple batch fermentations are 0.6 g of ethanol per l per hour and 1.3 g of ethanol per g cell dry weigh per hour, respectively [15]. Further improvements have resulted in volumetric productivities of as high as 1.8 g per hour of ethanol. A recent study focused on the technical and economic analysis of the production of fuel ethanol by fermentation of a pentose-rich hydrolysate using the recombinant *E. coli* strain KO11A [25]. The calculations were based on a feed capacity of 10 metric tons of dry willow per hour to the pretreatment stage, providing 35 metric tons of hydrolysate per hour, consisting of 45 g of sugars per l. The economic analysis predicted an ethanol production cost of \$0.48 per l in the pentose fermentation plant, which was considered economically good.

#### ***Klebsiella oxytoca***

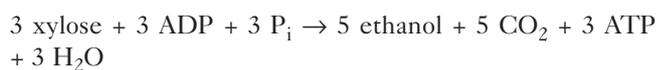
Ohta *et al.* [17] investigated the expression of the *pdh* and *adh* genes of *Z. mobilis* in a related enteric bacterium, *Klebsiella oxytoca*. The native organism has the capability to transport and metabolize cellobiose, thus minimizing the need for extracellular additions of cellobiase. In *Klebsiella* strains, two additional fermentation pathways are present compared with *E. coli* (Figure 3), converting pyruvate to succinate and butanediol. As in the case of *E. coli*, it was possible to divert more than 90% of the carbon flow from

sugar catabolism away from the native fermentative pathways and toward ethanol (strain P2). Overexpression of recombinant PDC alone produced only about twice the ethanol level of the parental strain; however, when both PDC and ADH were elevated in *K. oxytoca* M5A1, ethanol production was both very rapid and efficient: volumetric productivities >2.0 g per l per hour, yields 0.5 g of ethanol per g of sugar, and final ethanol of 45 g per l for both glucose and xylose carbon sources were obtained.

### **Zymomonas mobilis**

Xylose also could be a useful carbon source for the ethanol producer *Z. mobilis*. This is a bacterium that has been used as a natural fermentative agent in alcoholic beverage production and has been shown to have ethanol productivity superior to that of yeast. Overall, it demonstrates many of the desirable traits sought in an ideal biocatalyst for ethanol, such as high ethanol yield, selectivity and specific productivity, as well as low pH and high ethanol tolerance. In glucose medium, *Z. mobilis* can achieve ethanol levels of at least 12% (w/v) at yields of up to 97% of the theoretical value. When compared to yeast, *Z. mobilis* exhibits 5–10% higher yields and up to fivefold greater volumetric productivities. The notably high yield of this microbe is attributed to reduced biomass formation during fermentation, apparently limited by ATP availability.

As a matter of fact, *Zymomonas* is the only genus identified to date that exclusively utilizes the ED pathway anaerobically. The stoichiometry of ethanol production from xylase through the ED pathway (e.g. in the recombinant *Z. mobilis* organism described below) can be summarized as follows (neglecting the NAD[P]H balances):



Thus, the theoretical yield on ethanol is 0.51 g of ethanol per g of xylose (1.67 mol per mol). It is important to note that the metabolically engineered pathway yields only 1 mole of ATP from 1 mol of xylose, compared with 5 and 3 moles typically produced through a combination of the pentose phosphate and EMP pathways, respectively. When converting glucose to ethanol, this organism produces only 1 mole of ATP per mole of glucose through the ED pathway compared with 2 moles produced via the more common EMP pathway. The energy limitation is expected to result in a lower biomass formation and, thus, a more efficient conversion of substrate to product.

Furthermore, glucose can readily cross the cell membrane of this organism by facilitated diffusion, efficiently be converted to ethanol by an overactive PDC–ADH system, and is generally recognized as a safe (GRAS) organism for use as an animal feed. As discussed earlier, the main drawback of this microorganism is that the wild type can only utilize glucose, fructose, and sucrose and is thus unable to ferment the widely available pentose sugars.

