

Engineering microbial surfaces to degrade lignocellulosic biomass

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Abbreviations: CBP, consolidated bioprocessing; CEM, cellulose-enzyme-microbe; PASC, phosphoric acid swollen cellulose; RAC, regenerated amorphous cellulose; IL, ionic liquid; GPI, glycosylphosphatidylinositol; CWS, cell wall sorting signal; CBM, cellulose binding module

Renewable lignocellulosic plant biomass is a promising feedstock from which to produce biofuels, chemicals, and materials. One approach to cost-effectively exploit this resource is to use consolidating bioprocessing (CBP) microbes that directly convert lignocellulose into valuable end products. Because many promising CBP-enabling microbes are non-cellulolytic, recent work has sought to engineer them to display multi-cellulase containing minicellulosomes that hydrolyze biomass more efficiently than isolated enzymes. In this review, we discuss progress in engineering the surfaces of the model microorganisms: *Bacillus subtilis*, *Escherichia coli*, and *Saccharomyces cerevisiae*. We compare the distinct approaches used to display cellulases and minicellulosomes, as well as their surface enzyme densities and cellulolytic activities. Thus far, minicellulosomes have only been grafted onto the surfaces of *B. subtilis* and *S. cerevisiae*, suggesting that the absence of an outer membrane in fungi and Gram-positive bacteria may make their surfaces better suited for displaying the elaborate multi-enzyme complexes needed to efficiently degrade lignocellulose.

Introduction

Dwindling supplies of petroleum and the need to reduce net carbon emissions have driven the search for innovative and cost-effective methods to produce biofuels, chemicals, and materials from lignocellulosic biomass.¹ In the United States alone, it is estimated that over 1 billion tons of non-food lignocellulosic biomass can be produced annually on a sustainable basis at costs of only \$40–50 per ton.^{2,3} However, a major obstacle limiting the use of lignocellulose as feedstock is its recalcitrance to degradation.^{2,4} While a number of technologies are being explored in industry to degrade lignocellulose, enzyme-based methods predominate, and are currently used to produce cellulosic ethanol (Fig. 1A).⁴ In

this hydrolytic method, plant biomass is degraded in a two-step process in which it is first pretreated with various chemicals (e.g., acids or ionic liquids) to expose and partially degrade the cellulose and hemicellulose sugar polymers, and then hydrolyzed by adding a consortium of purified cellulase enzymes.^{4–9} Yeast then ferments the sugars into ethanol. To produce biomass-derived commodities cost-effectively, several groups are developing consolidated bioprocessing (CBP) microbes that combine cellulase production, cellulose hydrolysis, and fermentation into a single process (Fig. 1B). In principle, their use would significantly lower costs, as it would circumvent the need for adding purified cellulase enzyme cocktails and hydrolysate separation procedures.^{10–13} Avoiding the use of purified enzyme cocktails would be particularly advantageous as it is currently the single largest contributor to overall costs (\$0.68–1.47 per gallon of cellulosic ethanol).¹⁴ An ideal CBP-enabling microbe would catabolize biomass efficiently and completely, utilize all of the sugars released from the biomass, and generate products at good yields, rates, and titers. It would also require minimal nutrient supplementation, be tolerant to low pH and high temperatures, and possess generally regarded as safe (GRAS) status.^{2,13} Many promising CBP-enabling microbes possess several of these characteristics, but they are unable to degrade and use biomass as a nutrient. To overcome this limitation, several groups have devised methods to create recombinant cellulolytic microbes that deconstruct plant biomass using surface displayed cellulases.

In order to degrade the complex structure of plant biomass, naturally cellulolytic microbes produce an array of cellulases that have different substrate specificities. Although a variety of plants are being considered as industrial feedstocks (corn stover, straw, *Miscanthus*, switchgrass, poplar, sugarcane bagasse, etc.), their cell walls all contain lignocellulose which is comprised of varying amounts of cellulose (25–55%), hemicellulose (8–30%), and lignin (18–35%) (Fig. 2).¹⁵ The most abundant component, cellulose, is a homopolymer of β -1,4-linked glucose molecules that are hydrogen bonded with other cellulose polymers to form both amorphous and crystalline regions, the latter of which is particularly recalcitrant to degradation.¹⁶ Naturally cellulolytic microorganisms produce three main types of cellulases that function synergistically: endoglucanases, exoglucanases,

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and β -glucosidases.¹⁷ Endoglucanases hydrolyze internal β -1,4-glucosidic bonds in the polymer, creating reducing and non-reducing ends that are further hydrolyzed by exoglucanases.¹⁸ Working together, the enzymes create shorter cellodextrins, including the disaccharide cellobiose, which is degraded into its component sugars by β -glucosidases.¹⁸ The hemicellulose component of lignocellulose is a heterogeneous polymer of pentose and hexose sugars.¹⁹ To liberate these sugars, microbes employ a variety of hemicellulases that have distinct substrate specificities, including exoxylanases, endoxylanases, arabinases, and mannanases, among others.²⁰ Finally, the cellulose and hemicellulose carbohydrate polymers are embedded in lignin, a complex polymer containing a mix of phenolic compounds connected by a variety of linkages.²¹ Microbial lignin degradation remains poorly understood, but in white-rot fungi, it is mediated by a combination of extracellular peroxidases and laccases.²²

Recent work has engineered microorganisms to display multicellulase containing complexes called minicellulosomes (Fig. 3). These complexes are miniaturized versions of the cellulosomes used by naturally cellulolytic anaerobes to degrade plant biomass. Native cellulosomes contain a variety of cellulases that function synergistically to degrade biomass more efficiently than isolated enzymes. The cellulosome from the cellulolytic thermophile *Clostridium thermocellum* is archetypal (Fig. 3A). It contains a central scaffoldin protein, CipA, which coordinates the binding of nine cellulases.²³ Binding is mediated by type-I cohesin modules within CipA that interact with sub-nanomolar affinity with type-I dockerin modules present in the cellulases.²⁴ CipA also contains a carbohydrate-binding module (CBM) that tethers the cellulosome complex to its substrate, as well as a type-II dockerin module located at its C-terminus that anchors the cellulosome complex to cell wall associated proteins.²⁵ Other species of anaerobic bacteria also display cellulosomes, which can adopt more elaborate structures that contain as many as 96 enzymes.²⁴

