The potential of cellulases and cellulosomes for cellulosic waste management

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Lignocellulose is the most abundant plant cell wall component of the biosphere and the most voluminous waste produced by our society. Fortunately, it is not toxic or directly harmful, but our major waste disposal facilities — the landfills — are rapidly filling up with few realistic alternatives. Because cellulose is pure glucose, its conversion to fine products or fuels has remained a romantic and popular notion; however, the heterogeneous and recalcitrant nature of cellulosic waste presents a major obstacle for conventional conversion processes. One paradigm for the conversion of biomass to products in nature relies on a multienzyme complex, the cellulosome. Microbes that produce cellulosomes convert lignocelluose to microbial cell mass and products (e.g. ethanol) simultaneously. The combination of designer cellulosomes with novel production concepts could in the future provide the breakthroughs necessary for economical conversion of cellulosic biomass to biofuels.

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Current Opinion in Biotechnology 2007, **18**:237–245

This review comes from a themed issue on
Environmental biotechnology
Edited by Eliora Z Ron and Philip Hugenholtz

Available online 25th April 2007

0958-1669/$ – see front matter

DOI 10.1016/j.copbio.2007.04.004

Introduction

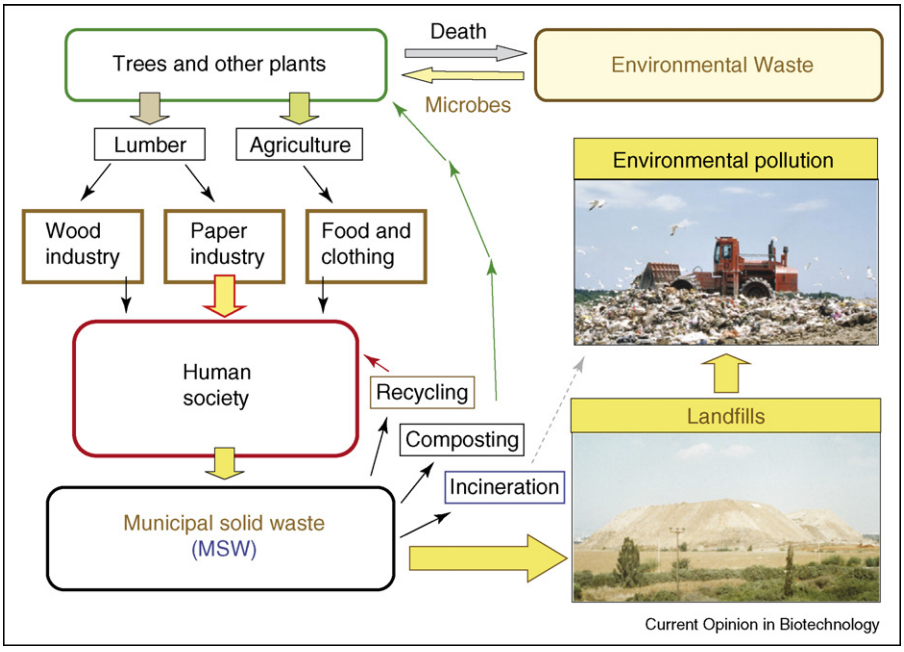
Of the 150 billion kilograms of industrial and domestic waste generated in the United States each year, approximately 100 billion kilograms are estimated to be biodegradable [1]. Municipal solid waste (MSW) consists primarily of cellulose in the form of newspaper, wood and cardboard [2], the majority of which is deposited in landfills and subject to natural anaerobic processes (Figure 1). These anaerobic processes are complex, with a consortium of microorganisms responsible for the production of specific enzyme mixtures necessary for the effective breakdown of the polymeric substrates. The uncontrollable landfill environment results in variable populations of microorganisms and/or enzyme systems, as well as suboptimal environments, which contribute to the characteristically slow and ineffective rates of anaerobic degradation in landfills. Moreover, landfills typically contaminate groundwater and rapidly fill to capacity; old sites have to be rendered environmentally friendly and new sites are unavailable, thus prompting evaluation of alternative disposal options. We are thus faced generally with two choices regarding the disposition of MSW: to leave landfill waste in place and attempt to contain new landfill waste *in situ* or, preferably, to mine old landfill waste and divert new waste to processing before internment.

