A paradigm shift in biomass technology from complete to partial cellulose hydrolysis: lessons learned from nature

Rachel Chen*
School of Chemical and Biomolecular Engineering; Georgia Institute of Technology; Atlanta, GA USA

A key characteristic of current biomass technology is the requirement for complete hydrolysis of cellulose and hemicellulose, which stems from the inability of microbial strains to use partially hydrolyzed cellulose, or cellodextrin. The complete hydrolysis paradigm has been practiced over the past 4 decades with major enzyme companies perfecting their cellulase mix for maximal yield of monosaccharides, with corresponding efforts in strain development focus almost solely on the conversion of monosaccharides, not cellodextrin, to products. While still in its nascent infancy, a new paradigm requiring only partial hydrolysis has begun to take hold, promising a shift in the biomass technology at its fundamental core. The new paradigm has the potential to reduce the requirement for cellulase enzymes in the hydrolysis step and provides new strategies for metabolic engineers, synthetic biologists and alike in engineering fermenting organisms. Several recent publications reveal that microorganisms engineered to metabolize cellodextrins, rather than monomer glucose, can reap significant energy gains in both uptake and subsequent phosphorylation. These energetic benefits can in turn be directed for enhanced robustness and increased productivity of a bioprocess. Furthermore, the new cellodextrin metabolism endows the biocatalyst the ability to evade catabolite repression, a cellular regulatory mechanism that is hampering rapid conversion of biomass sugars to products. Together, the new paradigm offers significant advantages over the old and promises to overcome several critical barriers in biomass technology. More research, however, is needed to realize these promises, especially in discovery and engineering of cellodextrin transporters, in developing a cost-effective method for cellodextrin generation, and in better integration of cellodextrin metabolism to endogenous glycolysis.

Critical Barriers of Biomass Technology

Rapid depletion of finite petroleum reserve and environmental concerns associated with its use are 2 major motivations that drive technology innovations toward a more sustainable economy. It is widely recognized many products currently made from petroleum can be produced from abundant renewable plant biomass (or lignocellulose). Particularly, transportation fuels based on lignocellulosic biomass represent the most scalable alternative fuel source. However, despite recent progresses, currently no low-cost technology is available to transform this abundant resource into useful products. All three aspects of biomass technology (pretreatment, enzymatic hydrolysis, and microbial technology) need further improvement before a widespread use. Several critical barriers are frequently cited in recent literatures. These include:

1.) Enzyme cost: The cost of enzyme used for depolymerization continued to be a major constraint to cost-effective processing of cellulosic biomass. Recent low estimate of enzyme cost, 50 cents per gallon ethanol, is comparable to the cost of feedstock, which is widely considered as the most important cost contributor.

2.) Process robustness: Numerous inhibitors, either derived from pretreatment or biofuel product itself, dramatically

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*Correspondence to: Rachel Chen; Email: rchen@chbe.gatech.edu
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impact biofuel processes. The inhibitive compounds exert adverse effects by limiting cell growth or even cell death; they also slow, reduce or completely prevent product formation. As a result, product titer, productivity, and yield are all affected.\textsuperscript{7}

3.) Low productivity: The complex nature of lignocelluloses, which include glucose, xylose, arabinose, and other minor sugars, requires that a fermenting strain converts all sugars rapidly to biofuels. This could only be achieved by simultaneous metabolism of all sugars present. Yet due to carbon catabolite repression, a sequential use of sugars (one sugar at a time), is a more common occurrence, which prolongs fermentation steps and dramatically lowers the productivity in the fermentation stage.\textsuperscript{8} Sequential use of sugars also leads to more undesired consequence than just prolonged fermentation. For example, in mixed glucose and xylose fermentation, xylose consumption takes place when glucose is exhausted and when accumulation of fuel and other byproducts is significant enough to be inhibitive. Consequently, xylose utilization is often slow and not complete, leading to low yield and low productivity.\textsuperscript{9}

These problems require innovative approach to overcome. The requirement of a complete hydrolysis of lignocelluloses is based on the inability of microbes to use celloextrin in the fermentation stage. This requirement is central to some of the problems analyzed above. The mode of complete hydrolysis is believed to be followed by most aerobic cellulolytic fungi such as \textit{Trichoderma reesei} and has been the paradigm being practiced for biomass research over the last 4 decades. As such, the major enzyme companies have developed enzyme cocktails that aim to hydrolyze lignocellulose completely to glucose, xylose and other monosaccharides. Correspondingly, strain developments in the past have primarily focused on conversion of glucose and other monosaccharides to biofuel and other products. Only recently, an alternative paradigm, referred here as Partial Hydrolysis Paradigm, has emerged. This paradigm is characterized as requiring only partial hydrolysis of cellulose yielding oligomers or celloextrins, which in turn are directly assimilated by microbial catalysts and converted to biofuels or other products. Although currently only 2 microorganisms are the focus of this approach, \textit{E. coli} and \textit{Saccharomyces cerevisiae}.\textsuperscript{10,11} nature provides several examples that follow the partial hydrolysis paradigm. Both bacteria such as \textit{Clostridium thermocellum}\textsuperscript{12} and fungi such as \textit{Neurospora crassa} \textsuperscript{13} follow this paradigm in cellulose metabolism.

