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In vitro assembly of minicellulosomes with two scaffoldins on the yeast cell surface for cellulose saccharification and bioethanol production

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ABSTRACT

The present paper reports *in vitro* strategies for assembly of minicellulosomes with two miniscaffoldins on the *Saccharomyces cerevisiae* cell surface. It was carried out through incubation of the yeast cells displaying scaffoldins with *Escherichia coli* lysates containing recombinant cellulases, or using a four-population yeast consortium. The results showed that the display level of miniscaffoldin II was distinctly increased by moving the cellulases production into *E. coli* or other yeast cells, indicating that the metabolic burden of the yeast host was decreased. The yeast consortium did not show any cellulolytic activity, while the *E. coli* lysates-treated yeast, whose anchoring miniscaffoldin length was optimized, was able to produce ~1138 mg/L ethanol from microcrystalline cellulose within 4 days. We also confirmed that the yeast-associated minicellulosome moreover showed both higher thermal stability and lower protease accessibility than free minicellulosome. This research promotes the application of *S. cerevisiae* as a consolidated bioprocessing (CBP) microorganism in cellulosic bioethanol production.

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1. Introduction

Bioethanol is widely used as a biofuel additive for gasoline. It is usually produced by the action of microorganisms and enzymes through the fermentation of sugar or starch or cellulose, of which, cellulosic biomass is particularly well-suited for bioethanol production because of its large-scale availability, low cost and environmental benignancy [1–3]. However, industrially deriving ethanol from cellulose is still challenging, since the saccharification of such material into fermentable sugar by addition of the cellulases from a dedicated process step is costly and time-consuming [4–6]. One of the most creative developments for solving this problem is consolidated bioprocessing (CBP) [7].

CBP requires a microorganism with the ability of simultaneous saccharification and fermentation of cellulose to ethanol. Engineering a non-cellulolytic organism, which exhibits high ethanol yield and titer, is believed promising. *Saccharomyces cerevisiae* is an ideal engineered candidate, since it has high ethanol productivity, strong ethanol tolerance and well-developed tools for genetic manipulation [8,9].

Recent studies reported the functional display of complexed cellulase systems (cellulosomes) using single miniscaffoldins on the yeast cell surfaces. Tsai et al. [10] assembled functional minicellulosomes *in vitro* on the yeast cell surface by incubation of the yeast cells displaying a chimeric scaffoldin with *Escherichia coli* lysates containing recombinant cellulases. The engineered yeast was able to directly hydrolyze and ferment phosphoric acid-swollen cellulose (PASC) to ethanol, producing up to 2.6-fold higher ethanol levels than that achieved by using free enzymes. Wen et al. [11] achieved the *in vivo* assembly and display of trifunctional minicellulosomes on the yeast cell surface by coexpressing the cellulosomal components in the host. The yeast cells showed ~8.8-fold enhanced activity than lower order complexes and had the ability to simultaneously break down and ferment PASC to ethanol with a titer of ~1.8 g/L. Moreover, Tsai et al. [12] and Goyal et al. [13] also exploited a yeast consortium composed of four different populations capable of either displaying the single miniscaffoldin or secreting one of the three cellulases. The optimized consortium produced almost twice the level of ethanol (~1.87 g/L) as a consortium with an equal ratio of the different populations using PASC as the sole carbon source, and the final ethanol production was 3-fold that of a similar yeast consortium secreting only the three cellulases.

In our previous study [14], trifunctional minicellulosomes with two individual miniscaffoldins on the yeast cell were *in vivo* displayed in order to increase the display level of the cellulosomes. The recombinant yeast showed a significant hydrolytic activity toward Avicel. Although the yeast first succeeded in conversion of microcrystalline cellulose into bioethanol, the difficulty level for expression of cellulases and miniscaffoldins (metabolic burden) for the host caused by the *in vivo* assembly mode was increased.

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Table 1
The recombinant *S. cerevisiae* EBY100, *S. cerevisiae* YS58 and *E. coli* BL21 (DE3) used in this study.

