0273-2289/95/5152—0169$05.80

Cellulase Lessons Revealed Through the Microbe's Perspective†

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ABSTRACT

Cellulose, the world's most abundant organic material, is central to the world's ecosystem. This energy resource is mobilized and recycled by microbial action. The inspired microbe has developed both considerable insight and efficient strategies for optimizing the use of this energy resource. Thus, cellulase is produced under strict regulatory control, is located optimally with respect to the microbe's habitat, and furthermore, the enzyme components act cooperatively in a synergistic manner. This microbial genius has been integrated into the Fuels from Biomass Programs and is illustrated with examples of cellulase production from *Microbispora bispora, Thermotoga neapolitana,* and *Trichoderma reesei*.

Index Entries: Cellulase; cellulose; *Microbispora; Thermotoga; Trichoderma*.

INTRODUCTION

The basic premise of the Microbiological Program of Fuels from Biomass, which Chuck Scott has espoused and developed for many years, is the possibility of using microbes to effect transformation of cellulose, hemicelluloses, and lignins to yield chemicals, *in toto* this being an energy-saving process. The products should have industrial application with focus on an alternative liquid transportation fuel—ethanol—in addition to consideration of energy saving through production of chemical

†This article is dedicated to Charles D. Scott on the occasion of his retirement.
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Table 1
Economy of Operation: The Cell vs the Industrial Microbiologist

	The cell	Industrial microbiologist
Substrate utilization	+++	+++
Enzyme overproduction	No	Yes
Enzyme synthesis		
Regulation by induction	Yes	No
Enzyme release		
(insoluble substrate)	Perhaps	Yes
Enzyme regulation		
End-product inhibition	Yes	No
High specific enzyme		
activity	Perhaps	Yes

feedstocks, such as acetone or acetic acid. This article considers the production of fuels and chemicals from cellulose, but from the microbe's perspective. Lacking direct communication with the microbes, this approach is conceptually teleologic. However, the microbes do "speak," and it is germane to be aware of their messages and heed them. How does industrial fermentative production compare with natural systems? This question is considered with illustrative examples of the potential of microbes, of attack of the insoluble substrate cellulose, and of optimizing the microbe's physiology to the advantage of the fermentation engineer.

As a starting point, it is well to remember Bernard Davis' comment (*1*): "I am sure that Nature takes account of slight differential growth rates even more minutely than a banker compares the interest rates on bonds. No device that improves the economy of operation of a cell will be neglected, including of course, not only the speed of reproduction in a given environment but also adaptability to fluctuating environments."

RESULTS AND DISCUSSION

The economy of operation of a cell often embodies efficient conversion of substrates and resultant high levels of product, such as ethanol, which implies strict regulation combined with no excess production of cell products. This is the antithesis of the industrial microbiologist's approach.

It is clear from Table 1 that the implied "objective" of the efficient cell and the industrialist are divergent. The only agreement appears to be with substrate utilization, where in addition to efficient conversion, homofermentative anaerobes are favored since they produce a single product in high yield.

With ethanologens, this limits the industrial microbes principally to two organisms, *Saccharomyces cerevisiae* and *Zymomonas mobilis*. However,

Table 2
Common Myths Associated with Cellulase Research

Myth 1	There are relatively few cellulolytic microorganisms.
Myth 2	Microorganisms produce cellulase mixtures that are deficient in a particular enzyme component.
Myth 3	Enzymes degrading insoluble substrates must be released to the environment.
Myth 4	Humans know more than Mother Nature.
Myth 5	The culture giving the greatest yield of cellulase is the best.

since both microbes have restricted substrate ranges and neither use starch, cellulose, or hemicellulose, the Fuels from Biomass industrialist immediately faces a challenge. The alcohol industry has solved this limitation by first enzymatically hydrolyzing starch substrates to glucose as a "pretreatment" step, with the released sugar being subsequently fermented to alcohol. Analogous approaches are being developed with cellulose as a substrate (2), through enzymatic prehydrolysis of cellulose or, alternatively, cloning studies in which either the cellulase genes are incorporated into an ethanologen or the genes of a fermentative pathway (the PET operon) are transferred into a cellulolytic microbe (2). However, in each instance, the parameters that regulate the efficiency of the cell (Table 1) are quite distinct. These are given focus through consideration of certain myths (Table 2).

