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Advances in consolidated bioprocessing using synthetic cellulosomes

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The primary obstacle impeding the more widespread use of biomass for energy and chemical production is the absence of a low-cost technology for overcoming their recalcitrant nature. It has been shown that the overall cost can be reduced by using a 'consolidated' bioprocessing (CBP) approach, in which enzyme production, biomass hydrolysis, and sugar fermentation can be combined. Cellulosomes are enzyme complexes found in many anaerobic microorganisms that are highly efficient for biomass depolymerization. While initial efforts to display synthetic cellulosomes have been successful, the overall conversion is still low for practical use. This limitation has been partially alleviated by displaying more complex cellulsome structures either via adaptive assembly or by using synthetic consortia. Since synthetic cellulosome nanostructures have also been created using either protein nanoparticles or DNA as a scaffold, there is the potential to tether these nanostructures onto living cells in order to further enhance the overall efficiency.

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Introduction

Because of its wide availability and low cost, lignocellulosic biomass has attracted interest as a fermentation feedstock to produce fuels and other commodity chemicals [1]. Various physical and chemical pretreatment methods have been developed to reduce the recalcitrance of lignocellulose [2]. However, these processes are often environmentally or economically challenging. In contrast, nature has created different solutions to tackle recalcitrant biomass. Aerobic microbes (such as *Trichoderma reesei*) produce copious amounts of soluble hydrolytic enzymes that synergistically break down cellulosic materials [3]. In contrast, anaerobic organisms, due to energetic constraints, can only produce a limited amount of enzymes. Therefore, in response, they have developed an elaborately structured enzyme complex, called the cellulosome, to maximize catalytic efficiency [4,5]. This self-assembled system brings multiple enzymes in close proximity to the substrate and provides a structure that ensures high local concentration and the correct ratio and orders of the enzymes, thereby maximizing synergy.

In order to make biological methods competitive in the market, past research efforts have been devoted to the development of consolidated bioprocessing (CBP) of lignocellulose into high-value products without the addition of exogenous enzymes [6]. The primary motivation behind this strategy is that the process cost is substantially lower compared with conventional configurations [7]. However, to date, native cellulolytic microorganisms that have been isolated are not capable of efficiently producing high-value compounds on the commercial scale. On the other hand, microorganisms capable of producing high-value compounds cannot efficiently utilize lignocellulose. Therefore, new strategies that grant chemical-producing microorganisms the ability to hydrolyze lignocellulose have been frequently studied for the creation of CBP microorganisms. In this review, we summarized recent efforts using synthetic biology strategies to create artificial (mini)cellulosome structures for a broad range of applications. While most reports focused on engineering heterologous hosts to display complex cellulosomal structures, recent progress in the creation of noncell-based cellulosomes is also discussed (Table 1).

Yeast surface display of synthetic cellulosomes for consolidated bioprocessing

Inspired by the natural ability of cellulosome to substantially enhance biomass hydrolysis, synthetic biologists have begun to create engineered microorganisms endowed with similar desirable features. One attractive candidate is the baker yeast *Saccharomyces cerevisiae*, which is widely used for industrial applications due to its high ethanol productivity as well as tolerance toward low pH and various inhibitors released from lignocellulosic

Table 1

Different CBP approaches and their synergy effects.

