NREL Advances in Research at the National Renewable Energy Laboratory

Research Brief

"Mini-Manhattan Project" for Cellulases

NREL Pursues Enzyme Development Critical to Viability of Biofuels

The National Renewable Energy Laboratory (NREL) is working to make ethanol America's automotive fuel of the future by domestically producing it from lignocellulosic biomass, the most abundant renewable resource on earth. Extensive production of this environmentally compatible fuel is contingent on the ability to convert wood, grasses, agricultural wastes, wastepaper, or other biomass to ethanol at prices competitive with gasoline. A key element of achieving competitive production costs is the ability to economically produce the cellulase enzymes that convert cellulose into sugar so yeasts can ferment the sugar into ethanol.

Cellulose and hemicellulose, the fibrous and woody material that constitutes the bulk of most plants, are carbohydrates. But unlike starches and sugars, they are ones that humans and most animals cannot digest and most yeasts cannot ferment. The common enzymes that convert starch to sugar are ineffective on the bonds that hold together the chains of sugar molecules in cellulose and hemicellulose. In cellulose, there are also hydrogen bond interconnections between the chains of sugars. The resulting crystalline structure is not soluble in water, which makes enzymatic attack difficult.

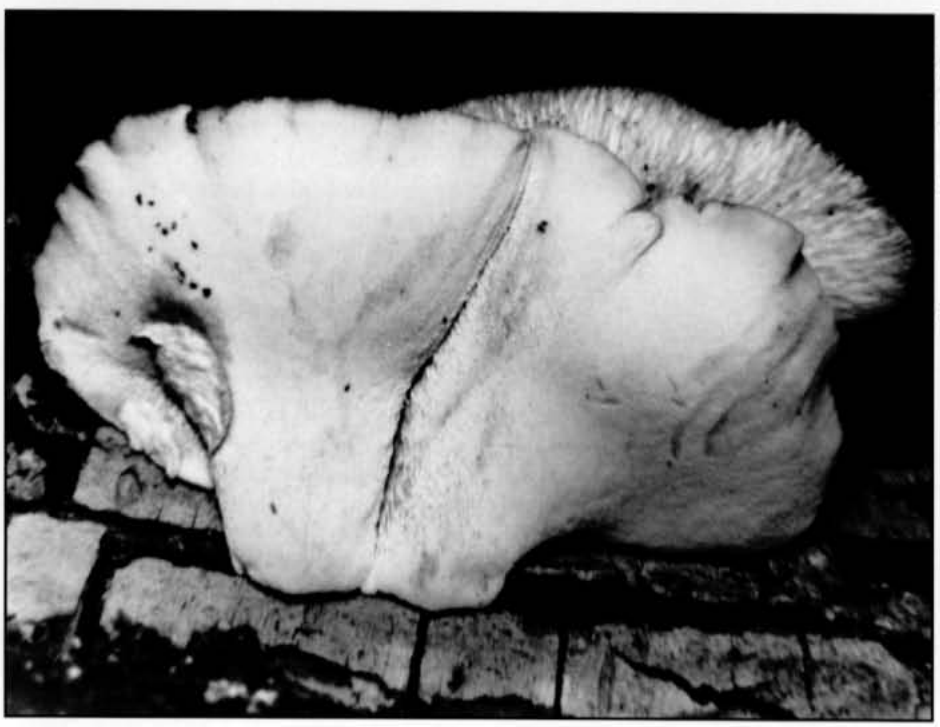


Photo courtesy of Denver Botanic Gardens

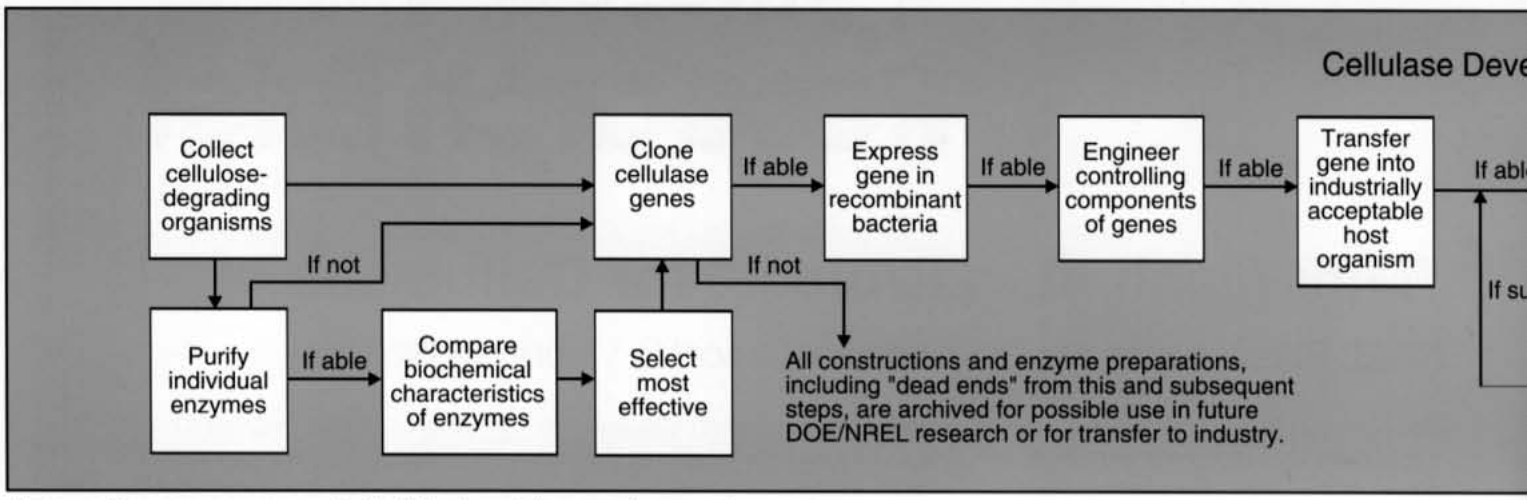
This shelf fungus (*Ganoderma applanatum*) produces cellulase enzymes that enable it to digest cellulose. NREL scientists are using cellulases from microscopic bacteria and fungi to produce ethanol from lignocellulosic biomass.

Carbohydrate chains are broken down to sugars by a chemical reaction called hydrolysis. Dilute acids are commonly used to hydrolyze hemicellulose, but for cellulose hydrolysis, enzyme catalysts called cellulases produce the best results. Cellulase enzymes are needed, however, in large amounts to be effective. This requirement is a major obstacle to cellulose hydrolysis because of the high cost of the enzymes. That high cost is caused by the slow growth and production rates of the fungi generally used to make the cellulases.

Fungi Versus Bacteria