Surface displayed minicellulosomes exhibit enhanced cellulolytic activity. Studies have shown that co-localizing cellulases with different substrate preferences into a cellulosome facilitates enzyme-enzyme synergism; the enzymes in the complex collectively exhibit greater cellulolytic activity than the sum of the activities of the isolated enzymes.²⁶ Synergy occurs because the enzymes have complementary activities, and their spacing and relative abundance is presumably optimal. The presence of both hemicellulolytic and cellulolytic activities in the cellulosome is also advantageous, since by working together these enzymes can remove “physical hindrances” blocking substrate access (e.g., hemicellulolytic enzymes degrade hemicellulose polysaccharides that might otherwise block access to cellulose). The displayed cellulosomes also tether the microbe to the biomass, thereby promoting cellulose-enzyme-microbe (CEM) synergistic interactions that increase the rate of hydrolysis.²⁷ CEM interactions minimize the distance over which the hydrolysis products must diffuse to the cell, facilitating more efficient sugar uptake and preventing the build-up of potential enzyme inhibitors (e.g., cellobiose and glucose).²⁸ It may also facilitate biomass degradation by promoting favorable substrate channeling of long-chain hydrolysis products to proximally bound cells. Thus, CBP-enabling microbes

that display minicellulosomes should degrade biomass more rapidly and thoroughly than microbes that only secrete cellulases.

There have been many excellent reviews describing efforts to create cellulolytic and consolidated bioprocessing microorganisms.^{2,10,13,29} In this review, we focus solely on recent synthetic biology efforts to construct microbes that display cellulases and miniaturized cellulosomes (minicellulosomes). Specifically, we review progress in engineering three microorganisms: *Saccharomyces cerevisiae*, *Escherichia coli*, and *Bacillus subtilis*. Because they are well studied and robust genetic tools are available to manipulate them, they serve as model organisms for eukaryotes, and Gram-negative and Gram-positive eubacteria, respectively. Here we discuss the distinct approaches used to display cellulase complexes on their structurally unique surfaces, and we compare the cellulolytic activities that have been thus far achieved. This exciting work may lead to the direct use of these microbes in consolidated bioprocessing and it promises to facilitate the engineering of other industrially useful microbes.

Engineering Yeast to Display Cellulase Enzymes

Since *S. cerevisiae* is already used industrially to produce ethanol from corn, considerable effort is being put forth to create recombinant cellulolytic strains that can degrade and utilize non-edible lignocellulose as a nutrient. While cellulase secreting yeast strains have been constructed, recent work is focused on generating strains that display cellulases and minicellulosomes in order to obtain improved cellulolytic activity. Cellulases are displayed on the cell surface using two related approaches. In the first approach, they are expressed as fusion proteins that contain a glycosylphosphatidylinositol (GPI) anchor signal sequence that is typically derived from the yeast Aga1 or Cwp2 proteins (Fig. 4A). After protein synthesis, the GPI anchor is added to the signal sequence's ω -site amino acid by the GPI transamidase complex in the endoplasmic reticulum.³⁰⁻³² GPI attachment initially targets the protein to the lipid bilayer, however, the protein is subsequently processed so as to become covalently linked to outer cell wall β -1,6-glucan, resulting in its display.³²⁻³⁴ In a second related approach, proteins are expressed as fusions to the yeast Aga2 protein, which associates with the endogenous Aga1 protein naturally displayed on the cell surface. Using these display systems, 1×10^4 – 1×10^5 proteins can be displayed per cell.³⁵

Displaying non-complexed enzymes

Initially, the Aga1 fusion system was used to display non-complexed cellulases. In pioneering work by Tanaka and colleagues, non-complexed endoglucanases and β -glucosidases from *Aspergillus aculeatus* were displayed by expressing each as a fusion protein containing a C-terminal GPI-anchor signal sequence.³⁶ Cells displaying these enzymes could degrade cellodextrins, soluble glucose polymers that are more readily degraded by enzymes than the insoluble cellulose present in lignocellulose.³⁶ During the past decade, Kondo and colleagues created cells with significantly improved cellulolytic activity and explored their ability to ferment cellulose into ethanol. They initially constructed strains that displayed two cellulases via a C-terminal GPI molecule, the *T. reesei* EGII endoglucanase and the *A. aculeatus* BGL1 β -glucosidase

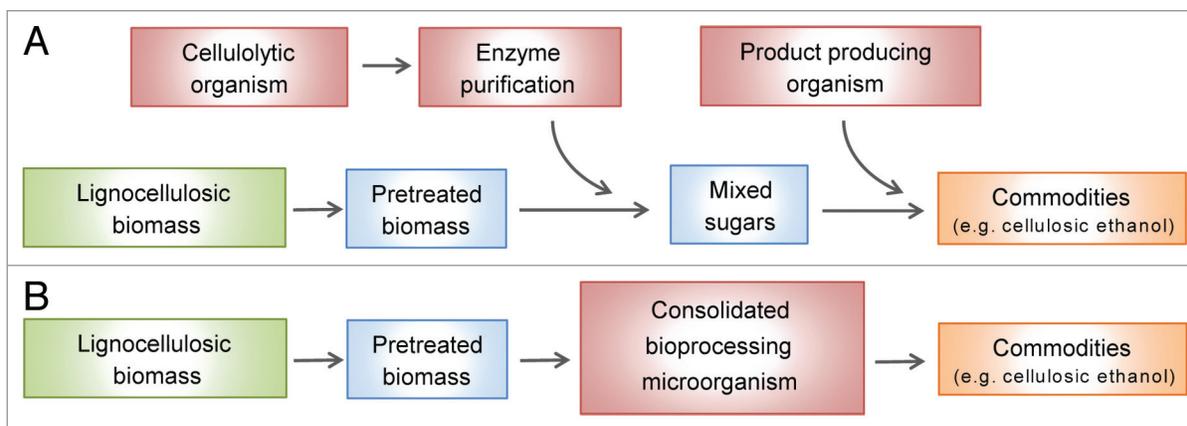


Figure 1. Consolidated bioprocessing of lignocellulosic biomass by cellulase displaying microbes. **(A)** The current steps involved in the industrial processing of plant biomass into ethanol using cellulase enzymes. Biomass degradation involves thermochemical pretreatment to expose its cellulose polymers, followed by exposure to purified cellulases to degrade cellulose into its component sugars. This is followed by a fermentation step in which yeast convert the sugars into ethanol. In principle, many other biocommodities can be produced from plant biomass using similar methods. **(B)** Steps in the consolidated bioprocessing (CBP) of biomass. CBP-enabling microbes would produce cellulase enzymes that degrade the cellulose and hemicellulose components of biomass, and then convert the resultant sugars into useful biocommodities. Microbes that naturally or recombinantly display cellulase enzymes are well suited for this process, as they are highly cellulolytic.