Host/matrix	Approach	Substrate	Product titer	Synergy	Reference
S. cerevisiae	Enzyme secretion	β-glucan	9.15 g/L ethanol	–	[10]
S. cerevisiae	Enzyme secretion	Corn cob	28.2 g/L ethanol	–	[11]
S. cerevisiae	Enzyme secretion	Corn cob	4.05 g/L ethanol	–	[12]
S. cerevisiae	Surface display cellulases	PASC	~3 g/L ethanol	–	[13]
S. cerevisiae	Surface display cellulases	PASC	3.4 g/L ethanol	2.9	[14]
S. cerevisiae	Surface display minicellulosome (lysate)	PASC	3.5 g/L ethanol	2.6	[16]
S. cerevisiae	Surface display minicellulosome (single strain)	PASC	~1.8 g/L ethanol	5.5	[17]
S. cerevisiae	Surface display minicellulosome (consortium)	PASC	1.87 g/L ethanol	2	[19]
S. cerevisiae	Surface display minicellulosome (single strain)	PASC	1.52 g/L ethanol	–	[21]
S. cerevisiae	Surface display complex cellulosome (lysate)	PASC	~2 g/L ethanol	4.2	[26]
S. cerevisiae	Surface display complex cellulosome (single strain)	Avicel	1.4 g/L ethanol	3.6	[27]
K. marxianus	Surface display trimeric xylanosomes	Avicel	3.09 g/L ethanol	–	[28••]
S. cerevisiae	Surface display trimeric xylanosomes (concentrated medium)	Xylan	1.6 g/L reducing sugar	3.3	[30]
S. cerevisiae	Surface display trifunctional minihemicellulosome (single strain)	Arabinoxylan	0.95 g/L ethanol	1.4	[31]
Cell-free	Chimeric cellulosome	Wheat straw	0.9 mM reducing sugar	2	[32]
E. coli	Surface display a BG	Cellobiose	17 g/L ethanol	–	[37]
E. coli	Surface display cellulases	PASC	3.59 g/L ethanol	–	[38]
P. putida	Surface display a BG via unifunctional scaffoldin	Cellobiose	–	–	[40•]
B. subtilis	Secrete and assemble minicellulosome in the medium	–	–	–	[43•]
B. subtilis	Surface display cellulosome	Napier grass	476.69 g/L reducing sugar	–	[44]
L. lactis	Surface display cellulosome-inspired enzyme complexes	–	–	–	[48]
L. plantarum	Secretion of cellulase and xylanase (consortium)	Wheat straw	3 mM reducing sugar	1.8	[49]
L. plantarum	Surface display bifunctional minicellulosome (consortium)	Wheat straw	~8 mM reducing sugar	–	[50]
L. plantarum	Surface display tetrafunctional minicellulosome (consortium)	Wheat straw	17 mM reducing sugar	–	[51•]
MNPs	Display multiple cellulase complexes	Avicel	~0.65 mM reducing sugar	–	[53]
CdSe–ZnS core–shell QDs	Display multiple cellulase complexes	PASC	0.8 g/L reducing sugar	5.6	[54]
CdSe nanoparticles	Display cellulase and CBD complexes	PASC	0.115 g/L reducing sugar	7.2	[55]
Polymeric micelles	Capture His6-tagged cellulases	PASC	0.193 g/L reducing sugar	2.2	[56]
Self-assembling protein nanocages	Conjugate cellulase and CBD complexes	PASC	~45 g/L reducing sugar	3.3	[59]
DNA scaffolds	Conjugate cellulase and CBD complexes	PASC	0.07 g/L reducing sugar	1.7	[62]
Rolling circle amplification DNA template	Conjugate cellulase and CBD complexes	PASC	~0.9 g/L reducing sugar	5.1	[63]
Synthetic DNA template	Five-component enzyme cascade	PASC	6.5 mol H2O2	1.5	[64]
Programmable DNA strand	Conjugate cellulase and CBD complexes	PASC	~0.9 g/L reducing sugar	1.5	[67]
DNA scaffold	dCas9-guided two-component cellulosome	PASC	45 g/L reducing sugar	2.6	[68•]
Molecular brush scaffold	Biomimetic polymeric cellulosomes	CMC	~0.52 mg/L reducing sugar	3	[70]

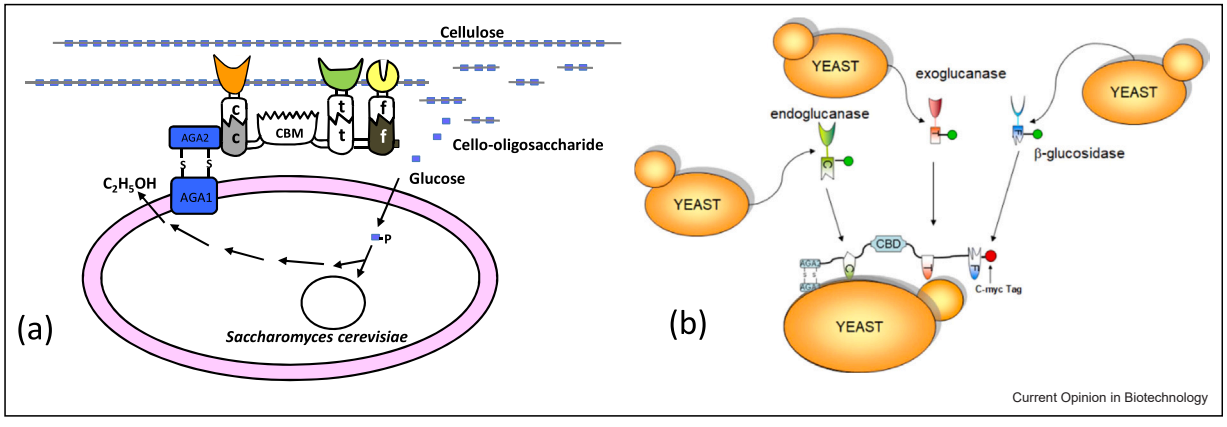
hydrolysates [8]. More importantly, advanced genetic tools are available to easily manipulate yeast with the desirable phenotypes [9].