Biomass is the only domestic, sustainable, and renewable primary energy resource that can provide liquid transportation fuels. In addition, it is estimated that the US, for example, has the potential to produce up to 1.4 billion tons of biomass annually on a sustainable basis without affecting food, feed and fibre uses [3]. To put this in perspective, almost 60% of 2004 motor gasoline demands could be met with ethanol from grain and biomass. New technology may permit a significant fraction of this biomass to be derived from MSW and landfills.

The conversion of cellulosic waste to useful byproducts has long been recognized as a desirable endeavour but has been neglected over the years. The presidential announcement of the Advanced Energy Initiative (www.whitehouse.gov/stateoftheunion/2006/) has now rekindled enormous interest in the development of new and cost-efficient processes for converting plant-derived biomass to liquid fuels [4,5•], as delineated in a 'research roadmap' published by the US Department of Energy [6••]. Disposal of cellulosic MSW through processes that would also derive energy production are thus of particular interest. The benefits would be two-fold: firstly, the amount of cellulose waste (the largest single waste byproduct of our society) would be diminished and its effects on our environment will be reduced, and secondly the pollutant would be converted to an alternative source of energy to help displace our growing dependence on fossil fuels.

In this review, we survey efforts that have been developed in the past for the conversion of lignocellulose substrates to useful products. Before a practical approach can be implemented, however, a revolution in our current thinking is required. Possible future directions include designer cellulosome and microbial cell-based strategies, which are summarized herein.

Figure 1



The fate of cellulose in our environment.

Conventional lignocellulose conversion technologies

Anaerobic hydrolysis of MSW polymers

The biological decomposition of organic matter principally to methane and carbon dioxide by anaerobic digestion is a natural process that occurs readily in MSW landfills. In natural anaerobic digestion processes, some members of the microbial consortia collectively produce fermentable sugars from polysaccharides and others specialize in converting sugars to methane and carbon dioxide. Such mixed fermentations are notoriously difficult to establish and maintain at large scale. MSW, herbaceous crops and woody biomass share the same rate-limiting step for bioconversion processes: the hydrolysis of complex polysaccharides to fermentable sugars [7]. The primary biodegradable polymer, cellulose, is often shielded by lignin — a relatively inert, polyphenylpropane, three-dimensional polymer [8,9] — and by hemicelluloses [10]. We note that evidence does not exist today to indicate that under anaerobic conditions lignin is degraded biologically; however, under aerobic conditions, white rot fungi and moulds readily perform this role.

Pretreatments of the polymeric feedstocks to enhance hydrolysis of the major components, such as cellulose and hemicellulose, include size reduction of the polymeric substrate particles, thermal-chemical pretreatments, and treatments with specific enzyme cocktails [7,10]. The use of a two-phase digestion system serves to separate the hydrolysis/acid-producing stage from the methanogenic stage [11] so that both processes can be optimized to environmental conditions of temperature and pH.

The anaerobic biological conversion of the major polymeric components of MSW requires appropriate microorganisms and hydrolytic enzyme systems. Extracellular hydrolytic enzymes, such as cellulases and lipases, have been shown to be effective in the post-hydrolysis of anaerobic digester effluent solids [12] or pretreatment of complex organic polymers before the digestion process [13]. The titers of cellulase activities found in anaerobic digesters, when compared with the few other 'hydrolytic environments' for which analytical data are available, were strikingly low. This evidence seems to indicate that the cellulose-degrading enzymes in MSW-fed digesters are operating under less than optimal enzyme titers. Only later were the types, activities, and relative concentrations of hydrolytic enzymes from MSW-fed digesters examined in detail [14,15].