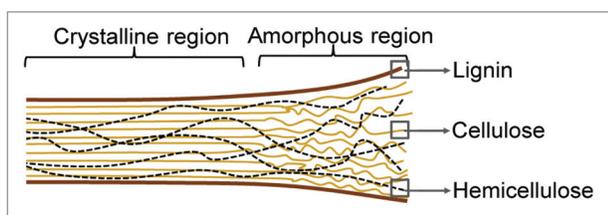


Figure 2. Schematic showing the structure of lignocellulose. Lignocellulose is composed of three components, including cellulose (solid tan lines), hemicellulose (dotted black lines), and lignin (solid brown lines). Cellulose is composed of β -1,4-linked glucose polymers, while hemicellulose is composed of a variety of pentoses. Lignin, which provides structural support for lignocellulose, coats these polymers.

enzymes.³⁷ After pre-culturing in nutrient-rich media, these strains fermented soluble β -glucan into ethanol. Later, the investigators improved activity by adding a third enzyme and several cellulose binding modules (CBM).^{38,39} These cells are capable of fermenting amorphous phosphoric acid swollen cellulose (PASC) into ethanol, which is a better lignocellulose mimic than soluble celloextrins. The authors also demonstrated the industrial utility of the cells by showing that they could produce ethanol from acid pretreated rice straw using a simultaneous saccharification and fermentation (SSF) process.⁴⁰ Although ethanol production still required the addition of a cellulase cocktail, as compared with native yeast strains that do not display cellulases, the amount of purified enzymes that needed to be added to hydrolyze the lignocellulose in the SSF process was reduced 10-fold. In addition, 1.4-fold more ethanol was produced (43.1 g/L ethanol from 200 g/L cellulosic material). The cellulase displaying cells could also be recycled between lignocellulose digestions, further demonstrating their practicality.⁴¹ Most recently, improved ethanol production from PASC was achieved by co-expressing a celloextrin transporter, an intracellular β -glucosidase and three non-complexed displayed

enzymes (endoglucanase, cellobiohydrolase, and β -glucosidase).⁴² Although the amount of ethanol produced was still low compared with industrial production levels (4.3 g/L ethanol from 20 g/L PASC), the results of this study highlight the benefits of optimizing both cellulase display and product import.

Displaying minicellulosomes

Because enzymes in cellulosome complexes degrade cellulose more efficiently than non-complexed enzymes, several groups have created yeast strains that display minicellulosomes. These complexes resemble the CipA cellulosome from *C. thermocellum* and are composed of a surface-displayed scaffoldin that contains cohesin modules that non-covalently bind to dockerin-cellulase fusion proteins (Fig. 3A). In 2009, two groups independently demonstrated that it was possible to display minicellulosomes on the surface of *S. cerevisiae* using an ex vivo assembly method in which yeast cells displaying a scaffoldin are incubated with a solution of purified cellulase-dockerin fusion proteins produced in *E. coli* (Fig. 3B).^{43,44} In these complexes, the scaffoldin is either directly fused to a GPI anchor signal sequence or it is fused to the Aga2 protein.^{43,44} Chen and colleagues constructed a minicellulosome that contained three enzymes targeted to specific sites within the complex via species-specific cohesin-dockerin interactions.⁴⁴ By incorporating endoglucanase, exoglucanase, and β -glucosidase enzymes into the complex, the investigators generated yeast cells that could produce ethanol from insoluble PASC, a notable improvement over older-generation yeast strains that displayed non-complexed enzymes.⁴⁴ In their systematic analysis, they demonstrated that the enzymes acted synergistically to hydrolyze cellulose, one of the first times enzyme-enzyme synergy was demonstrated in a cell surface displayed complex. Later, to eliminate the need to add purified enzymes to their cells, the investigators constructed surface-displayed minicellulosomes using a consortium of four yeast strains.^{45,46} In this ex vivo assembly method, cells displaying a

