Cellulases and biofuels

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David B Wilson

There is a major international effort to develop renewable alternatives to fossil fuels. One approach is to produce a liquid fuel by enzymatically hydrolyzing carbohydrate polymers in biomass to sugars and fermenting them to ethanol. Cellulose is the main polymer in biomass and cellulases can hydrolyze it to cellobiose, which can be converted to glucose by β-glucosidase. Extensive research is being carried out to try to obtain cellulases with higher activity on pretreated biomass substrates by screening and sequencing new organisms, engineering cellulases with improved properties and by identifying proteins that can stimulate cellulases. Despite extensive research on cellulases there are major gaps in our understanding of how they hydrolyze crystalline cellulose, act synergistically, and the role of carbohydrate binding modules.

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Introduction

This article will focus on the use of cellulases for the production of renewable liquid fuels, such as ethanol, butanol, and hydrocarbons, from biomass sugars. The most studied process involves some form of chemical or physical pretreatment that disrupts the hemicellulose-lignin-cellulose in plant cell walls and modifies the cellulose fibers to make the cellulose more accessible to enzymes, followed by enzymatic hydrolysis to produce sugars, which are fermented to ethanol, butanol, or a hydrocarbon mixture [1]. Most processes are designed to produce ethanol, as the technology for fermenting glucose to ethanol is very robust and high concentrations of ethanol can be achieved (15%). However, DuPont is trying to produce butanol as it mixes better with gasoline and gives better mileage. While there are well-studied organisms that produce butanol from glucose the concentration is only about 2% because of its toxicity. At this time research is continuing on all three steps, as none of them have been fully optimized. In addition, it is not clear if the very different substrates that are being considered for this process: corn stover, straw, various grasses, sorgum, willow, poplar, and so on will respond similarly to pretreatment or if a number of different pretreatments will be required for the different substrates. A list of industrial activity in the production of renewable liquid fuels is published in each issue of the journal, *Industrial Biotechnology*.

SHF and consolidated bioprocessing

There are two approaches being studied for producing liquid fuels from biomass with cellulases: in one, separate hydrolysis and fermentation (SHF), plant cell wall polymers are hydrolyzed by free enzymes in one step and the resulting sugars are fermented in a second step while in the other, called consolidated bioprocessing, the biomass will be converted to biofuel in a single step by either using an anaerobic bacterium that can hydrolyze plant cell walls and ferment the resulting sugars to ethanol [2] or by simply combining the two SFH steps in a single vessel. At this time there is no anerobic organism available that can carry out consolidated bioprocessing efficiently but efforts are underway to use metabolic engineering to create some. It is claimed that consolidated bioprocessing would be the more efficient process, as it can be carried out in a single vessel and does not require added enzymes, furthermore, sugars should not build up so that inhibition of cellulases by them should not occur. However, there are two problems with this process that will affect higher efficiency. One is that the optimum temperatures for plant cell wall hydrolysis and fermentation are quite different and if the rate-limiting step is reduced in the overall process, the vessel for consolidated bioprocessing needs to be larger than those used in a two-step process, thus, reducing the advantage of a single vessel in SSF. The other problem is that anerobic bacteria need much more carbon source to produce a given amount of protein then do aerobic organisms, so that it is difficult to produce the vast amount of bacteria needed for an industrial process.

Commercial cellulases

Cellulases are currently the third largest industrial enzyme worldwide, by dollar volume, because of their use in cotton processing, paper recycling, as detergent enzymes, in juice extraction, and as animal feed additives. However, cellulases will become the largest volume industrial enzyme, if ethanol, butanol, or some other fermentation product of sugars, produced from biomass by enzymes, becomes a major transportation fuel. Currently, industrial cellulases are almost all produced from

aerobic cellulolytic fungi, such as *Hypocrea jecorina* (*Trichoderma reesei*) or *Humicola insolens* [3]. This is due to the ability of engineered strains of these organisms to produce extremely large amounts of crude cellulase (over 100 g per liter), the relatively high specific activity of their crude cellulase on crystalline cellulose, and the ability to genetically modify these strains to tailor the set of enzymes they produce, so as to give optimal activity for specific uses.