Aerobic hydrolysis and fermentation processes

In conventional technologies for the aerobic degradation of lignocellulose, lignocellulosic sugars are typically released by thermal chemical pretreatment followed by aerobic enzymatic hydrolysis of chopped or milled biomass. The pretreated soluble fraction of biomass is known as the hydrolysate, and the hydrolysate containing the insoluble material is referred to as the slurry. In diluted acid pretreatment, most of the hemicellulosic sugars (xylose, arabinose, galactose and mannose) are solubilized;

however, the glucose component remains in the solid form as cellulose, where it is eventually depolymerized by cellulases. In the case where enzymes are added to the slurry and the saccharification process is allowed to proceed independent of fermentation, the process is referred to as separate hydrolysis and fermentation (SHF). When cellulases are combined with anaerobic fermentation (usually yeast) to relieve the enzymes from product inhibition, the process is called simultaneous saccharification and fermentation (SSF). A process based on the fermentation of pentose sugars (derived from the hydrolysate) combined with the saccharification of cellulose and fermentation of glucose (derived from cellulose) is referred to as simultaneous saccharification and co-fermentation (SSCF). Alternatively, a hybrid process with partial enzymatic hydrolysis to obtain high cellulose hydrolysis rates by operating at high temperature and co-fermentation can be used to achieve high overall conversion rates of biomass sugars to ethanol. This process, known as hybrid saccharification and fermentation (HSF), takes advantage of enzymes that are tolerant to high temperatures and able to function under conditions where known ethanologens cannot (i.e. $>75\ ^{\circ}C$). After several days of saccharification and fermentation, most of the major and minor sugars will have been converted to ethanol. The resulting beer is sent to product recovery, which involves distilling the beer to separate the ethanol from the water and residual solids. In a typical fermentation process, the mixture of nearly azeotropic water and ethanol is purified to pure ethanol using vapour-phase molecular sieves. Solids from the distillation bottoms are separated and sent to the boiler or sold as animal feed. Concentration of the distillation bottoms liquid is performed by evaporation using waste heat. The evaporated condensate is returned to the process, and the concentrated syrup is sent to the burner.

Although several microorganisms can efficiently ferment glucose to ethanol, only recently has conversion of the pentose sugars in the hemicellulosic fraction become feasible [16]. The few organisms that were known to utilize either D-xylose or L-arabinose typically grow slowly on pentoses and achieve relatively low ethanol yields and productivities [17]. Because of this, the identification and development of microorganisms capable of selectively converting D-glucose, D-xylose, and L-arabinose to ethanol at high yield has been the focus of extensive research over the past 10–15 years. For example, *Escherichia coli* and *Klebsiella oxytoca* have been engineered to be highly effective ethanol producers by introducing the genes for ethanol production from *Zymomonas mobilis* [18–20]. Extensive evaluation of these ethanologenic strains has been carried out, both in media containing pure sugars and in pretreatment hydrolysates derived from a variety of feedstocks [21–24]. Also, an engineered *Z. mobilis* strain able to ferment xylose to ethanol at high yield has been reported [25,26].