This led Zhang *et al.* [26] to attempt to introduce a pathway for pentose metabolism in *Z. mobilis*. Early attempts by other groups using the XI (*xyIA*) and XK (*xyIB*) genes (Figure 2) from either *Klebsiella* or *Xanthomonas* were met with limited success, despite the functional expression of these genes in *Z. mobilis*. It soon became evident that such failures were due to the absence of detectable transketolase and transaldolase activities in *Z. mobilis*, which are necessary to complete a functional pentose metabolic pathway (Figure 4). After the transketolase *E. coli* gene was cloned and introduced in *Z. mobilis*, there occurred a small conversion of xylose to CO<sub>2</sub> and ethanol [27]. The next step was to introduce the transaldolase reaction, as this strain accumulated significant amounts of sedoheptulose-7-phosphate (a substrate for transaldolase) intracellularly. Sophisticated cloning techniques were therefore applied to the construction of a chimeric shuttle vector (pZB5) that carries two independent operons: the first encoding the *E. coli xyIA* and *xyIB* genes and the second expressing transketolase (*tktA*) and transaldolase (*tal*), again from *E. coli*. The two operons, which included the four xylose assimilation and non-oxidative pentose phosphate pathway genes, were expressed successfully in *Z. mobilis* CP4. The recombinant strain was capable of fast growth on xylose as the sole carbon source, and, moreover, it efficiently converted glucose and xylose to ethanol with 86 and 94% of the theoretical yield from xylose and glucose, respectively.

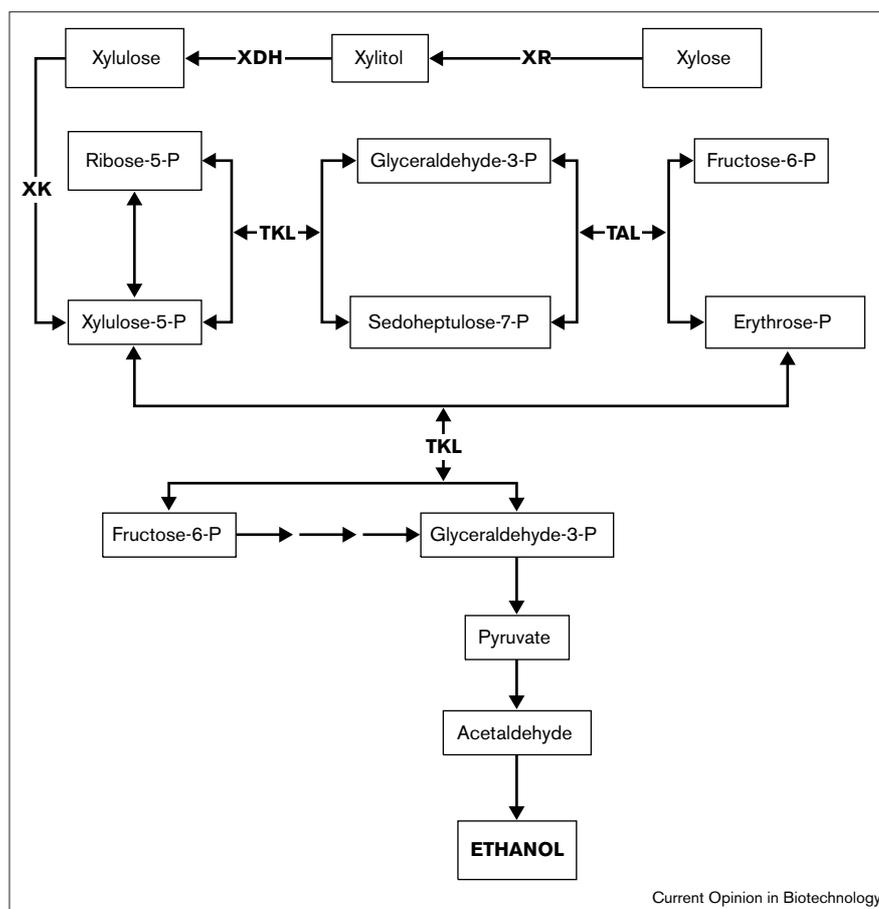
In a subsequent article [28], the same laboratory reported the construction of a *Z. mobilis* strain with a substrate fermentation range expanded to include the pentose sugar L-arabinose, which is commonly found in agricultural residues and other lignocellulosic biomass. Five genes, encoding L-arabinose isomerase (*araA*), L-ribulokinase (*araB*), L-ribulose-5-phosphate-4-epimerase (*araD*), transaldolase (*talB*), and transketolase (*tktA*), were isolated from *E. coli* and introduced into *Z. mobilis* under the control of constitutive promoters. The engineered strain grew on and produced ethanol from L-arabinose as a sole carbon source at 98% of the maximum theoretical ethanol yield, indicating that arabinose was metabolized almost exclusively to ethanol as the sole fermentation product. The authors indicate that this microorganism may be useful, along with the previously developed xylose-fermenting *Z. mobilis* [26], in a mixed culture for efficient fermentation of the predominant hexose and pentose sugars in agricultural residues and other lignocellulosic feedstocks to ethanol.

