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Session 2

Applied Biological Research

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The applied biological research session has traditionally emphasized novel approaches to long-standing problems. This year most of the novelty emerged from metabolic and enzyme engineering. Increasingly, genetically engineered enzymes or organisms with altered traits are being integrated into processes. Fermentation of xylose by genetically engineered bacteria and yeast continues to attract attention. The high ethanol yield *Zymomonas mobilis* characteristically attains on glucose was shown to extend to the fermentation of glucose/xylose sugar mixtures in continuous cultures with strains that had been genetically engineered to express a xylose metabolic pathway. This consisted of xylose isomerase, xylulokinase, transketolase, and transaldolase. These studies indicate that there is no inherent technical barrier to further scale-up of continuous fermentations with this organism. Other research in this field has resulted in the development of an engineered Z. *mobilis* strain that will coferment arabinose. Increasing attention is being given to the use of hydrolysates by Z. *mobilis* and other ethanologens. Also, a number of yeasts have been screened and adapted to the fermentation of softwood prehydrolysates. The nature of the adaptation is unknown, but fermentation performance increases with successive batch transfers.

Genetic engineering of xylose fermentations by yeasts is also progressing. In the case of mixed sugar fermentations by recombinant *Saccharomyces*, two of the principal barriers have been the excess production of xylitol, and the diminished consumption of xylose under anaerobic conditions. Both of these factors could be related to redox imbalances arising from cofactor preferences. One of the key enzymes enabling xylose fermentation by *Saccharomyces* is the xylose reductase of *Pichia stipitis*. This enzyme is capable of using either NADH or NADPH for the reduction of xylose to xylitol, but NADPH is strongly favored. The next enzyme in the pathway, xylitol dehydrogenase, uses NADH exclusively, so the excess consumption of NADPH can lead to cofactor can block assimilation. One paper described genetic engineering of xylose reductase to use NADH preferentially. In other research, xylose reductases from *Neurospora crassa* and *Candida guillierondii* were characterized.

To date, the bulk of genetic approaches to improved xylose fermentations have been carried out in *Escherichia coli*, Z *mobilis* and *Saccharomyces sp.*, but the development of an effective genetic system for P. *stipitis* is beginning to result in improvements there as well. Even though wild type strains of P. *stipitis*, *Candida shehatne*, and *Pachysolen tannophilus* will ferment xylose to ethanol, their product yields have been limited by an oxygen requirement for growth. The biochemical basis for that requirement was poorly understood, because it cannot be satisfied simply by

supplying essential lipids (as in the case of *Saccharomyces).* However, *Saccharomyces* has adapted to anaerobic growth in other ways. One of the most critical, is its ability to produce uracil under anaerobic conditions. This capacity is conferred by a novel form of the enzyme, dihydroorotate dehydrogenase. In most eukaryotic organisms, activity of this enzyme is necessarily tied to respiratory metabolism. In *Saccharomyces,* it can be coupled to the reduction of fumarate. By transforming P. *stipitis* with the *Saccharomyces* gene for this enzyme, anaerobic growth was conferred on this pentose-fermenting yeast.

Pretreatment and saccharification of lignocellulosic substrates remain significant problems in bioconversion. Steam pretreatment is an inexpensive means to prepare low- lignin content materials, such as willow, for enzymatic dissolution. Steam pretreatment releases hemicellulose as oligomers and opens up the wood pore structure. Enzymatic saccharification is rapid on the "amorphous" cellulose, but the bulk of the substrate is found in crystalline structures. Cellulose binding domains (CBD) are an integral part of most cellulases, but since they do not actively participate in the hydrolytic reaction, one can ask what role they play in saccharification. Removal of the CBD by proteolytic cleavage decreased the hydrolytic capacity of the enzyme on polymeric substrates. Presumably, the CBD keeps the catalytic domain in the vicinity of the substrate as it progresses along the chain. Cellulolytic microbes almost always form cellulases in mixtures of beta-glucosidases, endoglucanases, and exoglucanases. The relative portions of these enzymes vary from one organism to another, and it has been unclear as to whether the native mixtures maximize cellulose hydrolysis. Research reported in this section shows that synergism among various enzyme preparations can be increased, but that aside from deficiencies in beta-glucosidase, the native mixtures are nearly optimal for natural (untreated) substrates.

Lignocellulosic substrates for initial commercial use will most likely come from low-value agricultural byproducts. Corn fiber is one of the most interesting because it is present in large quantities in most ethanol and starch processing plants, and it has few competing uses. However, bioconversion of corn fiber faces several challenges. Most particularly, its hemicellulose is highly substituted and resistant to enzymatic degradation. Likewise, pretreatment must be carried out in an exacting manner, because the bulk of the hemicellulosic sugars, xylose and arabinose, are subject to degradation. Current research reported here has identified optimal conditions for pretreatment and several novel enzyme systems for saccharification.

While the bulk of bioconversion research reported in this symposium has focused on ethanol production, many other products are attracting increasing interest. These include bioplastics, enzymes, extractives, oils, xylitol, and oligosaccharides. Transglycosylation products are often problematic during starch saccharification, because they reduce ethanol yields. Cyclodextrins; however, are among the more valuable and versatile transglycosylation products from starch. They form hydrophobic molecular cages into which various small molecules can be entrapped for later diffusion and slow release. They are formed by cyclodextrin glycosyltransferases (CGTase). Cyclodextrins have a market of several thousand metric tons per year and a value of around $5.00/kg. Cyclodextrin glycosyltransferase is produced by various alkalophilic *Bacillus sp.* and research reported in this session identified a CGTase that can convert starch to cyclodextrins in a 21% yield.

Overall, this symposium session remains one of the most important in the field.