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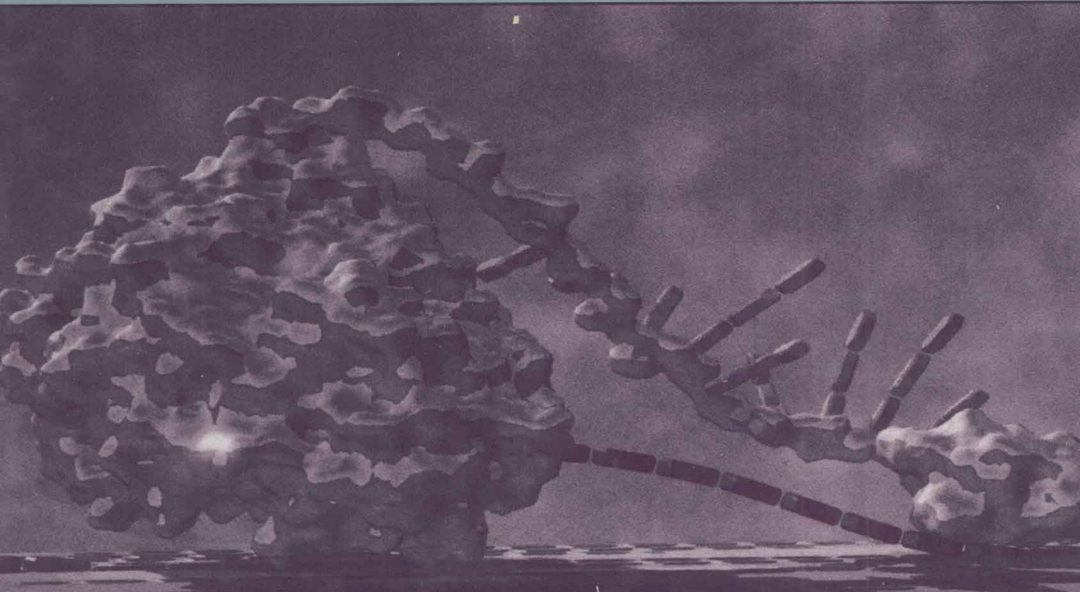
Glycosyl Hydrolases for Biomass Conversion



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Glycosyl Hydrolases for Biomass Conversion

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Glycosyl Hydrolases for Biomass Conversion

About the Cover

Computer-generated rendering of *Trichoderma reesei* cellobiohydrolase I enzyme depicts its action on cellulose. Animation created by Pixel Kitchen in Boulder, Colorado.

Foreword

The ACS Symposium Series was first published in 1974 to provide a mechanism for publishing symposia quickly in book form. The purpose of the series is to publish timely, comprehensive books developed from ACS sponsored symposia based on current scientific research. Occasionally, books are developed from symposia sponsored by other organizations when the topic is of keen interest to the chemistry audience.

Before agreeing to publish a book, the proposed table of contents is reviewed for appropriate and comprehensive coverage and for interest to the audience. Some papers may be excluded in order to better focus the book; others may be added to provide comprehensiveness. When appropriate, overview or introductory chapters are added. Drafts of chapters are peer-reviewed prior to final acceptance or rejection, and manuscripts are prepared in camera-ready format.

As a rule, only original research papers and original review papers are included in the volumes. Verbatim reproductions of previously published papers are not accepted.

ACS BOOKS DEPARTMENT

Preface

The depolymerization of biomass polysaccharides by glycosyl hydrolases has for some time been recognized as *the* critically important step in the conversion of biomass components into fermentable feedstocks for production of fuels and chemicals. For this reason, such enzymes have become the focus of studies ranging from the most fundamental, "purely scientific" investigations of the molecular and catalytic properties of individual enzyme molecules themselves, to strictly applied experiments at the pilot-plant or near-pilot-plant scale. The particular focus of this volume is on studies that have been carried out at what might be termed "large bench-scale" in order to address the ultimate application of the enzymes in industrial processes. Five of the chapters deal directly with the use of mixtures of glycosyl hydrolases in the saccharification of cellulose- and mannan-containing biomass. Topics include adsorption to macromolecular substrates of specific enzymes in a mixture, the effects on enzyme efficiency of producing enzyme mixtures in the presence of the target biomass, factors influencing enzymatic hydrolysis of lignocellulosics, and a study of enzymes acting on soluble and insoluble mannans. Two additional chapters describe explorations of solid-substrate fermentations and expression in transgenic plants as means of producing glycosyl hydrolases at an industrial scale.

