Clostridium thermocellum

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Clostridium thermocellum, a Gram-positive, thermophilic anaerobic bacterium, exhibits an outstanding capability for degrading cellulolytic biomass to release fermentable sugars of different lengths by means of its powerful cellulosomes.

Keywords: cellulase booster ; artificial cellulosomes ; designer cellulosomes ; laccases ; LPMOs

1. Introduction

During evolution, cellulolytic microbes developed an extracellular multienzyme complex called the cellulosomes to boost the cellulose degradation rate at maximum levels [1]. Since the polysaccharide compositions in plant cell walls vary substantially in both quality and quantity [2], *C. thermocellum* needs to mediate the composition of saccharolytic enzymes in the cellulosome complex to cope with the complexity and recalcitrance of a specific cell wall [3]. More than 70 cellulolytic enzymes-borne type-I dockerin (DocI) are noncovalently assembled onto the primary non-catalytic protein, termed scaffoldin (Scaf) CipA, through a calcium-dependent high-affinity interaction (i.e., dissociation constant K D < 10 −11 M) with the nine type-I cohesin (CohI) domains located on the CipA structure [4]. In addition, the CipA protein also contains a type-II dockerin (DocII) domain at its C-terminal to mediate the attachment of the cellulosome complex to the bacterial cell surface [5]. Correspondingly, C. thermocellum possesses three types of surface-anchoring Scaf(s), namely SdbA, Orf2p, OlpB, which contain one, two, and seven type-II cohesins (CohII), respectively, responsible for the binding of the cellulosome complex to the cell surface through CohII–DocII interaction.

Inspired by the Lego-like architecture of the *C. thermocellum* cellulosome, various research groups have been seeking to construct designer cellulosomes (DCs) for basic and applied studies [6][7][8]. Although there are several conjugation techniques to design artificial cellulosomes [9], the present review mainly focuses on the CohI– DocI interaction-based artificial cellulosome construction. Furthermore, the related issues of this approach, such as enzymatic unit positions, types of enzymes, linkers between DocI and catalytic domain, and number of carbohydrate-binding modules (CBMs), are updated and discussed. Another strategy is to recombinantly produce a library of individual catalytic enzymes with diverse hydrolytic reaction modes and then formulate the specified enzymatic cocktails for specific substrates [10]. In addition, to convert non-cellulolytic biofuels-producing microbes into consolidated bioprocessing (CBP)-enabling microbes, which can conduct enzyme production, substrate saccharification, and fermentation of the released sugars into biofuels in a single step, various studies have been carried out to express heterologous cellulolytic enzymes in heterologous host cells to make them genetically engineered cellulolytic microbes [11]. These engineered microbes use a cell-surface display or secretion system to display their novel hydrolytic capability. These strategies, with strengths and weaknesses, are discussed in the present review.

2. Conversion of Non-Cellulolytic Biofuel Microbes into Consolidated Bioprocessing Microbes

2.1. Creation of Cellulolytic *Bacillus subtilis*

Although well-known for the robust cellulosome machinery, the slow growth rate of *C. thermocellum* with strictly anaerobic culturing conditions, the highly cost of cellulosome production due to the low productivity, and its metabolic intermediates likely appear undesirable for industrial applications [12]. The Gram-positive bacterium *Bacillus subtilis* has been commonly used as a workhorse to produce numerous recombinant proteins [13]. As a super CBP microbe remains unfound, a co-culturing system that harmoniously combines one cellulolytic *B. subtilis* and another potent bioethanol-producing yeast is likely a feasible approach. In the study of Chang et al. [14], two polycistronic operons with the same gene collection but different orders, namely Type-I: *cipA–cel9K–cel48S–cel9R–sdbA–cel8A–xyn10C–xyn10Z* and Type-II: *cipA–xyn10Z–xyn10C–cel8A–sdbA–cel9K–cel9R–cel48S*, were constructed using the ordered gene assembly in *B. subtilis* (OGAB) method [15] to evaluate the influence of enzyme position on catalytic performance (**Figure 1**). Genes in these two operons were driven by a strong, thermo-inducible Pr promoter from phage lambda [16]. To measure the enzyme activity, 50 µL of

