Engineering Pichia pastoris with surface-display minicellulosome for carboxymethyl cellulose hydrolysis and ethanol production

Ce Dong
Hubei University
Jie Qiao
Hubei University
Xinping Wang
Hubei University
Wenli Sun
Hubei University
Lixia Chen
Hubei University
Shuntang Li
Hubei University
Lixin Ma
Hubei University
yi liu (yiliu0825@hubu.edu.cn)
hubei university https://orcid.org/0000-0002-2148-3596

Research

Keywords: Cellulosome, Pichia pastoris, Consolidate bioprocessing (CBP), Carboxymethyl cellulose (CMC), Bioethanol

Posted Date: February 12th, 2020

DOI: https://doi.org/10.21203/rs.2.23317/v1

Version of Record: A version of this preprint was published on June 15th, 2020. See the published version at https://doi.org/10.1186/s13068-020-01749-1.

Abstract

Backgrounds: Engineering yeast with cell surface immobilized cellulosome is a promising strategy for consolidated bioprocessing (CBP) to produce bioethanol from the conversion of cellulose. However, previous studies mostly focused on utilization of Saccharomyces cerevisiae , which was able to directly convert phosphoric acid-swollen cellulose (PASC) or microcrystalline cellulose (Avicel) but not carboxymethyl cellulose (CMC) to ethanol, with an average titer below 2 g/L.

Results: Harnessing an ultra-high-affinity IM7/CL7 protein pair, here we describe a method to engineer Pichia pastoris with minicellulosome through in vitro assembly of various recombinant cellulases on the cell surface. For the first time, the yeast can efficiently convert CMC to bioethanol, achieving an impressive ethanol titer of 5.1 g/L. Further, the engineered yeasts were lyophilized to powders that can be utilized as compound cellulases.

Conclusions: This research promotes the application of P. pastoris as CBP cell factory in cellulosic ethanol production and provides a promising platform for screening optimal cellulase species and ratios to construct celluosome on the yeast cell surface.

Background

Cellulosic biomass derived from low-value agricultural and wood pulping wastes is likely the most abundant renewable resource in the world [1–3]. In the past decades, production of bioethanol from cellulose has increasingly attracted attention due to its low cost and environmental friendliness. However, conversion of cellulose into fermentable sugars capable of utilization by microbes is still challenging [2, 4], largely limiting the industrial production of cellulosic bioethanol. In traditional ways, cellulosic biomass is degraded by commercial cellulases followed by microbial fermentation, leading to the process of production is time-consuming and costly [5–7]. Recently, several new approaches have been developed such as secretory expression of cellulases by bacteria [8], in vivo assembly of cellulosome within microorganisms[9, 10], and a consolidated bioprocessing (CBP) that combines enzyme production, cellulose hydrolysis, and biological fermentation into a single process[11, 12].

Yeast, especially for Saccharomyces cerevisiae, has been widely considered as a CBP candidate for bioethanol production due to its high ethanol productivity and strong ethanol tolerance [13]. In early studies, cellulases were cell-secreted in S. cerevisiae culture medium or independently displayed on the cell surface [14, 15], yet the conversion and bioethanol yield was low. Currently, the works demonstrated that in vivo or in vitro assembly of multiple cellulases on the S. cerevisiae cell surface in a structure termed cellulosome [16, 17] can enhance the ethanol yield [18–21]. These methods required displaying multiple components on the yeast surface, including heterogeneous dockerin-cohesion pairs, carbohydrate binding domains (CBDs) and appropriated bacterial cellulases, thereby often leading to low displaying efficiency. To date, microcrystalline cellulose (Avicel) or phosphoric acid-swollen cellulose (PASC) has been successfully utilized as the substrate for ethanol production by yeast fermentation,

though the yields were below the requirement of large-scale production. Moreover, carboxymethyl cellulose (CMC) is still hard to be converted by S. cerevisiae because its ultra-high viscosity would weaken the diffusion of hydrolysis products and influence the ethanol fermentation [22, 23]. So, more effort is needed to archive the goal of industrial production of ethanol from cellulose when harnessing yeast as the CBP cell factory.