In addition to the applications-oriented studies described above, the volume also includes accounts from the more fundamental and exploratory regions of the glycosyl hydrolase research spectrum. Direct structure-function studies address the roles of four conserved aspartate residues in *Thermomonospora fusca* endoglucanase E2, and the roles of four apparently distinct functional domains in a single molecule of a bifunctional (endoglucanase/exoglucanase) cellulase from *Teredinobacter turnerae*. Other chapters describe newly discovered enzymes (thermostable cellulases and xylanases from *Thermoascus aurantiacus* and alkalophilic dextranases from *Streptomyces anulatus*) that may extend the limits of useable reaction conditions. In another chapter, molecular mechanics calculations have been applied to the effect on substrate binding of a point mutation in E1 from *Acidothermus cellulolyticus* and to the generalized interaction between a glucose molecule and an alanine side-chain. Both the applied and fundamental studies are put in perspective relative to the entire bioconversion process by the opening chapter's comprehensive strategic analysis of the U.S. Department of Energy's National Ethanol Program.

This book includes several chapters describing "rational design" approaches to enhancing enzyme performance, and it is apparent that the application of random mutation strategies to glycosyl hydrolases will also be the subject of future studies. Site-directed-mutagenesis (SDM) is an informational approach to protein engineering and relies on high-resolution crystallographic structures of target proteins and some stratagem for specific amino acid changes. Resurgence in SDM technology has followed the recent advent of computational methods for identifying these site-specific changes for a variety of protein engineering objectives. Non-informational mutagenesis techniques (referred to generically as "directed evolution"), in conjunction with high-throughput screening, allows testing of statistically meaningful variations in protein conformation. Directed evolution technology has undergone significant refinement from initial error-prone PCR methodology and now includes Gene Shuffling, site-saturation mutagenesis, staggered extension process (StEP) technology, and others. In our opinion, the primary challenge in the application of directed evolution technology to cellulase improvement lies almost entirely in (1) adaptation of robotic screening methods to accurately select transformed host cells (clones) producing enzymes displaying enhanced performance on microcrystalline cellulose, and (2) the development of hosts able to express active and industrially relevant recombinant enzymes from shuffled or otherwise modified eukaryotic genes. One wonders where promising new directed evolution technologies can and will take us. Efforts have been made to predict the probability of successful engineering by estimating the proportion of total protein sequence space already visited, and perhaps rejected, in nature. Certainly, many protein forms not selected, or even encountered, in natural evolutionary processes are of interest to humankind. However, where do actual structural boundaries exist—those imposed by laws of thermodynamics, for example—and can we learn to recognize situations lying outside of these natural laws a priori? In the case of cellulase engineering, perhaps the low specific activities of known cellulases reflect more the inherent difficulty of the protein/substrate boundary, than problems of pure protein design. Improving cellulase efficiency, then, may only come with directed *co*-evolution of (or modification to) *both* enzyme and substrate (which may then not be cellulose as we know it). This is an experiment nature has probably had no incentive to conduct, and may thus represent a completely unexplored block of protein sequence space!

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INTRODUCTION

Chapter 1

The Road to Bioethanol: A Strategic Perspective of the U.S. Department of Energy's National Ethanol Program

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As the Bioethanol Program at the Department of Energy (DOE) nears the end of two decades of research, it is time to take a hard look at where we have been and where we are going. This paper summarizes the status of bioethanol technology today and what we see as the future directions for research and development. All of this is placed in the perspective of strategic national issues that represent the drivers for our program—the environment, the economy, energy security and sustainability. The key technology pathways include the use of new tools for protein engineering and directed evolution of enzymes and organisms, as well as new approaches to physical/chemical pretreatment of biomass.

Ethanol is used today as an alternative fuel, a fuel extender, an oxygenate and an octane enhancer. From just over 10 million gallons of production in 1979, the U.S. fuel ethanol industry has grown to more than 1.8 billion gallons of annual production capacity (*1*). Almost all of this capacity is based on technology that converts the starch contained in corn to sugars, which are then fermented to ethanol.