Future lignocellulose conversion process scenarios

The cellulosome: a means for increasing enzyme synergy

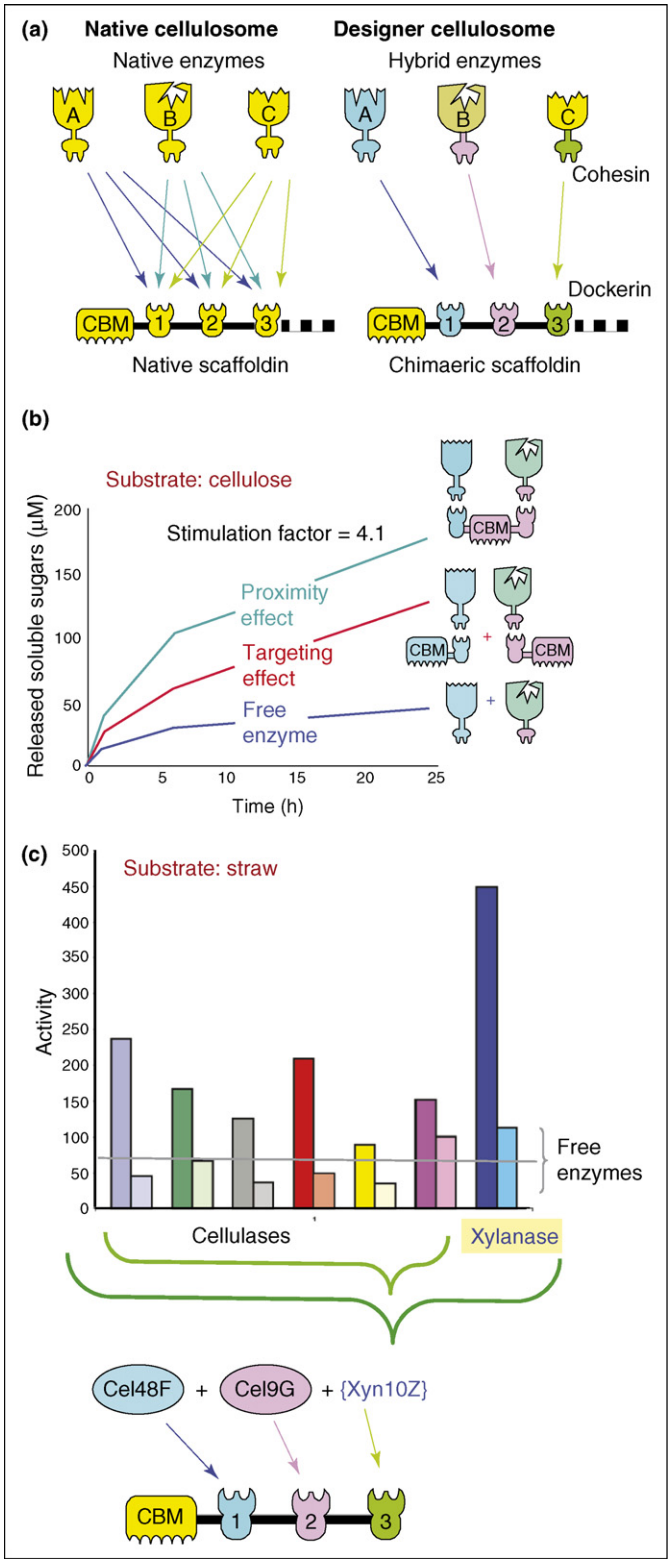
An intriguing paradigm for the conversion of biomass to products in nature relies on the multienzyme complex, the cellulosome [27,28•,29]. Microbes that produce the cellulosome convert lignocellulose to microbial cell mass and products simultaneously. Moreover, because these processes essentially occur within one cell, an opportunity is born to engineer these microbes to be more efficient and reliable than multienzyme or multicellular processes for MSW conversion.

The cellulosome concept was originally proposed using the cellulase system of the thermophilic anaerobe *Clostridium thermocellum*. In this bacterium, the cellulosome is composed of a primary scaffoldin subunit that can integrate up to nine enzymes into the complex, a process mediated by very strong intermodular interactions. The cellulosome differs from free cellulase systems, which generally contain individual enzymes that bear a catalytic module together with a cellulose-binding module (CBM). Instead, in cellulosomal systems the scaffoldin subunit contains a single CBM together with numerous cohesin modules. The cohesion modules, in turn, bind strongly to a dockerin module borne by each cellulosomal enzyme. Cellulosomes derived from different bacteria show a divergent type of architecture, owing to the number of interacting scaffoldins and the content and specificities of their resident cohesins [27]. Cellulosomes of some bacteria, notably *Acetivibrio cellulolyticus* and *Ruminococcus flavefaciens*, can be much more intricate than those of *C. thermocellum* [30,31•]; the heterogeneity of cellulosome composition and assembly is still a mystery. On the basis of genomic and proteomic analyses, such species could contain numerous cohesins and about 200 different dockerin-bearing enzymes and non-enzymatic components (BA White, MT Rincon, unpublished). The reason for such high levels of complexity is not entirely clear.