Strain	Plasmid(s)	Description	Reference
EBY (Control)	pYD1 and pRS425	No surface display (negative control)	[14]
EBY (C4doc-1)	pYD-ScaI-ScaII-1	Two scaffoldins with one CohII on EBY100	In this work
EBY (C4doc-2)	pYD-ScaI-ScaII-2	Two scaffoldins with two CohIIs on EBY100	[14]
EBY (C4doc-3)	pYD-ScaI-ScaII-3	Two scaffoldins with three CohIIs on EBY100	In this work
EBY (C4doc-4)	pYD-ScaI-ScaII-4	Two scaffoldins with four CohIIs on EBY100	In this work
EBY (C4doc [−]-2)	pYD-ScaII-2	Scaffoldin II with two CohIIs on EBY100	In this work
YS58 (E)	pYES2-celE	Expression and secretion of CBH in YS58	In this work
YS58 (A)	pYES2-celA	Expression and secretion of EG in YS58	In this work
YS58 (2454)	pYES2-2454	Expression and secretion of BGL in YS58	In this work
E. coli (E)	pETduet-celE	Intracellularly expression of CBH in *E. coli*	[14]
E. coli (A)	pET28-celA	Intracellularly expression of EG in *E. coli*	[14]
E. coli (2454)	pET28-2454	Intracellularly expression of BGL in *E. coli*	[14]
E. coli (C4doc)	pET28-ScaI	Intracellularly expression of scaffoldin I in *E. coli*	[14]

Interestingly, Tsai et al. [10,12] and Goyal et al. [13] showed that the ethanol production using cellulosomes constructed *in vitro* or through the yeast consortium was better than that of using *in vivo* assembled cellulosomes, maybe because the metabolic load was lowered.

In this work, we demonstrated the functional assembly of cellulosomes *in vitro* with two miniscaffoldins on the yeast cell surface. The miniscaffoldins were self-surface displayed by *S. cerevisiae*, while the recombinant cellulases were intracellularly expressed in *E. coli* or secreted by yeasts. The assembly of cellulosomes was accomplished by incubation of the induced yeasts with *E. coli* lysates or carried out through a yeast consortium. The displayed minicellulosome had higher stabilities than the free cellulosomes, and the recombinant *S. cerevisiae* showed the capability of directly converting microcrystalline cellulose to bioethanol.

2. Materials and methods

2.1. Strains and media

E. coli Top10 was used for genetic manipulations, and *E. coli* BL21 (DE3) was the host for intracellular expression of cellulases. *S. cerevisiae* EBY100 (Invitrogen, Carlsbad, CA) was used for yeast cell surface display, and *S. cerevisiae* YS58 (Lab stored) was the host for secretion of cellulases. *E. coli* cultures were grown in Luria–Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 1% NaCl) supplemented with either 100 μg/ml ampicillin or 50 μg/ml kanamycin. Yeasts were grown in YPD medium (1% yeast extract, 2% peptone, and 2% glucose). *S. cerevisiae* EBY100 transformants were selected on minimal dextrose plates (0.67% yeast nitrogen base with ammonium sulfate and without amino acids [YNB], 2% glucose, 0.01% leucine, 1.5% agar). Recombinant *S. cerevisiae* EBY100 cells were precultured in YNB-CAA (glucose) medium (0.67% YNB, 0.5% Casamino acid, 2% glucose), and were induced in YNB-CAA (galactose) medium (0.67% YNB, 0.5% Casamino acid, 2% galactose, 10 mM $CaCl_2$). *S. cerevisiae* YS58 transformants were selected on SC-U (glucose) minimal plates (0.67% YNB, 2% glucose, 0.01% [adenine, arginine, cysteine, lysine, threonine, tryptophan, leu], 0.005% [aspartic acid, histidine, isoleucine, methionine, phenylalanine, proline, serine, tyrosine, valine]). Recombinant *S. cerevisiae* YS58 cells were precultured in SC-U (glucose) minimal medium, and were induced in SC-U (galactose) minimal medium. The recombinant strains used in this study are summarized in Table 1.