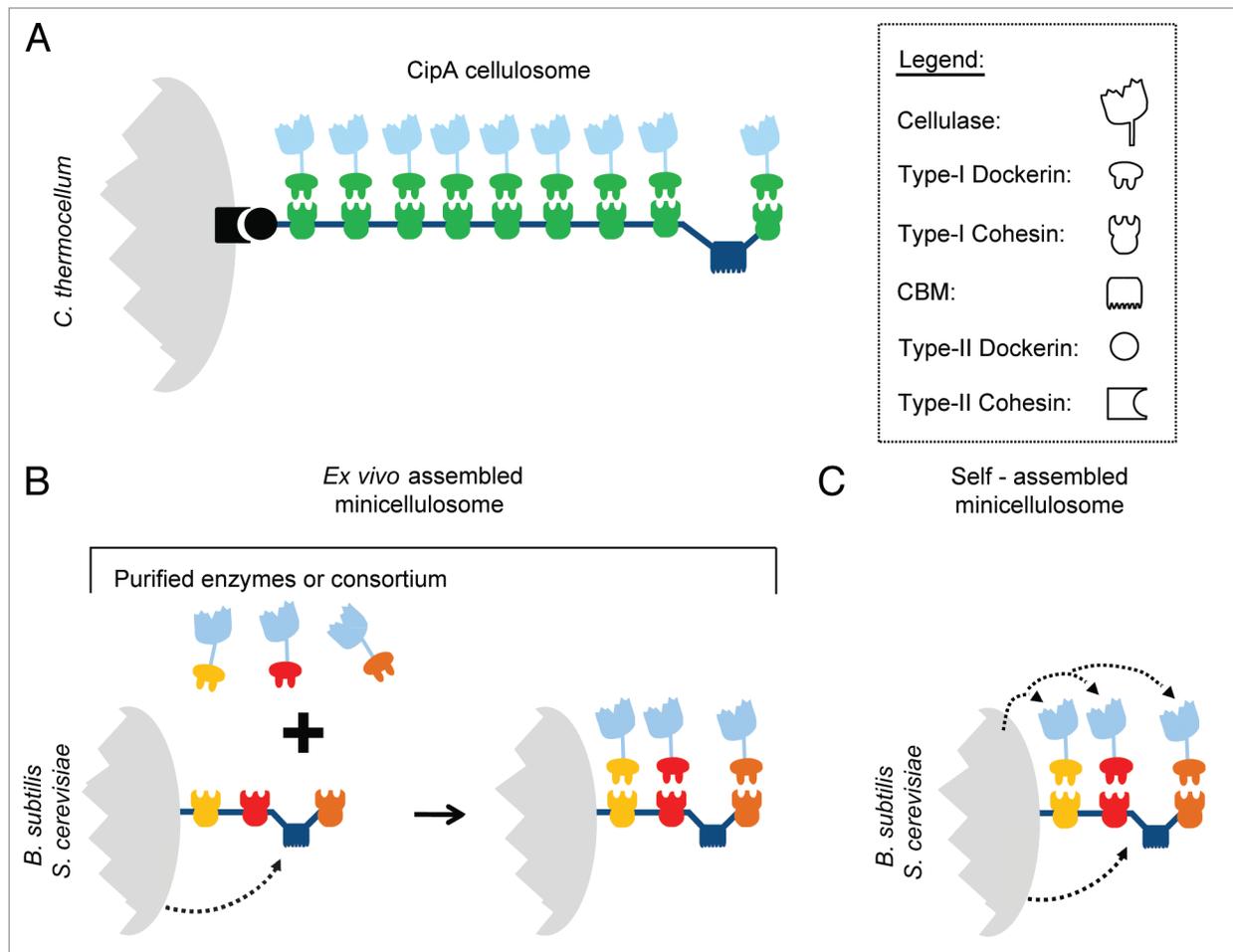


Figure 3. The prototypical CipA cellulosome and methods used to recombinantly display miniaturized cellulosomes (minicellulosomes). **(A)** Architecture of the prototypical CipA cellulosome produced by *C. thermocellum*. It houses 9 cellulase enzymes that are bound to the central scaffoldin protein, CipA.²³ Binding is mediated by type-I cohesin modules within CipA that interact with sub-nanomolar affinity with type-I dockerin modules present in the cellulases. CipA also contains a carbohydrate-binding module (CBM) that tethers the cellulosome complex to its substrate, as well as a type-II dockerin module located at its C-terminus that anchors the cellulosome complex to the cohesin module of cell wall associated proteins. **(B)** Ex vivo approach used to display minicellulosomes on the surfaces of *B. subtilis* or *S. cerevisiae*. The microbes secrete and display a scaffoldin protein that is displayed on their surface. Cellulase enzymes containing the appropriate type-1 dockerin module are incubated with the cells to construct the minicellulosome. The enzymes that are added to the cells are either purified enzymes or secreted by other microbes as part of a microbial consortium. Distinct colors are used to indicate species-specific type-1 dockerin and cohesin domains that selectively interact with one another to construct the minicellulosome. **(C)** Self-assembled approach used to display minicellulosomes on the surfaces of *B. subtilis* or *S. cerevisiae*. All of the components of the minicellulosome (scaffoldin and enzymes) are produced by the microbe and spontaneously assemble on the cell surface.

scaffoldin are co-cultured with strains capable of secreting cellulase-dockerin fusion proteins, eliminating the need to add purified enzymes.⁴⁶ After finding the optimal ratio of strains to maximize ethanol production, they demonstrated that ~1.87 g ethanol/L could be produced from PASC. Interestingly, in this system, the mechanism of scaffoldin anchoring appears to affect the efficiency of display, as an increased population of cells displaying minicellulosomes were observed when the scaffoldin was directly modified with a GPI anchor signal sequence instead of the Aga1-Aga2 anchoring system.⁴⁵ Very recently, Hahn and colleagues used a similar consortium approach to construct cells that display ex vivo assembled minicellulosomes and they showed that the cells produce similar amounts of ethanol from PASC (1.8 g/L ethanol).⁴⁷

Ex vivo assembly may be impractical for industrial applications, because it requires that purified enzymes be added to cells or that a consortium of different strains is used to construct the cellulosome. Two research groups have overcome this problem by constructing yeast cells that spontaneously assemble minicellulosomes on their surface (Fig. 3C).^{26,48} Zhao and colleagues were the first to achieve this milestone by constructing yeast cells that displayed a spontaneously assembling three-enzyme minicellulosome.²⁶ This strain produced all of the components of the complex, including an Aga1-Aga2 tethered scaffoldin derived from *C. thermocellum* CipA and the *T. reesei* endoglucanase EGII, cellobiohydrolase CBHI, and *A. aculeatus* β -glucosidase BGLI enzymes. By systematically comparing the activities of uni-, bi-, and tri-functional minicellulosomes, they demonstrated that