Myth 1. There Are Relatively Few Cellulolytic Microorganisms

With cellulose being the world's most abundant organic material, there should be myriads of diverse cellulolytic microbes, and yet one can routinely find statements that relatively few cellulose degraders occur. This myth was probably created through industrial screening for cellulolytic species with resultant rejection of positive isolates that did not overproduce soluble enzyme.

A good number of cellulolytic microorganisms have been described, but we presume that in reality there is a dearth of information. This can be illustrated at several levels. Torsvik et al. (3) have estimated, through comparative DNA hybridization techniques, that 1 g of Norwegian deciduous forest soil contains between 4 and 5000 microbial species. Equivalent numbers were found for other ecologic sites. Yet the compendium of bacterial species, *Bergey's Manual of Systematic Bacteriology, 1989*, records only a total for all bacterial species of about 4000, i.e., the full world listing. Furthermore, symbiotic associations between cellulolytic microbes and higher organisms have received meager study. The general ruminant-microbe and ant-fungal garden symbioses are well known (*4,5*), but there must be diverse analogous associations. Obligate associations, such as the shipworm-bacterium association (*6*) and perhaps

plant–endomycorrhizal associations (for the fungus does penetrate the plant cell wall), are essentially untouched fields of study. With use of DNA probes based on cellulase gene sequences, a major re-evaluation of the occurrence of cellulolytic microbes is predicted that will also result in a major increase in both their numbers and variety. With such diversity, there are probably no major theoretical restrictions with regard to efficiency of conversion in relation to the substrate utilization component (Table 1).

Myth 2. Microorganisms Produce Cellulase Mixtures That Are Deficient in a Particualr Enzyme Component

One classic statement is that the cellulase of *Trichoderma reesei* is deficient in β-glucosidase, but in teleologic terms, nobody bothered to tell the fungus that this was a fact. Indeed, much of this enzyme is propitiously associated with the cell membrane, thus ensuring associated cleavage of cellobiose followed by immediate uptake of glucose with no loss of the monomer to surrounding "freeloading" competitors. In contrast, for most commercial cellulase production, a soluble product is preferred, and if the normal mycelial mass is discarded, the β-glucosidase would be lost. There are a variety of solutions to this dilemma if a balanced cellulase is to be produced commercially. The most direct approach is the selection of mutant strains that both secrete and overproduce the β-glucosidases as illustrated with *T. reesei* (*7*). Current commercial cellulases are not lacking in β-glucosidase.

With a range of efficient screening protocols at hand for both total and individual cellulase components (*8*), selection of hypercellulolytic mutants should not prove to be a major challenge. Thus, enzyme overproduction (Item 2, Table 1), especially in light of regulatory aspects of protein synthesis, should not be problematic.

Myth 3. Enzymes Degrading Insoluble Substrates Must Be Released to the Environment

This statement is made to re-emphasize that early studies of cellulase failed to address cell-bound cellulases, perhaps, as noted above, in part because of the commercial requirement for soluble enzyme. Additionally there is some confusion regarding the status of the role of constitutive cellulase components in the induction process, and whether they are released or not.