An ideal yeast strain for CBP should possess the capability of simultaneous cellulose saccharification and ethanol fermentation. To produce ethanol from cellulose, the sequential action of three different enzymes — endoglucanase, exoglucanase (or cellobiohydrolase), and β-glucosidase (BG) — is required for the complete hydrolysis of cellulose to glucose [3]. Initial attempts to adapt yeast as a CBP organism focused on the co-secretion of enzymes for cellulose hydrolysis [10,11]. In addition to lab strains, super-secretion strains were isolated and engineered to co-secrete endoglucanase and BG for ethanol production [12]. Unfortunately, only a limited amount of cellulases could be secreted under anaerobic conditions due to energetic constraints. An alternative strategy is to display enzymes directly onto the cell surface. Up to four different cellulases were displayed on the yeast cell surface, permitting a synergistic hydrolysis of cellulose with concomitant ethanol production [13]. Codisplay of expansin-like proteins, which have a cellulose-loosening effect, further enhanced overall ethanol production [14]. While these works point to a valid strategy of combining the ethanol-producing capability with cellulose hydrolysis, the efficiency of hydrolysis must be substantially improved before it can be employed in commercial processes [15]. Clearly, significant improvement in cellulose hydrolysis is needed to make this strategy practical.

The key to improving hydrolysis is, perhaps, to increase the catalytic efficiency by maximizing the synergy with limited amount of enzymes. This has been realized by displaying designer cellulosomes onto yeast cell surfaces to efficiently degrade cellulose in an energy-limited environment. A miniscaffoldin that consisted of three divergent cohesin domains from *C. thermocellum (t)*, *C. cellulolyticum (c)*, and *Ruminococcus flavefaciens (f)* was functionally displayed on the yeast surface (Figure 1a) [16]. Incubation with *E. coli* lysates containing an endoglucanase (CelA), an exoglucanase (CelE), and a β-glucosidase (BG; BglA) fused to the corresponding dockerin domains resulted in the assembly of a functional minicellulosome on the cell surface. Cells displaying the minicellulosome exhibited substantially enhanced glucose liberation and produced 2.6-fold higher level of ethanol than that obtained by using the same amounts of added purified enzymes. Wen et al. also displayed a trivalent scaffoldin onto the yeast surface. By secreting three cellulases tagged with the corresponding dockerins, the resulting recombinant strain produced ethanol with a titer of 1.8 g/L [17]. Scaffoldin display can be further improved by disruption of glycosylation genes located in the endoplasmic reticulum [18].

Unfortunately, the use of a single yeast strain for surface anchoring and cellulase secretion has met with bioenergetic limitations as increasing amounts of cellular machinery are rerouted toward heterologous protein synthesis. Instead, the minicellulosomes were reassembled using a synthetic yeast consortium [19]. Four engineered strains were employed to either display the trifunctional scaffoldin or express individual enzymes tagged with the corresponding dockerin for self-assembly (Figure 1b). The level of cellulose hydrolysis and ethanol production can be optimized simply by fine-

Figure 1



Yeast surface display of synthetic cellulosomes. **(a)** Simultaneous hydrolysis of cellulose and ethanol production by an engineered yeast strain displaying a trifunctional cellulosome. **(b)** Functional display of a trifunctional cellulosome using a four-member yeast consortium. The basic design consisted of four different engineered yeast strains capable of either displaying a trifunctional scaffoldin or secreting one of the three corresponding dockerin-tagged enzymes (endoglucanase, exoglucanase, or BG).

tuning the ratio of different yeast populations in the consortium [20]. In addition to exploiting the interaction between the cohesin–dockerin pairs for cellulosome assembly, synthetic cellulosome structures have also been created based on enzyme immobilization via disulfide bonds with enhanced stability [21]. It is possible that even bioconjugation strategies such as the SpyCatcher–SpyTag system may be used for either covalent [22,23] or light-responsive assembly [24•].