### **Genetically engineered yeast**

*Saccharomyces* spp. are the safest and most effective microorganisms for fermenting sugars to ethanol and traditionally have been used in industry to ferment glucose-based (or hexose sugar-based) agricultural products to ethanol. Yeasts produce ethanol efficiently from hexoses by the PDC–ADH system. *S. cerevisiae*, has the intrinsic limitation of not being able to ferment pentoses such as xylose or arabinose. Even though certain types of yeast, such as *Pachysolen tannophilus*, *Pichia stipitis*, or *Candida shehatae*, are xylose-fermenting,

Figure 4

Ethanol production from pentose sugars in metabolically engineered *Z. mobilis*.  
TAL, transaldolase; TKL, transketolase.



they have poor ethanol yields and low ethanol tolerance compared with the common glucose-fermenting yeasts, such as *S. cerevisiae*.

### ***Saccharomyces cerevisiae***

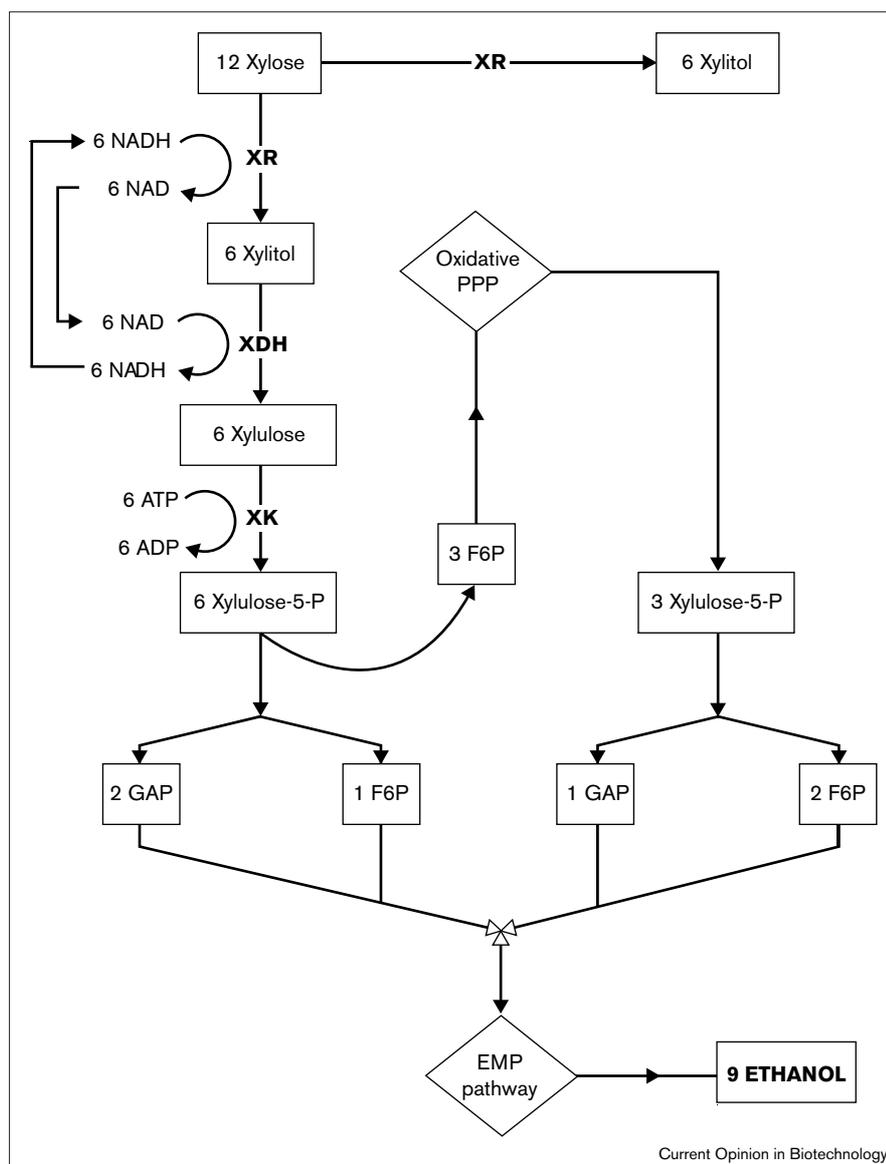
Many attempts to introduce the one-step pathway of xylose metabolism by cloning the gene coding for XI from either *E. coli* [29] or *B. subtilis* [30] in *S. cerevisiae* were unsuccessful due to the inactivity of the heterologous protein in the recombinant host cell. Later on, the *Thermus thermophilus xylA* gene encoding xylose (glucose) isomerase was cloned and expressed in *S. cerevisiae* under the control of the yeast *PGK1* promoter [31]. The recombinant XI showed the highest activity at 85°C with a specific activity of 1.0 U per mg protein. This study also demonstrated a new functional, yet low-throughput, metabolic pathway in *S. cerevisiae* with ethanol formation during oxygen-limited xylose fermentation.

