Chapter 18

Progress Towards Engineering Microbial Surfaces to Degrade Biomass

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Additional information is available at the end of the chapter

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Abstract

Lignocellulosic biomass is a promising feedstock to sustainably produce useful biocommodities. However, its recalcitrance to hydrolysis limits its commercial utility. One attractive strategy to overcome this problem is to use consolidated bioprocessing (CBP) microbes to directly convert biomass into chemicals and biofuels. Several industrially useful microbes possess desirable consolidated bioprocessing characteristics, yet they lack the ability to degrade biomass. Engineering these microbes' surfaces to display cellulases and cellulosome-like structures could endow them with potent cellulolytic activity, enabling them to be used in CBP. In this chapter, we discuss recent progress in engineering the surfaces of *Saccharomyces cerevisiae, Escherichia coli, Bacillus subtilis, Corynebacterium glutamicum*, and lactic acid bacteria. We discuss the techniques used to display cellulases on their surfaces, their recombinantly achieved cellulolytic activities, and current obstacles that limit their utility.

Keywords: lignocellulose, consolidated bioprocessing, cellulase, minicellulosome, cell surface display

1. Introduction

As concerns over limited petroleum supplies rise, the momentum to produce renewable fuels, chemicals, and other materials from biomass has increased [1–3]. Second-generation biofuels derived from sustainable feedstocks such as lignocellulosic biomass are attractive, as plant biomass is cheap and highly abundant; over 1 billion tons of lignocellulosic biomass are produced annually in the United States, while an estimated 10–50 billion tons of waste lignocellulose are produced worldwide [4–6]. However, the resistance of lignocellulose to

hydrolysis limits its use in biofuel production and has driven the search for new technologies to cost-effectively exploit this valuable resource [7, 8]. To produce fermentable sugars to generate cellulosic ethanol, conventional industrial processes utilize a multistep procedure to degrade lignocellulose (**Figure 1A**) [7, 9]. Typically, the biomass is first pretreated with strong acids and high temperatures to remove lignin and to partially degrade its cellulose and hemicellulose components [7]. It is then exposed to purified cellulase enzymes that hydrolyze the remaining polysaccharides into shorter polysaccharides and monosaccharides that can be fermented into ethanol by yeast. Significant effort has been put forth to optimize these steps, including the development of a range of new pretreatment approaches and enzyme cocktails [9–11]. It is generally believed that the cost of converting biomass into useful biocommodities could be greatly reduced by using a consolidated bioprocessing (CBP) microbe, a single microorganism that produces all of the necessary enzymes to degrade lignocellulose and then utilizes the resulting sugars to produce high levels of the biocommodity (**Figure 1B**) [12–14]. A CBP microbe would decrease costs by reducing the number of processing steps required to generate the final product and avoid the use of costly purified cellulase enzymes that are estimated to contribute $0.68–1.47 to the per gallon cost of cellulosic ethanol [15]. Given their great potential, a number of research groups have sought to develop a lignocellulose-utilizing CBP microbe using native and recombinant strategies [12]. In the native strategy, product production pathways are engineered into naturally cellulolytic microbes, while in the recombinant strategy, genetically well-studied microorganisms that may already be capable of producing a desired product are engineered to possess cellulolytic activity. Here, we describe progress towards creating recombinant cellulolytic microbes to convert biomass into useful commodities by engineering their surfaces to display cellulase enzymes.

Lignocellulose is recalcitrant to degradation and requires the action of many different types of enzymes to break it down [15]. It is composed of varying amounts of cellulose (25–55%), hemicellulose (8–30%), and lignin (18–35%) [7, 16]. Cellulose is a polymer of β-1,4-linked glucose molecules that can hydrogen-bond with other cellulose polymers to form both amorphous and crystalline regions [17]. It is synergistically degraded by three types of cellulases: endoglucanases, exoglucanases, and β-glucosidases [7]. Endoglucanases attack within a cellulose strand to hydrolyze the β-1,4-glucosidic bonds, producing new reducing and nonreducing ends that can be further broken down by exoglucanases [18]. The shorter cellodextrin chains that are produced by these enzymes, including the disaccharide cellobiose, are then degraded into glucose monomers by β-glucosidases [18]. Hemicellulose is a sugar polymer that is composed of a number of different types of pentose and hexose sugars [16]. Xylan is its main component and is the second most abundant polysaccharide in lignocellulose. As compared to cellulose, hemicellulose is more accessible to degradation by a range of enzymes with different substrate specificities, including among others: xylanases, arabinases, and mannanases. Finally, lignin surrounds and blocks enzyme access to cellulose and hemicellulose and is a complex polymer containing a mixture of phenolic compounds linked through radical coupling reactions [19]. A large number of enzymes are needed to degrade it, including peroxidases and laccases [20].

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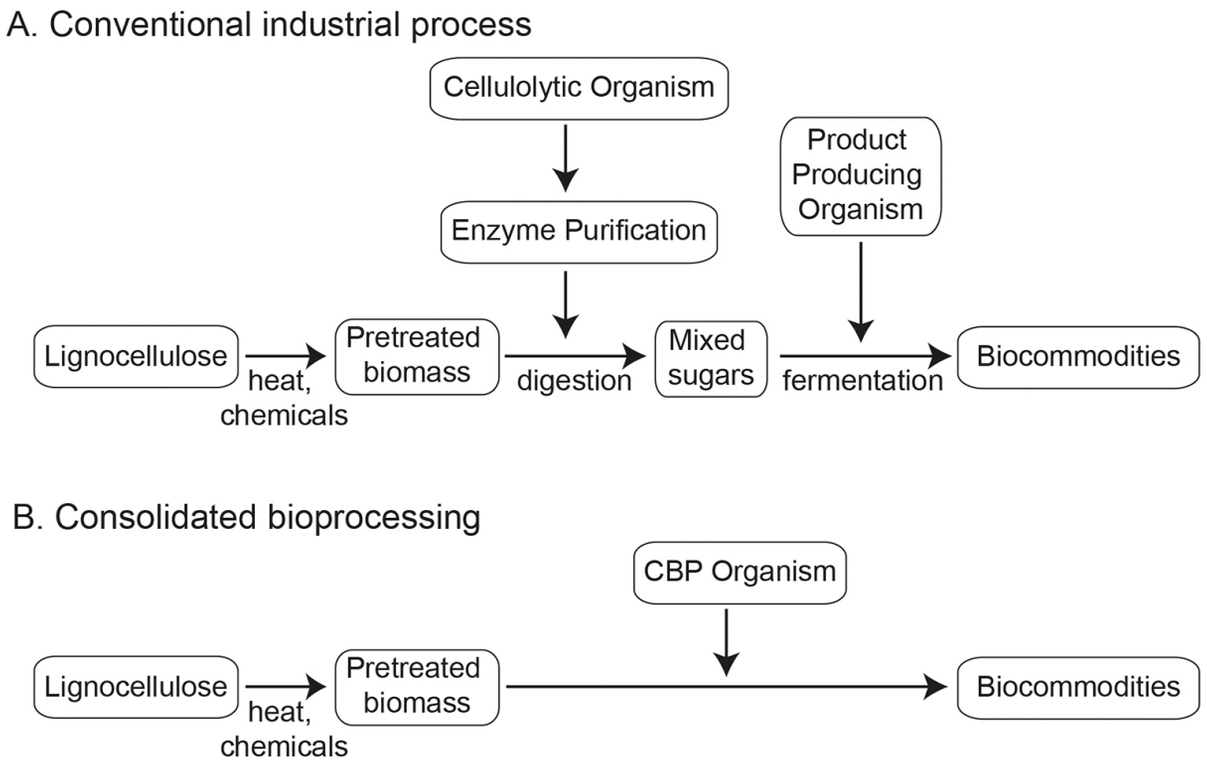

Figure 1. Conventional and consolidated bioprocessing (CBP) methods to convert biomass into biocommodities. (A) Many conventional industrial methods first pretreat biomass using heat and chemicals to remove lignin and to partially digest cellulose and hemicellulose. Complex enzyme mixtures are then added to the pretreated biomass to degrade its cellulose more fully. The resultant sugars are then fermented by a microorganism to generate the final product (currently only cellulosic ethanol). (B) A consolidated bioprocessing (CBP) microorganism could directly convert either pretreated or untreated lignocellulose into a biocommodity. It would bypass multiple steps in the conventional process, including the use of purified enzymes to degrade the biomass.