From its first days, this industry has been looking for ways to expand the available resource base to include many other forms of biomass. The U.S. Department of Energy has, throughout this period, invested in research and development on technology that will allow the fuel ethanol industry to achieve its goal of expanded production using a diversified supply of biomass feedstocks.

We refer to ethanol made from these as-yet untapped biomass resources as "bioethanol." This paper provides a strategic perspective on this new bioethanol technology.

Strategic Issues

There are several major strategic issues that motivate and influence DOE's research program for bioethanol. These include:

- national security,
- the environment, and
- the marketplace.

Though each of these issues has shifted in importance over the years, all three remain consistent drivers for our plans. Let me touch on each of these issues briefly.

National Security.

Oil Supply. A recent *Science* article summarized the strategic situation with regard to oil supply this way:

> "Nature took half a billion years to create the world's oil, but observers agree that humankind will consume it all in a 2-century binge of profligate energy use."(*2*)

Our dependence has been growing at an alarming rate since the early 1980s, ironically a time when public concern about petroleum has been very low. DOE's Energy Information Administration paints a dismal picture of our growing dependence on foreign oil (*3*). Consider these basic points:

1. Petroleum demand is increasing, especially due to new demand from Asian markets
2. New oil will come primarily from the Persian Gulf
3. As long as prices for petroleum remain low, we can expect our imports to exceed 60% ten years from now
4. U.S. domestic supplies will likewise remain low as long as prices for petroleum remain low

Not everyone shares this view of the future, or sees it as a reason for concern. The American Petroleum Institute does not see foreign imports as a matter of national security (*4*). Others have argued that the prediction of increasing Mideast oil dependence worldwide is wrong (*5*). Nevertheless. the International Energy Agency (IEA) recently announced that it sees annual petroleum supplies reaching a peak some time between 2010 and 2020. The IEA is one more voice in a growing chorus of concern about the imminent danger of shrinking oil supplies (*2*). While some disagree with this pessimistic prediction, concern about our foreign oil addiction is widely held by a broad range of political and commercial perspectives (*6*).

While there may be uncertainty and even contention over when and if there is a national security issue, there is one more piece to the puzzle that influences our perspective on this issue. Put quite simply, 98% of the energy consumed in the U.S. transportation sector comes from petroleum (mostly in the form of gasoline and diesel fuel). The implication of this indisputable observation is that even minor hiccups in the supply of oil could have crippling effects on our nation. This lends special significance to the Bioethanol Program as a means of diversifying the fuel base in our transportation sector.

Energy Diversity. An important corollary to the notion of increasing energy security is the concept of energy diversity. Today, in the U.S., natural gas, propane, and biodiesel are establishing a place in the transportation fuel market. Bioethanol is yet another option in the fuel mix that we seek to provide. J.S. Jennings, the Chairman of Royal Dutch Shell, a company recognized as a leading strategic thinker in the energy industry, has stated that "...the only prudent energy policy is one of diversity and flexibility" (*7*).

Economic Security. Our view of national security today must include questions about the health and robustness of our economy. Energy today plays an essential role in our economy. Petroleum imports represent 20% of our growing trade deficit. This cannot help but have an impact on our economy. A diverse portfolio of fuels, including bioethanol, would bring money and jobs back into the U.S. economy built on this new renewable energy technology. The associated development of energy crops will likewise provide a needed boost to our agricultural sector, a mainstay of the U.S. economy.

The Environment.

Air Pollution. A life cycle study conducted by DOE in 1993 evaluated the overall impact of bioethanol on several key regulated pollutants targeted by the Clean Air Act Amendments of 1990 (1990 CAAA) (*8*). This study found that, compared with reformulated gasoline (RFG), a 95% ethanol/5% gasoline blend (E95) reduced sulfur oxide emissions by 60 to 80%. Volatile organic emissions from E95-fueled vehicles are 13 to 15% lower. Net (life cycle) emissions of NOx and carbon monoxide are essentially the same.