The first step in yeast xylose metabolism is carried out by xylose (aldose) reductase (Figure 5). The gene coding for this enzyme has been given the designation *XYL1*. In most yeasts and fungi, this enzyme has a cofactor specificity for NADPH, but in *P. stipitis* the enzyme shows 70% as much activity with

NADPH as with NADH [32]. The *P. stipitis XYL1* gene has been cloned by at least three groups independently [33–35]. More recently, the equivalent genes from *Kluyveromyces lactis* [36], *Pachysolen tannophilus* [37], *Trichoderma reesei* (M Saloheimo, M Penttilä, unpublished data) and even *S. cerevisiae* [38] have been cloned. The relative affinity of various XRs for NADH and NADPH vary widely, and it has recently been reported that the enzyme from *Candida boidinii* has higher activity with NADH than with NADPH [39].

The second step in xylose metabolism is carried out by XDH (*XYL2*), which, unlike XR, is always specific for NAD [12]. Attempts have been made to modify the XDH cofactor specificity [40]. The mutation D207→G (amino acid single letter code) and the double mutation D207→G/D210→G within the binding domain (GXGXXG) increased the apparent  $K_m$  for NAD ninefold and decreased the XDH activity to 47% and 35%, respectively, as compared to the unaltered enzyme. The introduction of the potential NADP-recognition sequence (GSRPVC) of the ADH from *Thermoanaerobium brockii* into the XDH allowed the mutant enzyme to use both NAD and NADP as cofactor with equal apparent  $K_m$  values. The mutagenized *XYL2* gene could still mediate growth of

Figure 5



Anaerobic xylose utilization and cofactor regeneration in recombinant *S. cerevisiae*. F6P, fructose-6-phosphate; GAP, glyceraldehyde-3-phosphate; P, phosphate; PPP, pentose phosphate pathway.

*S. cerevisiae* transformants on xylose minimal-medium plates when expressed together with the *XYL1* gene. More recently, the gene coding for a *Saccharomyces* XDH enzyme was also discovered [41], as well as one from *T. reesei* (T Wong, M Penttilä, unpublished results).

Several laboratories have attempted to engineer a xylose-fermenting *S. cerevisiae* through the expression of *XYL1* or both *XYL1* and *XYL2*. Expression of *XYL1* alone has not proven sufficient to enable *S. cerevisiae* to ferment or even to grow on xylose, but in the presence of glucose, *S. cerevisiae* strains expressing *XYL1* will produce xylitol from xylose at high yield [33,42,P2]. Production of xylitol appears to be a consequence of redox imbalance in the cell, and is affected by glycerol production [43–45].

Expression of both *XYL1* and *XYL2* has proven to be more successful [12]; however, although it made it possible for *S. cerevisiae* to grow on xylose, the amounts of ethanol formed by such strains are typically low (10 mM). Kötter and Ciriacy [13] studied the xylose fermentation in *S. cerevisiae* more extensively, and compared its fermentative activities to those of *P. stipitis*. In the absence of respiration, *S. cerevisiae* transformed with both *XYL1* and *XYL2* converts about half of the xylose present in the medium into xylitol and ethanol in roughly equimolar amounts. By comparison, *P. stipitis* produces only ethanol. They proposed, as had Hahn-Hägerdal and co-workers [43], that in *S. cerevisiae*, ethanol production is limited by cofactor imbalance. Additional limitations of xylose utilization in *S. cerevisiae* were also attributed to the inefficient capacity of the non-oxidative pentose-phosphate

pathway, as indicated by the accumulation of sedoheptulose-7-phosphate [46–48].

