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Engineered microbial systems for enhanced conversion of lignocellulosic biomass

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In order for plant biomass to become a viable feedstock for meeting the future demand for liquid fuels, efficient and cost-effective processes must exist to breakdown cellulosic materials into their primary components. A one-pot conversion strategy or, consolidated bioprocessing, of biomass into ethanol would provide the most cost-effective route to renewable fuels and the realization of this technology is being actively pursued by both multi-disciplinary research centers and industrialists working at the very cutting edge of the field. Although a diverse range of bacteria and fungi possess the enzymatic machinery capable of hydrolyzing plant-derived polymers, none discovered so far meet the requirements for an industrial strength biocatalyst for the direct conversion of biomass to combustible fuels. Synthetic biology combined with a better fundamental understanding of enzymatic cellulose hydrolysis at the molecular level is enabling the rational engineering of microorganisms for utilizing cellulosic materials with simultaneous conversion to fuel.

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Introduction

Improvements in overall efficiency and yield of producing soluble sugars from lignocellulosic biomass are being pursued from several different angles including selective breeding and genetic engineering of more labile energy crops [1,2], improved pretreatment technologies [3], and directed evolution of hydrolytic enzymes for improved activity and stability [4,5]. It is anticipated that the largest gains in cost competitiveness in terms of producing fuels from biomass could be realized through the consolidation of several production steps into a streamlined process where hydrolytic enzymes are simultaneously produced *in situ* by a solventogenic, fermentative microbe [6••]. In contrast to starch ethanol production where robust biocatalysts are available (*Saccharomyces cerevisiae*) a consolidated bioprocess (CBP) requires a highly engineered microbial workhorse that has been developed for several different process-specific characteristics (Figure 1). These desirable traits include enzyme production/stability, balanced growth on hexoses and pentoses, tolerance to pretreatment inhibitors, maximal product yield and production rates, solvent tolerance, and the ability to persevere through process fluctuations. Naturally occurring microbes possess many intrinsic qualities (i.e. cellulase production) that may advance them as candidates for use in an industrial scale consolidated processing scheme; but, deficits in other necessary phenotypes must be evolved or directly engineered into a host. This review will focus on recent advances in engineering microorganisms for cellulose utilization through the heterologous expression of non-native cellulases and hemicellulases for growth on biomass.

Multiple microbial strategies to breakdown biomass

Multiple enzymatic activities are required to hydrolyze cellulose into soluble sugars that can be metabolized by the cell. These activities include endoglucanases (EG) of the enzyme family EC 3.2.1.4; exoglucanases including D-cellodextranases within EC 3.2.1.74 and cellobiohydrolases (CBH) within EC 3.2.1.91; and finally, β-glucosidases (BGL; EC 3.2.1.21) which cleave cellobiose units into glucose monomers [7•]. Fungi of the genus *Trichoderma* express free, non-complexed (hemi)cellulases and are known to produce high titers of extracellular enzymes reaching up to 100 g l^{-1} [8]. *Trichoderma reesei* produces a cocktail of hydrolytic enzymes for degrading plant biomass including at least two cellobiohydrolases (CBH1–2), five endoglucanases (EG1–5), as well as BGL, and hemicellulases [9,10]. However the bulk of the cellulase activity in *T. reesei* is attributed to CBH1, CBH2, and EG2 [11].

The cellulosome system of complexed hydrolytic enzymes appears to be exclusively a strategy employed by anaerobic microorganisms for biomass degradation [12]. The cellulosomal architecture involves multiple catalytic components assembled together on the structural scaffoldin(s) subunit through strong non-covalent protein–protein interactions (type-I interaction) between the cohesin modules on the scaffoldin and the dockerin module borne by the individual cellulosomal enzymes [13••]. In contrast to free enzymes, which contain individual carbohydrate binding domains (CBM), cellulosomal enzymes

