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Ethanolic Fermentation of Lignocellulose Hydrolysates

A Minireview

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Lignocellulosic raw materials, such as forest products and agricultural residues, constitute a major renewable energy source for the fermentative production of liquid fuels, such as ethanol. Using transportation fuels from renewable resources should not contribute to global warming, generally referred to as the "greenhouse effect," and would thus assure sustainable development of the transportation sector. This argument is based on the assumption that the production and harvest rates of lignocellulosic raw materials used for the production of transportation fuels are balanced by the assimilation rate of combustion carbon dioxide in the photosynthesis of green plants.

Lignocellulosic raw materials are composed of hemicellulose, cellulose, and lignin. The hemicellulosic and cellulosic fractions are made up of the monosaccharides glucose, mannose, galactose, xylose, and arabinose, the composition being dependent on the nature of the raw material. In soft woods and in agricultural residues, the pentose sugars xylose and arabinose account for 5–25 and 0.5–3%, respectively, of the raw material *(1)*. Prior to fermentation, lignocellulosic materials are pretreated and/or hydrolyzed with acids and/or enzymes *(2)* to make the sugars accessible to fermenting microorganisms. The sugars can be fermented to ethanol with bacteria, yeast, and filamentous fungi (for a recent review, *see* ref. *3*). During pretreatment and hydrolysis, a number of microbial inhibitors are released and produced. The nature and composition of these compounds are dependent on the pretreatment and hydrolysis conditions, and will have a profound influence on the efficiency of fermentation process. Furthermore, in environmentally sustainable processes, the utilization of fresh water has to be minimized by circulation of process streams, which leads to an accumulation of the inhibitory compounds *(4)*.

Compounds inhibitory to fermentation are both inherent in the lignocellulosic materials and produced during pretreatment, hydrolysis, and fermentation. The degree of inhibition varies with raw material and pretreatment, and the presence of two or more compounds is usually more inhibitory than one single compound alone *(5)*. On pretreatment and/or hydrolysis, acetic acid—commonly used as an antimicrobial food preservative—is released from acetyl groups in the hemicellulose fraction *(6,7)*. Sugar degradation products, such as furfurals *(8)*, and lignin degradation products, such as phenolics *(9–11)*, are formed owing to the chemical reactions in pretreatment and/or hydrolysis. In addition, the product ethanol and the fermentation byproducts acetic acid, acetaldehyde, formic acid, and lactic acid

are well-known microbial inhibitors *(12,13)*. Finally, heavy metals released from the equipment have an inhibitory effect *(14)*.

Lignocellulose hydrolysates have been detoxified to enhance their fermentability. Several methods have been suggested: calcium hydroxide in combination with sulfite *(15)*, ion exchange *(16)*, molecular sieves *(10)*, and steam stripping *(17)*. Using calcium hydroxide in combination with sulfite, also called overliming, contributes as much as 22% to the production costs of ethanol produced from a pentose-rich, steam-pretreated willow hydrolysate using the recombinant bacterium *Escherichia coli* KO11 *(18,19)*. The fact that detoxification potentially allows all sugars in a lignocellulose hydrolysate to be fermented to ethanol may justify its relatively high cost. When the liquid stream from steam-pretreated willow was fractionated by evaporation, the fermentation inhibitors were localized to the nonvolatile fraction *(20)*. The nonvolatile fraction also inhibited the enzymatic hydrolysis of the steam-pretreated raw material, however, to a lesser extent. Therefore, in addition to the detoxification methods mentioned, the inhibitors could also be removed by evaporation of the recirculating liquid streams. The volatile fraction would be recirculated, and the nonvolatile fraction containing organic material would be burned in a steam boiler to provide energy for the process. Economic analyses will determine whether or not detoxification should be used and which method to choose.