Tantirungkij *et al.* [49] took the approach one step further by subcloning *P. stipitis* *XYL1* into *S. cerevisiae* under the control of the enolase promoter on a multicopy vector. This achieved 2–3 times the level of *XYL1* expression observed in *P. stipitis*. *XYL2* was also cloned and co-expressed in *S. cerevisiae* at about twice the level achieved in induced *P. stipitis*. Despite these higher levels of expression, only low levels of ethanol (on the order of 5 g per l) were observed under optimal conditions after 100 hours. These researchers also selected mutants of *S. cerevisiae* carrying *XYL1* and *XYL2* that exhibited rapid growth on xylose medium [50]. The fastest growing strain showed a lower activity of XR, but a higher ratio of XDH to XR activity. Southern hybridization showed that the vector carrying the two genes had integrated into the genome resulting in increased stability of the cloned genes. The yield and production rate of ethanol increased 1.6- and 2.7-fold, respectively, but the maximum concentration of ethanol reported was only 7 g per l after 144 hours.

The effect of the relative levels of expression of the *XYL1* and *XYL2* genes from *P. stipitis* in *S. cerevisiae* has also been investigated [51]. These two genes were placed in different directions under the control of the alcohol dehydrogenase I (*ADHI*) and phosphoglycerate kinase (*PGK*) promoters and inserted into the yeast multicopy vector. Different recombinant *S. cerevisiae* strains were constructed with different specific activities of XR and XDH. The XR:XDH ratio (ratio of specific enzyme activities of XR and XDH) in these recombinant *S. cerevisiae* strains varied from 17.5 to 0.06. In order to enhance xylose utilization in the *XYL1*–*XYL2* containing *S. cerevisiae* strains, the native *TKL1* gene encoding transketolase and the *TAL1* gene encoding transaldolase were also overexpressed, which resulted in considerably good growth on the xylose plate. Fermentation of the recombinant *S. cerevisiae* strains containing *XYL1*, *XYL2*, *TKL1*, and *TAL1* were studied with mixtures of glucose and xylose. The strain with a XR:XDH ratio of 0.06 consumed 3.25 g per l xylose and formed no xylitol and less glycerol and acetic acid, but more ethanol compared with the strains with a higher XR:XDH ratio.

Ho *et al.* [52] have recently reported the construction of a recombinant *Saccharomyces* strain expressing the genes for the three xylose metabolizing enzymes: the XR-encoding gene *XYL1*, XDH-encoding gene *XYL2* (both from *Pichia stipitis*), and XK-encoding gene *XYL3* (from *S. cerevisiae*). Cloning of the *XYL3* gene from *Pachysolen tannophilus* was first reported in 1987 [53], and cloning of *S. cerevisiae* *XYL3* by complementation of a XK-deficient mutant of *E. coli* was first reported in 1989 [52], and was recently verified [54]. Ho *et al.* [55\*] group developed recombinant multicopy vector plasmids that can transform *Saccharomyces* spp. into xylose-fermenting yeasts. In addition to the geneticin resistance and

ampicillin resistance genes that serve as dominant selectable markers, these plasmids also contain 3 xylose-metabolizing genes, an XR gene, an XDH gene (both from *P. stipitis*), and an XK gene (from *S. cerevisiae*). The parental yeast strain *Saccharomyces* 1400 is a fusion product of *Saccharomyces diastolicus* and *Saccharomyces uvarum*. It exhibits high ethanol and temperature tolerance and a high fermentation rate. Overexpression of *XYL3* in the *Saccharomyces* 1400 fusant along with *XYL1* and *XYL2* results in production of about 47 g per l of ethanol in 84% of theoretical yield from a 1:1 glucose : xylose mixture [55\*].