Recently, we reported an indirect Pichia pastoris surface display method [24] that can simply display various enzymes with an efficiency ten times higher than that of traditional S. cerevisiae surface display methods. Additionally, P. pastoris has been reported to achieve a much higher cell density than S. cerevisiae in fermentation [1]. In practice applications, P. pastoris has been successfully applied in whole-cell biocatalysis for biodiesel production [25]. Therefore, we were thinking that the P. pastoris might be suitable for catalyzing the reactions in greater viscosity, for example, achieving conversion of CMC to ethanol. In this work, we sought to expand our approach for construction of minicellulosome on the P. pastoris cell surface, aiming to hydrolyze cellulose and produce bioethanol. Other than use of the traditional cellulosomal dockerin-cohesion pair, we harnessed the IM7/CL7 protein pair [26] with an ultra-high binding affinity (K_d ~10^{-14} to 10^{-17} M). According to the design (Fig. 1), the IM7 proteins were repeatedly displayed on the P. pastoris cell surface for twice or three times. Diverse cellulases including exo-mode cellobiohydrolases (CBH) from Yarrowia lipolytica [27], endoglucanases (EG) from Clostridium thermocellum DSM1237 [28], glucose-tolerant β-glucosidase (BGL) from Thermoanaerobacterium thermosaccharolyticum DSM 571 [29], and CBD from Thermobifida fusca [30] were fused with N-terminal CL7 tags and recombinantly expressed in Escherichia coli. The engineered P. pastoris yeast with displayed IM7 proteins was then in vitro incubated with E. coli lysates containing various recombinant cellulases, leading to the assembly of minicellulosome on the yeast cell surface. The data indicated that such kind of P. pastoris was able to hydrolyze microcrystalline cellulose (Avicel), phosphoric acid-swollen cellulose [PASC (86.2)] or carboxymethyl cellulose (CMC) to reducing sugars, with the enzyme activity comparable to or higher than that of free cellulases. Moreover, the P. pastoris with surface-display minicellulosome was lyophilized as compound cellulases, showing great commercial potential in industrial applications. Last and most importantly, we employed the engineered P. pastoris as CBP cell factory to directly break down and ferment Avicel, PASC or CMC to ethanol. The ethanol yield is 2.5 g/L for Avicel and 1.2 g/L for PASC respectively, which are comparable to or better than the previous yields (< 2 g/L) observed by S. cerevisiae. Surprisingly, the CMC is preferred for bioethanol fermentation by our engineered P. pastoris, with the highest titer of 5.1 g/L. To the best of our knowledge, this is the first example of yeast that can efficiently transfer CMC to ethanol. Together, we develop a promising CBP platform for cellulose hydrolysis and bioethanol production by engineering the P. pastoris with cell-surface minicellulosome.

Results

Repeatedly displaying IM7 proteins on the P. pastoris cell surface

In traditional yeast cell-surface display methods, the dockerin-cohesion pair from bacterial cellulosomes is adopted, in which the dockerin is roughly a 10 kDa calcium-binding module that non-covalently associates with the scaffoldin cohesion at affinity in the sub-nM (~ 10^{-6} M) range [16]. In this work, the IM7/CL7 protein pair (Fig. 1) with a much higher binding affinity (K_D ~10^{-14}-10^{-17} M), was used instead [26]. In principle, the ultra-strong protein-protein interaction between IM7 and CL7 could significantly enhance the cellulosome assembly efficiency. Taking advantage of this IM7/CL7 system, we recently developed an indirect P. pastoris surface display method that can display ~ 2.8×10^6 enzyme molecules per yeast cell [24], observing a display efficiency ten times higher than that of traditional S. cerevisiae display methods. As shown in Fig. 1, the yeast surface anchor protein SED1 from S. cerevisiae without its signal sequence was fused to the repeated IM7 units. The surface localization of IM7 proteins was verified with immunofluorescence microscopy and flow cytometric analysis (FACS) in Fig. 2. As a control, the strain Y-IM0 without modification was not immunostained, whereas the variant Y-IM1, Y-IM2 and Y-IM3 were all in green color in the presence of mouse anti-HA monoclonal antibodies and FITC- conjugated goat anti-mouse antibodies. These results indicated that all recombinant yeasts displayed the IM7 proteins on the surface, and the display efficiency was elevated when increasing IM7 repeats. In addition, the engineered yeasts were all in red color in the presence of CL7 tagged mCherry fluorescent proteins (Fig. 3). The FACS data (Fig. 3) further demonstrated that repeatedly anchoring the IM7 proteins enhanced the display efficiency of CL7-mCherry. Compared with other yeast surface display systems, in which less than 50% of cells were positively stained by immunofluorescence [18], our yeast surface display system has a much better display level of over 90%, possibly owning to the ultra-strong protein-protein interaction between CL7 and IM7.

In vitro assembling minicelluolosome on the P. pastoris cell surface

Previously, researchers assembled functional minicellulosome in vitro on the S. cerevisiae yeast cell surface by incubation of the engineered yeast that has a chimeric scaffoldin [9, 10] or two miniscaffoldins [20, 21] with exogenous recombinant cellulases. In comparison to in vivo assembling mode, the in vitro assembled cellulosome showed higher bioethanol production, possibly because the metabolic load was lowered in these strains. In this work, we also chose the in vitro assembly strategy to construct minicelluolosomes on the P. pastoris cell surface. Specially, the different cellulases, including CBH from Yarrowia lipolytica, EG from Clostridium thermocellum DSM1237, BGL from Thermoanaerobacterium thermosaccharolyticum DSM 571, and CBD from Thermobifida fusca, were fused with a N-terminal CL7 tag and recombinantly expressed from E. coli. Those purified cellulases were finally incubated with yeast strain Y-IM2 or Y-IM3.