These results are encouraging, but of greater importance is the impact that bioethanol has directly on tailpipe emissions (as opposed to net pollutant levels across the life cycle of the fuel). Low blends of ethanol have some peculiar emission problems that go away at higher blend rates (mostly due to Reid vapor pressure increases that occur between 10% and 20% volume blends). A survey of the available emissions data for high blends of ethanol reveals that, while there is a fair amount of data, it is often not consistently obtained. Still, the survey found the following broad trends for ethanol used in high blend levels with gasoline: (*9*)

- CO levels may decrease as much as 20%, probably because of the oxygen content of ethanol
- Similar decreases in NOx can be anticipated as well.
- High blends of ethanol cut end-use emissions of volatile organic carbon (VOCs) by 30%.
- Aldehyde emissions from ethanol combustion in spark-ignited engines are, however, substantially higher for ethanol.

The first round of comprehensive emissions tests for flexible fueled ethanol vehicles used in federal fleets was completed in 1996. These tests included a comparison of 21 ethanol-fueled Chevrolet Luminas with an equal number of standard gasoline model Luminas (*10*). The results of the extensive study of exhaust emissions confirm the trends seen across the literature (see Figure 1).

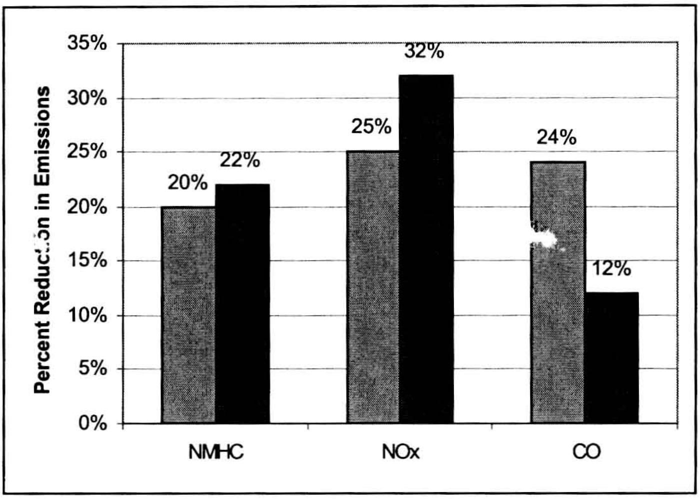

Figure 1: *Emission Reductions for E85-fueled Federal Fleet Vehicles. The two sets of data represent analytical results from two independent laboratories. (NMHC = non-methane hydrocarbons)*

Sustainable Development. Public concern about the quality of our environment has grown steadily over the past decade (*11*). Vice President Al Gore posits an environmental crisis that has been brought on by an exploding world population, a technology revolution that has led to over-exploitation of our natural resources and an apparent disregard for the future. He cites the 1992 "Earth Summit" in Rio de Janeiro as a major turning point in our thinking about the environment.

World-renowned naturalist Edward O. Wilson echoes these sentiments in his call for technology development that moves us away from fossil fuels and reduces the energy intensity of our economy. Wilson describes very eloquently his notion of an ethic of sustainability:

> "The common aim must be to expand resources and improve quality of life for as many people as heedless population growth forces upon Earth, and do it with minimal prosthetic dependence. That, in essence, is the ethic of sustainable development." (*12*)

Bioethanol technology represents just one approach to moving our economy to a more sustainable basis. We, like many others touting technological solutions, should heed his remonstration of over-dependence on what he calls "environmental prostheses" that will extend the capacity of our planet, but will not eliminate the risk of environmental catastrophe. Environmentalists and technologists must work together to provide balance and reason in our approach.

The biggest impediment to sustainable development is our economic system, which places no value on the environment or on the future. "The hard truth," writes Al Gore," is that our economic system is partially blind" (*13*). The blindness of the marketplace to environmental issues makes deployment of bioethanol technology problematic, but not impossible. It forces a discipline on our development efforts in which we seek out opportunities for bioethanol that meet multiple needs. Still, it is clear that something must change in our economic calculus if renewable and sustainable technologies are to take hold before a crisis forces the issue.