the 25-fold condensed culture supernatant of engineered *B. subtilis* was mixed with 50 µL of 50 mM sodium acetate (pH 5.0), 2% (*w*/*v*) PASC or 2% (*w*/*v*) xylan. The type-I engineered *B. subtilis* strain released higher reducing sugars than the type-II strain when growing on Avicel and filter paper, whereas the type-II strain exhibited more robust capability of degrading Napier grass powder than the type-I strain, suggesting an important role of xylanases enzyme in the decomposition of native plant biomass. The finding was also in agreement with the finding of Stern et al. [17], in that the enzyme position within a given enzyme complex, e.g., DCs, is of importance for the optimum solubilization of a specific substrate.

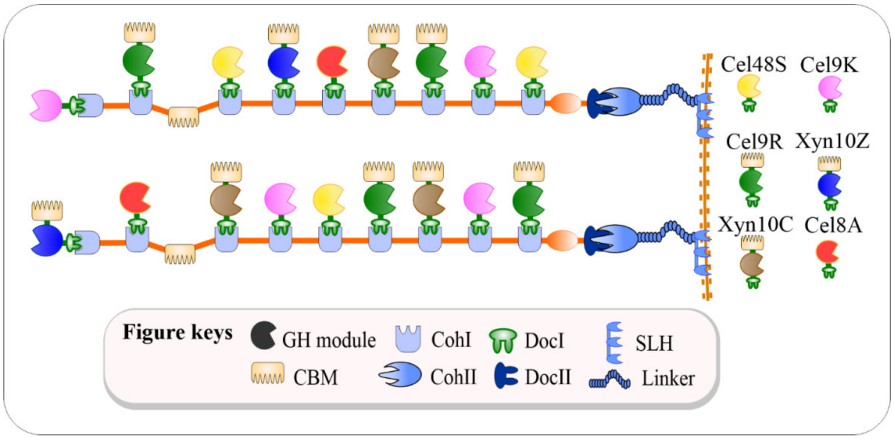

Figure 1. Two cell-surface displayed artificial cellulosomes anchored on *B. subtilis* cells. The upper operon represents the order of enzyme-encoding genes where the CBH and EG genes were placed near the Pr promoter. The lower operon displays enzyme-encoding genes where the *Xyn* genes were positioned near the Pr promoter.

2.2. CBP-Enabling Saccharomyces cerevisiae

A double-layered cellulosome was synthesized in a study by Tang et al. [18] where an artificial ScafAGA3 bearing the repeated N-terminus of Aga1p (tAga1p) was displayed on the cell surface of S. cerevisiae through the Aga1p C-terminal domain. The ScafAGA3 was used as an anchoring protein via Aga1p-Aga2p linkage and the ScafCipA3 functioned as the primary Scaf for cellulase assembly (**Figure 2**). The ScafCipA3, which contained three CohI(s) from *C. thermocellum* on its structure, carried three cellulases from divergent microbes via CohI– DocI interaction. For the conversion of free cellulases into cellulosomal mode, a BGL from *Saccharomycopsis fibuligera*, a CBH from T*alaromyces emersonii*, and an EG CelA from *C. thermocellum* were fused with the traditional *C. thermocellum* DocI(s). The novel disulfide bonds showed higher display efficiency of the ScafAGA3 on the cell surface in comparison with that of the conventional CtScafCipA3. The result demonstrates that the covalent disulfide bonds of tAga1p–Aga2 appeared to outperform the non-covalent bonds of the conventional CohI–DocI pair in cellulase assemblage. For enzyme activity measurement, the enzymes were mixed with 5 mM p-nitrophenyl-β-D-cellobiose (pNPC) or carboxymethylcellulose sodium salt (CMC-Na) as the substrates in 50 mM citrate buffer (pH 5.0) at 50 °C for 30 min for CBH and EG measurements, respectively. The resultant engineered S. cerevisiae carrying the double-layered cellulosome ScafAGA5–ScafCipA3: CBH1/CelA/BGLA produced 1.52 g/L ethanol on 1% (w / v) PASC. In summary, despite many efforts to create the CBP S. cerevisiae , the bioethanol produced by these engineered strains have been still modest due mainly to the lower efficiency of the surface-displayed cellulosomes. Some catalytic proteins assembled onto the DCs did not have sufficient ability to digest high substrate concentrations to supply an abundant fermentable sugar source for the CBP *S. cerevisiae*.