Potential for engineering cellulosomes

Designer cellulosomes have been proposed as a tool for understanding cellulosome action and for subsequent biotechnological application in waste management [32–34]. Small artificial cellulosomes have been constructed for the efficient degradation of specific substrates [35]. The rationale behind the designer cellulosome concept involves the construction of chimaeric scaffoldins that contain divergent cohesins and matching dockerin-bearing enzymes (Figure 2). This arrangement allows us to control the composition and spatial arrangement of the resultant designer cellulosomes as, in the native state, the cohesin–dockerin recognition qualities appear to be relatively nonspecific. Such designer cellulosomes could eventually find use in the processing of cellulose substrates. Currently, however, the controlled incorporation

Figure 2



Principles of designer cellulosome action. (**a**) Architecture of native versus designer cellulosomes. The cohesion–dockerin interaction is of uniform specificity in the native complex; the specificity is divergent in designer cellulosomes to facilitate controlled incorporation of enzyme components. (**b**) Enhanced synergism of binary designer cellulosomes, by combined targeting and enzyme proximity effects [36]. Targeting of enzymes to the substrate through the cellulose-binding module (CBM) results in enhanced synergistic action (red trace), compared to the free

of components afforded by designer cellulosomes provides a better understanding of the important factors for efficient cellulosome action. In this context, recent studies have defined two factors that serve to enhance deconstruction of recalcitrant forms of cellulose [36]. One is the well-established effect of targeting to the substrate surface by the scaffoldin-borne CBM, and the second, less well-recognized factor, is the consequent proximity of the enzyme components (Figure 2b). The resulting enhancement of deconstruction depends on the recalcitrance of the cellulosic substrate. For more complex lignocellulosic substrates, the contribution of a greater spectrum of enzymes specialized for the different subcomponents has been demonstrated for straw relative to crystalline cellulose alone (Figure 2c) [37••].

Doi and colleagues [38] have constructed minicellulosomes that use recombinant cellulosomal enzymes and truncated scaffoldin components from *Clostridium cellulovorans*. The reconstituted cellulosomes exhibited synergistic activity on cellulosic substrates. Despite the presumably nonspecific nature of the cohesin–dockerin interaction in a single bacterial scaffoldin, the action of minicellulosomes on different types of celluloses and hemicelluloses suggested that the cohesin–dockerin interaction from *C. cellulovorans* might be more selective than originally believed [39•].

Cellulosomal enzymatic components can be further improved by recombinant means using combinatorial screening and robotic handling, as has been carried out for single cellulase enzymes [40]. However, the defining characteristics of cellulase and cellulosome action is not the improvement of individual enzymes, but how the different cellulases work in concert to counteract the recalcitrant properties of the substrate. In this context, the rate-limiting step in cellulase and cellulosome hydrolysis is not the catalytic cleavage of the β-1,4 bond of cellulose, but the disruption of a single chain of the substrate from its native crystalline matrix, thereby rendering it accessible to the active site of the enzyme. Thus, before engineering individual enzyme components, it is crucial to identify efficient individual enzymes, and spatial combinations thereof, to direct subsequent rounds of screening towards the most optimal combinations. For this reason, the perfection of relevant substrates and assay systems, appropriate for high-throughput analyses, are essential for identifying superior sets of synergistically acting enzymes. Screening and selection procedures should be based on relevant solid substrates, such as paper or plant cell walls [41•]. To date, only moderate success has been achieved, such as improvements in the thermostability of a given cellulase [42], using protein engineering techniques. The future construction of designer cellulosomes will concentrate on several fronts: the incorporation of currently available enzymes into designer cellulosomes; the development of novel cellulosomal components — scaffoldins, cohesins, dockerins and CBMs; and the rational design or directed evolution of improved hybrid enzymes and assessment of their enhanced synergistic action within designer cellulosomes. The desired result will be improved biomass degradation with concurrent increased understanding of the structure–function relationship of cellulosome components.