To prove the successful construction of minicellulosome, Avicel, PASC (86.2), or CMC was utilized as the substrate for enzyme activity assay [31]. We adjusted the ratio of EG, CBH, BGL and CBD at 1:1:1:1, 2:4:2:7 and 1:3:6:10, respectively. Meanwhile, the free cellulases (1:1:1:1) were used as a control [31]. The data (Fig. 4) indicate that the enzyme activity of Y-IM2 and Y-IM3 is comparable to or higher than that of free cellulases. Interestingly, both minicellulosome and the free cellulases showed higher activity toward CMC and PASC than Avicel. However, the improvement of the enzyme activity causing by

minicellulosome toward Avicel was more obviously (~ 2.6-fold). Based on these results, we chose 1:1:1:1 and 2:4:2:7 as the optimized ratios for the following ethanol fermentation experiments.

Lyophilization of the engineered P. pastoris as compound cellulases

The commercial cellulase is often supplied as the compound of three-type cellulases including EG, CBH and BGL. The annual consumption of cellulases is huge in various industrial fields [1], especially for breeding industry. As it is known to all, P. pastoris is considered as a GRAS (generally recognized as safe) microorganism by FDA and has been employed to produce diverse human peptides and proteins. In addition, P. pastoris has a strong cell wall and outer membrane in structure capable of serving as a stable biomaterial for enzyme immobilization. Inspired by these works, we thought that the above engineered P. pastoris with surface-display minicellulosome might be suitable for industrial applications. Initially, we tested if the induced but dead yeast with cellulosome has an enzyme activity to hydrolyze the cellulose. The results (Fig. S1) demonstrated that the catalytical activity was unchanged within at least three months when the yeast strains were stored at -20^{o}C. Thereafter, the Y-IM2 or Y-IM3 variant with minicellulosome was lyophilized as powders for long-term storage. When recovered in solution, no cellulase activity changes were observed (Fig. S1), further proving that the lyophilized P. pastoris can be used as the compound cellulases. Most importantly, such kind of lyophilized P. pastoris with cellulosome can be rapidly produced at large scale and low cost, showing great commercial potential in industry.

Direct fermentation of the cellulose to ethanol

Direct ethanol fermentations from Avicel, PASC (86.2) or CMC was examined using E. coli lysates treated Y-IM2 and Y-IM3 (Fig. 5a and Fig. 5b). The data demonstrated that the ethanol titer quickly increased within 60 hours for all the substrates. Besides, Y-IM2 was better as the ethanol producer than Y-IM3 toward Avicel and PASC, with the highest ethanol yield of 2.5 g/L for Avicel and 1.2 g/L for PASC (Fig. 5c), respectively. In the previous S. cerevisiae fermentation studies, Avicel or PASC was always the better substrate than CMC, yet the average ethanol production was lower than 2 g/L. Our bioethanol production is higher than or at least comparable to those of works. Surprisingly, CMC was the best carbon source for both Y-IM2 and Y-IM3 variants, achieving a highest ethanol yield of 5.1 g/L. With an ultra-high viscosity, CMC was thought to weaken the diffusion of hydrolysis products and influence the S. cerevisiae fermentation. Very low ethanol production was observed when CMC was used in yeast CBP studies [18, 20, 21]. Comparingly, the P. pastoris has been found able to achieve a very high cell density in fermentation. Therefore, it may utilize the high-viscosity CMC for ethanol fermentation better than S. cerevisiae, which is consisted with the observations here. Meanwhile, the Y-IM0 variant without minicellulosome was found to have no ability to produce ethanol.

Discussion

Plenty of works have demonstrated that assembly of cellulosome on the S. cerevisiae cell surface can enable it as CBP cell factory to produce ethanol from cellulose [9, 10, 18–21, 32]. However, the application

of such engineering strategy was limited largely due to the low display efficiency of cellulases and/or the high metabolic burden of the host yeast. To enhance the bioethanol production, several attempts have been made such as screening the dockerin-cohesion pairs[19], utilizing the double-layered scaffoldins [20, 21], and adjusting the cellulase species and ratios [18, 32], etc. However, the minicellusomes in these works were constructed through hydrophobic interaction, hydrogen bond, or disulfide bond, achieving relatively low surface display efficiency and stability. Additionally, the engineering S. cerevisiae only produced less than 2 g/L ethanol from the conversion of Avicel or PASC. Moreover, no obvious bioethanol was produced from CMC due to its ultra-high viscosity causing problems in fermentation.

In this study, to improve the display efficiency and availability of CMC, we describe a new strategy by the employment of engineering P. pastoris with tightly linked cell-surface minicellulosome. The repeatedly anchoring IM7 units on the P. pastoris cell surface demonstrated significantly increased display efficiencies. Through the introduction of CL7 protein which has an ultra-high binding affinity toward IM7, the minicellulosome was efficiently assembled. Specially, four cellulosome components including EG, CBHI, BGL and CBD from distinct bacteria were fused with CL7 tags, purified from E. coli, and in vitro incubated with the Y-IM2 or Y-IM3 variant at different ratios, resulting in comparable or better catalytic activity than that of free enzymes. Moreover, the engineered P. pastoris strains were lyophilized as powders for long-term storage without cellulase activity losses, which shows great potential as the compound cellulases instead of commercial cellulase in breeding industry.