One promising recombinant strategy to create a useful CBP microbe is to engineer it to display a range of enzymes that degrade lignocellulose, thereby allowing it to produce sugars that can be further metabolized by the microorganism into useful chemicals [21–26]. In this approach, the goal was to engineer the microbial surface to effectively mimic the activity of naturally cellulolytic anaerobic bacteria. These microbes have the impressive capacity to adhere to, and degrade, untreated biomass and are typified by the cellulolytic anaerobic bacterium *Clostridium thermocellum* [27]. This eubacterium efficiently degrades biomass using a surface displayed complex called a cellulosome (**Figure 2A**) [27]. The cellulosome contains a central scaffolding protein that coordinates the binding of different enzymes. The primary scaffoldin, CipA, contains nine type-I cohesin modules that bind to cellulases harboring type-I dockerin modules [28]. CipA also contains a carbohydrate-binding module (CBM) that tethers the cellulosome complex to its cellulose substrate and a C-terminal type-II dockerin module [29]. The type-II dockerin module anchors the cellulosome complex to cell surface proteins by interacting with their type-II cohesin domains. As these surface proteins can contain multiple cohesin domains (1, 2, or 7 domains), large polycellulosomal structures can be displayed. Even more complex polycellulosomal structures exist in other species of bacteria and can contain over 100 enzymes [28].

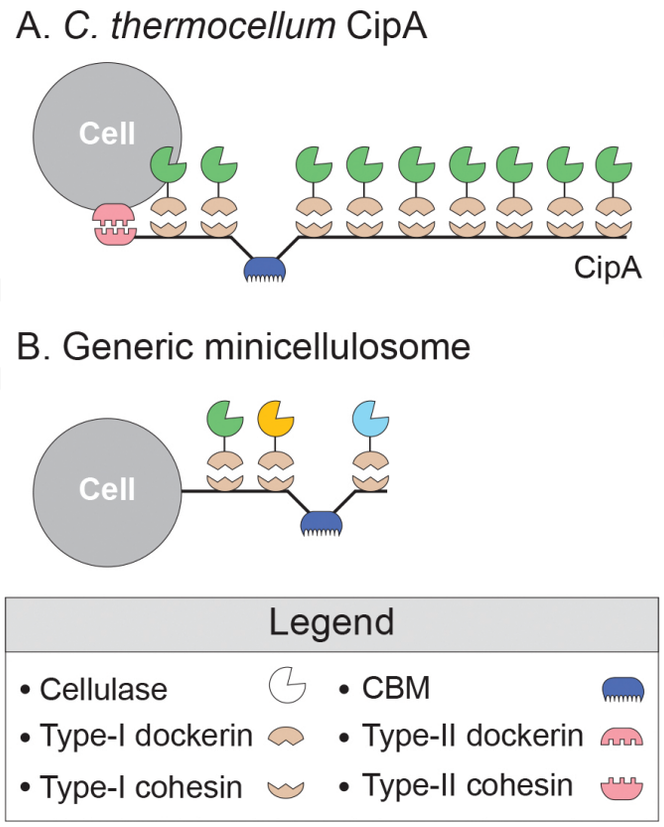

Figure 2. Natural cellulosomes and recombinant minicellulosomes. (A) The prototypical cellulosome from *C. thermocellum*. It contains the CipA scaffoldin that is capable of coordinating the binding of nine cellulases. CipA contains nine type-I cohesin domains that bind to cellulases containing type-I dockerin domains. It also contains a carbohydrate-binding module (CBM) that can bind to cellulose, holding the enzymes and the microorganism in close proximity to the substrate. CipA is anchored to the cell surface through its type-II dockerin domain that interacts with type-II cohesin modules present in the cell wall bound SdbA, Orf2, or OlpB proteins. (B) Basic structure of a minicellulosome that is displayed on a recombinant microbe. It is similar to the cellulosome, but contains fewer binding sites for enzymes. Color and symbol code: grey circles, cells; pink units, type-II cohesin-dockerin pairs; tan units, type-I cohesin-dockerin pairs; dark blue comb, CBM; and partial circular units: cellulases.

Microbes displaying cellulosomes are believed to degrade lignocellulose much more efficiently than microbes that degrade biomass by secreting cellulases [30]. There are three main reasons why increased efficiencies may be obtained. First, secreting enzymes presumably imposes a higher metabolic burden upon the microorganism as compared to displaying the enzymes on the cell surface. This is because the secreted enzymes can be lost to the environment with no guarantee that the sugars that they will produce will ultimately be accessible to the microbe for use as nutrients. As a result, larger quantities of the enzymes must be produced if they are to be secreted. For example, aerobic fungi (such as *Trichoderma reesei*, from which many purified industrial enzyme cocktails are derived) secrete 1–10 g of enzymes per liter of culture to degrade biomass, while cellulolytic anaerobic bacteria (such as *C. thermocellum*) need to produce only ~0.1 g of enzymes per liter of culture [31]. Second, colocalization of enzymes with different substrate preferences within a cellulosome promotes synergistic enzyme-enzyme and enzyme-proximity interactions, where the cellulolytic activity of the complexed enzymes is greater than that of individual enzymes due to their complementary activities and

optimal enzyme spacing [32]. The presence of both hemicellulases and cellulases in a cellulosome complex also enables hemicellulose and cellulose fibers to be removed simultaneously, thereby overcoming potential physical hindrances. The benefits of the *C. thermocellum* cellulosomal system have been quantified: its specific activity against crystalline cellulose is 15-fold higher than the secreted enzyme system from *T. reesei* [33]. Finally, the placement of the cellulosome on the microbial surface increases the rate of hydrolysis by promoting cellulose–enzyme–microbe (CEM) synergy [34]. In this process, sugar uptake by the microbe presumably becomes more efficient by promoting import of the products into cells and removing potential enzyme inhibitors such as glucose and cellobiose from the environment [35].

Microbe-based CBP technologies are not currently used industrially to produce biocommodities from lignocellulosic biomass. However, a major step towards CBP of starch into ethanol has recently been demonstrated by Lallemand and Mascoma. These companies created TransFerm®, a genetically modified yeast strain that secretes a glucoamylase and that is optimized to produce higher yields of ethanol. Although the cells do not fully degrade starch on their own, they reduce by 20–45% the amount of exogenous enzymes that needs to be added to process starch. At present, microbe-based CBP of lignocellulose is not being performed industrially, and ongoing research is primarily focused on constructing and identifying microorganisms with optimal cellulolytic and biocommodity production capabilities. Furthermore, detailed cost analyses of CBP versus conventional pretreatment and saccharification approaches have not been reported [36]. This is because the specific costs associated with CBP will vary based on the biocommodity produced, and the microbe and biomass source that is employed. However, the greatest cost savings associated with CBP will likely be obtained by reducing the costs of saccharification. A detailed cost analysis has been performed for cellulosic ethanol production from corn stover using dilute acid pretreatment, enzymatic saccharification, and cofermentation [37]. In this analysis, on-site production of fungal enzymes was estimated to contribute $0.34 per gallon of ethanol (assuming enzyme loadings of 20 mg enzyme per gram of biomass), which could in principle be eliminated using a CBP microbe. Another analysis by Johnson explored the potential cost savings associated with altering the source of enzyme production from off-site to on-site cultivation, specifically on biomass as a primary substrate [38]. The estimated full cost of producing cellulosic ethanol was reduced by 19% if the enzymes were produced on-site because it eliminated the need for enzyme purification, formulation of the enzyme mixture to preserve stability, and transport. Similar substantial gains could be obtained using microbe-based CBP.

In this chapter, we review progress towards engineering microbes to display "minicellulosomes," smaller cellulosome-like complexes that can degrade biomass (**Figure 2B**). A list of the microorganisms engineered to display minicellulosomes discussed in this chapter is presented in **Table 1**. We discuss recent developments in displaying these structures on industrially useful microorganisms, including *Saccharomyces cerevisiae*, *Escherichia coli*, *Bacillus subtilis*, *Corynebacterium glutamicum*, and lactic acid bacteria. The mechanisms used to display enzymes, their cellulolytic activities, and current obstacles that limit their utility are discussed.