The overall performance can be further improved by displaying more complex cellulosomes composed of more than three enzymes. Instead of employing only a single scaffoldin for cellulosome formation, many bacteria exhibit a more complex cellulosome structure, in which several adaptor scaffoldins were found in addition to a surface-displayed anchoring scaffoldin [25]. These adaptor scaffoldins serve to amplify the number of enzymatic subunits that can be incorporated into the cellulosome. Several notable examples in creating complex cellulosome via adaptive assembly have been reported. In one example, a yeast strain displaying an anchoring scaffoldin containing two different cohesin domains was used to recruit two adaptor scaffoldins containing two additional cohesin domains to amplify the number of dockerin-tagged cellulase loadings to four per cell [26]. Cells displaying the tetravalent cellulosome on the surface exhibited a 4.2-fold enhancement in the hydrolysis of phosphoric acid-swollen cellulose (PASC) than free enzymes and produced twofold more ethanol compared with cells displaying the divalent cellulosomes. A similar strategy using adaptive assembly was reported by employing a single yeast strain for both surface display and secretion. As expected, increasing the number of dockerin domains on the displayed scaffoldin has a detrimental effect on expression due to burden on the secretory pathway [27]. Recently, a synthetic cellulosome consisting up to 63 enzymes was displayed onto the probiotic yeast *Kluyveromyces marxianus*, allowing the production of 8.61 g/L of ethanol from PASC [28••]. This impressive result again suggests that feasibility to efficient CBP via complex cellulosome display, a feature highlighted by nature. The recent discovery of fungal cellulosomes provides additional research opportunities to design more elaborate chimeric cellulosomal structures consisted of components from both bacteria and anaerobic fungi [29].

Yeast surface display of synthetic cellulosomes for hemicellulose processing

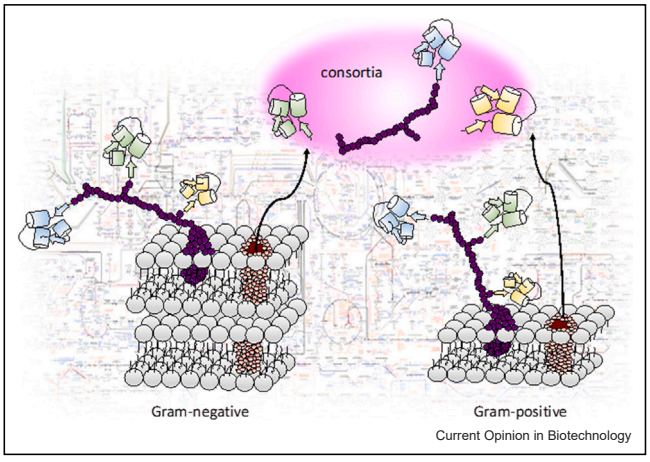
Hemicellulose is the second most prevalent source of fermentable sugars from plant biomass. Many natural cellulosomes are known to degrade xylan and other hemicelluloses [5], and contain different types of hemicellulases in addition to cellulases. While only a few reports have successfully displayed synthetic cellulosomes targeting hemicellulosome, similar levels of enhanced hydrolysis were observed. In an early report, five trimeric xylanosomes were successfully assembled on the yeast cell surface using three dockerin-tagged fungal endoxylanase, β-xylosidase, and acetylxylan esterase [30]. Inclusion of a xylan-binding domain is important as this addition improved hydrolysis by 2.1-fold relative to a scaffoldin without a binding domain, and 3.3-fold over free enzymes. Placement of the xylan-binding domain adjacent to the endoxylanase provided the highest level of enhancement, highlighting the importance of enzyme–substrate synergy. Similar enhancements have also been demonstrated by displaying a trifunctional cellulosome consisted of an arabinofuranosidase rather than an acetylxylan esterase [31]. A single engineered strain was used to display the scaffoldin and to secrete all three enzymes for assembly. While it has not been demonstrated, the addition of an enzyme active on lignin will further expand the applicability of synthetic cellulosomes toward combined delignification and saccharification [32].

Assembly of synthetic cellulosomes by other microorganisms

In addition to the development of CBPs that convert lignocellulose to ethanol, the production of other valuable solvents and fine chemicals from lignocellulosic biomass by an organism or a mixed culture without the external addition of cellulolytic or hemicellulolytic enzymes has also attracted substantial research interests [33]. Motivated by this highly ambitious strategy, several Gram-negative and Gram-positive microorganisms with engineered cellulolytic activity are being developed for CBP. As shown in Figure 2, this configuration can be achieved either in a single strain or a consortium.

Escherichia coli is the most commonly used host mainly due to its capability to produce recombinant proteins in high yields and its ease of genetic manipulation, allowing the production of a wide range of different fine chemicals [34]. However, due to the complex cell envelope, *E. coli* is generally not considered a good candidate for secreting or displaying heterologous proteins as compared with Gram-positive bacteria [35]. Therefore, the assembly or display of cellulosomal structures on the *E. coli* surface is surprisingly rarely reported. While the cellulases and scaffoldins are considered large and complex passengers, their display systems are mainly based on autotransporter proteins and ice nucleation proteins (INPs) [36]. Displaying a BG from *Thermobifida fusca* on the surface of an ethanologenic *E. coli* Ms04 via autodisplay yielded a cellobiose-degrading whole-cell biocatalyst that directly fermented cellobiose into ethanol in the mineral medium. Remarkably, the cells could produce 15–17 g/L ethanol from a 40 g/L carbon source, representing more than 80% of the