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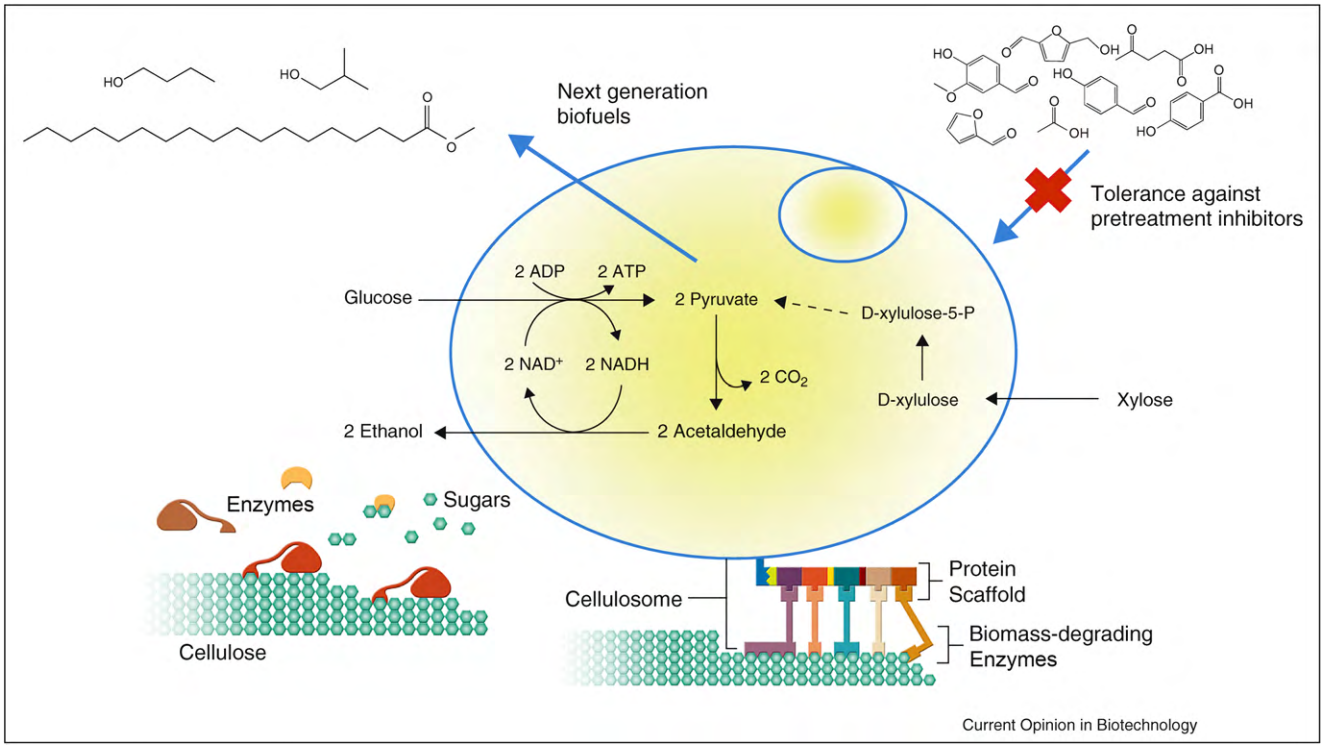
Figure 1



A conceptual whole-cell biocatalyst for producing liquid transportation fuels. The optimal organism would express a synergistic mixture of highly active exo- and endoglucanses, glucosidases, and xylanases. Both hexose and pentose sugars would be metabolized in a highly robust, balanced fermentation. The strain would exhibit a high tolerance to toxic inhibitors from pretreated biomass. In addition, next generation fungible fuels could be produced through the expression of synthetic pathways. Cellulosome and free-enzyme system images are from the U.S. Department of Energy Genome Programs image gallery (http://genomics.energy.gov) [52].

primarily rely on the cellulose-specific CBM of the scaffoldin for their collective binding to lignocellulosic substrates. Cellulosome-based complexation thus provides for spatial proximity of enzymes with complimenting catalytic activities, through their assembly on the scaffoldin, and also for targeting of the enzymes to the insoluble substrate via the CBM on the scaffoldin for concerted and efficient hydrolysis of plant biomass [14]. Cellulosomal organisms encode a wide array of catalytic subunits with diverse hydrolytic capabilities (e.g. *Clostridium thermocellum* possesses more than 70 dockerin-containing subunits), which have evolved to specifically tackle the chemical heterogeneity and structural complexity of their natural target substrate, the plant cell wall. Catalytic diversity among subunits also allows the organisms to modulate the composition and activity of cellulosomes according to the growth substrate available, as has been recently demonstrated for several *Clostridia* [15•,16,17].