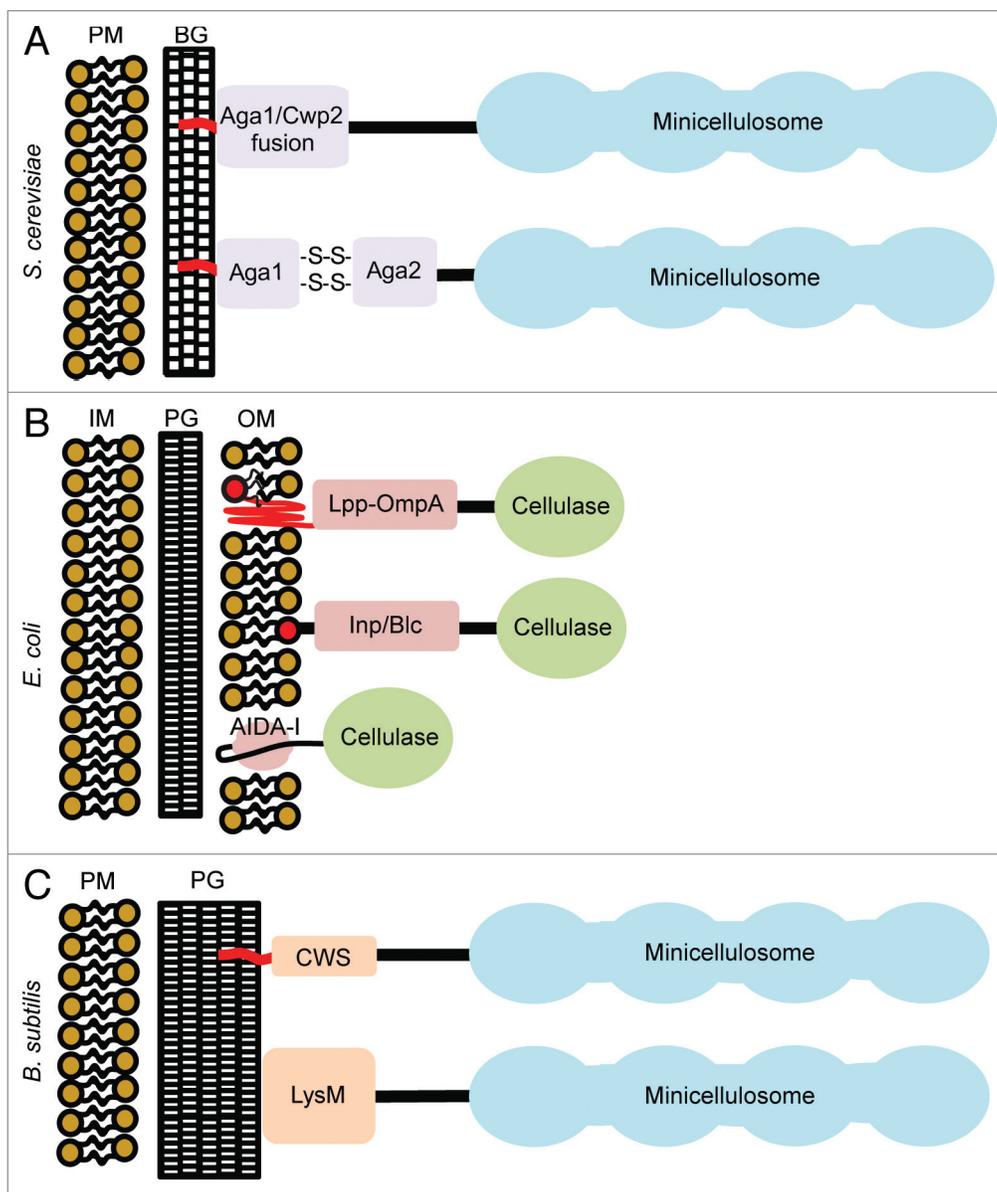


Figure 4. Approaches used to display cellulases and minicellulosomes on different types of microbes. **(A)** *S. cerevisiae*: Proteins are displayed on the cell surface by embedding them into the lipid bilayer via a covalently attached C-terminal glycosylphosphatidylinositol (GPI) molecule. Heterologous proteins are displayed by either appending a GPI anchor signal sequence to their C-terminus (typically derived from the Aga1 or Cwp2 proteins) or by expressing them as fusion proteins with Aga2, a yeast protein that associates with the Aga1 protein naturally displayed on the cell surface. $\sim 1 \times 10^4$ – 1×10^5 proteins are attached per cell.³⁵ This approach has been used to display a minicellulosome. **(B)** *E. coli*: Heterologous proteins are expressed as fusion proteins with lipoproteins (e.g., Lpp-OmpA, Inp, and Blc) or to the autotransporter AIDA-I. This results in the protein being embedded in the outer membrane (OM). Sixty thousand proteins are attached per cell using the Lpp-OmpA display system.⁵³ At present, only non-complexed cellulases have been displayed using this approach. **(C)** *B. subtilis*: Two methods are used to display proteins in this microbe. They are displayed non-covalently by expressing them as fusions with the LysM protein that interacts with cell wall N-acetylmuramic acid and N-acetyl-D-glucosamine.⁶⁴ Alternatively, proteins containing a C-terminal cell wall sorting signal (CWS) are covalently linked to the peptidoglycan cross-bridge by the sortase transpeptidase enzyme.⁶⁶ The LysM and sortase approaches are estimated to result in the display of 1.2×10^7 and 2.4 – 3×10^5 proteins per cell, respectively.^{63,67,68} Both approaches have been used to display minicellulosomes. Key: PM, plasma membrane. BG, β -glucan. IM, inner membrane. PG, peptidoglycan. OM, outer membrane.

enzyme-enzyme synergistic interactions improved activity up to 1.6-fold. Moreover, cells displaying tri-functional minicellulosomes exhibited improved growth on PASC and could use it as a carbon source to produce 1.8 g/L of ethanol.

Tan and colleagues have displayed the largest and most complex self-assembling minicellulosome reported to date.⁴⁸ To

avoid having to display a long scaffoldin protein, the minicellulosome was constructed using two scaffoldins. The Aga1-Aga2 attached scaffoldin II protein is associated with the cell wall and coordinates the binding of scaffoldin I, mimicking nested architectures found in nature. Scaffoldin II contains four type-II cohesin modules from *C. thermocellum*, which coordinate