At last, we harnessed the engineered P. pastoris as CBP cell factory to directly convert Avicel, PASC and CMC to ethanol. The highest ethanol yield was 2.5 g/L for Avicel and 1.2 g/L for PASC, which were comparable to or higher than the reported values obtained by use of S. cerevisiae. More importantly, the results indicated that the CMC was preferred for bioethanol fermentation in our P. pastoris system, achieving a highest titer of 5.1 g/L. To the best of our knowledge, it is the first successful work that realized efficient production of ethanol from CMC by using yeast as CBP cell factory. Compared with the traditional bioethanol producer S. cerevisiae, P. pastoris has been proven to be able to reach a higher cell density in fermentation. Thus, it should be suitable for conversion of high-viscosity substrate such as CMC, which is consisted with what we observed. In addition, we found that optimizing the ratio of various recombinant cellulases would influence the ethanol production quite a lot. In future, higher bioethanol production can be achieved by further combinatorial optimization of cellulase species and ratios.

Conclusion

Taking advantage of the ultra-high-affinity IM7/CL7 system, here we develop an efficient method capable of in vitro assembling minicellulosome on the P. pastoris cell surface. The engineered yeast with cellulosome can be cost-effectively produced at large scale and lyophilized as compound cellulases, showing great potential in industrial applications. Most importantly, this yeast was able to produce ethanol from direct conversion of CMC, which was unable to be converted well by S. cerevisiae, achieving an impressive ethanol titer of 5.1 g/L. Collectively, the research promotes the application of P. pastoris as CBP cell factory in cellulosic ethanol production.

Methods

Strains and media

E. coli DH5α was used as the host for DNA manipulations, and E. coli BL21(DE3) was the host for recombinant expression of CL7 tagged cellulases or CBD domains. P. pastoris strain GS115 and the vector pPICZαA were obtained from Invitrogen (Carlsbad, CA, USA). The vectors pET23a-T, pET23a-CL7, and pCDNA3.1-mCherry were constructed and stored in our laboratory previously [24]. The genes encoding exo-mode cellobiohydrolases (CBH) from Yarrowia lipolytica, endoglucanases (EG) from Clostridium thermocellum DSM1237, glucose-tolerant β-glucosidase (BGL) from Thermoanaerobacterium thermosaccharolyticum DSM 571, as well as CBD from Thermobifida fusca were synthesized by Sangon Biotech (Shanghai, China). E. coli strains were grown in LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl) supplied with 100 μg/ml ampicillin. P. pastoris yeasts were grown firstly in YPD plates (1% yeast extract, 2% peptide, and 2% glucose) supplemented with 100 μg/mL of Zeocin, and then cultured in BMGY/BMMY medium base (20.0 g/L peptone, 10.0 g/L yeast extract, 100 mmol PBS broth, pH 6.0).

Plasmid construction

The plasmids pET23a-CL7-EG, pET23a-CL7-CBHI, pET23a-CL7-BGL, and pET23a-CL7-CBD were constructed by insertion of the corresponding genes into pET23a(+) vectors (Invitrogen, USA). The plasmid pPICZαA-HA-Im7-SED1 that produces P. pastoris Y-IM1 was described in our previous work [24]. The Y-IM2 and Y-IM3 plasmids were constructed based on this plasmid by repeating IM7 for twice or three times, namely pPICZαA-HA-2XIm7-SED1 and pPICZαA-HA-3XIm7-SED1, respectively. In addition, a "GGGGS"$_2$ liker was added between each IM7 units. Information regarding these yeast plasmids were illustrated in Figure S2.

Yeast surface display and E. coli expression

All the P. pastoris yeast plasmids were digested with Pme1 and transformed into GS115 competent cells. Transformants were firstly isolated by incubation at 28^{o}C for 48 h on YPD plates supplemented with 100 μg/mL of Zeocin. Then, five to 10 single colonies of transformants were inoculated in 20 mL of BMGY in 250 mL flasks and cultivated at 28^{o}C under 200 rpm. After 24 h, the cells were centrifuged at 5000 × g for 5 min, resuspended in 20 mL of BMMY medium containing 1% (v/v) methanol and continued to grow at 28^{o}C, 200 rpm for 24 h. All CL7 fusion proteins were induced by 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 18^{o}C for 12 h when the cells were grown to an OD_{600} of 0.6. The E. coli cells collected were resuspended in PBS buffer containing 200 mM NaCl and 10 mM $CaCl_2$ (pH 7.4) and then sonicated on ice for 20 minutes. The cell lysates were either purified by Ni-NTA affinity columns, or directly incubated with the engineered yeast strains in fermentation experiments.