Non-complexed cellulase expression in yeast

Perhaps the dominant strategy for engineering an efficient biocatalyst for ethanol production from CBP is to express multiple components of a cellulotyic system from either fungi (non-complexed) or bacteria (either non- or complexed) in *S. cerevisiae*. *S. cerevisiae* is an attractive host for developing a CBP biocatalyst given its capability of robust fermentative growth and widespread use as an industrial ethanologen. *S. cerevisiae* is amenable to genetic manipulation and highly efficient recombineering tools have been developed for constructing synthetic, multigene pathways directly within the cell [18,19,20•]. Expression and secretion of an effective cellulase system in *S. cerevisiae* has been pursued for over 20 years. Van Zyl *et al.* provided a table summarizing reports of 74 attempts to express different non-complexed cellulases in *S. cerevisiae* including CBH (exo-glucanses), EG and BGL [7]. The most common sources for non-native cellulase genes are fungi, especially *T. reesei*, however mesophilic and thermophilic bacteria also provide a source. In their review, the authors noted that cellulase (CBH) titers and specific activities are generally low from heterologous expression in yeast thus making this a focus area for future improvements. Den Hann *et al.* systematically evaluated the expression of four individual CBHs in *S. cerevisiae* originating from *T. reesei* (*cbh1* and *cbh2*), *Aspergillus niger* (*cbhB*) and *Phanerochaete chrysosporium* (*cbh1-4*) [21•]. They developed a sensitive ELISA technique that allowed a direct comparison of the specific activities of

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yeast-derived recombinant cellulases versus native enzymes. They concluded that heterologous expression of fungal CBHs in *S. cerevisiae* does not significantly reduce enzyme-specific activity but extracellular titers were insufficient to allow direct conversion of cellulose to ethanol without a ~20- to ~120-fold improvement in expressed activity. A truly cellulolytic yeast must efficiently express multiple enzymes which act synergistically to hydrolyze polymeric cellulose into monomers. While high titers continue to be a challenge, the direct yeast-driven production of ethanol from amorphous cellulose using combinations of enzymes has been demonstrated. Co-expression of an endoglucanase from *T. reesei* (*EGI*) and a β-glucosidase from *Saccharomycopsis fibuligera* (*BGL1*) allowed 1.0 g l^{-1} of ethanol to be produced from 10 g l^{-1} of phosphoric acid-swollen cellulose (PASC) as the sole carbon source [22]. Jeon *et al.* expressed a cellulosomal endoglucanase from *C. thermocellum* (*EgE*) in conjunction with *BGL1* from *S. fibuligera* which resulted in 9.67 g l^{-1} and 8.56 g l^{-1} of ethanol being produced from 20 g l^{-1} CMC and β-glucan, respectively [23•]. In another report, a non-cellulosomal endoglucanase from *Clostridium cellulovorans* produced similar results compared to *C. thermocellum EgE* expression with ethanol production reaching 9.15 g l^{-1} from 20 g l^{-1} of barley β-glucan after 50 h of incubation [24]. Yanase *et al.* also demonstrated direct ethanol production from PASC up to 2.1 g l^{-1} when EG and CBH enzymes from *T. reesei* and BGL from *Aspergillus aculeatus* were expressed and displayed on the surface of *S. cerevisiae* which produced higher ethanol yields that EG- and CBH-secreting strains [25].