the binding of four scaffoldin I proteins via its type-II dockerin. Scaffoldin I also contains a CBM and three type-I cohesin modules from different bacterial species that enable species-specific placement of dockerin fused enzymes.⁴⁸ The cellulases and scaffoldin I are secreted using α -factor, and therefore their assembly and attachment to the cell presumably occurs extracellularly, avoiding export problems that would occur if the complex were assembled intracellularly. This approach enabled up to 12 enzymes to be displayed, four copies each of the *C. cellulolyticum* celCCA endoglucanase, celCCE cellobiohydrolase, and Ccel_2454 β -glucosidase. However, there may be a limit to the size of the scaffoldin that can be attached to the cell wall, as the investigators discovered that the percentage of cells displaying scaffoldin II decreased when longer scaffoldin II polypeptides were expressed. In the end, they chose to work with yeast displaying only 6 enzymes, two copies each of the endoglucanase, exoglucanase, and β -glucosidase, which produced 1.4 g/L of ethanol from insoluble Avicel. Very recently, Chen and colleagues used a similar “adaptive assembly” strategy to display a four enzyme containing minicellulosome that required ex vivo assembly.⁴⁹ It also contains two scaffoldins enabling the display of four enzymes; two copies each of an endoglucanase and a β -glucosidase. Although the requirement for ex vivo assembly limited the ability of these cells to grow using cellulose as a nutrient, they could produce 1.9 g ethanol/L from PASC, which was double the amount of ethanol produced by cells displaying a related minicellulosome that contained a total of only two enzymes.

Recombinant Cellulolytic Eubacteria

Many species of eubacteria are promising consolidated bioprocessors because they are already used industrially to produce chemicals (e.g., amino acids, vitamins, solvents, etc.). They can be divided into Gram-negative and Gram-positive groups, whose distinct cell surface architectures necessitate that different approaches be used to display proteins. Work thus far has concentrated on the model Gram-negative and Gram-positive microorganisms *Escherichia coli* and *Bacillus subtilis*, respectively. They are not naturally cellulolytic, but contain robust genetic systems that enable their genetic manipulation. The most progress has been made with *B. subtilis*, leading to the display of minicellulosomes that enable it to grow on untreated lignocellulose, while only non-complexed cellulases have been displayed on the surface of *E. coli*. Below we describe this work, which could lead to their direct use in the consolidated bioprocessing of biomass, and facilitate the introduction of cellulolytic activity into less well studied industrially useful eubacteria.

Single Cellulase Display on the Surface of *E. coli*

The cell wall in Gram-negative bacteria consists of inner and outer membranes separated by peptidoglycan. In the model Gram-negative organism *E. coli*, a variety of approaches have been developed to display heterologous proteins in the outer membrane (Fig. 3B).⁵⁰ Two general approaches have been used

to display cellulase enzymes on the surface of *E. coli*. In the first approach, the cellulase is expressed as a fusion protein with an *E. coli* lipoprotein. For example, display has been achieved by fusing to the N-terminus of a cellulase the signal sequence and the first nine amino acids from the major outer membrane lipoprotein (Lpp) and the transmembrane domain from the outer membrane protein (OmpA).⁵¹ The Lpp component targets and anchors the protein to the outer membrane, while the OmpA segment is required for surface expression of the passenger cellulase.⁵² Using an Lpp-OmpA display system, $\sim 6 \times 10^4$ proteins can be displayed per cell.⁵³ Similar display strategies fuse cellulases to either the Ice nucleation protein (Inp) or the bacterial lipocalin (Blc) lipoproteins that are in turn embedded in the outer membrane.⁵⁴⁻⁵⁸ In the second approach, the cellulase is displayed on the bacterial surface using the type V secretion system.⁵⁹ Specifically, the enzyme is expressed such that it contains an N-terminal signal sequence and the C-terminal translocator domain derived from the autotransporter AIDA-I protein which is embedded in the outer membrane.

At present, only non-complexed cellulases have been displayed on the surface of *E. coli*.^{51,54-59} As only a single type of enzyme was displayed, these cells exhibit limited cellulolytic activity. Although multi-enzyme display is desirable to maximize cellulolytic activity, *E. coli* cells that secrete cellulases can also degrade biomass. Recently, Keasling's group engineered a consortium of enzyme-secreting *E. coli* cells that can degrade ionic liquid (IL) pretreated switchgrass and produce a range of chemicals (butanol, fatty acid ethyl esters, and pinene).⁶⁰ After 48 h, cell densities of 140×10^7 CFU/mL were obtained, which is $\sim 50\%$ lower than *E. coli* cells grown in minimal media containing glucose as a carbon source. The rate of lignocellulosic degradation may be growth limiting, as the consortium digested only $\sim 5-6\%$ of the cellulose and hemicellulose. An endoglucanase was also displayed on the surface of the Gram-negative bacterium *Zymobacter palmae* by fusing it to the ice nucleation protein from *Pseudomonas syringae*. However, the activity of these cells was only verified using soluble CMC as a substrate.⁶¹

Cellulolytic *Bacillus subtilis*

Gram-positive bacteria typified by *B. subtilis* contain a single membrane surrounded by a thick peptidoglycan cell wall. Two approaches are used to display cellulases and minicellulosomes on its surface (Fig. 4C). Proteins are displayed non-covalently by expressing them as fusions with the LysM protein that contains binding modules that interact with N-acetylmuramic acid and N-acetyl-D-glucosamine within the cell wall, or they are covalently attached to the peptidoglycan using sortase transpeptidase enzymes.⁶²⁻⁶⁵ In the latter procedure, the protein is expressed as a fusion protein that contains a C-terminal cell wall sorting signal (CWS), which is then covalently linked to the peptidoglycan cross-bridge by the sortase.⁶⁶ Although direct comparisons have not been made, Chen et al. concluded that 1.2×10^7 proteins are attached to each cell using the LysM fusion approach, whereas it is estimated that $2.4-3 \times 10^5$ proteins can be displayed per cell using sortase transpeptidases.^{63,67,68}