Figure 2



Assembly of synthetic cellulosomes by Gram-positive and Gram-negative microorganisms. Owing to the double-cell-membrane structure, the secretion and display of cellulosomal components are more difficult in the Gram-negative strains than those in the Gram-positive ones. Alternatively, the assembly of cellulosome structures can be achieved by creating a synthetic consortium.

theoretical yield [37]. Although the result is impressive, more essential cellulolytic enzymes are needed in order to achieve CBP. To completely hydrolyze cellulose, three enzymes from *Clostridium cellulolyticum* have been displayed on the cell surface of ethanologenic *E. coli* LY01 via the PgsA-anchoring motif. Remarkably, the resulting whole-cell biocatalyst yielded 3.59 g L^{-1} ethanol after 60 hours of fermentation, which corresponds to a theoretical yield of 95.4 ± 0.15% calculated from the sugar consumed [38]. Aside from autotransporter proteins and INPs, the trivalent scaffoldin has also been successfully displayed on the *E. coli* surface by fusing with the curli protein CsgA. The scaffoldin merged to CsgA is correctly exposed at the *E. coli* surface and each cohesin module is fully operational to trap a large number of cellulases. The strain displaying the minicellulosome exhibited a sixfold higher activity on crystalline cellulose hydrolysis as compared with the free enzyme system [39].

Another promising Gram-negative microorganism to become a host of choice for CBP is *Pseudomonas putida*. However, similar to the majority of other domesticated microbial platforms, it cannot degrade lignocellulosic biomass alone due to the lack of an efficient extracellular cellulolytic apparatus. To address this issue, three scaffoldin variants were optimally delivered to the surface of *P. putida* with the Ag43 autotransporter protein from *E. coli*. The display and functionality of cohesins were confirmed by the extracellular attachment of chimeric BG and fluorescent proteins. The recombinant strains after incubating with the chimeric BG were able to grow on the cellobiose [40•]. It has been shown that the heterologous expression of extracellular proteins (e.g. scaffoldin, cellulases, or hemicellulases) is the key feature of synthetic cellulosome strategies [41]. However, due to the fact that protein secretion in Gram-negative bacteria actually faces the challenge of translocation across the double-membrane system, surface display of scaffoldins and subsequent assembly of a designer cellulosome of any size has not yet been reported. This may also help explain why most cellulolytic bacteria in nature are Gram-positive.

More studies have been conducted on the surface display and secretion of various enzymes for minicellulosome assemblies in Gram-positive bacteria. *Bacillus subtilis*, *Lactococcus lactis*, and *Lactobacillus plantarum* all have been used as the hosts for assembling the cellulosome on the bacterial surface. *B. subtilis* is one of the most popular Gram-positive model bacteria and is considered a promising workhorse candidate for the industrial production of various recombinant proteins, amino acids, and fine chemicals [42]. As early as 2004, the first report showed EngB endoglucanase and mini-CbpA1 scaffoldin could be coexpressed heterogeneously in *B. subtilis* [43•]. Although the miniscaffoldin used in this study only possessed one cohesin, the exciting part is that the two components could be successfully secreted into the medium to form a minicellulosomal structure. To mimic the complete cellulosome structure of *C. thermocellum*, an elegant study successfully expressed eight cellulosomal genes of *C. thermocellum* in *B. subtilis* [44]. It demonstrated that the full-length scaffoldin could be successfully displayed on the cell surface. The CBD preserved its cellulose-binding ability and the cohesins retained their cellulase-docking ability. Impressively, the engineered *B. subtilis* was able to liberate 476.69 g/L of reducing sugar from the saccharification of raw biomass Napier grass in 48 hours. This is, by far, the best result reported using genetically engineered microorganisms.

L. lactis is another Gram-positive microorganism of specific interest as it is generally regarded as safe and has been used to produce valuable commodity chemicals such as lactic acid and bioactive compounds [45]. A notable characteristic of the strain is that it has been successfully engineered to secrete or display a wide variety of proteins ranging from 9.8 to 165 kDa [46]. Using *L. lactis* as a surrogate host, researches have successfully displayed fragments of scaffoldins with different sizes on the cell surface through the incorporation of the C-terminal anchor motif of the streptococcal M6 protein or the three SLH modules of the *Clostridium cellulovorans* EngE protein [47,48]. However, while the cohesins in the fragmented scaffoldin originated from *C. thermocellum* were capable of functionally associating with engineered enzymes, those originated from *C. cellulovorans* were not.

Unfortunately, neither of these two studies demonstrated that the minicellosomes they constructed could be used to hydrolyze cellulose likely due to the low level of display. Further optimization is required to improve the display efficiency.