The designer cellulosome

Designer cellulosomes have provided a 'bottom-up' approach for dissecting the role of various domain modules, evaluating the synergistic interplay between different Carbohydrate Active enZymes (CAZymes) [26,27,28•,29]; and, for successfully delineating the positive contributions of enzyme 'proximity' and CBM-mediated 'targeting' effects to the catalytic efficiency of cellulosomes [27,30]. The discovery of intra-species specificity in type-I cohesin–dockerin interactions, albeit with a few exceptions [31], led Bayer *et al.* to propose the idea of constructing designer cellulosomes with tailored subunit composition and defined spatial arrangement of enzymes for potential biotechnological applications [32]. By using recombinant chimeric scaffoldin(s), desired enzymes engineered to bear appropriate complimentary dockerins could be selectively incorporated in precise locations on the scaffoldin [33]. In addition to cellulosomal enzymes, several free enzymes (lacking the dockerin domain) have been engineered for designer cellulosome construction. For example, non-cellulosomal cellulases from *C. thermocellum* [34], free enzymes from *Thermobifida fusca* [29,35–37] and the fungus, *Neocallimastix patriciarum* [38•], have been successfully grafted with dockerin and converted to cellulosomal mode for incorporation onto bacterial chimeric scaffoldins. Tri-functional chimeric cellulosomes consisting of cellulosomal family 9 endoglucanase and family 48 exoglucanase from *Clostridium cellulolyticum* and xylanase XynZ from *C. thermocellum* were found to be 6-fold more efficient in hydrolyzing straw than bi-functional complexes containing only the cellulases. Complexed enzymes also displayed 4-fold higher activity than the same enzymes free in solution [27]. A similar synergistic cooperation was also observed between chimeric *T. fusca* xylanases (Xyn11A and Xyn10B) and cellulase Cel5A within designer cellulosome complexes in the hydrolysis of hatched wheat straw [37]. These results demonstrated the importance of enhanced synergy resulting from physical proximity of enzymes in complexed systems and the need for catalytic diversity, particularly in the degradation of recalcitrant lignocellulosic substrates.

Engineering cellulosomal yeast

The past year has seen several noteworthy developments in the quest for extending cellulolytic capability to yeast via cell surface display of mini-cellulosomes. Lilly *et al.* reported successful heterologous expression and surface attachment of a functional chimeric scaffoldin from *C. cellulolyticum*, containing a CBM and two divergent cohesins from *C. thermocellum* and *C. cellulolyticum*, in *S. cerevisiae* [39]. Kondo and colleagues, instead of using divergent cohesins, availed the affinity between protein A and immunoglobulin G (IgG) to construct chimeric scaffoldin containing both cohesins (from *Clostridium cellulovorans*) and Z domains (from *Staphylococcus aureus* protein A) for directed assembly and display of enzymes on the yeast cell surface [40]. Recombinant *T. reesei EG2* and *A. aculeatus BGL1*, bearing engineered Fc (from human IgG) and dockerin domains, respectively, were heterologously expressed and assembled *in vivo* into functional complexes on the cell surface, via interactions with complimentary domains on the scaffold, yielding yeast strains capable of hydrolyzing β-glucan [40]. Two recent studies reported the functional display of tri-functional mini-cellulosomes on yeast cell surfaces for simultaneous saccharification and fermentation of cellulose to ethanol. In the first study by Tsai *et al.*, functional mini-cellulosomes were assembled *in vitro* on the yeast cell surface by incubation of yeast cells displaying a chimeric scaffoldin, containing divergent cohesion domains from *C. thermocellum*, *C. cellulolyticum* and *Ruminococcus flavefaciens*, with *Escherichia coli* lysates containing recombinant endoglucanase, one each from *C. thermocellum* (CelA) and *C. cellulolyticum* (CelG) and an exoglucanase from *C. cellulolyticum* (CelE), appended with complimentary dockerin domains [41]. The displayed mini-cellulosomes synergistically hydrolyzed cellulose and by replacing one of the endoglucanases with a *C. thermocellum* BGL, the engineered yeast strain was able to directly hydrolyze and ferment PASC to ethanol, producing up to 2.6-fold higher