Two research groups reported the construction of *B. subtilis* cells that display an ex vivo assembled minicellulosome (Fig. 3B).^{63,65} Zhang and colleagues created cells that display a minicellulosome that is non-covalently associated with the cell wall. These cells secrete the mini-CipA scaffoldin, which associates with the cell wall via its LysM domain. Mini-CipA also contains three cohesin modules and a CBM derived from *C. thermocellum* CipA.⁶⁵ To construct the minicellulosome, cells displaying mini-CipA were incubated with three *E. coli* purified cellulases (*B. subtilis* endoglucanase Cel5, *C. phytofermentans* exoglucanase Cel48, and *C. thermocellum* endoglucanase Cel9). Interestingly, similar to *C. thermocellum* that naturally displays a cellulosome, the recombinant *B. subtilis* cells exhibited cellulolytic CEM synergy, as the activity of the surface-displayed complex was superior to a purified minicellulosome that contained the same enzymes; the cells degraded RAC and microcrystalline cellulose 2.3- and 4.5-fold better than the isolated complex, respectively. It was proposed that interactions between cells might also contribute to CEM synergy as presumably adjacent cells can assimilate long-chain hydrolysis products before they diffuse into the bulk phase, which prevents product inhibition of the cellulases and cellulosomes. Independently, Anderson and colleagues engineered *B. subtilis* cells to display an ex vivo assembled minicellulosome that is covalently attached to the cell wall.⁶³ In this system, a scaffoldin (called Scaf) is joined via a peptide bond to the cross-bridge peptide of the peptidoglycan by a heterologous sortase enzyme.⁶⁶ Tri-functional minicellulosomes are then assembled when the Scaf displaying cells are incubated with the appropriate purified cellulase-dockerin fusion proteins (the *C. thermocellum* endoglucanase Cel8A and the *C. cellulolyticum* exoglucanase Cel9E and endoglucanase Cel9G enzymes). These cells degraded RAC, and exhibited enzyme-enzyme synergism that increased cellulolytic activity 1.3-fold.⁶³ Importantly, the sortase displayed minicellulosome exhibited stable activity for up to 70 h when the WprA cell wall protease was genetically eliminated, presumably because the complex is covalently linked to the cell wall. A sortase-utilizing system was also used to display miniaturized scaffoldins on the cell surface of *L. lactis*, which could then bind purified β -glucuronidase UidA.⁶⁹

B. subtilis cells displaying a self-assembled minicellulosome grow on untreated lignocellulose

To overcome the requirement for ex vivo assembly, we recently engineered *B. subtilis* cells that display a covalently attached minicellulosome that assembles spontaneously (Fig. 3C).⁶² The minicellulosome was constructed by co-expressing five proteins: the SrtA sortase from *B. anthracis*, a chimeric scaffoldin (Scaf) composed of three cohesin modules that are covalently attached to the cell wall by SrtA, and three dockerin-cellulase fusion proteins that bind to the scaffoldin non-covalently via species-specific dockerin-cohesin interactions. Three enzymes derived from the mesophile *C. cellulolyticum* were displayed (endoglucanase/xylanase Cel5A, exoglucanase/endoglucanase Cel9E, and the processive endoglucanase Cel48F) and based on immunoblot analyses, they are present in the complex at saturating levels. The cells exhibit potent cellulolytic activity enabling growth on dilute acid-pretreated corn stover to densities similar to those

achieved by cells cultured in minimal media containing glucose. Recombinant azide-treated *B. subtilis* cells required ~96 h at 37 °C to degrade 62% of a 5 g/L solution of pretreated corn-stover biomass. Azide-killed cells supplemented with β -glucosidase released 21% and 33% of the glucose and xylose found in corn stover after 48 h. This result is promising, since when assayed under similar conditions at 37 °C, cells displaying a tri-functional minicellulosome exhibit ~1/3 the cellulolytic activity of a Ctec2/Htec2 enzyme cocktail (Novozymes) that contains dozens of enzymes. Importantly, bacteria displaying the tri-functional minicellulosome could also grow on untreated plant biomass (corn stover, straw, or switchgrass) to high cell densities. To the best of our knowledge, this is the first such demonstration of this capability by a recombinant organism. The specific growth rates of cells cultured in minimal media containing 0.5% wt/volume of glucose, acid-treated corn stover, and untreated corn stover were ~0.17, ~0.08, and ~0.05 per hour, respectively. Additional improvements in their activity are needed if they are to rival naturally cellulolytic microbes such as *C. thermocellum*, which at 60 °C degrades 2 g/L of microcrystalline Avicel in 20 h.⁷⁰ Creating recombinant *B. subtilis* that display more than three types of enzymes can be expected to lead to even more potent cellulolytic microbes with better growth properties.

Comparing Activities of Recombinant Cellulolytic *B. subtilis*, *E. coli*, and *S. cerevisiae*

Direct comparisons of the cellulolytic activities of the microbes discussed in this review are not possible because different experimental approaches and cellulose substrates have been used to assess their activities. The methods range from detailed analyses of the amount of biomass degraded and the sugars produced from hydrolysis, to less informative approaches that monitored only microbial growth or ethanol production. Further hindering direct comparisons, the identities and numbers of enzymes displayed can vary between each study and there are differences in the abilities of each microbe to import and metabolize the lignocellulosic degradation products. A variety of substrates have been used to assess the cellulolytic activity of recombinant microbes. They vary in their recalcitrance to enzymatic degradation because they differ in their crystallinity, degree of polymerization, fraction of reducing ends, and presence of hemicellulose and lignin.²⁹ Based on these properties, the substrates range from easy to difficult to degrade as follows: PASC/RAC < microcrystalline cellulose < pretreated lignocellulose < untreated lignocellulose. With these considerations in mind, below we compare the cellulolytic activity of the recombinant microbes that have been discussed in this review.

The data shown in Table 1 compares the cellulolytic activities of the recombinant microbes discussed in this review. For simplicity, only strains capable of degrading insoluble forms of cellulose without the need for adding purified enzyme cocktails are considered. *B. subtilis* cells displaying a covalently attached self-assembling minicellulosome have the highest demonstrated activity.⁶² They degrade the most complex forms of cellulose, both untreated and acid-treated lignocellulose. Notably, these