Fluorescence microscopy and FACS

The yeast strains including Y-IM0, Y-IM1, Y-IM2, and Y-IM3 were harvested and washed twice by ice-cold water, resuspended and blocked in 1 mL PBS buffer (200 mM NaCl, pH 7.4) with 1 mg/mL BSA for 1 h at 4^{o}C with rotation. Then, 1 μL of mouse anti-HA tag monoclonal antibodies or 5 μg of CL7-mCherry proteins was added to the cell suspension of 1000 μL and then incubated at room temperature with rotation for 2 h. In the next, the cells were washed three times with PBS and resuspended in 200 μL of PBS with the addition of 1 μL of FITC–conjugated goat anti-mouse IgG(H + L) antibodies, followed by incubation of them at room temperature for 1 h with rotation. Finally, the cells were washed three times with PBS, resuspended in 1 mL of PBS and examined by a fluorescence microscopy (IX73, Olympus, Tokyo, Japan). FACS was analyzed with a flow cytometer (CytoFLEX, Beckman Coulter, Suzhou, China) to estimate the percentage of the fluorescence positive yeast cells.

In vitro assembly of minicellulosome and enzyme activity assay

The induced Y-IM2 and Y-IM3 strains and purified recombinant CL7 tagged cellulases were mixed in 100 mM Tris-HCl buffer with 10 mM $CaCl_2$ (pH 8.0) at various ratios, and kept for 2 h at 4^{o}C for minicellulosome assembly. The enzyme activity of cellulosome or free cellulases against Avicel or PASC or CMC was detected by 3, 5-dinitrosaloculoc acid (DNS) assay [31]. The PASC (86.2) was prepared from Avicel (Sangon Biotech, Shanghai, China) as described previously [33]. Minicellulosome or free cellulases were incubated with 0.1% cellulose substrate in 50 mM citrate buffer (pH 4.8) with 10 mM $CaCl_2$ at 50^{o}C for 30 min. After addition of DNS and boiling for 10 min, the reducing sugars were detected at 540 nm. One unit of the enzyme activity was defined as the amount of enzyme that released 1 mol of product from the cellulose substrate at 50^{o}C in 1 min.

Fermentation

After induction, the strains Y-IM2 and Y-IM3 were washed twice with YP medium (1% yeast extract, 2% peptone, 10 mM $CaCl_2$). Then, they were incubated with E. coli lysates containing cellulases at various ratios in the same buffer, and kept for 4 h at 4^{o}C to allow cellulosome assembly. Next, yeast cells with minicellulosome were cultivated in YP medium with 1% cellulose substrate (Avicel, PASC, or CMC) to an OD_{600} of 50. Fermentation was performed anaerobically 100 mL flask at 30^{o}C with agitation at 250 rpm. The ethanol concentration was analyzed by an ethanol biosensor M-100 (Shellman Life Science, Shenzhen, China) supplied with polyaniline film immobilized alcohol oxidase, which had been proven and in good agreement with the standard method (gas chromatography) results [34].

Abbreviations

PASC
acid-swollen cellulose; Avicel:microcrystalline cellulose; CMC:carboxymethyl cellulose (CMC); CBP:consolidated bioprocessing; CBD:carbohydrate binding domain; CBH:exo-mode cellobiohydrolases; EG:endoglucanases; BGL:glucose-tolerant β-glucosidase; FACS:flow cytometric analysis.

Declarations

Acknowledgements

This work was supported by the National Natural Science Foundation of China (NO. 2190070220), and Project funded by China Postdoctoral Science Foundation (NO. 2019M662573)

Authors' contributions

LXM and YL designed the experiments. CD, JQ and XPW performed the enzyme activity assay and detection experiments of bioethanol production. LXC, WLS and STL constructed and plasmids and purified the recombinant enzymes. CD and YL analyzed the data. YL wrote the paper, All authors read and approved the final manuscript.

Funding

This work was supported by the National Natural Science Foundation of China (NO. 2190070220), and Project funded by China Postdoctoral Science Foundation (NO. 2019M662573)

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its additional files.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

A patent involved in ethanol production using *Pichia pastoris* with surface-display minicellulosome was submitted by the institute of authors.

Author details

1. State Key Laboratory of Biocatalysis and Enzyme Engineering, School of Life Sciences, Hubei University, Wuhan, 430062 Hubei, China.
2. Hubei Key Laboratory of Industrial Biotechnology, School of Life Sciences, Hubei University, Wuhan, 430062 Hubei, China.