Climate Change. Climate change is a particular example of the kind of risks that are involved in ignoring the "ethic" of sustainable development. Political and public concern about climate change varies with the time of day and day of the week. A year with El Niño certainly promotes the cause. One reason for the seemingly arbitrary nature of our views on climate change is that it involves a discussion of relative risks, rather than explicit cause-and-effect problems. The reason for this is simple: understanding the climatic implications of global warming is *not* simple. Some have even suggested that we can never understand the complex interaction of variables involved in understanding our climate (*14*). The salvos continue to go back and forth among the scientific experts as to the degree of warming that has occurred and its impact (*15*, *16*). For example, many critics of climate change claim that satellite data on global temperature contradict claims of increased temperature over the past decade. Researchers have recently demonstrated that decreasing temperature trends seen in satellite data are actually due to errors caused by not accounting for changing altitude of the satellite. When corrected for this change, the satellite data is consistent with other surface temperature measurements showing an increase in average temperature (*17*).

What the policymakers and the public need to do is to make some rational choices about risk. The research reported in 1957 that confirmed CO_2 accumulation in the atmosphere couched the question of climate change in exactly these terms (*18*), and there is still no better way to look at the problem. Given the catastrophic nature of the implications related to climate change, how much risk is too much? The potential risk associated with climate change has gotten the attention of the insurance industry, a group all too familiar with the damage and expense that could be involved (*19*). E.O. Wilson's take on the kind of risk associated with our environment is along similar lines:

> "In ecology, as in medicine, a false positive diagnosis is an inconvenience, but a false negative diagnosis can be catastrophic. That is why ecologists and doctors don't like to gamble at all, and if they must, it is always on the side of caution. It is a mistake to dismiss a worried ecologist or a worried doctor as an alarmist." (*12*)

In other words, can we afford a false negative diagnosis regarding climate change? Technologies like bioethanol are insurance. Prudence dictates that we take some forward movement in encouraging the use of such sustainable technologies.

The current political setting for discussing climate change frames the question as an all or nothing proposition. Either climate change is a real problem or it is not. If it is real, then we should treat it as a "crisis"; otherwise, we are wasting our time. The Kyoto agreement signed by representatives of countries from around the world is doomed to fail if we continue to view the issue in this ill-conceived framework. A group of prominent energy and environmental leaders recently met at the highly respected Aspen Institute to address the issue of climate change. In a letter to the White House, they urged the Clinton administration not to send the Kyoto agreement to Congress, where it will too readily be dismissed (*20*). Instead, they suggest that the U.S. take a leadership role in establishing a *long-term* strategy for dealing with climate change. "Climate change," they wrote, "is a long term problem, and the focus should be on achieving sustainable levels of greenhouse gas concentrations at the least cost, not only on near-term emission reductions." This approach recognizes climate change as a question of risk rather than a black and white problem that must be dealt with using Draconian measures. In the end, renewable energy options like bioethanol benefit from this type of longer-term strategy. Reasonable and sustained support is what is needed if bioethanol is to play a part in our energy future.

The Market. The bottom line for bioethanol is what, if any, market opportunities exist for this fuel. It can be used as a fuel additive or extender in blends of around 10%, or it can be used as a fuel substitute. In today's U.S. fuel market, ethanol can be used in flexible fuel vehicles that can operate using blends of 85% ethanol (and 15% gasoline).

Alternative Fuels Market. For a long time, the greatest impediment to ethanol's use as an alternative fuel was the lack of ethanol-compatible vehicles in the U.S. This has changed dramatically. Today, both Ford and Chrysler offer standard models designed to run on either 85% ethanol (E85) or gasoline. They are offering this fuel flexibility at no additional cost to the consumer (*21*, *22*). While the availability of vehicles is no longer an issue, there is still a paucity of fuel stations and fuel distribution infrastructure for E85. Today, 45 publicly available E85 stations are available in the U.S. Thirty more limited access stations are available (*23*). The lack of basic infrastructure and the higher price of ethanol versus gasoline are major constraints on this market.

Fuel Additive Market. Use of ethanol as an additive in gasoline has become a major market. Starting from literally nothing a little over 20 years ago, ethanol as a fuel additive has become a billion gallon per year market. It has value as an oxygenate in "CO nonattainment" markets, and as a fuel extender and octane booster. The value of ethanol in the oxygenate and octane booster market is around 70 to 80 cents per gallon.