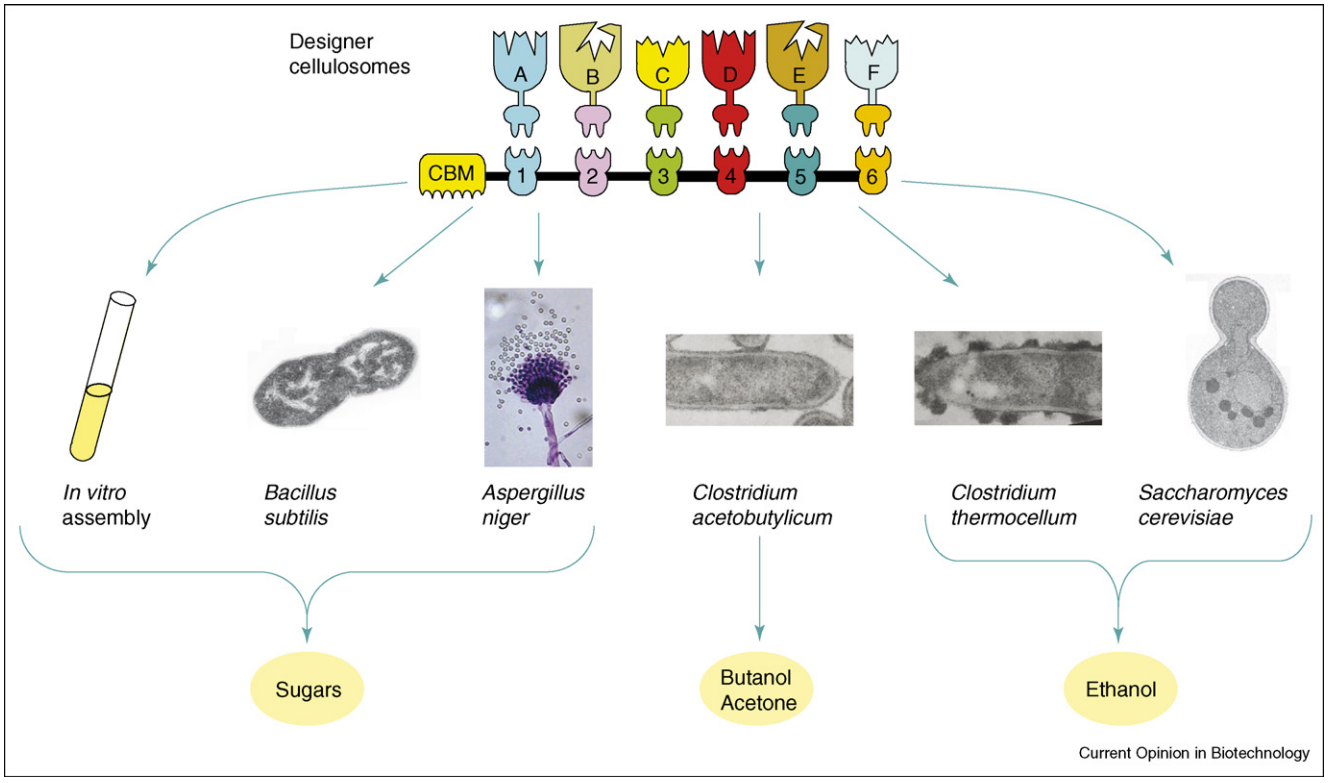
Recent cell-based development strategies: a means to reduce production costs

Over the past few decades, various approaches have been considered to engineer microbial cells for the efficient deconstruction of plant cell wall cellulosic materials. In light of the renewed popularity of the biomass-to-bioenergy initiative, such approaches have been extended and focused in relatively new directions. Several strategies might be used. Firstly, native cellulose-degrading microbes could be engineered further to improve the profile of useful products (particularly ethanol). Alternatively, non-cellulolytic microbes that produce high levels of a desired product could be engineered to include a secreted or cell-surface cellulase or cellulosome system. Finally, the combination of both approaches (engineering both deconstructing enzymes and product profile) could be achieved in a single cell.