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Figures

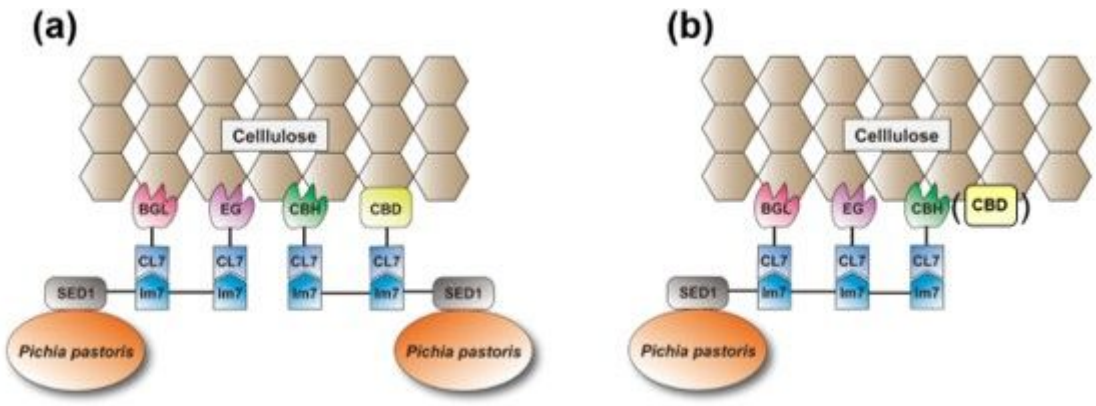

Figure 1

In vitro assembly of minicellulosome on the P. pastoris cell surface through the ultra-strong protein-protein interaction between IM7 and CL7. The IM7 proteins were repeatedly displayed for (a) twice or (b) three times. The CL7 tagged cellulases were expressed and purified from E. coli.

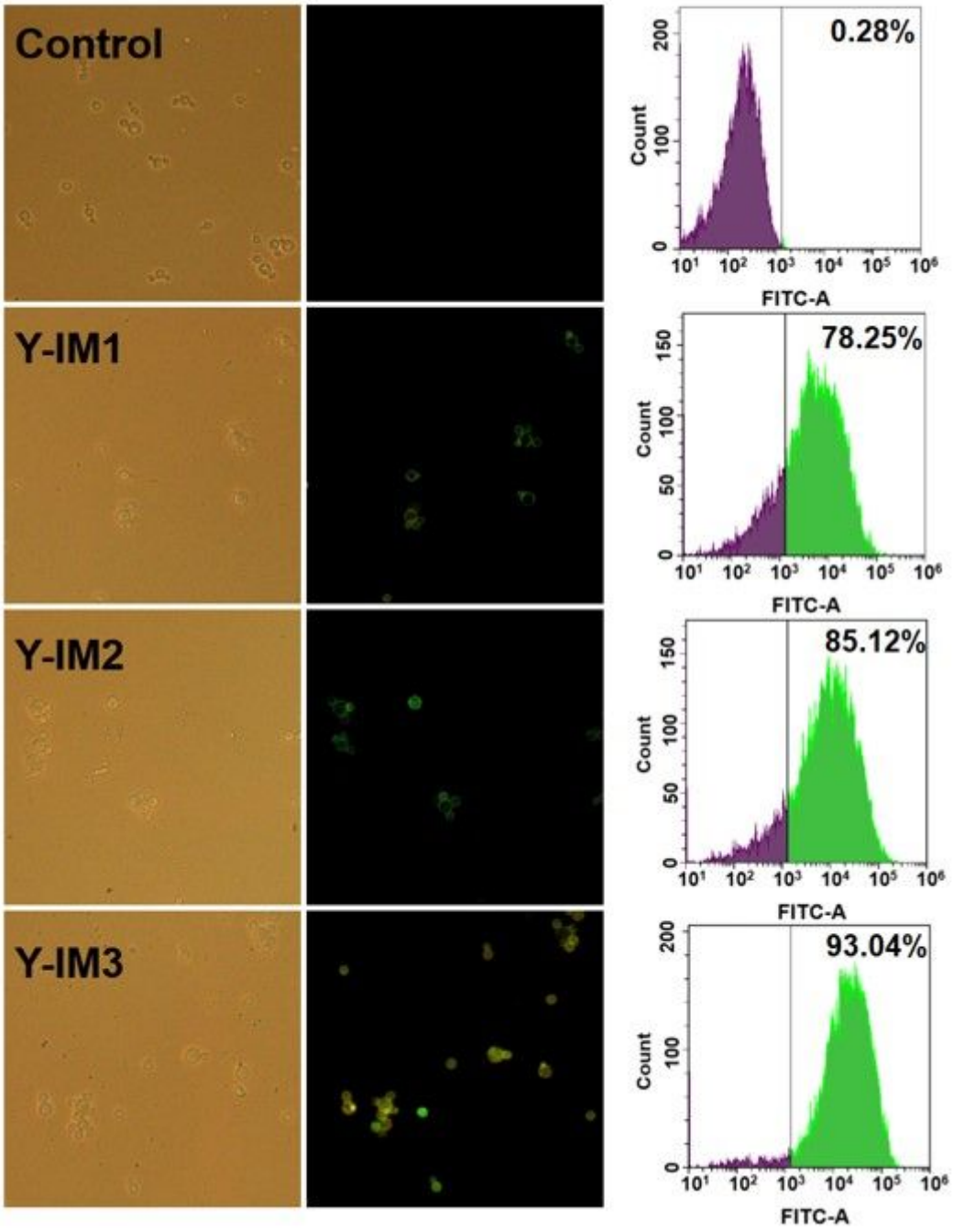

Figure 2

Immunofluorescence micrographs and FACS analysis of surface display of repeated IM7 units.