Anchor	Assembly (# enzymes/ complex)	Enzymes displayed		References
S. cerevisiae				
Aga2	*Ex vivo* (3)	*C. cellulolyticum*: *C. thermocellum* and *C. cellulolyticum* or *C. thermocellum*:	Exoglucanase (CelE) Endoglucanase (CelA) Endoglucanase (CelG) β-Glucosidase (BglA)	[73]
Aga2	*Ex vivo* consortium (3)	*C. thermocellum* *T. aurantiacus* and *C. cellulolyticum* or *T. reesei*:	Endoglucanase (CelA) β-Glucosidase (Bgl1) Exoglucanase (CelE) Cellobiohydrolase (CBHII)	[74]
α-Agglutinin	*Ex vivo* consortium (3)	*C. thermocellum*: *T. aurantiacus*: *T. reesei*:	Endoglucanase (CelA) β-Glucosidase (Bgl1) Cellobiohydrolase (CBHII)	[75]
Aga2	*Ex vivo* consortium (2)	*C. thermocellum*: *T. reesei*:	Endoglucanase (CelA) Exoglucanase (CBHII)	[76]
Aga2	*Ex vivo* adaptive assembly (4)	*C. cellulolyticum*: *C. thermocellum*:	Endoglucanase (CelG) β-Glucosidase (BglA)	[77]
Aga2	*In vivo* (3)	*A. aculeatus*: *T. reesei*:	β-Glucosidase 1 (BGL1) Cellobiohydrolase II (CBHII) Endoglucanase II (EGII)	[32]
Aga2	*In vivo* (5)	*A. aculeatus*: *T. reesei*: *H. insolens*: *T. aurantiacus*:	β-Glucosidase 1 (BGL1) Cellobiohydrolase II (CBHII) Endoglucanase II (EGII) Cellobiose dehydrogenase (CDH) Lytic polysaccharide monooxygenase (LPMO)	[78]
Aga2	*In vivo* adaptive assembly (12 max)	*C. cellulolyticum*:	Endoglucanase (CelCCA) Cellobiohydrolase (CelCCE) β-Glucosidase (Ccel_2454)	[79]
α-Agglutinin	*Ex vivo* (3)	*A. awamori*: *A. niger*: *T. lanuginosus*:	Acetylxylan esterase (AwAXEf) β-xylosidase (XlnDt) Endoxylanase (XynAc)	[87]
Aga2	*In vivo* (3)	*A. niger*:	Arabinofuranosidase (AbfB)	[88]

Anchor	Assembly (# enzymes/ complex)	Enzymes displayed		References
S. cerevisiae				
		T. reesei:	β-Xylosidase (XlnD) Endoxylanase (XynII)	
α-Agglutinin	*In vivo* (4)	*A. aculeatus*: *T. reesei*:	β-Glucosidase 1 (BGL1) Endoglucanase II (EGII)	[89]
Aga2	*Ex vivo* (N/A)	*C. cellulolyticum:*	Endoglucanase (CelA)	[90]
B. subtilis				
LysM	*Ex vivo* (3)	*B. subtilis*: *C. phytofermentans*: *C. thermocellum*:	Endoglucanase (Cel5) Cellobiohydrolase (Cel48) Endoglucanase (Cel9)	[31]
LPXTG from *S. aureus*	*Ex vivo* (3)	*C. cellulolyticum*: *C. thermocellum:*	Exoglucanase (Cel9E) Endoglucanase (Cel9G) Endoglucanase (Cel8A)	[118]
Lactic acid bacteria				
LPXTG from *S. pyogenes*	*Ex vivo* (1-2)	*E. coli*:	β-Glucuronidase (UidA)	[127]
LPXTG from *S. pyogenes*	*Ex vivo* (2)	*E. coli*:	β-Glucuronidase (UidA) β-Galactosidase (LacZ)	[128]
LPXTG from *L. plantarum*	*Ex vivo* consortium (2)	*T. fusca*:	Endoglucanase (Cel6A) Xylanase (Xyn11A)	[129]
C. glutamicum				
MscCG	*In vivo* (2)	*C. thermocellum*:	Endoglucanase (CelE) β-Glucosidase (BglA)	[133]

Table 1. Multi-enzyme display in minicellulosomes.

2. Displaying enzymes on *Saccharomyces cerevisiae*

Significant effort has been put forth to display cellulolytic enzymes on the surface of *S. cerevisiae* because of its established role in producing bioethanol [39]. The yeast cell surface is comprised of β-glucans, mannoproteins, and small amounts of chitin [40]. As β-glucan is the major constituent, displayed proteins are typically anchored to this fibrous scaffold. The most widely used approach to display cellulases and minicellulosomes employs a glycosylphosphatidylinositol (GPI)-anchoring system (**Figure 3A**). The protein of interest is expressed as a fusion with a polypeptide GPI attachment signal that is typically derived from the α-agglutinin protein or other cell wall proteins such as Sed1 and Cwp2 [41, 42]. After protein synthesis, GPI is added to the ω-site amino acid in the anchor signal sequence by the GPI transamidase complex in the endoplasmic reticulum [43, 44]. The GPI-linked protein is then directed towards

the lipid bilayer and subsequently covalently linked to the cell wall β-1,6-glucan by the putative Dfg5 and Dcw1 cross-linkers [45–48]. Cellulase can also be displayed indirectly. In this approach, the enzyme is expressed as a fusion protein with the a-agglutinin Aga2 subunit, which in turn, forms disulfide bonds to the Aga1 subunit that is covalently linked to the cell wall via a GPI anchor. An estimated 1×10^4 to 1×10^5 proteins per cell are displayed using the Aga2 subunit [49]. The number of proteins displayed on the surface can be increased by genetically modifying the yeast strain. For example, deletion of a major endogenous GPI-anchored cell wall protein, SED1, can greatly improve heterologous protein display by reducing competition for cell wall-anchoring sites [50]. Display levels can also be improved by lowering the mannan content of the cell surface [51] and by employing the SED1 signal peptide, promoter and anchor [52, 53]. Reducing protein glycosylation also improves minicellulosome display [54]. The general strategies used to enhance enzyme display in yeast may also be useful in optimizing the other recombinant microbes that are discussed below.

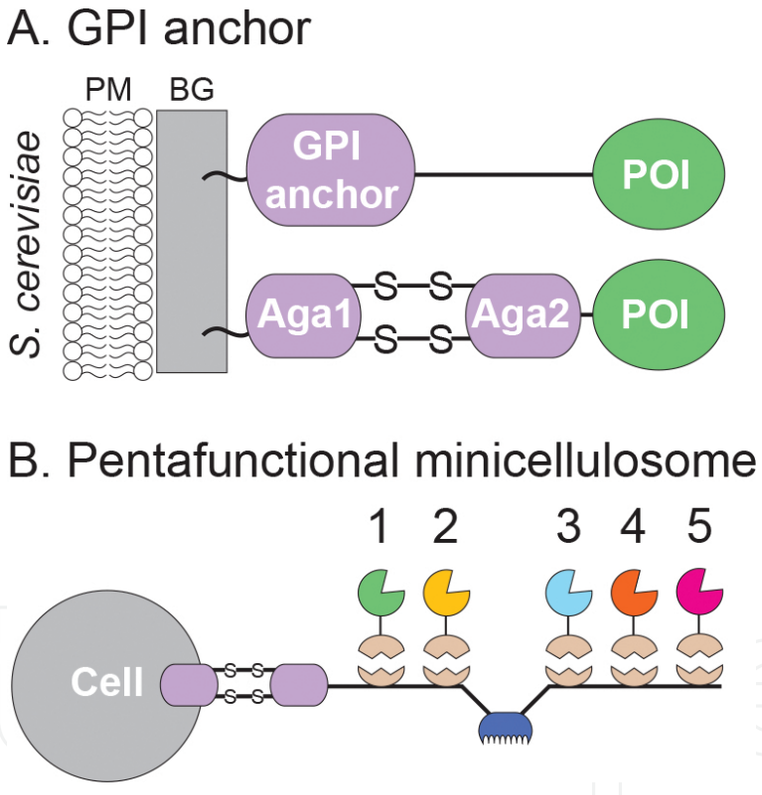

Figure 3. Cellulase and minicellulosome display in *S. cerevisiae*. (A) Methods used to display cellulases and their complexes on the cell surface. Proteins of interest (POI) are attached to the cell surface by a GPI anchor that is covalently linked to the β-glucan of the cell wall. The GPI anchor is attached to the displayed protein by expressing it as a fusion protein that contains the GPI attachment signal from either the α-agglutinin, SED1, or Cwp2 proteins. Proteins can also be displayed indirectly by expressing them as a fusion with the Aga2 protein that in turn interacts with the GPI anchored a-agglutinin Aga1 located on the cell surface. (B) One of the most active cellulolytic minicellulosomes that has been displayed on the surface of *S. cerevisiae*. It contains five enzyme functionalities and is displayed through fusion of the miniscaffoldin to the Aga2 protein. Symbols have been described in **Figure 2**. Enzymes 1–5 correspond to *T. reesei* endoglucanase (EGII), *T. reesei* cellobiohydrolase (CBHII), *A. aculeatus* β-glucosidase (BGL1), *T. aurantiacus* GH61a lytic polysaccharide monooxygenase (LPMO), and *H. insolens* cellobiose dehydrogenase (CDH), respectively. Abbreviations: POI, protein of interest; PM, plasma membrane; BG, β-glucan.