It is now quite clear that certain microbes synthesize massive cellulase complexes comprised of up to 26 proteins, the cellulosome (*9,10*), that are immobilized on the outer cell wall, and through which the cells are attached to the cellulose substrate. Such cellulase complexes are extremely effective, and fermentor cultures of *Clostridium thermocellum* can completely utilize cellulose substrates (1% suspension) overnight. In spite of the cellulosome's multiple nature, molecular biological techniques allow labor-

atory dissection into the subcomponents, and this approach has indicated that in addition to the standard cellulase components, cellobiohydrolase, endoglucanase, and β-glucosidase, there are novel binding proteins (for review, *see 11*). In teleologic interpretation, since protein synthesis is a major metabolic drain on the cell, the surface attachment of the cellulase complex allows efficient use, especially in aquatic niches in which the cellulase would be lost if released from the cell. In contrast, a terrestrial microbe burrowing through wood does not have this restraint, and it can be advantageous to release enzymes from the hyphal tip to facilitate progress through the wood. This teleologic pronouncement is supported in that most cellulolytic hyphal microbes (fungi and actinomycetes) release their cellulases. Interestingly, the exact site of secretion of extracellular fungal enzymes, amylases, cellulases, chitinases, and so on, is quite ill-defined. In contrast to teleologic analysis, anaerobic aquatic chytrids that occur in the rumen can potentially use either approach, since they have rhizoids that penetrate the woody fibers, combined with the requirement of not forfeiting cellulase simply by secreting enzyme into the rumen milieu (*12*).

Understanding the induction of cellulases is problematic. It has been assumed that low levels of constitutive cellulase components in the presence of substrate release cellobiose or oligosaccharides that act as the true inducers of cellulase (*13,14*). In *T. reesei*, our studies using antibodies to block the action of constitutive cellulase components showed that cellobiohydrolase I mRNA synthesis became blocked even in the presence of a cellulose substrate (*13*). In contrast, the feeding of a soluble "inducer," sophorose, resulted in specific mRNA synthesis, since this inducer was not dependent on the presence or activity of the constitutive cellulase components (*13*). However, for *T. reesei*, Kubicek and coworkers (*14*) were only able to demonstrate the presence of constitutive forms of cellobiohydrolase I and cellobiohydrolase II, and that these two enzymes were found principally with the spores and not the mycelium. Kubicek et al. (*14*) reason the conidial CBHI and CBHII are principally involved in the initial attack of cellulose by *T. reesei*. These studies are particularly well reviewed at the molecular level by Kubicek and coworkers (*14*).

Myth 4. Humans Know More than Mother Nature

This myth is clearly illustrated by our studies of resistance to end-product inhibition of cellobiases. There is continual reference to the potential use of cellulase to convert cellulose to highly concentrated glucose syrups (20%), which can be fermented to roughly 10% ethanol. Thus, it would be desirable to find a source of cellobiase that is resistant to end-product inhibition by glucose, in addition to being stable in 10% alcohol. This concept is a naturalist's enigma, for glucose repression of "adaptive" enzyme synthesis is long recorded (Karstrom in the 1930s),

Table 3
Screening for End-Product-Resistant Enzymes

β-Glucosidase: Cellobiase
Culture cellulolytic microorganisms on cellobiose medium
Following growth, overlayer the cultures with a thin agar layer containing *p*-nitrophenol-β-D-glucoside[a] plus 20% glucose
After 30 min at appropriate temperature (make alkaline if necessary) select strains with a yellow halo

[a] A variety of colorimetric substrates can be used, e.g., methylumbelliferyl-β-D-glucoside or arbutin plus ferric ion.

and thus, superficially there is no apparent evolutionary pressure to select for disaccharases when glucose is present. Furthermore, mechanistically the glucose product will by definition occupy the active site and thus exacerbate inhibition. Alternative approaches to circumvent end-product inhibition are to combine cellulose saccharification with fermentation, either with cellulase plus an ethanologen (yeast or *Z. mobilis*) or development of a cellulolytic ethanologen. The glucose generated is used immediately, and thus, problems of end-product inhibition do not arise. In spite of these restrictions, and in addition to the practical inherent problem of preventing infection of such glucose syrups, it is easy to screen additionally in the primary selection for end-product-resistant species. Reports of such an approach appear rarely, but our experience gives further insight.

In the selection of *Microbispora bispora* as a cellulolytic model organism, high-temperature (60°C) enrichment culture with crude cellulosics as a substrate was employed, using soils that had been collected worldwide from thermal zones and additionally from composts (*15*). Promising cellulolytic cultures were then further screened for β-glucosidases.