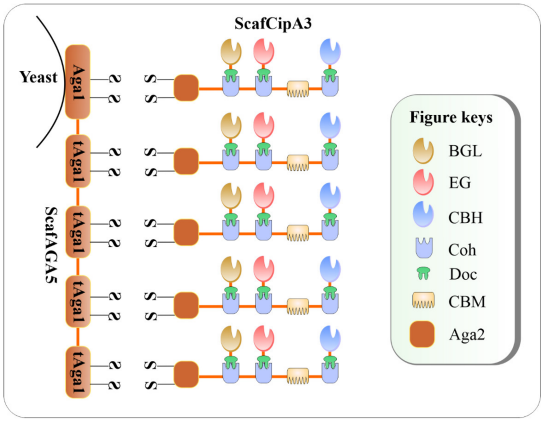

Figure 2. The attachment of trivalent Scaf on *S. cerevisiae* cell surface via covalent disulfide bonds of tAga1p–Aga2. This approach helps to improve surface display efficiency relative to the common CohII–DocII binding.

2.3. CBP-Enabling Pichia pastoris

Pichia pastoris has been widely used for the expression of diverse recombinant proteins. The limited production of endogenous secretory proteins in *P. pastoris* makes the purification of recombinant proteins easy [19]. Additionally, an appropriate posttranslational modification is an advantage in producing proteins with correct folding and proper biological activity. Recently, an indirect *P. pastoris* surface-display method was developed to create a CBP cell factory [20]. Instead of using the usual non-covalent interactions between CohI and DocI from *C. thermocellum*, the colicin E7 DNase (CE7) and its matching immunity protein 7 (Im7) was used as a CE7–Im7 protein pair. The CE7 was mutated to inactivate DNase activity but retained its full binding affinity to generate CL7 tag, and the Im7, a cognate inhibitor of CE7, was employed to form an ultra-high-affinity IM7–CL7 protein pair (K D ~10 −14 –10 −17 M) [21]. The IM7–CL7 protein pair was used as an alternative to the conventional Coh-Doc pair for cellulosome assembly (**Figure 3**). A CBH from *Yarrowia lipolytica*, an EG Cel9D from *C. thermocellum* DSM1237, a BGL from *Thermoanaerobacterium thermosaccharolyticum*, and a CBM from *T. fusca* were fused with N-terminal CL7 tags and recombinantly expressed in *E. coli.* The surface anchoring protein SED from *S. cerevisiae* was fused to the IM7 scaffoldin protein to mediate the attachment of the enzyme complex onto cell surface. In turn, the IM7 proteins were engineered to display for two or three times to carry two to three catalytic modules, thus generating Y-IM2 and Y-IM3 yeasts, respectively. Subsequently, the engineered P. pastoris yeasts were incubated with the *E. coli* lysates containing cellulosomal-mode-cellulases and CBMs to promote the assembly of minicellulosomes on cell surface via IM7–CL7 interaction. On Avicel and PASC, the engineered Y-IM2 performed better than Y-IM3 with 2.5 g/L and 1.2 g/L ethanol, respectively, whereas the Y-IM3 surpassed Y-IM2 on CMC substrate with up to 5.1 g/L ethanol. Moreover, a synergistic effect on CMC hydrolysis was also observed in Y-IM3, indicating a positive relationship between the number of Scaf IM7 and the catalytic efficiency.