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2.1. Individual cellulases

Individual cellulases were originally displayed on the cell surface by the Tanaka group [55]. They engineered cells to codisplay individual carboxymethylcellulase (CMCase) and β-glucosidase (BGL1) enzymes derived from *Aspergillus aculeatus* by fusing them to a C-terminal GPI anchor sequence from α-agglutinin. The cells could degrade cellodextrins, soluble glucose polymers that contain up to six glucose residues. Subsequently, the Kondo laboratory significantly advanced this technology by developing *S. cerevisiae* strains that display additional enzymatic activities. Initially, they constructed strains that codisplayed the *A. aculeatus* BGL1 and *T. reesei* endoglucanase II (EGII) enzymes, which when cultured in nutrient-rich media fermented β-glucan into ethanol [56]. Later, the inclusion of the *T. reesei* cellobiohydrolase II (CBHII) enzyme allowed the strains to ferment more complex amorphous phosphoric acid swollen cellulose (PASC) into ethanol [57]. Separately, the Kondo group also demonstrated that cellulose degradation can be further improved using a variety of approaches, including engineering a displayed cellulase to contain multiple cellulose-binding domains, integrating multiple copies of the cellulases genes into the yeast chromosome, and by strain diploidization [58–60]. Increasing the number and density of the displayed cellulases significantly improves the ability of *S. cerevisiae* to degrade cellulose. For example, a strain displaying the BGL1, EGII, and CBHII enzymes produces more ethanol from PASC than a strain that displays only BGL1 and secretes EGII and CBHII [61, 62]. Enzyme proximity on the cell surface also appears to have a major effect on activity, as the cellulolytic properties of cells displaying one or more cellulases improved as the enzyme density increased [63]. Interestingly, in these studies, good activity was observed when the enzymes were estimated to be separated on the cell surface by 10–100 nm, an enzyme spacing that is presumably similar to the spacing found in certain types of bacterial cellulosomes [64].

Yeast displaying cellulases may be industrially useful. For example, strains displaying the BGL1, CBHII, and EGII enzymes produced 43.1 g/L ethanol from 200 g/L liquid hot water pretreated rice straw in 72 h [65]. While supplementation with a purified cellulase cocktail at 10 filter paper units (FPU)/g-biomass was necessary to achieve this high ethanol yield, a control strain that did not display the enzymes required 10-fold more added cellulase to produce similar quantities of ethanol. Attractively, the cells displaying the enzymes could be reused in five fermentation cycles without significantly losing their activity [66]. Displaying enzymes also reduces the amount of purified cellulase lost through irreversible adsorption onto crystalline cellulose, facilitating more efficient cellulose degradation and higher ethanol yields [67]. Recently, studies using cellulase displaying cells have further reduced the amount of purified cellulase cocktail that needs to be added to convert biomass into ethanol [68]. These newer generation cells require 44% less commercial enzyme supplementation to degrade pretreated biomass by displaying four enzymes using the Sed1 anchor: *A. aculeatus* BGL1, *T. reesei* EGII, *Talaromyces emersonii* cellobiohydrolase I (CBHI), and *Chrysosporium lucknowense* cellobiohydrolase II (CBH2). When supplemented with 1 FPU of commercial enzyme cocktails, the cells yielded 18 g/L ethanol from 100 g/L of pretreated and milled rice straw in 96 h, obtaining 80% of the theoretical yield. In contrast, without enzymatic supplementation, only 7% of the theoretical yield was obtained using the same cells. Therefore, while the use of

cellulase displaying yeast cells can significantly reduce the amount of commercial enzyme that needs to be added to degrade pretreated biomass, current generation cells still require enzyme supplementation.

Towards the goal of improving their cellulolytic activity, several studies have engineered cellulase displaying yeast cells to also produce complementary enzymes and transporters that improve cellulose solubilization and utilization. The Kondo group constructed cells that co-expressed three displayed cellulases, as well as the *Neurospora crassa* cellodextrin transporter (CTDI) and intracellular BGL1 enzyme [69]. This strain produces 1.7-fold more ethanol from PASC than a strain that only displays the cellulases, presumably by reducing the build-up of enzyme inhibitory products on the cell surface. Additionally, strains displaying cellulases as well as proteins that disrupt the structure of cellulose show improvements in ethanol production; cells codisplaying three cellulases and an *Aspergillus oryzae* expansin-like protein (AoelpI) produce 1.4-fold more ethanol from PASC than a control strain that only displayed the cellulases [70]. Finally, the Ueda group demonstrated that sequential exposure of biomass to engineered yeast cells could be beneficial. In this work, pretreatment of hydrothermally processed rice straw with yeast displaying the *Trametes* sp. Ha1. laccase I enzyme enabled 1.21-fold more ethanol to be produced from the biomass after it was subsequently exposed to yeast displaying three cellulases [71]. These studies highlight interesting enzyme functionalities that should be considered for inclusion in other recombinant microorganisms.

2.2. *Ex vivo* assembled minicellulosomes

Several research groups engineered *S. cerevisiae* to display minicellulosomes that are smaller than natural cellulosomes (**Figure 2B**). In the minicellulosome, a cell-surface displayed "miniscaffoldin" protein that contains cohesin domains noncovalently binds cellulases via their dockerin modules. These recombinant miniscaffoldins are either attached directly to the cell wall by fusing them to a GPI anchor sequence (from α-agglutinin or Cwp2) or indirectly by fusing them to the a-agglutinin Aga2 subunit. Two general methods are used to display minicellulosomes, an *ex vivo* approach in which cellulases need to be added to cells that display a miniscaffoldin (**Figure 4A**, **4B**), or an *in vivo* approach in which the microbe produces all of the protein components that are necessary to assemble and display the minicellulosome (**Figure 4C**).

In 2009, two groups demonstrated *ex vivo* minicellulosome assembly by creating cells that display a miniscaffoldin and then adding to the cells cellulases that contained a dockerin domain. The Volschenk group displayed a miniscaffoldin by fusing two cohesin modules and a CBM to the Cwp2 GPI anchor. They visually demonstrated binding of this yeast strain to filter paper via the CBM and constructed a minicellulosome by adding a purified endoglucanase–dockerin enzyme to the cells [72]. Concurrently, the Chen laboratory constructed an Aga2-fused recombinant minicellulosome that contained three cohesin modules derived from three different bacterial species [73]. This more elaborate minicellulosome used species-specific cohesin–dockerin interactions to target β-glucosidase, exoglucanase, and endoglucanase enzymes to specific sites within the complex. Quantitatively, the researchers demonstrated that incorporation into the minicellulosome enabled the enzymes to function synergistically to

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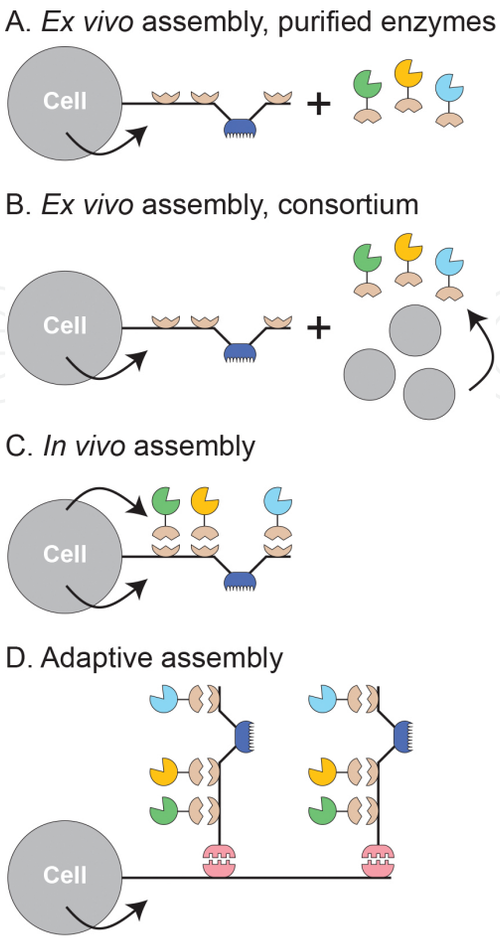