The Technical Research Center of Finland (VTT) group has addressed the redox imbalance of the XR and XDH reactions by introducing artificial transhydrogenase cycles in xylose-utilizing *S. cerevisiae* [56,P3]. This work was based on the simultaneous expression of dehydrogenase enzymes that have different cofactor specificities (e.g. the yeast glutamate dehydrogenase 1 and 2 — *GDH1*, *GDH2*), and also in combination with enzymes that can be driven by ATP (e.g. malic enzyme), thus overcoming intrinsic limitations due to the physiological redox cofactor concentrations. Results of such genetically engineered organisms so far have been encouraging in terms of improving xylose utilization rates and ethanol productivities. In addition to *S. cerevisiae*, these ideas were further successfully applied to the fission yeast *Schizosaccharomyces pombe*, which also over-expresses the *Pichia* XR and XDH [P3].

#### ***Pichia stipitis***

*P. stipitis* has also received significant attention in the past few years, in terms of developing and applying genetic engineering techniques to address metabolic imperfections, such as the oxygen requirements for efficient xylose utilization. Respiratory and fermentative pathways coexist to support growth and product formation in *P. stipitis*. This yeast grows rapidly without ethanol production under fully aerobic conditions, and it ferments glucose or xylose under oxygen-limited conditions, but it stops growing within one generation under anaerobic conditions.

Expression of *S. cerevisiae* *URA1* (*ScURA1*) in *P. stipitis* enabled rapid anaerobic growth in minimal defined medium containing glucose when essential lipids were present. *ScURA1* encodes a dihydroorotate dehydrogenase that uses fumarate as an alternative electron acceptor to confer anaerobic growth [57\*•]. Initial *P. stipitis* transformants grew and produced 32 g per l ethanol from 78 g per l glucose. Cells produced even more ethanol faster following two anaerobic serial subcultures. Control strains without *ScURA1* were incapable of growing anaerobically and showed only limited fermentation. *P. stipitis* cells bearing *ScURA1* were viable in anaerobic xylose medium for long periods, and supplemental glucose allowed cell growth, but xylose alone could not support anaerobic growth even after serial anaerobic subculture on glucose. These data imply that *P. stipitis* can grow anaerobically using metabolic energy generated through fermentation but that it

exhibits fundamental differences in cofactor selection and electron transport with glucose and xylose metabolism. This is the first report of genetic engineering to enable anaerobic growth of a eukaryote.

The *P. stipitis* XR gene (*XYL1*) was inserted into an autonomous plasmid that *P. stipitis* maintains in multicopy [58]. This plasmid with the *XYL1* insert or a control plasmid without *XYL1* was introduced into *P. stipitis*. When grown on xylose under aerobic conditions, the strain with pXOR had up to 1.8-fold higher xylose reductase (XOR) activity than the control strain. Oxygen limitation led to higher XOR activity in both recombinant and control strains grown on xylose; however, the XOR activities of the two strains grown on xylose were similar under oxygen limitation. When grown on glucose under aerobic or oxygen-limited conditions, the experimental strain had XOR activity up to 10 times higher than that of the control strain. Ethanol production was not improved, but rather it decreased with the introduction of pXOR compared to the control, and this was attributed to nonspecific effects of the plasmid.

Jeffries's (see e.g. [59]) group also studied the expression of the genes encoding group I ADs (*PsADH1* and *PsADH2*) in the xylose-fermenting yeast *P. stipitis* CBS 6054. The cells expressed *PsADH1* ~10 times higher under oxygen-limited conditions than under fully aerobic conditions when cultivated on xylose. Transcripts of *PsADH2* were not detectable under either aeration condition. The *PsADH1::lacZ* fusion was used to monitor *PsADH1* expression and found that expression increased as oxygen decreased. The level of *PsADH1* transcript was repressed about 10-fold in cells grown in the presence of heme under oxygen-limited conditions. Concomitantly with the induction of *PsADH1*, *PsCYC1* (the *P. stipitis* gene encoding cytochrome c) expression was repressed. These results indicate that oxygen availability regulates *PsADH1* expression and that regulation may be mediated by heme. The regulation of *PsADH2* expression was also examined in other genetic backgrounds. Disruption of *PsADH1* dramatically increased *PsADH2* expression on non-fermentable carbon sources under fully aerobic conditions, indicating that the expression of *PsADH2* is subject to feedback regulation under these conditions.