Assembly of synthetic cellulosomes by bacterial consortia

To make a functional cellulosome or even a minicellulosome, the recombinant microorganisms have to secrete cellulases and display scaffoldins to a sufficient level. This could be a burden to cells due to either the limitation of available resources or the obstruction of translocation machinery. To overcome drawbacks associated with CBP using a single microbe, synthetic consortia are attracting increasing interest recently. By doing this, the labor of displaying and secreting the cellulosomal components was therefore divided among the members of the microbial community. Apart from the synthetic yeast consortium mentioned previously, another elegant example is the creation of synthetic *L. plantarum* consortia for minicellulosome assembly.

L. plantarum is also an attractive candidate for metabolic engineering as its natural characteristics include high ethanol and acid tolerance and the ability to metabolize pentoses and hexoses. An attempt to create cellulolytic *L. plantarum* strain was via the separate introduction of cellulase and xylanase into different strains. By creating a synthetic consortium of the two engineered *L. plantarum* strains, it could synergistically degrade cellulosic biomass [49]. Later on, a designer minicellulosome containing a xylanase, a cellulase, and a cellulose-binding module (CBM) was successfully assembled on the *L. plantarum* surface by a synthetic consortium containing two strains for cellulase and xylanase secretion, respectively, and one for producing the scaffoldin [50]. Comparing the two different strategies (secretion enzymes and designer cellulosomes), the enzyme-secretion system appeared to be less stable than the designer cellulosome system, suggesting an advantage of the latter over longer operations. However, due to the obstacles encountered when trying to display large scaffoldins on the *L. plantarum*, only a limited number of enzymes could be incorporated into the minicellulosomal complex. This trims the degradation capability of the engineered consortium. To overcome these issues, the 'adaptor scaffoldin' strategy, which mimics natural elaborated cellulosome architectures, was employed to compensate for the low levels of protein displayed on the bacterial cell surface [51•]. The resulting consortium was able to display large and fully active self-assembling cellulosomal complexes on the cell surface *in vivo*. This consortium also demonstrated that the enzyme stability and performance of the cellulosome machinery are superior to those seen with the equivalent secreted free enzyme system.

Although the two examples, the *S. cerevisiae* consortium and the *L. plantarum* consortium, reviewed here were using the same species, creating synthetic consortia composing cross-species microorganisms is also a promising strategy. However, challenges, such as controlling population dynamics and optimizing the compromised culture conditions, must be overcome before they can be applied in industries.

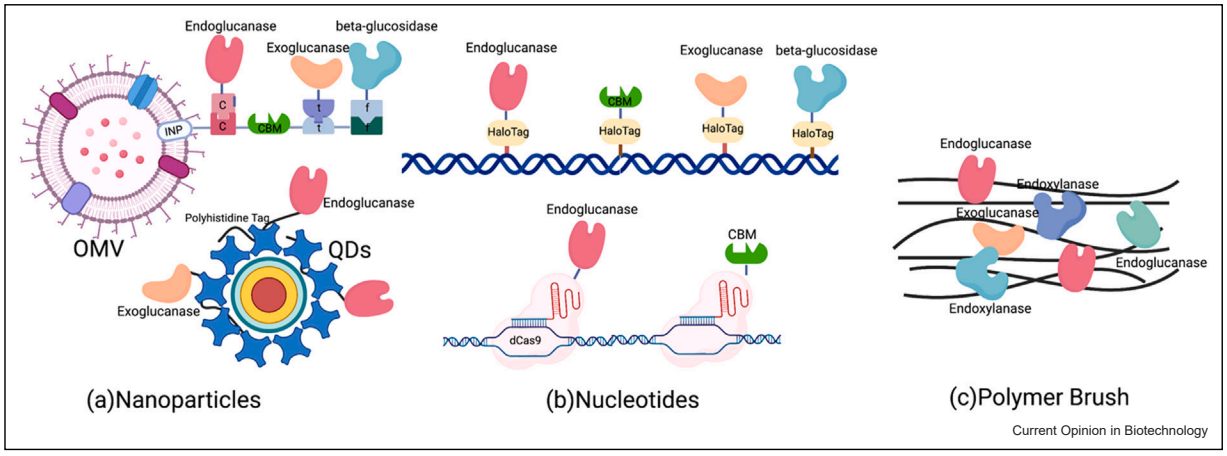
Nanoparticle-based cellulosome assembly

Living organisms, including yeast and bacteria, have been well engineered for cellulosome assembly and CBP applications. Compared with whole-cell-based strategy, which needs sophisticated environment for maintenance, non-living organism-based cellulosome assembly has the advantages of less maintenance cost and potentially longer shelf life for CBP applications.