Figure 4. Summary of strategies used to assemble minicellulosomes on microbial surfaces. (A) *Ex vivo* assembly. Exogenous cellulase-dockerin fusion proteins (either purified or derived from cell lysates) are added to cells engineered to display a miniscaffoldin that binds to the cellulases. (B) *Ex vivo* assembly using a microbial consortium. Similar to (A), but miniscaffoldin displaying cells are cultured with a consortium of microbes that have been engineered to secrete cellulase-dockerin fusion proteins. (C) *In vivo* assembly. A single microorganism is engineered to express all of the components of the minicellulosome, enabling its spontaneous assembly on the cell surface. (D) Adaptive assembly method used to construct larger minicellulosomes. The miniscaffoldin contains two basic parts, a non-catalytic anchor-anchoring scaffoldin that attaches the structure to the cell wall and a primary scaffoldin that binds to the cellulase-dockerin fusion proteins. Color and symbol code as described in **Figure 2**.

produce ethanol from PASC. Later, the Chen laboratory bypassed the need to add purified enzymes or cell lysates to cells to produce the minicellulosome by using a consortium of yeast strains to produce the cellulases [74]. This was accomplished using four strains: one that displayed the miniscaffoldin and three strains that secreted each of the enzymes needed to form the complex. After determining the optimal ratio of strains, they demonstrated that ~1.9 g/L of ethanol could be produced from 10 g/L of PASC in 48 h. Additionally, they demonstrated that fusing the miniscaffoldin directly to the cell wall via an attached α-agglutinin GPI anchor instead of indirectly through the Aga2 subunit improved minicellulosome display [75]. Similarly, the Hahn laboratory utilized a consortium approach to build an *ex vivo* assembled minicellulosome that contained three identical cohesin–dockerin pairs and randomly incorporated enzymes [76]. Cells displaying this structure also obtained similar ethanol yields, 1.8 g/L of ethanol from 10 g/L of PASC in 94 h.

In order to build larger surface structures with higher enzyme densities, the Chen laboratory used an adaptive assembly approach [77]. In this procedure, the minicellulosome is built using two separate scaffoldin pieces: a primary scaffoldin that binds to the catalytic components and an anchoring scaffoldin that is attached to the cell surface and only binds to the primary scaffoldin (**Figure 4D**). They produced a yeast strain that displayed an Aga2-fused anchoring scaffoldin that contained two cohesin domains from *Acetivibrio cellulolyticus* and *Bacteroides cellulosolvens*. Two *E. coli* strains were then used to produce two primary scaffoldins that each contained the *C. thermocellum* and *R. flavefaciens* cohesins, and either the dockerin module from *A. cellulolyticus* or *B. cellulosolvens*. Thus, the final complex contained a total of four enzymes, with two copies each of the *C. cellulolyticum* endoglucanase (CelG) and *C. thermocellum* β-glucosidase (BglA) enzymes. This strain produced only 1.9 g/L of ethanol from 10 g/L of PASC in 72 h, presumably because it lacked exoglucanase activity.

2.3. *In vivo* assembled minicellulosome

The need to add enzymes to cells displaying a miniscaffoldin may make the use of *ex vivo* assembled minicellulosomes industrially impractical. To overcome this problem, two research groups engineered *S. cerevisiae* to express all of the components of the minicellulosome such that it could form spontaneously (*in vivo* assembly). The Zhao laboratory constructed a yeast strain that produced an Aga2-fused miniscaffoldin, *T. reesei* EGII and CBHII, and *A. aculeatus* BGL1 enzymes [32]. By comparing the display of a single enzyme to the simultaneous display of two or three noncomplexed enzymes, they demonstrated enzyme-enzyme synergy, which improved activity by 5.5-fold. They also demonstrated enzyme-proximity synergy, as the trifunctional minicellulosome degraded biomass better than cells containing three independently displayed enzymes (1.6-fold improvement). Quantitative measurements indicated that each cell displays 3×10^4 unifunctional miniscaffoldins, whereas 1.8×10^4 tri-functional minicellulosomes are displayed per cell. *In vivo* construction of increasingly more elaborate minicellulosomes decreased protein expression, likely due to metabolic burden. Compared to unifunctional minicellulosomes, the production of the miniscaffoldin and two enzymes caused the expression of all components to decrease slightly, and overexpression of an additional third enzyme caused protein expression levels of CBHII to drop significantly. This suggests that it will be increasingly more challenging to display larger, more complex enzyme structures on the cell surface. This is unfortunate, because adding additional enzyme functionalities is advantageous to cellulose solubilization. Although difficult to achieve, for example, the trifunctional displayed minicellulosome constructed by the Zhao group produces 1.8 g/L of ethanol from 10 g/L of PASC in 70 h. However, pentafunctional minicellulosomes are more effective, as they produce 2.7 g/L of ethanol from 10 g/L of PASC in 96 h and 1.8 g/L of ethanol from 10 g/L of Avicel in 96 h [78]. This larger pentafunctional complex contains two additional enzymes with new functionalities, the *Thermoascus aurantiacus* GH61a lytic polysaccharide monooxygenase (LPMO) and *Humicola insolens* cellobiose dehydrogenase (CDH) (**Figure 3B**). Even though fermentative growth was reduced because of the presence of oxygen needed for monooxygenase function, greater ethanol yields were still obtained.

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The largest *in vivo* assembled minicellulosome reported thus far was constructed by the Tan group using an adaptive assembly approach [79]. This structure could display up to 12 enzymes and was formed using a cell surface-associated Aga2-fused anchoring scaffoldin that contained type-II cohesin domains that could bind four primary miniscaffoldins harboring type-II dockerin domains. Each primary scaffoldin also contained a CBM and three species-specific type-I cohesin modules for controlled cellulase binding. Combined, this strain displays in a single complex four copies each of the *C. cellulolyticum* endoglucanase, cellobiohydrolase, and β-glucosidase enzymes. However, the investigators found that as longer anchoring scaffoldins were used, a smaller percentage of cells displayed the scaffoldin. For this reason, they demonstrated the production of 1.4 g/L ethanol from 10 g/L Avicel in 96 h from a smaller six enzyme displaying strain.

2.4. Hemicellulases and hemicellulosomes

In order to develop xylose-fermenting strains of yeast, similar strategies have been employed to display hemicellulases and hemicellulosomes on the cell surface. The Kondo laboratory displayed an individual *T. reesei* endoxylanase II (XYNII) enzyme using the α-agglutinin anchor and demonstrated that the cells degraded birchwood xylan into xylobiose, xylotriose, and xylotetraose [80]. Subsequently, the cells were further engineered to produce ethanol from birchwood xylan by adding the *A. oryzae* β-xylosidase (XylA) to the cell surface and by expressing intracellular xylose utilization proteins (the *Pichia stipitis* xylose reductase (XYL1) and xylitol dehydrogenase (XYL2), as well as the *S. cerevisiae* xylulokinase (XKS1)) [81]. In a separate set of studies, the investigators later demonstrated cellodextrin degradation and xylose assimilation by yeast displaying *A. aculeatus* BGL1 enzyme, which enabled the microbe to produce ethanol from the sulfuric acid hydrolysate of wood chips [82]. Interestingly, the ability of this strain to co-utilize cellobiose and xylose avoided carbon catabolite repression [83]. Additional engineering created cells that displayed the XylA, XYNII, and BGL1 enzymes, enabling the xylose-utilizing strain to produce 8.2 g/L of ethanol from 80% (v/v) rice straw hydrolysate [84]. When *P. stipitis* xylose reductase (XR) was produced intracellularly in place of the three aforementioned xylose utilization proteins, xylitol accumulated, which is a starting material for chemical production of some pharmaceuticals [85]. Using an alternative pathway to utilize xylose, the Ueda group produced α-agglutinin anchored *Clostridium cellulovorans* xylose isomerase (XI) which converted xylose to xylulose extracellularly before fermenting it to ethanol [86].