Table 1. Cellulolytic activities of recombinant cellulase displaying microbes

Recombinant Microorganism	Cellulase(s) ^a Displayed	Insoluble Cellulose Substrate			
		PASC/RAC	Microcrystalline Cellulose	Pretreated Lignocellulose	Untreated Lignocellulose
<i>B. subtilis</i>					
Anderson et al. 2011	Ex vivo mini. ^a	+	-	-	-
You et al. 2012	Ex vivo mini.	+	+	-	-
Anderson et al. 2013	Self-assembled mini. ^a	-	-	+	+
<i>S. cerevisiae</i>					
Tsai et al. 2009	Ex vivo mini.	+, E ^b	-	-	-
Yanase et al. 2010	Non-complexed cellulase. ^a	+, E	-	-	-
Tsai et al. 2010	Ex vivo mini. Consortium. ^a	+, E	-	-	-
Wen et al. 2010	Self-assembled mini.	+, E	-	-	-
Goyal et al. 2011	Ex vivo mini. Consortium.	+, E	-	-	-
Fan et al. 2012	Self-assembled mini.	+, E	+, E	-	-
Tsai et al. 2013	Ex vivo mini.	+, E	-	-	-
Kim et al. 2013	Ex vivo mini. Consortium.	+, E	-	-	-

^aEx vivo mini: minicellulosome assembled ex vivo. Self-assembled mini: minicellulosome assembled spontaneously. Non-complexed cellulase: individually displayed enzyme. Consortium: multiple strains required to assemble minicellulosome. ^bE, ethanol.

bacteria grow in minimal media containing industrially relevant forms of untreated lignocellulosic biomass as a primary nutrient source (corn stover, hatched straw, and switchgrass) to densities that are similar to those achieved by cells that are cultured in glucose. *B. subtilis* cells displaying an ex vivo assembled complex can degrade microcrystalline cellulose, but the need to add purified enzymes to construct the complex limits their ability to replicate using cellulose as a nutrient.^{62,65} The most cellulolytic yeast strains reported to date display a complex that contains 6 enzymes (two copies of three types of enzymes).⁴⁸ These cells degrade microcrystalline cellulose, but their ability to metabolize more complex lignocellulose has not been tested. In addition, growth of these cells on microcrystalline cellulose required supplementation with rich nutrients unlike recombinant *B. subtilis* grown on biomass. Notably, the same group reported cells that display 12 enzymes, but these microbes were less cellulolytic because fewer enzyme complexes were displayed per cell, presumably because of the increased metabolic burden of displaying this large complex. Thus far, the surface of *E. coli* has only been engineered to display non-complexed cellulases, with only a single type of enzyme displayed on each cell.^{51,55,56,71} As expected, these cells have limited degradative capacity, as they only demonstrated significant activity on CMC. The limited progress that has been made thus far in engineering the surface of *E. coli* to display multi-enzyme complexes may be due to difficulties associated with exporting proteins across its two membranes and may explain why cellulosomes are predominantly found in Gram-positive species.

Because these model organisms are still under development, the cellulolytic activity that can ultimately be obtained by engineering their surfaces remains unknown. However, *B. subtilis* may have the greatest potential for further development because a higher density of enzyme complexes can presumably be displayed on its surface. This may result in increased rates of cellulolysis,

as more enzymes will be available to degrade the cellulose fibers bound to each cell. Specifically, based on documented levels of heterologous protein display, *B. subtilis* can display $\sim 60\text{--}3.2 \times 10^4$ times more proteins per micron² of surface area as compared with *S. cerevisiae* (*B. subtilis* and *S. cerevisiae* display $\sim 2.4 \times 10^5\text{--}1.2 \times 10^7$ and $\sim 1 \times 10^4\text{--}1 \times 10^5$ heterologous proteins per cell resulting in surface densities of $\sim 1.9 \times 10^4\text{--}9.6 \times 10^5$ and $\sim 30\text{--}300$ proteins per micron², respectively).^{35,67,68} The surface of *E. coli* can also be densely coated with proteins ($\sim 6.4 \times 10^3$ proteins/micron² assuming 6×10^4 proteins displayed per cell), but as of yet, only non-complexed cellulases have been displayed.⁵³ In order to develop improved minicellulosome display methods, it will be important to rigorously quantify the number of complexes displayed per cell. Thus far, the number of minicellulosome complexes displayed on the surface of *S. cerevisiae* has not been quantified, while in *B. subtilis*, experimental measurements have shown that 2×10^4 and 1.5×10^5 minicellulosomes can be attached to each cell using LysM and sortase-mediated approaches, respectively.^{62,65} These studies suggest that the number of minicellulosomes displayed per cell may decrease as the size of the complex increases. For example, in *B. subtilis*, quantitative studies have shown that it is possible to display 1.2×10^7 individual LysM fusion proteins, but only 2×10^4 LysM anchored minicellulosomes.^{65,68} Similar decreases in anchoring efficiency also occur in *S. cerevisiae* with increasing protein size, suggesting that this is a general problem.⁴⁸

Future Directions

Surface engineering efforts thus far have created recombinant microbes with potent cellulolytic activities. However, even more elaborate structures will need to be grafted onto their surfaces if their activities are to rival those of naturally cellulolytic organisms or cellulase cocktails that are currently used in industry.

The composition and structure of lignocellulose varies depending upon its source and the method of pretreatment. Therefore, the number, type, relative abundance, and positioning of the surfaced displayed enzymes will need to be optimized to degrade different types of plant feedstocks. Displaying minicellulosomes that more closely resemble native cellulosomes is an obvious strategy to improve cellulolytic activity, as it will presumably maximize enzyme-enzyme and CEM synergy. However, constructing these large self-assembling complexes may prove difficult as the results of recent work in *S. cerevisiae* and *B. subtilis* indicate that the surface density of displayed complexes decreases as they become larger and more complex. Overcoming this problem may require minicellulosome construction using adaptive assembly strategies and/or using protein components produced from a microbial consortium. The activity of these displayed complexes may be further improved using directed evolution approaches using growth on biomass to select for cells that display minicellulosomes possessing the best enzyme compositions

and architectures. Combined, this work promises to yield potent recombinant surface-engineered microbes that can degrade biomass. Concurrently, many research groups are using metabolic engineering, synthetic biology, and systems biology approaches to construct microorganisms capable of producing next generation biofuels and useful chemicals.⁷²⁻⁷⁵ When paired with the novel lignocellulosic degrading platforms described in this review, these microbes could significantly reduce the world's dependency on oil by directly producing biofuels and other useful biocommodities from renewable and abundant plant biomass.

Disclosure of Potential Conflicts of Interest

No potential conflict of interest was disclosed.

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