The heterologous expression of designer cellulosome components in a suitable industrial host cell system (Figure 3) is an attractive approach to prepare large quantities of highly active cellulases or cellulosomes for the deconstruction of lignocellulosic substrates, employing SSF or related processes. The process would be even more advantageous if the host bacterium is, or can be rendered, cellulolytic and/or ethanologenic. In this context, a truncated scaffoldin and dockerin-containing endoglucanase from *C. cellulovorans* were recently co-expressed in *Bacillus subtilis* and isolated, although the resultant bacterium itself did not grow on cellulosic substrates [43•]. More recently, heterologous production, assembly, and secretion of a minicellulosome was accomplished in the solventogenic bacterium

(Figure 2 Legend Continued) enzymes (blue trace). Integration of the enzymes into a single complex generates an additional enhancement of synergy (cyan trace). (**c**) Enhanced cellulase-hemicellulase synergy of a ternary designer cellulosome on a crude cellulose substrate [37••]. Incorporation of an additional cellulase to a chimaeric complex containing two processive enzymes (*C. cellulolyticum* Cel48F and Cel9G) augments the activity of the complex on hatched straw. The different colored bars (pale blue, green, gray, red, yellow and violet) represent different *C. cellulolyticum* cellulases (from left to right, Cel5A, Cel8C, Cel9E, Cel9M, Cel9G and Cel48F). The adjacent bar in each case represents the activity of the three free enzymes alone (without the scaffoldin). By including a xylanase (*C. thermocellum* Xyn10Z) in the complex (dark blue bar), a dramatic increase in activity on hatched straw is observed.

Figure 3



Engineering potent cellulolytic microbes for the production of desired end products. Genes encoding for cellulases and/or designer cellulosome components (i.e. chimaeric scaffoldin and desired dockerin-containing hybrid enzymes) can be cloned into a desired bacterial or fungal host cell, and the secreted proteins can be overexpressed for the degradation of cellulosic biomass in an industrial reactor (*in vitro* assembly). Alternatively, the genes can be cloned into a suitable bacterial, fungal or yeast host, and the transformed cell with either *de novo* or improved cellulose-degrading capacity can be grown directly on cellulosic biomass to produce a desired end product.

Clostridium acetobutylicum. For this purpose, the genes encoding a truncated scaffoldin and cellulosomal (dockerin-containing) mannanase from *Clostridium cellulolyticum* were cloned into the host cell [44,45•,46•,47]. If expressed alone, the mannanase lost its N-terminal dockerin. When co-expressed together with the truncated scaffoldin the enzyme was stabilized, presumably through protection by the scaffoldin-borne cohesin. Interestingly, the *C. acetobutylicum* genome includes a complete, but essentially inactive, cellulosome gene cluster of its own [48].

Other types of carbohydrate-active enzymes can be expressed in alternative host cell systems in both a cellulosome and noncellulosome mode. Thus, *Aspergillus niger* was employed as a host-cell system for the expression of a hybrid enzyme, comprising a feruloyl esterase and C-terminal dockerin module [49]. The presence of such an enzyme in a cellulosome together with a xylanase would aid in the detachment of the hemicellulose from the lignin component of the plant cell wall. In a related work [50], the same authors succeeded in overexpressing a bifunctional noncellulosomal chimaeric enzyme that would serve the same purpose: a feruloyl esterase was fused to a xylanase and a CBM. The resultant chimaeric enzyme exhibited both catalytic activities and bound to cellulose; a synergistic effect on the degradation of complex substrates (i.e. corn and wheat brans) was observed.