The latter screening protocol is outlined in Table 3. The method depends on the use of indicator substrates, such as *p*-nitrophenol-β-D-glucoside, methylumbelliferyl-β-D-glucoside, or arbutin plus ferric ion. This selection only implies action toward β-D-glucosides and not cellobiose, and it is essential to know that a cellobiase is truly under study. As an aside, colorimetric substrates are especially convenient for monitoring enzyme activity in purification protocols, but do not necessarily reflect their apparent activity. Methylumbelliferyl-β-D-cellobioside, an apparent substrate for cellobiohydrolase, has been usefully employed in the selection of endo-xylanases (*16,17*)!

As a result of incubation at 60°C, thermophilic bacilli and actinomyetes were the primary isolates, and the latter were studied further. A surprising general result of the end-product inhibition screening was that several of the actinomycetes possessed β-glucosidases that were resistant to end-product inhibition by glucose, and quite markedly so in compari-

son with control fungi, such as *T. reesei*. This observation is most intriguing and has yet to be further clarified. After comparing the cellulolytic characteristics of several isolates (yield, enzyme spectrum, and so on), *M. bispora* was selected for further study based in part on the end-product resistance of its β-glucosidase. This β-glucosidase was studied further. Cloning into *Escherichia coli* using methylumbelliferyl-β-D-glucoside for selection led to two distinct clones, one a true cellobiase (*18*) and another that was characterized as an exo-splitting glucosidase (*19*). The cellobiase has a mol wt of roughly 50 kDa, and has the interesting properties of being activated by glucose three- to fourfold. The finding of a true exo-splitting glucosidase was most useful, since this enzyme has rarely been documented in cellulolytic organisms and reinforces the complexity of the cellulase process.

The initial rationalization that there is no *a priori* reason to expect to find an end-product-resistant cellobiase has been confounded. The corollary is that it is indeed a fallacy that humans know more than Mother Nature. Additionally a variety of end-product-resistant β-glucosidases occur in actinomycetes. The use of the artificial substrate methylumbelliferyl-β-D-glucoside in combination with cloning led to the discovery of a rarely reported enzyme, exo-splitting exo-glucanase, an enzyme that is exceedingly difficult to screen for directly in cellulase complexes.

Myth 5. The Culture Giving the Greatest Yield of Cellulase Is the Best

Celluloses are complex and diverse, and when apparently equal amounts of cellulase from different sources are added to them, the degree of attack can differ greatly, i.e., specific activity is a useful parameter, but does not offer a clear assessment of degradability in relation to different substrates. The high SA of 1200–1500 for endoglucanases of *Thermotoga neapolitana* (*20*) far outweigh those of *T. reesei* in the 50–75 range, but this does not guarantee the former is more effective than the latter when used with natural substrates. In this sense, screening should be based a substrate that is the focus of the study, for example, steam-exploded aspen chips rather than "purified" cellulosics, since there are a variety of extraneous parameters in addition to the cellulose molecule *per se* that control the efficiency of degradation. In this sense, the *T. reesei* cellulase has been remarkably resilient toward many attacks made against it with "bigger and better" claims. There is apparently no simple answer to selection of individual cellulase components that are most effective toward a specific substrate, and this is made even more complex if attempts are also made to assess their synergistic effectiveness. Focus should also be toward effecting 100% degradation, which is in stark contrast to the International Enzyme Commission definition of an enzyme unit based on initial rates of reaction. Furthermore, since the metabolic cost of producing a protein to a microbe is high, anaerobic systems in which energy availability is

more restricted than that of aerobic processes should realize pressure to develop more efficient enzymes (*21*). This appears true of high specific activity cellulases from anaerobic chytrids (*12*), whereas the high specific activity of up to 1500 for endoglucanases of the anaerobic *T. neapolitana* (*20*), admittedly in part of the result of assaying at 95°C, also gives further credence to this concept. Perhaps the most exciting aspect with regard to specific activity is that through protein engineering, it should be possible to design rationally cellulase components of higher activity.