**Advantages from an Alternative Paradigm**

The partial hydrolysis paradigm has several compelling advantages, which could be exploited to overcome critical barriers in today’s biomass technology.

1.) Reduced enzyme requirements and improved enzymatic hydrolysis: By engineering microbial biocatalysts to directly assimilate celloextrin, the need for β-glucosidase could be eliminated completely. This was indeed demonstrated by Lee et al in which a yeast strain engineered to use celloextrin intracellularly was shown to produce ethanol without exogenous β-glucosidase in a simultaneous saccharification and fermentation (SSF) process.\textsuperscript{14} Moreover, rapid removal of celloextrin relieves the strong inhibition of celloextrin on cellulase/xylanase activity. As a result, enzymatic hydrolysis could proceed at a much faster rate and significant savings on the enzymes could be expected. A recent study with a yeast strain displaying celloextrins on its surface showed that the additional capability of transporting and assimilating celloextrine intracellularly gave the strain 70% higher ethanol titer than the strain expressing cellulosases only. Since the cellulosates levels in both strains were similar, the beneficial effect of removing celloextrine could be attributed to the relief of celloextrine inhibition on cellulosates.\textsuperscript{15}

2.) Enhanced process robustness: Microbial cells engineered to follow partial hydrolysis paradigm are more energetic and thus are better able to tolerate stresses from inhibitors and other unfavorable conditions. Compared to monosaccharide metabolism, intracellular metabolism of celloextrin can utilize energy-saving mechanisms not available to glucose metabolism. For example, when cells uptake celloextrase through an ABC transporter, only one ATP is used for the transport. This compares to 4 ATPs if 4 glucose molecules are transported through the same mechanism one at a time. Besides the opportunity to engineer cells to use energy-saving mechanism for uptake, at the phosphorylation step, celloextrin phosphorylate could be additionally used to generate more energy savings. This is because the phosphorolytic cleavage of celloextrin catalyzed by celloextrin phosphophorylase yields glucose-1-phosphate using inorganic phosphate anion as donor (rather than ATP). As shown in Figure 1, the saving of ATPs for a celloextrase via phosphorylisis is 3 ATPs, compared to glucose phosphorylation using ATP or equivalent. The energy gain increases with the increase in DP (degree of polymerization) of celloextrin. The energy saving from celloextrin metabolism is significant and is expected to be particularly important for production processes under anaerobic conditions, such as cellulosic ethanol and butanol.

Overall bioprocesses could be more efficient following more energy-efficient

**Figure 1.** Celloextrin assimilation via phosphorylisis.

\begin{align*}
\text{Celloextrin phosphorylase:} & \text{ Celloextrase + Pi } \rightarrow \text{ Celloextrin + glucose-1-P (1)
}
\text{Celloextrin phosphorylase:} & \text{ Celloextrase + Pi } \rightarrow \text{ Celloextrin + glucose-1-P (2)
}
\text{Celloextrin phosphorylase:} & \text{ Celloextrase + Pi } \rightarrow \text{ Glucose + glucose-1-P (3)
}
\text{Phosphoglucose mutase:} & \text{ Glucose-1-P } \rightarrow \text{ Glucose-6-P (4)
}\end{align*}
pathways as better cell growth could be expected with overall reduced fermentation time. Additionally, tolerance of inhibitors generally requires expenditure of cellular resources especially energy, thus more energized cells could be expected to better tolerate stress and inhibitors, and leading to more robust biofuel production processes. Thus, the naturally-existing energy-saving mechanisms of cellodextrin uptake and phosphorylation offer synthetic biologists new tools to engineer biocatalysts for improved biofuel production. Engineering biocatalysts for cellodextrin phosphorylation can be achieved by coexpression of a cellodextrin transporter and a cellodextrin phosphorylase. A biocatalyst assimilating cellodextrin intracellularly via hydrolysis requires a cellodextrin transporter and a β-glucosidase. These engineering steps can be readily accomplished.