A whole-cell biocatalyst with the ability to induce synergistic and sequential cellulose degradation activity was constructed through the co-display of three types of cellulolytic enzymes on the cell surface of the yeast *Saccharomyces cerevisiae* [51•]. In addition, yeast strains displaying an endoglucanase, fused with several CBMs, showed heightened binding affinity and hydrolytic activity, indicating the importance of substrate targeting to enzyme activity [52•]. In a related work, combined surface and metabolic engineering was employed to construct a xylan-fermenting yeast strain, thus demonstrating the direct conversion of xylan to ethanol [53•]. In the future, it would be interesting to see whether the hemicellulolytic strain could be used in co-culture with a cellulolytic yeast strain for the synergistic degradation of more complex cellulosic substrates. In a similar vein, a recent work [54] employed a recombinant xylose- and cellooligosaccharide-assimilating yeast strain to produce ethanol from

acid-treated wood-chip hydrolysate. However, surface display of the enzyme(s) might impair their activity, and full functionality of the cellobiohydrolase component is especially crucial for the efficient hydrolysis of crystalline cellulose, which has yet to be achieved using such systems.

Future process scenarios have been proposed that combine key production steps, thus reducing overall process complexity and cost. One notable example is the combined biomass processing technology (i.e. consolidated bioprocessing, CBP), initially proposed by Lynd and colleagues [55••] for *C. thermocellum*. The bioenergetic benefits specific to growth on cellulose result from the efficiency of oligosaccharide uptake combined with intracellular phosphorolytic cleavage of β-glucosidic bonds [56•]. The authors propose that these benefits exceed the bioenergetic cost of cellulase synthesis, supporting the feasibility of anaerobic processing of cellulosic biomass without added saccharolytic enzymes. The CBP approach was extended to ethanol production in yeast cells by cloning an endoglucanase and a β-glucosidase in *S. cerevisiae*, and the resultant recombinant strain was capable of growing on phosphoric acid swollen cellulose [57]. It is hoped that experimental breakthroughs will meet expectations raised by theoretical calculations.

The designer cellulosome concept can be combined with any of the above-mentioned strategies, by employing either a separate enzymatic step to produce sugars, by converting a non-cellulosomal microorganism into a cellulosome producer, or by re-designing the resident cellulosome of a bacterium, such as *C. thermocellum*, *C. cellulovorans* or *C. cellulolyticum*. Likewise, a combination of CBP with a redesigned cellulosome of *C. acetobutylicum* can be employed to produce butanol or, perhaps preferably, cellulosomal genes can be encoded into its cellulosome-lacking relative, *Clostridium beijerinckii*, for butanol production. In the case of *C. thermocellum*, the advantages of direct contact of the cell surface to the substrate and concomitant absorption of cellodextrins could be maintained with enhanced activity towards the desired substrate. The microorganism could also be further engineered metabolically to produce better yields of ethanol or other products. In the future, it might be possible to engineer yeast surfaces with designer cellulosomes for ethanol conversion. Combining CBP with designer cellulosomes could provide optimized degradation of a specific waste substrate; paper waste, such as paper sludge [58], would appear to be a good primary substrate for assessing such a programme. In any event, it will be necessary to expand the study of the designer cellulosome concept and genetic engineering of *C. thermocellum* to properly evaluate and develop the benefits of this approach.

Conclusions

Nature solves the problem of removing recalcitrant plant cell wall material from the environment through the action of a broad consortia of bacteria in the various cellulosic ecosystems, but over extended time periods. Decades of intensive research have demonstrated that incremental advances in scientific and/or engineering approaches to the cost-effective conversion of plant cell wall biomass to biofuels will not suffice and that major breakthroughs are required. It will be interesting to see whether future approaches (e.g. combinations of designer cellulosomes with single-cell systems like CBP), will be able to provide the quantum leap necessary to solve the problem of accumulating cellulose waste and contribute to the biomass-to-biofuels challenge.

Acknowledgements

Work in the authors' laboratory was funded by the US DOE Office of the Biomass Programme, by Research Grants 394/03 and 442/05 from the Israel Science Foundation (Jerusalem), and by grants from the United States-Israel Bi-national Science Foundation (BSF), Jerusalem, Israel.

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