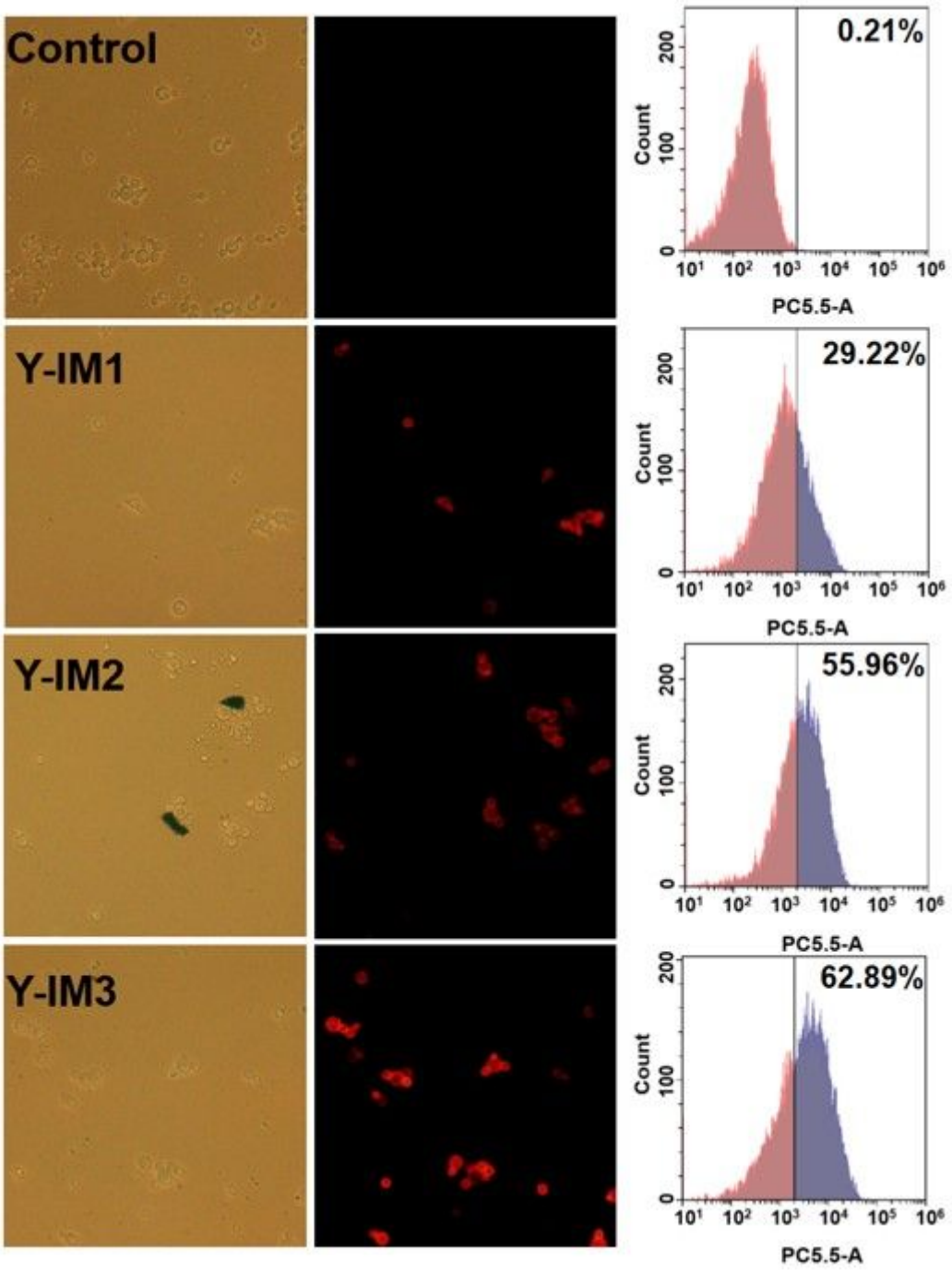

Figure 3

Fluorescence micrographs and FACS analysis of cell-surface immobilization of CL7-mCherry red fluorescent proteins.