The DOE has been supporting industrial research to reduce the cost of cellulases for production of ethanol for a number of years. The first grants went to the enzyme companies, Genencor and Novozyme, which allowed them to further improve their production strains and demonstrate that the cost of industrial cellulase could be dramatically reduced if the crude cellulase from the fermenter was used directly after production, so that it did not require modification to extend its shelf life. Without pilot plant studies it is difficult to determine the actual cost of cellulase after this research but it is estimated at about 50 cents per gallon of ethanol. Further work carried out by Novozyme led to the finding that addition of a family 61 protein from a thermophilic fungi could increase the activity of *T. reesei* crude cellulase on pretreated corn stover thus dropping enzyme cost severalfold [4,5•]. This result is surprising as *T. reesei* contains several family 61 genes and the structure of a family 61 protein does not contain any regions that resemble cellulose binding sites [5•]. A current round of grants were awarded to Novozyme, Genencor, and Verenium to lower the cost of cellulase even more. Another major DOE effort to help develop renewable liquid fuels is the funding of three Bioenergy Research Centers: one led by the Berkeley Argone National Laboratory, one led by Oak Ridge National Laboratory and one led by the University of Wisconson that will study all aspects of liquid biofuel production from biomass sugars. Novozyme has broken ground on a large new plant to produce enzymes for both corn and cellulose ethanol production in Blair, NE, showing their commitment to enzymatic production of biofuels.

Production of cellulases in plants

A different approach to reducing cellulase cost is to produce cellulases in plants rather then in fungi [6,7]. Plants produce the lowest cost protein, so that if cellulases could be produced at a high level without hurting the yield of a productive crop, their cost could be reduced. A major advantage of producing cellulases in plants over fungal production is that it is much easier to adjust the amount of cellulase that is produced to meet the demand, as the amount of land planted can be adjusted to the demand, whereas once a fermenter is built, its capacity is always there. A problem with plant production of cellulases is the multiplicity of proteins that are needed to efficiently degrade plant cell walls. Fungi produce large numbers of proteins and we do not always know which proteins are required to degrade a given substrate so that it is difficult to engineer a plant to produce a mixture with equal activity to that produced by a cellulolytic fungus.

Basic research on cellulases

Although there has been extensive research on cellulases since the end of World War II, there are still some major gaps in our understanding of the mechanism by which they catalyze the hydrolysis of crystalline cellulose [8]. One gap is information on the mechanism by which a cellulase binds a segment of a cellulose chain from a microfibril into its active site. This is probably the rate-limiting step for crystalline cellulose degradation, so that understanding the mechanism of this step is very important for trying to engineer cellulases with higher activity on real cellulose substrates. Another gap is our understanding of how cellulosomes are able to efficiently catalyze the hydrolysis of cellulose, despite their large size that restricts their ability to access much of the cellulose surface area that is available to smaller free cellulases. A third gap is an understanding of the way in which certain free cellulose binding modules (CBM) stimulate cellulase hydrolysis [9,10]. It is possible that these domains modify the cellulose but exactly how is not known. Finally, while there are some plausible mechanisms for cellulase synergism, there is still much more to be learned about this important process [11], particularly how mixtures of cellulases hydrolyze both crystalline and amorphous regions in bacterial cellulose while most individual enzymes only seem to degrade amorphous regions [12].

Genomic approaches

Genomic sequencing of cellulolytic organisms has been carried during the past decade and the genome sequences have provided important new information about how microorganisms degrade cellulose. The sequences of the aerobic microorganisms: *Hypocrea jecorina* (*Tricoderma reesei*), *Phanerochaete chrysosporium*, and *Thermofida fusca*, all contain multiple cellulase genes, most of which encode a carbohydrate binding module (CBM), and several processive cellulase genes are present in each organism [13,14]. The genome sequences of *Clostridium thermocellum*, *Ruminococcus albus* and *Ruminococcus flavifaciens* all contain scaffoldin genes and multiple cellulases genes that encode docerin domains, consistent with the presence of cellulosomes in these anaerobic bacteria and several processive cellulase genes are found among the docerin encoding genes [15]. There are three cellulolytic microorganisms whose genomes do not contain known genes for processive cellulases or docerin domains or scaffoldins: *Cytophiga hutchinsonii* is an aerobic cellulolytic bacterium that is tightly bound to cellulose fibers during growth on cellulose [16], while *Fibrobacter succinogenes* is an anaerobic cellulolytic bacterium that also is tightly bound to cellulose fibers [17]. From their genome sequences these organisms do not use either the free

cellulase or the cellulosomal mechanism to degrade cellulose, so they must use a novel mechanism [18]. Finally *Postia placenta* is an aerobic brown rot fungus that appears to produce hydrogen peroxide and Fe (II) ions that generate OH radicals that carry out cellulose depolymerization [19]. Further research is needed on each of these organisms to determine the detailed mechanisms that they use to completely metabolize cellulose. Metagenomics is also being used to try to identify new cellulases and a major study of DNA isolated from the microrganisms in termite guts was reported recently [20•]. About one hundred hydrolases related to cellulose degradation were identified including members of eight cellulose families; however, no members of families containing exocellulase genes were present. This might be due to the fact that termites chew up the biomass into very fine particles that may be easier to degrade then other forms of cellulose. It is interesting that screening of genes for cellulase activity, either from isolated organisms or from DNA libraries from various environmental samples has not identified any new cellase families in the past few years. One novel hydrolase containing both a glucanase and a xylanase was found in a library isolated from soil [21].