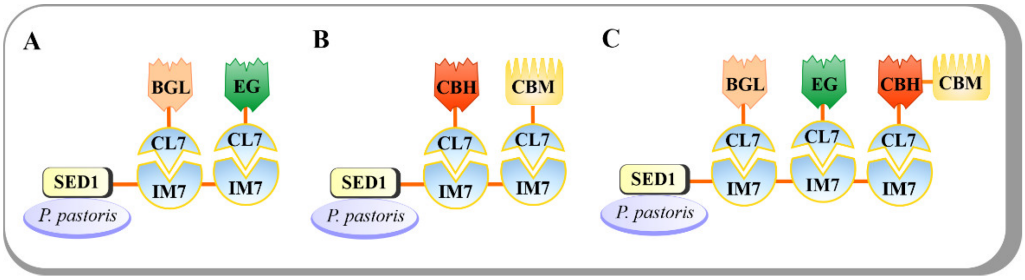

Figure 3. The novel ultra-high-affinity IM7–CL7 protein pair (K_D~10^{-14}–10^{-17} M) was used for cellulase assembly onto *P. pastoris* cell surface. (**A**) The engineered *P. pastoris* with one BGL and one EG enzyme on its surface. (**B**) The engineered *P. pastoris* with one CBH and one CBM on its surface. (**C**) The engineered *P. pastoris* with one CBH, one EG, one CBH, and one CBM on its surface.

2.4. Consolidated Bioprocessing-Enabling K. marxianus

Apart from the LPMO from *Thermoascus aurantiacus* (TaLPMO) and its electron donor cellobiose dehydrogenase from *Myceliophthora thermophila* (MtCDH), an EG from *T. reesei* (TrEgIII), a synthetic CBH (CBHII), and a BGL from *Neocallimastix patriciarum* (NpaBGS) were fused with a DocI of *C. thermocellum* to create DocI-fused enzyme subunits, namely TrEgIII–t, CBHII–t, NpaBGS–t, TaLPMO–t, and MtCDH –t, respectively (**Figure 4**). In addition, a cell-surface protein glycosylphosphatidylonositol (GPI) from *S. cerevisiae* (ScGPI) was used to facilitate the anchoring of the artificial cellulosome to the K. marxianus cell surface because the anchoring domain of *C. thermocellum* is not suitable for eukaryotic hosts. The supernatants of red fluorescent protein (RFP)-fused DocT (RFP–DocT), OlpB–ScGPI, and CipA1B9C were mixed to form the entire cellulosome complex, namely OlpB– ScGPI :CipA1B9C:RFP–DocT, and their assembly was confirmed using an epifluorescence microscopy. The hydrolytic activity of cellulosome on 1% (w / v) Avicel was quantified by mixing the concentrated supernatants of the YP culture broth with Avicel and incubated at 40 °C for 6 h. On the model micro-crystalline cellulose Avicel, the amounts of sugar release were significantly influenced by the number of CohI and the number of CBMs, whereas the effect of these components was less pronounced on amorphous cellulose phosphoric acid swollen cellulose (PASC) hydrolysis (**Figure 4** A,B). The results confirmed the decisive roles of the scaffoldin protein CipA in cellulose solubilization rate as elucidated in a previous study by Olson et al. [22]. To the best of our knowledge, this is the first time an assembly of 63 saccharolytic cellulosomal enzymes from different species was engineered and expressed on a heterologous host's surface, enabling a greater biomass degradation rate for future consolidated bioprocessing (CBP) microorganisms (**Figure 4** C).