Clustering hemicellulases within surface displayed complexes leads to improved enzymatic activity. The Silva group developed an *ex vivo* assembled xylanosome using the α-agglutinin anchor [87]. The xylanosome contained the *Thermomyces lanuginosus* endoxylanase (XynAC), *Aspergillus niger* β-xylosidase (XlnDt), *Aspergillus awamori* acetylxylan esterase (AwAXEf) enzymes, as well as the xylose-binding domain (XBD) from *Thermotoga maritima*. The xylanases in the complex functioned synergistically, with enzyme-enzyme synergy improving xylan hydrolysis by 1.6-fold, and enzyme-substrate synergy improving hydrolysis by 3.3-fold as compared to the free enzymes. Proximity of the endoxylanase to the XBD

improved xylan hydrolysis by 2.5-fold, suggesting that placement of substrate binding domains can contribute significantly to hydrolysis. The Zhao laboratory created an *in vivo* assembled minihemicellulosome that is structurally related to their minicellulosome complex described above [88]. The minihemicellulosome contained the *A. niger* arabinofuranosidase (AbfB), β-xylosidase (XlnD), and *T. reesei* endoxylanase (XynII) enzymes. The yeast strain also possessed *P. stipitis* xylose utilization enzymes and could hydrolyze arabinoxylan better than the uni- and bifunctional yeast strains. Surprisingly, against birchwood xylan, a bifunctional minihemicellulosome containing XynII and XlnD exhibited a higher hydrolysis rate than the tri-functional complex, producing 0.95 g/L of ethanol from 10 g/L of birchwood xylan in 80 h.

2.5. Artificial cellulosome structures

In order to better control enzyme placement and increase the number of displayed enzymes on the cell surface, several groups have created artificial cellulosomes that are structurally distinct from naturally occurring cellulosomes. These structures use unique protein-protein interaction domains to tether the enzymes to the scaffoldin instead of naturally occurring cohesin-dockerin interactions. The Kondo laboratory created an *in vivo* assembled minicellulosome using a miniscaffoldin that contained two cohesin domains and two Z domains derived from *Staphylococcus aureus* protein A [89]. This enabled codisplay of the *A. aculeatus* BGL1—dockerin fusion protein and the *T. reesei* EGII protein fused to the Fc domain of human IgG. The group suggested that this artificial construct enables tighter regulation of the display ratio of the enzymes. Similarly, the use of type-I and type-II cohesin-dockerin pairs, or cohesin-dockerin pairs from different species, allows control over enzyme incorporation. The advantages of this noncellulosome-like structure were not extensively investigated, but activity against β-glucan was demonstrated. The Su group has displayed enzymes using a novel *ex vivo* assembled complex that contains an amyloid-like scaffoldin [90]. A multistep process was required to assemble the complex. Initially, yeast expressed an Aga2 anchored GFP-specific antibody fragment. Then, cells were incubated with a GFP-dockerin fusion protein to prime the cells to bind the protein scaffold. The protein scaffold itself was then created through the fibrillation of a recombinant-purified protein that contained a cohesin and a hydrophilic linker region that was fused to the N-terminus of Ure2, an amyloid-like yeast protein. In this way, multiple cohesin domains were incorporated into a large protein scaffold. As compared to cells displaying a single cohesin-dockerin pair harboring the *C. cellulolyticum* endoglucanase (CelA), cells displaying the amyloid-like scaffoldin had 8.5-fold greater activity against CMC. However, the process of assembly is complex, and certain cohesin domains may be rendered nonfunctional during fibrillation. The use of extremely large supramolecular scaffoldins to coordinate enzyme binding may also reduce the potential benefits of CEM synergy. Whether the enzymatic activities of these artificial cellulosomes are superior to complexes that more closely resemble natural cellulosomes remains to be determined, as their ability to degrade complex cellulose substrates to produce ethanol was not reported.

3. *Escherichia coli* surface display of individual cellulases

The model Gram-negative bacterium *E. coli* has several features that are attractive for CBP, including a robust genetic system that enables surface and metabolic engineering, as well as the microbe's innate ability to utilize the main components of lignocellulose, glucose, arabinose, and xylose [91, 92]. Its cell envelope is comprised of an inner membrane, a peptidoglycan layer, and an outer membrane. Only individual, noncomplexed cellulases and xylanases have been displayed on the surface of *E. coli* by fusing them to lipoproteins or integral membrane proteins (**Figure 5**). Lipoprotein anchors that have been used to display cellulases include the *E. coli*-derived Lpp-OmpA fusion protein [93, 94], the *P. syringae* ice nucleation protein (INP) [95–97], and the *E. coli* bacterial lipocalin (Blc) protein [98–100]. Cellulases have also been displayed by fusing them to outer membrane proteins such as the: *B. subtilis* poly-ɣ-glutamate synthase A (PgsA) [101, 102], *E. coli* outer membrane protein C (OmpC) [103], and the *E. coli* outer membrane protein X (OmpX) [104]. Cellulases can also be displayed through a unique mechanism in which they are fused to the *E. coli* autotransporter protein (AIDA-I) [105]. Other less commonly used approaches to display cellulases include fusing them to the *E. coli* inner membrane protein HdeD [100], or to the *B. anthracis* BclA exosporal protein whose mechanism of display in *E. coli* is not well understood [106]. In most cases, except for the work reported by the Kondo and the Karim groups, only single enzymes have been displayed on the surface, leading to cells that have limited cellulolytic activity [100, 101].

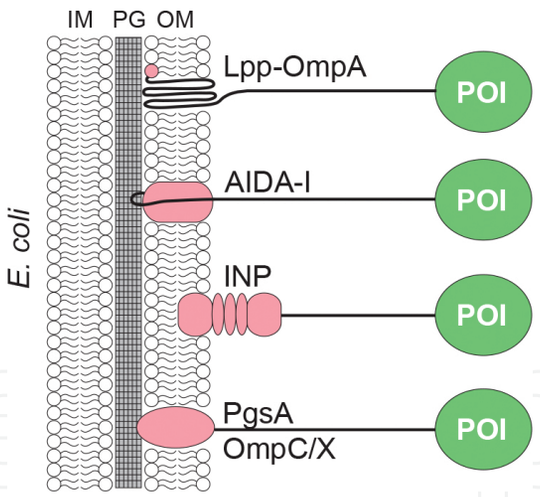

Figure 5. Methods used to display cellulases in *E. coli*. Proteins of interest are displayed as a result of fusion to lipoproteins (*E. coli* Lpp-OmpA, *P. syringae* INP, *E. coli* Blc), fusion to outer membrane proteins (*B. subtilis* PgsA, *E. coli* OmpC, *E. coli* OmpX), or fusion to the *E. coli* autotransporter AIDA-I. Not all anchoring methods are depicted here. At present, only individual enzymes have been displayed on the cell surface. Abbreviations: protein of interest (POI), inner membrane (IM), peptidoglycan (PG),and outer membrane (OM).

Pilot studies have shown that *E. coli* displaying cellulases can be used to produce ethanol or isopropanol from cellulosic substrates. Using the *E. coli* AIDA-I anchor, the display of the *T. fusca* β-glucosidase enzyme yielded cells that produced approximately 17–17.9 g/L of ethanol from 40 g/L of cellobiose in 72 h [105]. By utilizing the *E. coli* Blc anchor to display the *T. fusca*

β-glucosidase, cells could produce 69.0 ± 11.6 mM of isopropanol in a 21 h fermentation containing 50 g/L of cellobiose [99]. The low yield of isopropanol, as compared to when glucose was supplied, suggested that the β-glucosidase activity was too low to efficiently produce glucose from cellobiose. The most complex *E. coli* display system thus far achieved displayed three enzymes using the *B. subtilis* PgsA anchor [101]. This strain displayed endocellulase, exocellulase, and β-glucosidase enzymes derived from *C. cellulolyticum*. From 10 g/L of PASC, it produced 3.59 ± 0.15 g/L of ethanol in 60 h. It could also make ethanol from dilute sulfuric acid pretreated corn stover hydrolysate (2 g/L), producing 0.71 ± 0.12 g/L of ethanol in 48 h. One notable study performed by Bokinsky et al. demonstrated that *E. coli* strains that secreted cellulases could produce advanced biofuels from ionic liquid pretreated biomass [107]. A consortium of two *E. coli* strains was used to produce either the *Bacillus* sp. D04 endocellulase and the *Cellvibrio japonicus* β-glucosidase, or the *Clostridium stercorarium* endoxylanase and the *C. japonicus* xylobiosidase. The consortia could grow on ionic liquid (IL)-treated biomass, albeit to half the cell density of strains grown on glucose. Additional engineering enabled cells to produce fatty-acid ethyl esters, butanol, and pinene from IL-pretreated switchgrass. However, only 5 and 6% of the available cellulose and hemicellulose were digested, respectively. Improved cellulolytic activity could be achieved by displaying cellulase containing complexes, but *E. coli* cells that display minicellulosomes have yet to be constructed.