For optimal process economy, all sugars should be fermented to ethanol, preferentially simultaneously. Microorganisms fermenting all sugars in lignocellulosic hydrolysates to ethanol are either slow or highly susceptible to the inhibitors *(21)*. The efficient fermenting yeast, *Saccharomyces cerevisiae*, ferments only hexoses and the isomeric form of xylose, xylulose. It lacks the two initial enzymatic steps of the xylose metabolism, xylose reductase and xylitol dehydrogenase. The research efforts on the fermentation of all sugars in a lignocellulose hydrolysate have mainly focused on the efficient fermentation of the pentose sugar xylose (for recent reviews, *see* refs. *3,22–25*). A number of different organisms have been considered: filamentous fungi, such as *Mucor sp* 105 *(26)* and *Fusarium oxysporium (27)*; yeasts such as *Pichia stipitis*, *Candida shehatae*, *Pachysolen tannophilus* (for a review, *see* ref. *25*); Bakers's yeast *S. cerevisiae* in combination with xylose isomerase *(28,29)*; thermophilic anaerobic bacteria, such as *Clostridium thermohydrosulphuricum (30)*, *Clostridium thermosaccharolyticum (31)*, and *Thermoanaerobacter ethanolicus (32)* as well as lactate dehydrogenase mutants of *Bacillus stereothermophilus (33)*. Strains of *P. stipitis (34)*, *C. shehatae (17)*, and *S. cerevisiae (35)*, the latter in combination with xylose isomerase, have been adapted and screened for, respectively, in order to enhance their inhibitor tolerance. Present knowledge suggests that the yeasts together with the recombinant bacteria *(see below)* offer the greatest potential for the development of cost-effective fuel ethanol processes. With the filamentous fungi, the productivity is too low, and with the thermophilic anaerobic bacteria, both productivity and yield are too low.

Recombinant DNA technology makes it possible to introduce new pathways and to block unwanted metabolic pathways, so that novel microorganisms can be engineered for efficient ethanol production from all sugars present in lignocellulose hydrolysates. *E. coli* KO11, which can utilize all lignocellulose sugars, has been engineered to contain the ethanologenic genes from *Zymomonas mobilis (36,37)*. In addition, the acid-producing genes have been deleted. *Klebsiella oxytoca* M5A1 has been similarly genetically engineered *(38)*. In laboratory media, the recombinant strains ferment all sugars present in lignocellulosic raw materials to ethanol with

theoretical yields. However, the maximum fermentative performance of *E. coli* KO11 in a lignocellulose hydrolysate is greatly dependent on a cheap and efficient detoxification method *(18,19)*.

Recently, *Z. mobilis* CP4 has been genetically engineered with an entire xylose-utilizing pathway by introducing the *E. coli* genes for xylose isomerase, xylulokinase, transketolase, and transaldolase *(39)*. The fermentative performance of the recombinant strain has been established under selection pressure in laboratory media containing mixtures of glucose and xylose where it ferments both sugars with close to theoretical yields. The main drawback with *Z. mobilis* is that it does not ferment galactose, mannose, and arabinose present in most lignocellulosic raw materials *(1,40)*. The fact that *Z. mobilis* has been under consideration for large-scale fuel ethanol production from sugar feed stocks for over 15 yr without any process being established *(41–43)* throws some doubt on the usefulness of this organism in large-scale industrial applications.

With the aim to combine the xylose-fermenting properties of *P. stipitis* with the inhibitor tolerance of *S. cerevisiae*, the genes for the xylose-utilizing enzymes xylose reductase *(44–46)* and xylitol dehydrogenase *(47–51)* from *P. stipitis* have been cloned and expressed in *S. cerevisiae*. In addition, the genes for the enzyme xylolokinase from *P. tannophilus* and from *S. cerevisiae* have also been cloned and (over)expressed in *S. cerevisiae (52)*. All attempts to express a functional bacterial xylose isomerase in *S. cerevisiae* have so far failed *(53–55)*.

Transformants of *S. cerevisiae* harboring the gene for the enzyme xylose reductase convert xylose to xylitol with theoretical yields in the presence of cosubstrate for the generation of reduced cofactors and maintenance energy *(44,56–58)*. Xylitol is used in the food industry as an anticariogenic sweetener as well as a sugar substitute for diabetics *(59)*. Theoretically, *S. cerevisiae* transformed with the genes for the enzymes xylose reductase and xylitol dehydrogenase should ferment xylose to ethanol. Attempts in this direction met with limited success, so that only low yields of ethanol have been obtained *(49–51)*. However, when the *S. cerevisiae* gene for the enzyme transaldolase was simultaneously overexpressed, mixtures of xylose and glucose were fermented to ethanol *(60)*. Similarly, ethanol formation from xylose was enhanced when the gene for xylulokinase was overexpressed in transformants of *S. cerevisiae* harboring the genes for the enzymes xylose reductase and xylitol dehydrogenase (Ho, personal communication).

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