### ***Pichia pastoris***

A XR-encoding gene (*XYL1*) of *Candida guilliermondii* ATCC 20118 was cloned and characterized [60]. The derived amino acid sequence of *C. guilliermondii* XR was 70.4% homologous to that of *P. stipitis*. The gene was placed under the control of an alcohol oxidase promoter (*AOX1*) and integrated into the genome of a methylotrophic yeast, *P. pastoris*. Methanol induced the expression of the XR and the expressed enzyme preferentially utilized NADPH as a cofactor. The authors speculated that the different cofactor specificity between *P. pastoris* and *C. guilliermondii* XRs might be due to the difference in the numbers of histidine residues and their locations between the two proteins. The

recombinant was able to ferment xylose, and the maximum xylitol accumulation (7.8 g per l) was observed when the organism was grown under aerobic conditions.

### **Cellulose/hemicellulose depolymerization**

It would be desirable if microbes producing ethanol from lignocellulose also had means to depolymerize cellulose, hemicellulose, and associated carbohydrates. Many plant pathogenic bacteria (soft-rot bacteria), such as *Erwinia carotovora* and *Erwinia chrysanthemi*, have evolved sophisticated systems of hydrolases and lyases that aid the solubilization of lignocellulose and allow them to macerate and penetrate plant tissue [61]. Genetic engineering of these bacteria for ethanol production represents an attractive alternative to the solubilization of lignocellulosic biomass by chemical or enzymatic means. *E. carotovora* SR38 and *E. chrysanthemi* EC16 were genetically engineered with the PET operon and shown to produce ethanol and CO<sub>2</sub> efficiently as primary fermentation products from cellobiose and glucose [19]. Several genes encoding cellulolytic and hemicellulolytic enzymes have been expressed in *S. cerevisiae* in active secreted form, demonstrating that construction of hydrolytic yeast strains is conceivable [62,63]. Both ethanologenic *Erwinia* strains produced about 50 g per l ethanol from 100 g per l cellobiose in less than 48 hours with a maximum volumetric productivity of 1.5 g per l of ethanol per hour. This rate is over twice that reported for the cellobiose-utilizing yeast *Brettanomyces custersii* in batch culture [64].

Along similar lines, the incorporation of saccharifying traits into ethanol-producing microorganisms was also attempted. The gene encoding for the xylanase enzyme (*xynZ*) from the thermophilic bacterium *C. thermocellum* was expressed at high cytoplasmic levels in ethanologenic strains of *E. coli* KO11 and *K. oxytoca* M5A1(pLOI555) [65]. This is a temperature-stable enzyme that de-polymerizes xylan to its primary monomer xylose (99%). In order to increase the amount of xylanase in the medium and facilitate xylan hydrolysis, a two-stage, cyclical process was employed for the fermentation of polymeric feedstocks to ethanol by a single, genetically engineered microorganism. Cells containing xylanase were harvested and added to a xylan solution at 60°C, thereby lysing and releasing xylanase for saccharification. After cooling to 30°C, the hydrolysate was fermented to ethanol, in the meantime replenishing the supply of xylanase for the subsequent saccharification. *K. oxytoca* was found to be a superior strain for such an application, because, in addition to xylose (metabolizable by *E. coli*), it can also consume xylobiose and xylotriose. Even though the maximum theoretical yield of *K. oxytoca* M5A1(pLOI555) is in excess of 48 g per l ethanol from 100 g per l xylose, about one-third of that was achieved in this process because xylotetrose and longer oligomers remained un-metabolized by this strain. The yield appeared to be limited by the digestibility of commercial xylan rather than by the lack of sufficient xylanase activity or by ethanol toxicity.

## Conclusions

Although several obstacles still remain to be addressed, the production of fuels and chemicals from biomass is advancing with a rapid pace. Among other activities, significant efforts are being made by groups around the world to apply the tools of metabolic engineering to the generation of biocatalysts that can more efficiently and economically convert the various sugars present in the hydrolysate mixture to useful products. Most recent activities have focused on the bacteria *E. coli* and *Z. mobilis*, and the yeast *S. cerevisiae*. With the rapid advances of genetic tools and genome sequencing we are bound to see an expansion in the list of organisms that are genetically engineered for such purposes, with perhaps more emphasis on less conventional species, such as extremophiles (e.g. thermophilic bacteria) filamentous fungi or even photosynthetic organisms.

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## Patents

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