Non-cellulase protein stimulating cellulases

Several proteins have been identified that appear to modify cellulose and enhance its hydrolysis by cellulase. One is a class of plant proteins called expansins [22]. Another is a fungal protein with some homology to expansin called swollenin [23]. Recently an expansin like protein has been identified in *Bacillus subtilis* and its structure was determined. In another study, this protein was shown too stimulate corn stover hydrolysis by crude cellulase [24,25].

Several organisms secrete proteins that only contain CBMs and two *T. fusca* proteins (E7, E8) have been purified and shown to stimulate low concentrations of cellulases [10]. Finally there are the family 61 proteins mentioned earlier.

Modeling cellulase activity

Many attempts have been made to model the cellulase catalyzed hydrolysis of crystalline cellulose but we still do not know enough about this process to create a true mechanistic model. Peri *et al.* presented a detailed mechanistic model of amorphous cellulose hydrolysis by crude cellulase that fits their experimental results quite well [26].

Cellulase engineering

There are three main approaches that are being used to engineer cellulases with higher activity on crystalline cellulose: directed evolution, rational design, and increasing cellulase thermostability by either of the preceding methods, which can also lead to higher activity. Engineering more thermostable enzymes is relatively straightforward and there are some general approaches that can be applied to any enzyme for which a large number of related sequences are known, as is true for most cellulases [27•]. A recent paper describes evolving *T. reesei* Cel12A for enhanced thermostability while another evolved a family 5 endoglucanase with higher activity on CMC but it had no activity on crystalline cellulose [28,29]. At this time there are no published reports of engineered cellulases with major (greater than 1.5-fold) increases in activity on crystalline cellulose. Furthermore, to be useful in an industrial process the improved enzyme has to increase the activity of a synergistic mixture containing several cellulases and in several cases mutant enzymes with higher activity do not do this [30]. At this time, it is not clear why this is happening but it has been shown for several exocellulases. Another surprising result is that an improved processive endocellulase catalytic domain, produced by combining two site directed mutations, that showed higher activity in synergistic mixtures then the wild type catalytic domain, did not show higher activity on crystalline cellulose then wild type intact enzyme when the missing domains were added back to form the intact mutant enzyme. This result seems surprising but it shows that activity on crystalline cellulose may involve interactions between the catalytic domain and the carbohydrate binding module (CBM) that go beyond the CBM simply anchoring the catalytic domain to the cellulose [31].

Directed evolution of cellulases with improved activity on crystalline cellulose requires that the mutant cellulases be screened on a crystalline substrate not on CMC as most mutations that increase CMCase activity decrease activity on crystalline cellulose. Furthermore, the native enzyme should be utilized, not the catalytic domain given the above result. Finally any improved enzymes need to tested in the appropriate synergistic mixture on the actual substrate for the final process in order to be certain that they will be useful. A problem with directed evolution is that it can only be used to screen potential single or with a massive screen potential double mutations, since the mutant library size required to include most possible larger multiple mutations is too large.

Rational design does not have this limitation, but it does require a detail understanding of structure–functional relationships for cellulase crystalline cellulose activity that is still lacking. If we can gain a clear understanding of exactly how cellulases hydrolyze crystalline cellulose it should be possible to design enzymes with multiple changes that have higher activity on specific biomass substrates.

Designer cellulosomes

Another approach to engineering more active cellulose degrading enzymes is to create optimized cellulosomes by synthesizing hybrid scaffoldin molecules that contain

cohesins with different binding specificity from different organisms. The exact composition and geometry of the enzymes in a cellulosome can be controlled by attaching the approbriate docerin domain to each enzyme in the cellulosome. In one experiment, the six *T. fusca* cellulases produced during growth on cellulose were modified by removing their family 2 CBM domain and replacing it with a docerin domain, thus converting a free cellulase system into a cellulosomal system. There were a number of interesting findings from this approach but it did not produce a cellulosome with increased cellulase activity over the free cellulase system [32]. Another experiment involved adding CBM domains to two of the key cellulosomal enzymes. This inceased the activity of each enzyme when it was bound to an artificial scaffoldin containing one cohesin but various designer cellulosome containing the modified enzymes and other cellulases all had lower activity on crystalline cellulase than comparable designer cellulosomes containing the WT enzymes [33•]. These results do not invalidate the possibility of improving the activity of cellulosomes by the designer approach but we need to understand more about how the enzymes on cellulosomes interact to degrade crystalline cellulose before we can create better cellulosomes.

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