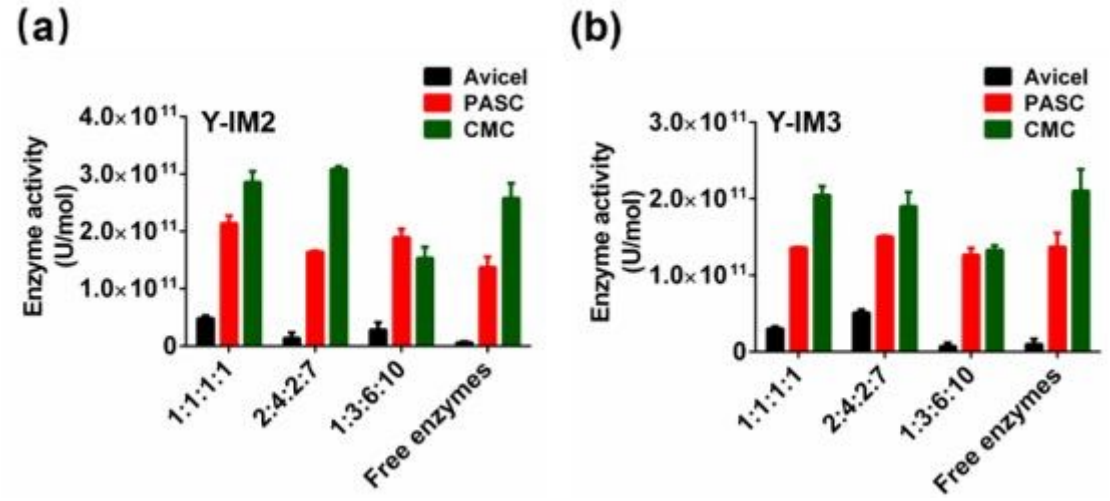

Figure 4

Enzyme activity of minicellulosome against celluloses in comparison to free cellulases.

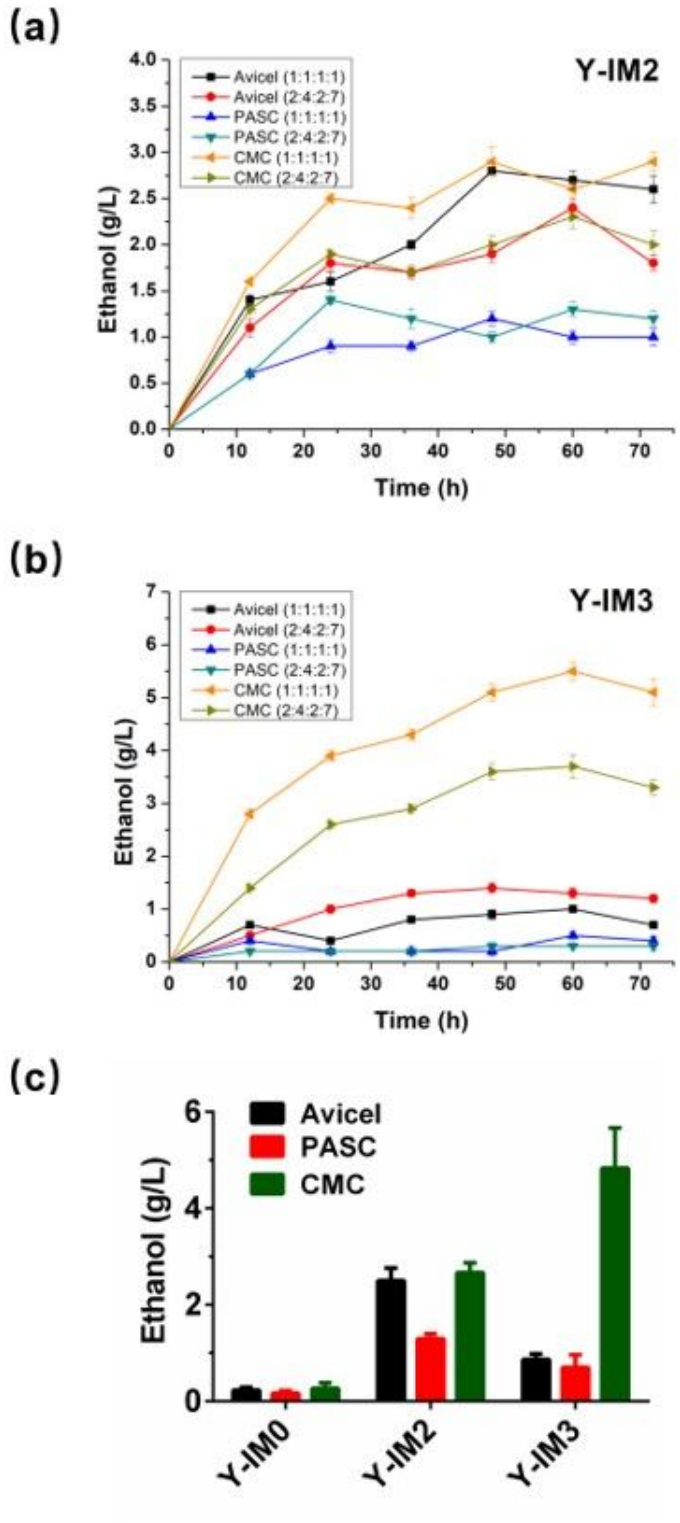

Figure 5

Direct ethanol production from different cellulose substrates. Time profiles of ethanol production by (a) Y-IM2 and (b) Y-IM3. The highest ethanol production of each substrates was shown in (c). Y-IM0 without modification was used as a control.

Supplementary Files

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