4. Displaying cellulases and minicellulosomes in Gram-positive bacteria

Many species of Gram-positive bacteria are used industrially to produce biocommodities and have great promise as agents to produce second-generation biofuels. However, they are not naturally cellulolytic, prompting efforts designed to decorate their surfaces with cellulases. Below, we discuss progress towards creating cellulolytic strains of *B. subtilis, C. glutamicum*, and several industrially useful species of lactic acid bacteria. A common feature of these microbes is the absence of an extensive outer membrane, as they are surrounded by a thick peptidoglycan layer that in some instances is further surrounded by additional protective layers. Thus, the mechanisms used to display proteins on their surfaces are distinct from those employed to decorate Gram-negative bacteria.

4.1. *Bacillus subtilis*

B. subtilis is a model Gram-positive bacterium that is used industrially to produce commercial enzymes. It has several desirable traits that could enable its use in consolidated bioprocessing including established genetic tools to manipulate its genome as well as its generally recognized as safe status (GRAS) [108, 109]. It is also tolerant to high concentrations of salts and solvents and has the ability to utilize both pentose and hexose sugars produced from lignocellulose [110]. Finally, *B. subtilis* naturally secretes large quantities of extracellular enzymes (20–25 g of protein per liter of growth culture), suggesting that it should be capable of robustly exporting the enzymes needed to build cellulase containing complexes [111, 112]. The Gram-positive cell wall offers many sites for both covalent and noncovalent protein anchoring. Three mechanisms have been used to display cellulase on vegetative *B. subtilis* cells: membrane association via a

lipoprotein anchor, noncovalent cell wall interactions using the LysM domain, and covalent attachment to the cell wall using sortase enzymes (**Figure 6A**). Covalent attachment occurs through sortase enzyme processing of a C-terminal cell wall sorting signal (CWSS) that contains a LPXTG motif and has been estimated to display $\sim 2.4 \times 10^5$ proteins per cell [113, 114]. Noncovalent methods enable $\sim 1.2 \times 10^7$ proteins to be displayed per cell when the protein of interest is fused to the LysM domain, a binding module that interacts with the N-acetyl-muramic acid and N-acetyl-D-glucosamine components of the peptidoglycan [115, 116]. Cellulases have also been displayed by expressing them as a fusion protein with the membrane-associated lipoprotein, PrsA [117]. However, this display mechanism requires lysozyme treatment of the cell to remove the peptidoglycan.

Two groups have displayed *ex vivo* assembled minicellulosomes on the surface of *B. subtilis*. The Zhang laboratory displayed a minicellulosome with a miniscaffoldin that contained three cohesin domains, a CMB, and three LysM domains [31]. An estimated 2×10^4 miniscaffoldins bound to the surface of each cell. Minicellulosomes were assembled by incubating the cells with purified *B. subtilis* endoglucanase, *C. thermocellum* endoglucanase, and *Clostridium phytofermentans* cellobiohydrolase enzymes. Against regenerated amorphous cellulose (RAC) and crystalline cellulose, respectively, cell-tethered minicellulosomes degraded substrate 2.3- and 4.5-fold better than free minicellulosomes. As compared to commercial fungal enzymes dosed at the same protein concentrations, minicellulosome-displaying cells degraded RAC to a similar extent after 72 h, but exhibited 30% greater hydrolytic activity on Avicel. The Clubb group also demonstrated *ex vivo* assembly of a minicellulosome complex using a LPXTG anchor that can be processed by a sortase enzyme [118]. Purified *C. cellulolyticum* endoglucanase and exoglucanase, and *C. thermocellum* endoglucanase enzymes associated with a covalently cell wall attached miniscaffoldin. A major challenge specific to *B. subtilis* is the large amount of proteases that this microbe produces that can degrade heterologous surface-exposed proteins [119]. The Zhang group addressed this problem by displaying the cellulosome in a *B. subtilis* strain in which six extracellular proteases had been deleted, while the Clubb group improved protein display by deleting WprA, a cell wall-associated protease. Display of an *in vivo* assembled minicellulosome has yet to be achieved. Notably, an *in vivo* assembled minicellulosome was reported that could degrade untreated biomass, but this work was later retracted.

B. subtilis sporulates to produce a highly stress-resistant, dormant spore cell that can be decorated with cellulases. During the process of spore formation within the mother cell, genomic DNA is encapsulated within multiple protective layers including a cortex, coat, and crust [120]. Attractively, proteins do not need to be translocated across the cytoplasmic membrane in order to be displayed on the spore. There has been a significant amount of research performed to optimize protein display on *B. subtilis* spores [121]. While spore coat proteins are typically used as carriers to display proteins, a recent study found that native, unmodified proteins could be overexpressed in the mother cell and absorbed to the spore surface for display (**Figure 6B**) [122]. Using this approach, monomeric *B. subtilis* carboxymethylcellulase (CelB) and multimeric *E. coli* β-galactosidase (LacZ) were successfully displayed on the spore surface, and vigorous physiochemical treatment was shown to be needed to

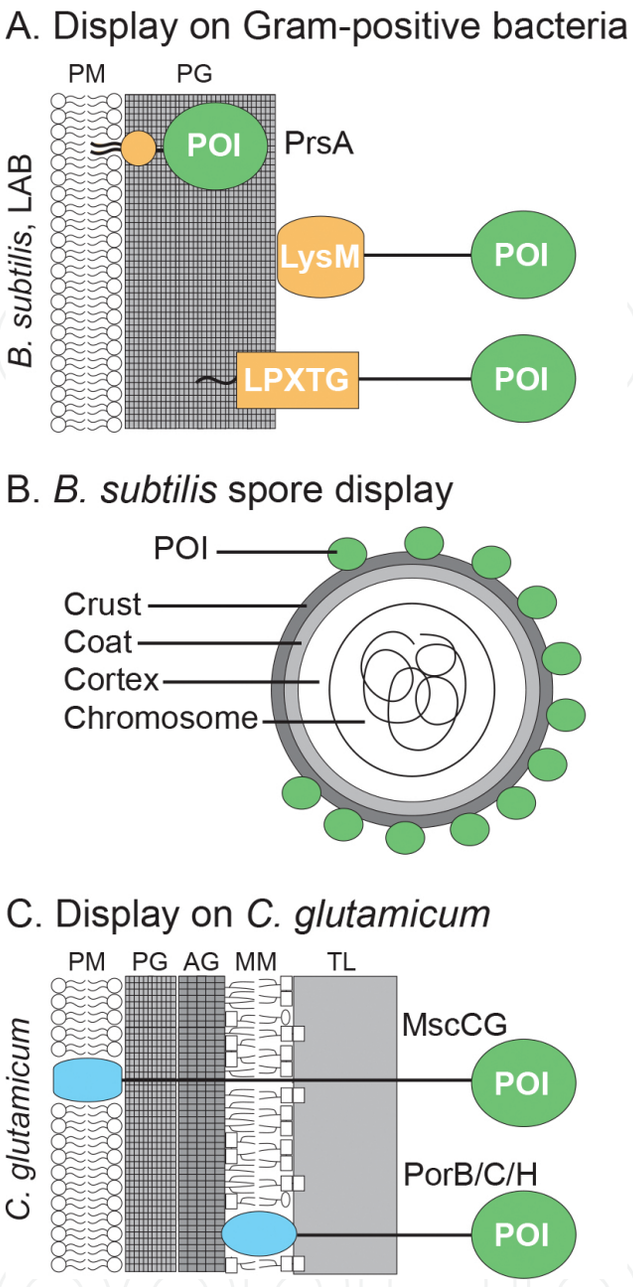

Figure 6. Cellulase and minicellulosome display in Gram-positive bacteria. (A) Methods used to display cellulases and their complexes. Proteins can be anchored to the plasma membrane through fusion to PrsA, but lysozyme treatment is necessary to expose this cellulase to the external environment. More conventionally, proteins are cell surface displayed either through noncovalent interactions with cell wall peptidoglycan by protein fusion to the LysM domain, or through covalent attachment to cell wall peptidoglycan by protein fusion to a cell wall sorting signal containing the LPXTG motif, which is processed by sortase transpeptidase. (B) Proteins can be displayed on the surface of *B. subtilis* spores. Native proteins, without fusion to anchor proteins, can be absorbed by the spore surface during spore formation. (C) Methods used to display cellulases and their complexes on the cell surface of *C. glutamicum*. Proteins of interest are displayed through fusion to membrane protein MscCG or porin proteins PorB, PorC, and PorH. Abbreviations: Protein of interest (POI), plasma membrane (PM), peptidoglycan (PG), arabinogalactan (AG), mycomembrane (MM), and top layer (TL).

remove the enzymes. Interestingly, proteins can also be directly adsorbed on the spore surface by incubating them with spores, resulting in 7.75×10^3 to 1.55×10^4 individual proteins being

displayed per particle [123]. Thus, cellulases that cannot be secreted or expressed as fusion proteins can be readily displayed on spores using this method.