2.2. Plasmid construction

The plasmids pYD-ScaI-ScaII-1, pYD-ScaI-ScaII-2, pYD-ScaI-ScaII-3, pYD-ScaI-ScaII-4, pYD-ScaII-2, pETduet-celE, pET28-celA, pET28-2454, and pET28-ScaI were described in our previous work [14]. Yeast secretion signal (α-factor, 270 bp) was amplified from pPICZαA (Invitrogen, Carlsbad, CA) using Hind III (5′-CCCAAGCTTAACACAATGTCTATGAGATTTCCTTC-3′) and Kpn I (5′-GGGGTA-CCATGGTGATGGTGATGATGAGCTTCAGCCTCTC-3′). The fusion gene celCCE-DocI-3 (2655 bp) was amplified with pETduet-celE as the template using Not I (5′-ATAAGAATGCGGCCGCAACACAATGTCTATGAGATTTCCTTC-3′) and Xba I (5′-GCTCTAGATTAGTTCTTGTACGGCAATG-3′). celCCA with native dockerin (DocI-2) (1350 bp) was cloned with pET28-celA as the template using primers Kpn I (5′-GGGGTACCAACACAATGTCTATGAGATTTCCTTC-3′) and Xba I (5′-TGCTCT-AGATTAGTTGCTTGGAAGCTTACTTACC-3′). Ccel_2454-DocI-1 (2313 bp) was cloned from pET28-2454 using Kpn I (5′-GGGGTACCAACACAATGTCTATGAGATTT-CCTTC-3′) and Xba I (5′-GCTCTAGATTAAGCAAGAAGTGCTTTCTTTAA-3′). The genes were ligated into pYES2 (Invitrogen, Carlsbad, CA) under the vector's native GAL1 promoter.

2.3. Yeast surface display, secretion and E. coli expression

The recombinant yeasts were precultured in YNB-CAA (glucose) medium or SC-U (glucose) minimal medium for 36 h at 30 °C. After washing with distilled water, the precultures were subinoculated into YNB-CAA (galactose) medium or SC-U (galactose) minimal medium at an OD_{600} = 1.0 and grown at 20 °C for over 60 h. Protein expression in *E. coli* was induced with 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 25 °C for 8 h when cells were grown to an OD_{600} of 0.5. The *E. coli* cells harvested were resuspended in 100 mM Tris–HCl buffer supplemented with 10 mM $CaCl_2$ (pH 8.0) at 20:1 and then disrupted by sonication on ice. Cellular debris was removed by centrifugation for 10 min at 11,000 × *g*.

2.4. Immunofluorescence microscopy and flow cytometry analysis (FACS)

The induced recombinant yeasts were harvested and treated as previously reported [14]. Cells were incubated in phosphate-buffered saline (PBS) containing 1 mg/ml bovine serum albumin (BSA) and 2 μg/ml mouse anti-V5-FITC antibody (Invitrogen, Camarillo, CA). After washed by PBS, the cell-antibody complex was analyzed by immunofluorescence microscope (OPTEC, Chongqing, China) and FACSAria II (BD, Franklin Lakes, NJ).

2.5. In vitro assembly of minicellulosomes

When using *S. cerevisiae* EBY100 cells and *E. coli* lysates, the yeasts with miniscaffoldins and the supernatants containing cellulases were mixed in 100 mM Tris–HCl buffer with 10 mM $CaCl_2$ (pH 8.0), and kept for 2 h at 4 °C. The yeasts obtained were named as eEA2-1, eEA2-2, eEA2-3, and eEA2-4, of which the miniscaffoldin IIs had 1, 2, 3, and 4 repeating CohIIs respectively. eEA2-2 (−) means the treated yeast had two repeating CohIIs on miniscaffoldin II and did not contain miniscaffoldin I. When using yeast consortium, the induced *S. cerevisiae* EBY100 and *S. cerevisiae* YS58 cells were mixed directly without removing the induction mediums, and kept for 2 h at 4 °C. The obtained yeast consortiums were named as yEA2-1, yEA2-2, yEA2-3, and yEA2-4.

2.6. Thermal stability and protease accessibility

Yeast-associated and free minicellulosomes were suspended in PBS with or without proteinase K (0.1 mg/ml) (Biomed, Beijing), and incubated at 30, 37, or 42 °C for 3 h. The residual cellulolytic activity was analyzed by 3,5-dinitrosalicylic acid (DNS) assay using Avicel (QXTD-Biotechnology, Beijing) as the substrate. Cellulosomes were incubated with 0.1% Avicel in 100 mM Tris–HCl buffer with 10 mM $CaCl_2$ (pH 5.5) for 16 h at 30 °C. After addition of DNS and boiling for 2 min, reducing sugars were quantified colorimetrically at OD = 540 nm.

2.7. Ethanol fermentation

After cellulosome assembly, yeast strain was washed twice with YP medium (1% yeast extract, 2% peptone, 10 mM $CaCl_2$) and resuspended in YP medium supplemented with 0.001% ergosterol, 0.042% Tween 80, and 1% cellulosic substrate to an OD_{600} = 50 (20 ml). The substrates used were Avicel, PASC [15], and carboxymethyl cellulose (CMC). The fermentation condition was similar with our previous study [14]. The ethanol concentration was determined by gas chromatography (GC-2010, Shimadzu, Japan).

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