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ethanol levels than that achieved by using free enzymes [41]. In the second study, Zhao and coworkers achieved co-expression of enzymes and scaffoldin for *in vivo* assembly and display of uni-, bi- and tri-functional mini-cellulosomes on yeast cell surfaces [42•]. Tri-functional complexes consisting of *T. reesei* EG2, CBH2, and *A. aculeatus* BGL1 enzymes showed ~8.8-fold enhanced activity than lower order complexes because of both enzyme–enzyme and enzyme proximity synergy [42]. While the engineered yeast strains generated in these studies were able to grow on amorphous cellulose, direct conversion of crystalline cellulose to ethanol remains a key challenge and requires further research.

Synthetic cellulosomes

Significant advancements have been made in construction, heterologous expression, and *in vivo* assembly of hydrolytic enzymes onto chimeric scaffolds in non-cellulolytic microorganisms. For example, *C. cellulovorans* cellulosomal endoglucanase (*EngB*) and a truncated form of the scaffoldin (mini-*CbpA1*, containing a CBM and one cohesin) were co-expressed in *Bacillus subtilis* leading to *in vivo* synthesis and secretion of mini-cellulosomes, although the complexes lacked Avicelase activity [43]. Using a similar approach, cloning of the cellulosomal mannanase (*Man5K*) from *C. cellulolyticum* along with a truncated scaffoldin (mini-*CipC1* containing a CBM and one cohesin) in the butanol producer, *Clostridium acetobutylicum*, resulted in secretion of functional mini-cellulosomes that bound to crystalline cellulose and displayed activity towards galactomannan [44,45].

The use of protein complexes as molecular scaffolds for construction of designer cellulosomes was demonstrated using the 12-subunit stress responsive protein, SP1, from aspen trees (*Populus tremula*). Recombinant *T. fusca* cellulases (Cel5A endoglucanase [46••] and Cel6B exoglucanase [47]) appended with a dockerin module from *C. thermocellum*, were successfully assembled onto the SP1 protein scaffold displaying complimentary cohesin modules. The endoglucanase complex showed 2-fold higher specific activity on carboxymethyl cellulose than free enzymes, possibly because of concentration effects resulting from their dense packing on the scaffold. The exoglucanase complex also displayed enhanced activity over non-complexed enzymes but required low levels of free wild-type Cel5A, presumably to internally cleave cellulose chains and create new free chain ends of the substrate, for synergistic degradation of cellulosic substrates [47]. Similarly, the 18-subunit homo-oligomeric rosettazome chaperonin complex from the archaeon, *Sulfolobus shibatae*, containing fused cohesins was used as the scaffold for assembling a mixture of four cellulosomal *C. thermocellum* cellulases (Cel9B/F, Cel9K, Cel9R, Cel48S) [48•]. The synthetic rosettazyme, as the authors described it, also displayed more than 2-fold enhanced activity on Avicel than the free enzymes in solution, due to proximity-based synergy between the cellulases in their complexed state. Synthetic cellulosomes constructed to-date, however, have been much less active than their natural counterparts. Higher order complexes that can display several different enzymes with diverse catalytic activity and at different molar ratios are needed to better mimic the heterogeneity of native cellulosomes in function, composition and modular organization.

Future outlook

Overall, the increased interest in biofuels research combined with a noteworthy increase in funding through various supporting agencies such as the U.S. Department of Energy (DOE) is significantly advancing research in this area [49]. Dedicated fundamental and applied research in both the public and private sectors will make renewable fuels and chemicals a reality in our future. Just as the petroleum industry has advanced through more than a century of development, biofuel production will become more widespread and economical with time. Engineering robust, industrial biocatalysts to efficiently hydrolyze cellulose with direct conversion to ethanol will be a major achievement in the path to renewable liquid transportation fuels. This could also be combined with engineered metabolic pathways to produce more fungible fuels that have direct compatibility with the existing fleet and infrastructure [50•,51•]. While important progress is being made in current research, future advances will ultimately lead to the development of a CBP organism that will allow significant savings in production costs and enable a sustainable biofuel industry.

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