4.2. Lactic acid bacteria (LAB)

Gram-positive lactic acid bacteria (LAB) are widely used in the food industry to ferment sugars into lactic acid [124]. They have potential in biomass processing, as they can utilize pentose and hexose sugars, and some members of this group, namely *Lactobacillus plantarum*, are tolerant to low pH and ethanol concentrations up to 13% [26, 125]. Similar methods are used to display proteins in LAB and *B. subtilis*, as their cell wall envelopes are structurally related (**Figure 6A**). In particular, proteins can be displayed noncovalently using LysM-binding modules, or covalently using LPXTG anchors that are processed by sortase enzymes. Comparison studies using *Lactococcus lactis* have shown that covalent attachment using sortase enzymes leads to the largest number of functionally displayed proteins on this microbe's surface, but the relative efficiencies of the sortase and LysM display systems in other species of LAB have not been determined [126].

Two groups have used sortases to assemble minicellulosomes *ex vivo* in LAB. The Martin group anchored a variety of nuclease A-fused miniscaffoldin constructs onto the surface of a single protease-deficient strain of *L. lactis* [127]. These constructs either contained one cohesin, two cohesins, a cohesin and a CBM, or only a CBM. Using a purified *E. coli* β-glucuronidase (UidA)-dockerin fusion protein, they estimated that $\sim 10^4$ complexes are displayed per cell. Interestingly, unlike work in yeast, scaffoldin size was not a limiting factor for display. Instead, it was suggested that protein secretion might be limiting, as scaffoldins containing a CBM showed improved secretion, presumably due to more rapid folding facilitated by the CBM. Using similar approaches, this group later produced a bifunctional minicellulosome using specifically associating type-I and type-II cohesin-dockerin pairs [128]. They demonstrated that the order of enzyme docking affected the activities of the *E. coli* UidA and LacZ enzymes, due to steric factors that influenced enzyme binding.

In separate studies, the Mizrahi group engineered *L. plantarum* to display a sortase attached miniscaffoldin and then used a consortium of cellulase secreting strains to assemble the minicellulosome *ex vivo* [129]. In these studies, recombinant *T. fusca* endoglucanase (Cel6A) and xylanase (Xyn11A) activities were studied in their free form, individually bound to cells, or bound to surface displayed minicellulosomes. Against hypochlorite pretreated wheat straw, secreted enzymes had the best initial activity, followed by the minicellulosome. However, the sugar production rate of the surface displayed minicellulosome slowly increased over time, while the rate for the secreted enzymes decreased. Eventually, cellulosomal activity overtook that of the secreted enzymes, presumably because the cell-associated enzymes are more stable. Interestingly, cells displaying individually anchored enzymes had minimal activity. This is consistent with results obtained in yeast that have shown that enzymes with different substrate specificities are most active against cellulose when they are densely clustered with one another on the cell surface to promote synergistic interactions [63].

4.3. *Corynebacterium glutamicum*

C. glutamicum is an industrially important microbe that produces several tons of glutamate and lysine annually. Although it is a Gram-positive bacterium, its peptidoglycan layer is covered by an arabinogalactan layer, a mycolic acid bilayer, and a top layer composed of polysaccharides, glycolipids, and proteins [130]. The presence of the outer mycolic acid layer confers Gram-negative characteristics to the bacterium and, along with the cytoplasmic membrane, is the primary point to which cellulases are attached (**Figure 6C**). The Kondo laboratory demonstrated the feasibility of displaying heterologous proteins by attaching them to the mycolic acid layer [131]. This was achieved by expressing the cellulase as a fusion protein in which it was joined to the PorC porin. *C. glutamicum* displaying a *Saccharophagus degradans* β-glucosidase-PorC fusion produced 1.08 g/L of lysine from 20 g/L of cellobiose in 96 h [132]. The Han group also displayed an *in vivo* assembled bifunctional minicellulosome that associated with the cytoplasmic membrane. This was accomplished by expressing the scaffoldin as a fusion protein with the mechanosensitive channel (MscCG) [133]. The complex contained the *C. thermocellum* endoglucanase E (CelE) and β-glucosidase A (BglA) enzymes and could release sugars from pretreated rice straw, miscanthus, and rape stem. In the future, engineering these cells to more efficiently degrade complex lignocellulose may lead to more cost-effective ways to produce lysine and glutamate from inexpensive biomass.

5. Conclusions

Towards the goal of cost effectively converting biomass into useful biocommodities, several research groups have developed creative ways to display cellulases on microbes to endow them with cellulolytic activity. Comparing the biomass degradation efficiencies of different types of recombinant microorganisms is difficult. This is because investigators have measured their activities using a variety of cellulosic substrates that can vary dramatically in their resistance to enzymatic degradation as they differ in their solubility, enzyme accessibility, crystallinity, degree of polymerization, fraction of reducing ends, and the presence of hemicellulose and lignin [134]. Moreover, different methods are frequently used to pretreat biomass and to measure the extent of degradation, which can be reported as enzymatic activity, sugar released, biomass remaining, or product produced [17, 135, 136]. However, despite these qualifiers, the results of studies reported to date enable several major conclusions to be drawn. In particular, they provide convincing evidence that clustering enzymes on the surface within minicellulosomes leads to improved microbial cellulolytic activity by promoting synergistic interactions, and in some instances, by improving enzyme stability [32]. Interestingly, synergistic enzyme interactions can also be obtained by displaying different types of individual enzymes, as long as they are densely clustered and have complementary activities [63]. Because complexed enzyme systems require smaller amounts of enzymes to be produced than secreted enzyme systems to achieve efficient degradation of lignocellulose, the cellulosomal system may be optimal for CBP microorganisms, since conserved energy may be directed towards product production.

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Displaying large, enzymatically diverse recombinant minicellulosomes remains a challenging problem, but progress has been made in *S. cerevisiae*, *B. subtilis*, *C. glutamicum*, and lactic acid bacteria (summarized in **Table 1**). Surface engineering in *S. cerevisiae* is the most advanced, with several groups developing strains that can assemble minicellulosomes *in vivo*, leading to significant improvements in biomass utilization [32, 78, 79, 89]. Complexes containing up to 12 enzymes have been displayed, but great challenges remain as expressing large structures with more enzymes reduces cellular display levels [79]. A variety of promising approaches may help to overcome this limitation, including adaptive assembly and host engineering methods. Furthermore, greater cellulolytic activity through the incorporation of new synergistic enzyme functionalities may offset the decreases associated with the production of larger proteins, as seen by the increased activity of a pentafunctional minicellulosome [78]. Approaches to display minicellulosome structures on eubacteria that have developed genetic systems are less advanced. Thus far, only *ex vivo* assembled complexes have been displayed in Gram-positive *B. subtilis* and LAB, and only individual enzymes have been displayed on the Gram-negative bacterium *E. coli*. The potential of displaying complexes on the cell surface of *E. coli* would seem to be bleak, as the presence of a second outer membrane in this microbe hampers protein secretion. However, studies of *C. glutamicum* are promising, as it is the only bacterium that has been engineered to display an *in vivo* assembled minicellulosome, although this complex contained only two enzymes. In *B. subtilis*, exogenous proteases appear to be a limiting factor, while in LAB protein expression levels are suboptimal [119, 137]. However, it would seem likely that these limitations can be overcome by optimizing display using genetic engineering and directed evolution approaches, as well as the use of cell consortiums to construct complexes *ex vivo*.

A great variety of surface engineering approaches have been developed to create ever more impressive recombinant cellulolytic organisms. However, it is clear that the lignocellulose hydrolysis rates of these recombinant microorganisms needs to be improved if they are to be used in CBP. Future studies may improve their cellulolytic activity by using directed evolution to enhance complex display and stability, by judiciously displaying cellulases that exhibit maximal enzyme synergy, and by devising new methods to stably attach proteins to the cell surface. Genetic engineering of the host will also be critical, enhancing expression, secretion, and display levels, and by eliminating proteins or factors affecting the stability and retention of anchored protein complexes over time. Displayed complexes will also have to be optimized to be maximally active against different types of biomass that have different sugar compositions and structures [138–140]. When these cells are further engineered to produce useful chemicals, their ability to cost-effectively produce biocommodities from biomass will be an important step towards reducing the world's dependency on oil [141–144].

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Nomenclature

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

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