NREL and its subcontractors are working with bacteria and fungi to find the most efficient means of producing highly effective cellulases. Most cellulose-digesting fungi and bacteria produce sets of at least three different kinds of cellulase enzymes to effectively attack the complex structure of cellulose. (They often also produce separate sets of hemicellulases, enzymes that attack hemicellulose.)

The three general components of cellulase enzyme systems are endoglucanases, exoglucanases, and beta-glucosidases. Endoglucanases attack the cellulose crystalline structure at random points, breaking the linear chains of glucose molecules to



Because there are so many potential "dead ends," researchers must process many cellulases (several of which may come from a single organism) and their genes through the pathway shown above.

produce shorter chains. Each break produces two new chain ends. Exoglucanases attach to these exposed ends of the chains and, working down the chains, release cellobiose and some glucose. Cellobiose, which consists of two connected glucose molecules, is then split by beta-glucosidases.

In nature, fungi tend to produce more cellulase enzyme than bacteria; however, cellulases produced by bacteria are often more effective catalysts. They may also be less inhibited by the presence of material that has already been hydrolyzed (feedback inhibition). Researchers have not definitively determined which organisms have greater effectiveness.

Fungi currently produce most commercial (extensively used by the food processing and detergent industries) and laboratory cellulase. But several factors suggest that bacteria may have greater potential. Bacteria grow more rapidly and easily. Also, some bacterial cellulases tolerate very high temperatures, a valuable characteristic because chemical reactions

Several factors suggest that bacteria may have greater potential, particularly the ease with which bacteria can be genetically engineered.

are generally faster and more cost effective at elevated temperatures. High-temperature tolerance often also indicates a greater tolerance of other physical stresses, such as shear forces. Of greatest potential importance, however, is the ease with which bacteria can be genetically engineered. This factor is likely to be invaluable in achieving cellulase production goals.