Outer membrane vesicles (OMVs) are 20–200 nm proteoliposomes derived from the outer membrane and periplasmic space of many Gram-negative bacteria, including *E. coli* as part of their natural growth cycle. By taking advantage of the fact that OMVs naturally contain proteins found in the outer cell membrane, a trivalent protein scaffold containing three divergent cohesin domains from *Clostridium cellulolyticum* (C), *Clostridium thermocellum* (T), and *Ruminococcus flavefaciens* (F) with an internal CBM was displayed on OMV surface through a truncated INP anchoring motif. This trivalent scaffold enabled the position-specific presentation of a three-enzyme cascade, including endoglucanase (CelA), an exoglucanase (CelE), and a BG (BglA) with each enzyme fused with a dockerin binding onto the scaffold (Figure 3a) [52•]. The enzyme-decorated OMVs provided synergistic cellulose hydrolysis, resulting in a 23-fold enhancement in glucose production than free enzymes. This substantially higher level of enzyme synergy may come from the much higher enzyme-to-volume ratio on this nanoscale OMV.

The magnetosome is a unique organelle in magnetotactic bacteria, consisting of a magnetite core covered by a lipid bilayer membrane with an average diameter of 75 nm. Magnetic nanoparticles (MNPs) from magnetotactic bacterium from strain AMB-1.12 were functionalized with cohesin domains from *Clostridium thermocellum* (T), *Ruminococcus flavefaciens* (F), and CBM by fusing them with mms13, a protein known to double-pass transmembrane protein and tightly bind to the magnetite core [53]. Dockerin-tagged endoglucase and BG were immobilized on the MNPs with improved hydrolysis activity against cellulose, demonstrating the

Figure 3



Assembly of synthetic cellulosome onto different synthetic materials. **(a)** Nanoparticle-based cellulosome assembly, including OMV (top) and QDs (bottom). **(b)** Nucleotide-based cellulosome, including HaloTag (top) and dCas9- (bottom) enabled cellulsome. **(c)** Polymer-based cellulosome assembly.

potential of using a biological approach to assemble cellulosome on MNPs.

Other than biology-derived nanoparticles, inorganic nanoparticles were explored as the scaffold for cellulosome assembly. Two different cadmium selenide (CdSe)–ZnS core–shell quantum dots (QDs) with desirable sizes of 5 and 10 nm in diameter, were explored respectively for cellulosome assembly. Polyhistidine fusion was employed to directly recruit two enzymes, endoglucanase (CelA) and exoglucanase (CelE), onto CdSe–ZnS QDs [54]. Although the two enzymes were randomly attached onto the QDs, both enzyme complexes showed roughly fivefold better initial hydrolysis rates compared with free enzymes. This demonstrates the advantage of enzyme proximity on nanosized particles while the influence of particle size is relatively modest. In addition to using a histidine tag, endoglucanase A (EglA) and CBM fused with a biotin acceptor peptide were immobilized onto streptavidin-conjugated CdSe nanoparticles with up to threefold enhanced cellulose degradation activity [55], suggesting the choice of enzyme immobilization may slightly impact orientation and overall enhancement. Other nanoparticles such as nickle NTA-functionalized micelles [56], MNPs [57], polystyrene nanospheres [58], and protein nanoparticles [59] were also used as scaffolds for cellulase and CBM immobilization, demonstrating the potential of using a range of nanoparticles for cellulosome assembly.

Nucleic acid-based cellulosome assembly

The ability to easily predict and manipulate the base-pairing property of nucleic acids along with the ease of synthesis has allowed researchers to create various nucleotide nanoscaffolds with one-dimensional, two-dimensional, three-dimensional, and dynamically programmed structures [60]. Artificial cellulosomes based on DNA scaffolds have been achieved using chemical conjugation [61], zinc finger protein-guided assembly [62], and self-labeling HaloTag [63] for enzyme immobilization. The self-labeling HaloTag that can form a covalent bond with synthetic chlorohexane-modified molecules, including DNA, was used to generate fusions to enable the formation of cellulosome component DNA complex [63]. By combining sequence-specific DNA hybridization for four cellulosome components, including endoglucanase (CelA), CBM, exoglucanase (CelE), and beta-glucosidase (BglA), a fully functional synthetic cellulosome was created on a DNA template to release glucose from cellulose (Figure 3b). Furthermore, by applying longer (> 100 repeats) DNA template produced by rolling circle amplification, 5.1-fold enhancement of glucose release was achieved compared with free enzymes. This strategy is advantageous over the other two methods as it offers less enzyme deactivation and improved colocalization efficiencies. A further extension of the HaloTag framework was demonstrated by using one additional enzyme, glucose oxidase (GOX), to the system by conjugating GOX with a DNA linker via the well-known N-ε-maleimidocaproyloxy sulfosuccinimide ester chemistry [64]. The five-enzyme assembly was able to achieve conversion of cellulose to gluconic acid and H_2O_2 with a 1.5-fold activity enhancement, suitable for enzyme fuel cell applications.