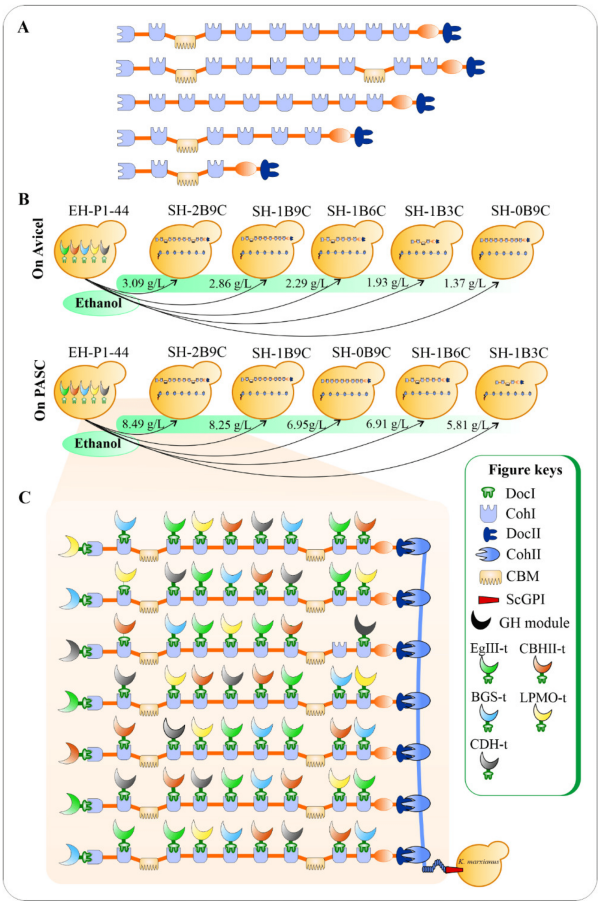

Figure 4. Engineered *K. marxianus* carrying the largest cellulosome complex on its cell surface. (**A**) (from the top) Full-length of CipA with 1 CBM3a (1B9C), full-length of CipA with 2 CBM3a(s) (2B9C), full-length of CipA without CBM3a (0B9C), truncated CipA with 6 CohI(s) + 1 CBM3a (1B6C), and truncated CipA with 3 CohI(s) + 1 CBM3a (1B3C), respectively. (**B**) Ethanol productivity of different engineered *K. marxianus* strains. On Avicel, the SH-2B9C strain yielded the highest ethanol titer, followed by the SH-1B9C strain, and the lowest ethanol concentration was found in SH-0B9C which does not have any CBM, indicating the decisive role of a CBM in crystalline cellulose hydrolysis. On PASC, however, the lowest ethanol titer was recorded in SH-1B3C strain, suggesting that the numbers of catalytic subunits, but not CBM3a(s), dictate the efficiency of amorphous cellulose solubilization. (**C**) The engineered *K. marxianus* with the whole cellulosome complex consisting of total 63 enzyme subunits from divergent species on its surface. Abbreviations in the **Figure 4**: B, Carbohydrate-binding module; C, Cohesin.

3. Promise, Limitations and Future Directions

The search more effective biomass-degrading DCs or cellulase blends is an onerous task. For further optimization of artificial cellulosomal compositions, more investigations are required to support a better understanding of the hydrolysis modes, especially synergistic effects between divergent cellulolytic enzymes in a whole complex. Despite some difficulties in the conversion of free enzymes into cellulosomal modes due to the incompatibility between host cells and the expression of the transgenes, this approach has been a common strategy in recent years because it enables scientists to exploit novel enzyme sources with advantageous characteristics (i.e., hyperthermal stability, lignin degrader, cellulase boosters). Furthermore, the reasons underlying unsuccessful cases of many *C. thermocellum* cellulosomal enzymes that could not be recombinantly produced in *E. coli* cells remain unknown but may be also related to the suboptimal heterologous protein expression in the host cells. Although O-linked α-1,2-galactose-containing oligosaccharides were found on the linkers of scaffoldin proteins from *C. thermocellum* and *Bacteroides cellulosolvens* for decades, the impact of glycosylation on the performance of artificial cellulosomes was recently confirmed as the glycosylated DCs exhibited enhanced saccharolytic effectiveness and thermal stability. The glycosylation of DCs should therefore be taken into consideration as a new parameter for DCs construction in future studies. Besides, mechano-stability of the CohI(s) is also an important parameter for preserving net saccharification activity under great mechanical load. Furthermore, the finding of new protein pairs other than CohI-DocI pairs is a new trend noticed in some recently published papers to improve binding affinity between saccharolytic domains, scaffoldin protein, and/or cell-surface-display effectiveness.

The development of robust microbes that can ferment cellulose to biofuels or other valuable products is an interesting but challenging proposition for the biofuels industry. Despite the many efforts that have been deployed thus far to convert the

non-cellulolytic ethanol producers into CBP microbes, it is likely that these engineered microbes, including *S. cerevisiae*, *P. pastoris*, and *K. marxianus*, could not perform well as we might expect. For instance, although well-armed with up to 63 cellulolytic enzymes on the surface, the engineered *K. marxianus* only produced 3.09 g/L and 8.61 g/L ethanol from microcrystalline cellulose Avicel and amorphous cellulose PASC, respectively, let alone the native recalcitrant biomass. These ethanol titers, though higher than any other engineered yeast cellulosome, are still quite low to make the artificial cellulolytic *K. marxianus* CBP ready.

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