A comparison between phosphorylation and hydrolysis of cellodextrin in 2 metabolically engineered E. coli cells showed that, relative to the isogenic hydrolysis cells, cells undergoing phosphorylation better tolerate inhibitors such as acetate, an inhibitor released in pretreatment of biomass, and butanol, an advanced biofuel. Interestingly, better tolerance to inhibitors in phosphorylation cells was observed in both anaerobic and aerobic conditions. For example, under aerobic condition, acetate at 5% (w/v) concentration completely inhibited cell growth for cells assimilating cellodextrin via hydrolysis. In stark contrast, cells undergoing phosphorylation could assume a normal growth after a lag time of about 6 hours. Similarly, phosphorylation cells could tolerate 0.8% (w/v) butanol and could grow to OD₆₀₀ of 2.5, whereas hydrolysis cells could only reach final cell density of 0.5 under the same aerobic condition. These results demonstrated significant advantages of using phosphorylolytic assimilation of cellodextrin, possible only when cellodextrin is metabolized intracellularly. The work illustrates a new tool for metabolic engineers and synthetic biologists to fashion a robust microbial strain in biomass conversion.

3) Increased productivity: As analyzed above, carbon catabolite repression prevents microbial cells to simultaneously convert different types of biomass-derived sugars to biofuels. Engineering cells to use cellodextrin directly could avoid this repression mechanism. This was indeed demonstrated successfully in yeast and in E. coli. In mixed sugar fermentation, the E. coli strain assimilating cellodextrin hydrolytically, could convert 5% cellodextrin and 5% xylose simultaneously to 4% ethanol, whereas the same strain left much of xylose unused in mixed monosaccharide fermentation (5% glucose and 5% xylose). By providing glucose in cellodextrin, glucose is made “invisible,” no longer able to trigger repression on xylose uptake and metabolism.

Beside ethanol, other product formation could also benefit from the more energetically favorable metabolism in phosphorylolytic cells. The Chen Lab demonstrated that phosphorylolytic cellodextrin allowed 3- and 5-fold more production of green fluorescent protein and a β-xylosidase, respectively, than hydrolysis cells.

**Challenges and Prospect**

These studies convincingly show the promises for this new paradigm. However, significant challenges remain in integrating these discrete synthetic modules to endogenous metabolic network and to product-specific pathways. Much work needs to be done to translate the potential of the new paradigm into real gains in terms of productivity, titer, yield, robustness, and reduced overall cost.

As the new approach requires transporters for cellodextrin uptake, discovery of new transporters for more efficient uptake is one area of research critical to the success of the new technology. So far naturally cellulolytic microorganisms such as *Neurospora crassa* provided several useful transporters for *Saccharomyces cerevisiae* to establish the new metabolism. Subsequent studies show that in-vitro engineering is necessary to improve the rate and capacity of transport. New discovery of cellodextrin transporters in cellulolytic fungus *Penicillium oxalicum* provides additional candidates in the engineering of eukaryotes. In prokaryotes, *E. coli* ‘s LacY was found to be adequate as a transporter for cellodextrin, which was subsequently used to engineer *E. coli* for cellodextrin conversion to lactic acid and meso 2,3-butanediol.

Discovery of other bacterial cellodextrin transporters that uptake cellodextrin with higher DP were made in Chen Laboratory, which opens up opportunity to engineer *E. coli* or other bacteria for cellodextrin assimilation (unpublished).

Cellodextrin (DP = 2) is the smallest cellobextrin. While the advantage of the new paradigm is evident even at the level of cellodextrin, to reap the benefits of energy gain from cellodextrin metabolism, it is necessary to engineer cells to use higher oligomers of glucose than cellodextrin. This creates a need to generate cellodextrin in a cost-effective manner. While natural cellulolytic organisms provide template about how this can be achieved, current knowledge about enzymatic hydrolysis is not tuned to generate large amounts of cellodextrin. New enzymes and enzyme cocktails are needed to effectively hydrolyze pretreated cellulose to a mix of cellodextrin. Until this issue gets resolved, the new paradigm will only be at the cellodextrin level.

While phosphorylolytic cellodextrin potentially have significant advantages over hydrolysis, such as allowing simultaneous assimilation of cellodextrin and xylose, complication could arise unexpectedly. For example, bacterial phosphorylase, when heterologously expressed in yeast, catalyzes a side reaction that lead to a byproduct, undesirable as it detracts the yield and inhibits the phosphorylase enzyme activity. This problem, however, does not seem to exist in *E. coli* in similar reaction conditions. Instead, a different problem emerges in mixed sugar fermentation. At low concentration, such as 2% each, complete consumption of xylose and cellodextrin was observed. However, at higher concentration 5%, only 60% xylose consumption was achieved. This is intriguing as cells engineered to assimilate cellodextrin hydrolytically could consume all xylose under the same fermentation conditions. More research on the cause of stoppage of xylose utilization at higher concentration for the phosphorylolytic cells and strategies to remove obstacles are needed to move the new paradigm to the realm of practice.

In conclusion, the new paradigm inspired by nature has promises to overcome several challenges with the biomass
technology. Significant work in both *E. coli* and Yeast in this regard, while still preliminary, illustrate the way that the paradigm can be followed to advance biomass technology. More research is needed to move the promising approach to fruition.

(b) a cost-effective method for cellobextrin generation; and
(c) better integration of cellobextrin transporters.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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