ACKNOWLEDGMENT

Support for this study has been provided through the National Renewable Energy Laboratory, Golden, CO, and the US Department of Energy, Washington, DC. Paper of the New Jersey Agricultural Experiment Station No. D-01111-01-94.

REFERENCES

1. Davis, B. D. (1961), *Cold Spring Harbor Quantitative Biol.* **26,** 1–10.
2. Béguin, P. and Aubert, J.-P. (1994), *FEMS Microbiol. Rev.* **13,** 25–58.
3. Torsvik, V., Salte, K., Sorheim, R., and Goksoyr, J. (1990), *Appl. Environ. Microbiol.* **56,** 776–781.
4. Hungate, R. E. (1966), in *The Rumen and Its Microbes.*, Academic, New York, p. 533.
5. Batra, S. W. T. and Batra, L. R. (1967), *Sci. Am.* **217(5),** 112–120.
6. Greene, R. V. (1994), *SIM News* **44,** 49–59.
7. Montenecourt, B. S. and Eveleigh, D. E. (1977), *Appl. Environ. Microbiol.* **34,** 777–782.
8. Wood, W. A. and Kellogg, S. T. (eds.) (1988), *Methods in Enzymology,* vol. 160, *Biomass Part A: Cellulose and Hemicellulose*. Academic, San Diego, p. 774.
9. Bayer, E. A., Morag, E., and Lamed, R. (1994), *Trends in Biotechnol.* **12,** 379–386.
10. Felix, C. R. and Ljungdahl, L. G. (1993), *Ann. Rev. Microbiol.* **47,** 791–819.
11. Lamed, R. and Bayer, E. A. (1988), in *Biochemistry and Genetics of Cellulose Degradation,* Aubert, J.-P., Béguin, P., and Millet, J. eds., Academic, New York, pp. 101–116.
12. Zhou, L., Xue, G.-P., Orpin, C. G., Black, G. W., Gilbert, H. J., and Hazelwood, G. P. (1994), *Biochem. J.* **297,** 369–374.
13. El-Gogary, S., Leite, A., Crivellaro, O., Eveleigh, D. E., and El-Dorry, H. (1989), *Proc. Natl. Acad. Sci.* **86,** 6138–6141.
14. Kubicek, C. P., Messner, R., Gruber, F., Mach, R. L., and Kubicek-Pranz, E. M. (1993), *Enzyme & Microbial Technol.* **15,** 90–99.
15. Waldron, C. R., Jr., Becker-Vallone, A., and Eveleigh, D. E. (1986), *Appl. Microbiol. Biotechnol.* **24,** 477–486.
16. Grepinet, O., Chebrou, M. C., and Béguin, P. (1988), *J. Bacteriol.* **170,** 4576–4581.

17. Wu, Y.-M. (1994), A new endoxylanase from *Microbispora bispora*. M. S. thesis, Rutgers—The State University of New Jersey, New Brunswick, NJ.
18. Wright, R. M., Yablonsky, M. D., Shalita, Z. P., Goyal, A. K., and Eveleigh, D. E. (1992), *Appl. Environ. Microbiol.* **58,** 3455–3465.
19. Goyal, A. K. (1993), Molecular and biochemical characterization of a glucan-glucohydrolase from *Microbispora bispora*. Ph.D. Dissertation, Rutgers—The State University of New Jersey. New Brunswick, NJ. p. 155.
20. Bok, J. D., Goers, S. K., and Eveleigh, D. E. (1994), The cellulase and xylanase systems of *Thermotoga neapolitana*, in American Chemical Society Symposium No. 566, Enzymatic Conversion of Biomass for Fuels Production, Himmel, M., Baker, J. O., and Overend, R. P., eds. American Chemical Society, Washington, DC, pp. 54–65.
21. Eveleigh, D. E. (1987), *Phil. Trans. R. Soc.* (Lond.) **A321,** 435–477.