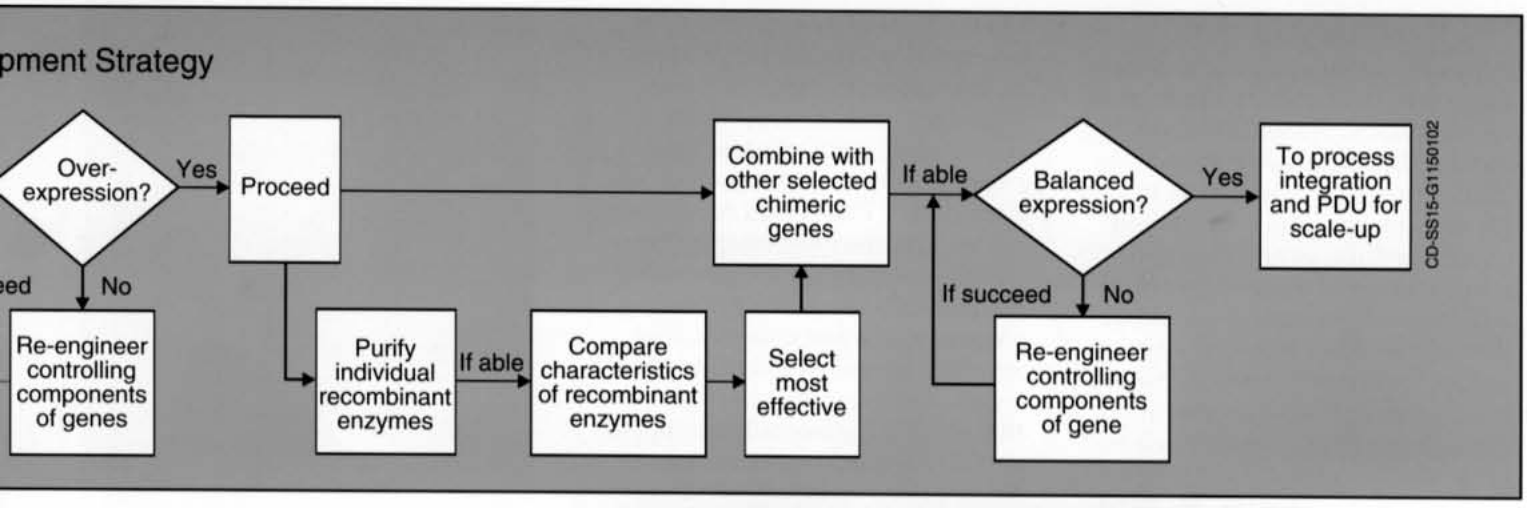
Improving Technology for Use in Pilot Plant

For the near term, NREL is concentrating on the extant fungal production system. That system generally uses the two endoglucanases and the two exoglucanases produced by the filamentous fungus *Trichoderma reesei*, and beta-glucosidase from the fungus *Aspergillus niger*. The immediate objective is to maximize the potential of the current system by late 1994. At that time, NREL plans to begin operating a fermentation process development unit (PDU) or pilot plant now under construction at its Golden, Colorado, site. The goal is for the new PDU to employ

Nature's Woody Biomass Recyclers

Although humans and yeasts cannot digest the cellulose and hemicellulose in trees and grasses, other organisms can. Yet many of the organisms that come to mind cannot do so inherently. Fungi, which derive all their energy from dead or living trees or other plant material, do have the ability to break cellulosic material down to sugars, as do the various soil and aquatic bacteria responsible for the decay of plant material. These two classes of organisms are nature's primary agents for recycling lignocellulosic biomass.

Nearly all other consumers of cellulosic material from beavers to grass-eating cows and horses, to wood or paper-eating termites and cockroaches, rely on the bacteria, fungi, or, in the case of termites, protozoa colonizing their digestive systems. These symbiotic guests break down bonds in the fibrous material and enable their hosts to live on a diet of biomass. Fungi and bacteria produce enzymes that attach to the chains of molecules in cellulose and hemicellulose and hydrolyze or break down this material into digestible or fermentable sugars. NREL researchers seek ways to produce and employ these enzymes on an efficient industrial scale so they can use lignocellulosic material as a feedstock for fermentation into ethanol.