One special property of using nucleic acid nanoscaffolds is the feasibility of dynamic assembly by toehold-mediated strand displacement, which involves revealing a new binding sequence in response to the presence of an initiator strand [65,66]. Taking advantage of strand

displacement, dynamic assembly of endoglucanase (CelA) and CBM was achieved [67]. In the ON state, both CelA and CBM are in close proximity for fast cellulose hydrolysis and when the NOT strand is added, CelA and CBM are separated onto noninteracting complexes resulting in slow hydrolysis. Similarly, dCas9 fusions were used to enable dynamic assembly of CelA and CBM [68•]. By exploiting the high-affinity and specific DNA-binding ability of dCas9, the enzymes were colocalized onto a double-strand DNA template (Figure 3b). Dynamic assembly was demonstrated by adding a toehold-gated gRNA design as an RNA-sensing conditional element [69]. Since more complex 2-D and 3-D DNA/RNA scaffolds can be created, further increase in the overall activity may be achieved by exploring different enzyme arrangements, leading to more favorable substrate channeling.

Polymer is another scaffold candidate for cellulosome assembly. The development of polymeric cellulosomes–enzyme-polymer conjugates (EPCs) was generated by covalent binding of cellulases onto a molecular brush polymer scaffold synthesized by radical copolymerization of poly(ethylene glycol) methyl ether methacrylate and glycidyl methacrylate [70]. Compared with natural cellulosomes, the EPC is generated based on random conjugation of different endoglucanases (Cel5A, Cel6B), exoglucanase (Cel7A), and/or endoxylanase (Xyl10 and Xyl11) (Figure 3c). EPCs exhibit 5–10-fold improved catalytic activity with both soluble and insoluble substrates, and the catalytic activity of the EPC conjugates improved with the increased diversity of enzymes included in the EPC structure. This work demonstrates that complex biomass structures will benefit from longer synergistic motifs. Consequently, a larger random EPC accommodating more enzymes in the structure could be beneficial for depolymerization of real biomass substrates such as those derived from poplar tree.

While all these assembly strategies were successful in creating synthetic cellulosomes, none has been combined with engineered microorganisms for CBP. It may be possible to include a cell-binding motif to the synthetic scaffolds to enable secreted enzymes to assembly into functional cellulosomes for CBP. The key benefit is the potential to create more complex cellulosome nanostructures that are different to achieve using a purely synthetic biology approach. In the future, such a combined chemistry and synthetic biology strategy may be another potential way to further enhance the overall CBP efficiency.

Discussion and future prospects

While the idea of CBP has been around for several decades, the first use of engineered organisms displaying synthetic cellulosomes has only been reported since 2010 [16]. However, there are still many hurdles remaining to translate this strategy into industrial scale. While a noncellulolytic host allows the hydrolysis sugars to be rerouted more easily toward product synthesis, the ability to create complex cellulsome structures for efficient biomass hydrolysis without compromising cell growth remains a key limitation. In some cases, the use of synthetic consortia has the potential to alleviate some of these issues. In addition, the incomplete conversion of crystalline cellulose to simple sugars suggests the requirement of higher ratios of certain enzymes such as cellobiohydrolases to accelerate the process. To this end, the use of cellulosome nanostructures with the appropriate enzyme cocktails may be an alternative solution to this problem. The SpyCatcher/SpyTag bioconjugation is particularly attractive as this strategy can be used to generate cellulosome structures using a one-pot reaction [71]. Highly stable protein nanoparticles such as the HVB capsid, which contains 240 conjugation sites [22], can be easily repurposed for this application as a large mixture of cellulases and hemicellulases can be combined together.

A second issue is that the composition of natural cellulosome is not static but dynamic in nature, depending on the level of cellulose and hemicellulose [72]. So far, our ability to fine-tuning the cellulosomal composition during CBP is still lacking. Natural cellulosome-forming bacteria such as *Clostridium thermocellum* based on the composition of cellulosomal enzymes change in response to different carbon sources and growth rates [73]. A membrane-bound sigma factor is released upon cellulose binding to provide the dynamic transcriptional regulation. While some dynamic cellulosome assembly has been reported [67,68•], new extracellular sensors are needed to detect and modulate the secretion of enzymes based on substrate availability [74]. This should be a new focus moving forward in the future.

Conflict of interest statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

No data were used for the research described in the article.

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- •• of outstanding interest.

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