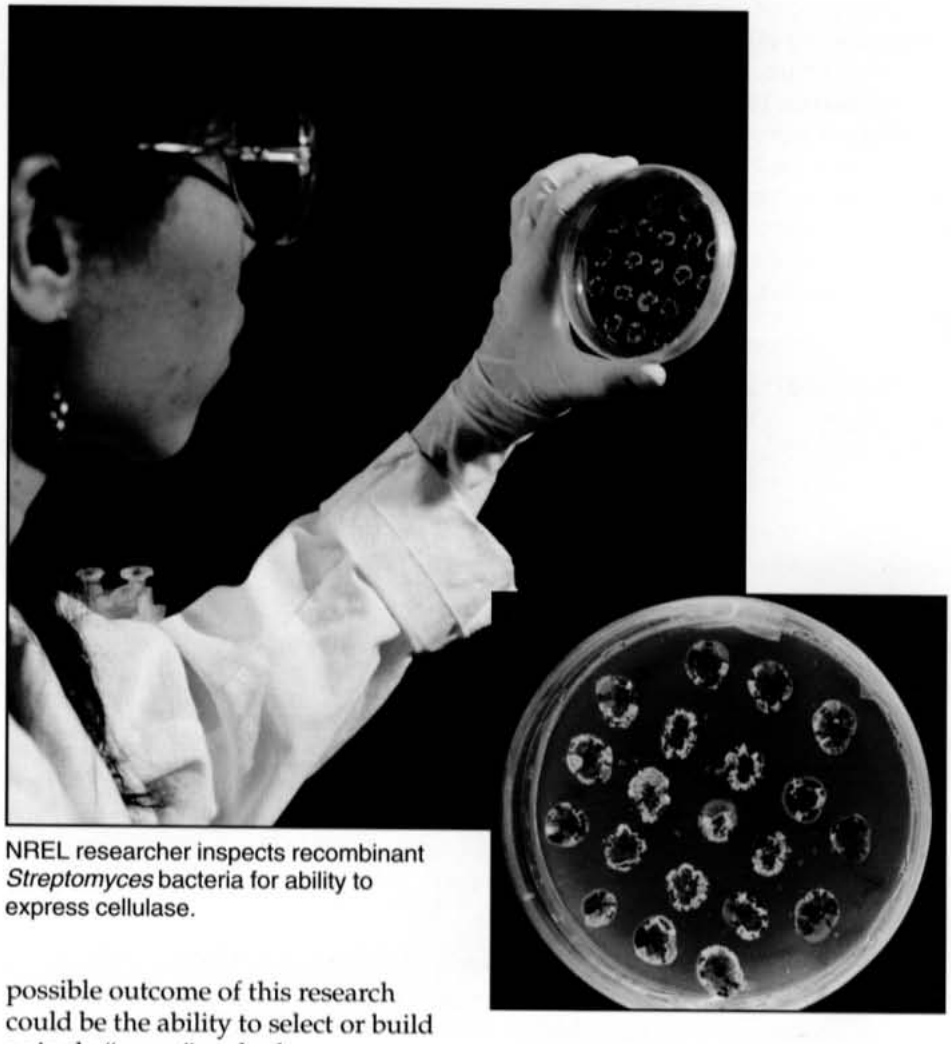


the best cellulase production technology available.

One current project strives to identify the optimum ratio of the four enzymes taken from *T. reesei*. Research has shown that the ratio of endoglucanases and exoglucanases is critical. However, that research has generally been done on refined feedstock materials, and NREL seeks to identify the best enzyme mix for several "real world" biomass feedstocks that the PDU will likely use.

A second project aims to improve assay techniques for identifying and measuring endoglucanase enzymes. This is important for screening and selecting the best enzyme producers. These projects are being conducted both in house and by subcontract.

A third project will focus on selecting alternatives to the *A. niger* fungal strain to more effectively produce beta-glucosidase. Beta-glucosidase plays a critical role because high concentrations of cellobiose (converted to glucose by beta-glucosidase) inhibit the action of the *T. reesei* exoglucanases.



NREL researcher inspects recombinant *Streptomyces* bacteria for ability to express cellulase.

Moving Beyond Current Technology

One advanced technology project that NREL has subcontracted is the investigation of cellulase/substrate surface interactions. The binding of cellulases to cellulose is crucial to the process, but the chemistry of this event is not understood in detail. A possible outcome of this research could be the ability to select or build a single "super" endoglucanase or exoglucanase.

Although genetic engineering is easier with bacteria, it can also be accomplished with some fungi. Cellulase technology could be advanced by transferring modified fungal or bacterial cellulase genes with desirable traits into *T. reesei*. A primary objective for such engineering would be to obtain enzymes that are less inhibited by the presence of cellobiose or glucose than are the enzymes currently used.

Mini-Manhattan Project

Central to all intermediate and long-range cellulase production efforts is one that NREL researchers think of as the "Mini-Manhattan Project." NREL is aggressively seeking to identify and purify various fungal and bacterial cellulases and their genes so the efficacy of these enzymes can be compared "head to head." More than 60 fungi and 50 bacteria are known to produce cellulase, but in the absence of central coordination, research has been unable to determine which organisms and enzymes are best for industrial use. By standardizing procedures, NREL plans to determine the effective activity level and other characteristics of the various cellulases. This multiple-task initiative is being carried out by in-house and subcontract researchers. Seven subcontracts contribute to enzyme development and supply, and one is for development of a standard testing procedure.

On the basis of these results, NREL will develop systems for production of the best-performing enzymes. Ultimately researchers seek to construct a "multicellulase gene expression system in an industrial bacterium." This would require identification of the most effective combination of cellulase enzymes and the genetic material for producing them in the largest quantity. That genetic material would then be transferred into one or two bacterial hosts that are environmentally acceptable and can be quickly and inexpensively grown on an industrial scale.

Critical Cost Factor for Ethanol and Much More

In both the short and long terms, cellulase improvement is one aspect of the ethanol-from-biomass process with great potential for cost reduction. The capability to efficiently produce cellulase—and thereby fermentable sugars and their derivatives from cellulosic polymers—also has tremendous potential beyond ethanol. Whole new industries could evolve from the ability to produce chemicals and materials from renewable biomass instead of fossil fuels.

NREL researcher collects cellulase-producing bacteria from hot springs. One of the heat-tolerant species collected shows great promise for cellulase production.

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