## Special Feature: Biotechnology for Sustainable and Aging Societies

*Research Report*

# Reconstitution of Artificial Cellulosome on Yeast Surface

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**ABSTRACT** We examined the ability of *S. cerevisiae* to produce and display artificial scaffolding proteins consisting of different numbers of cohesins on its cell surface, mimicking the cellulosome. By analysis of recombinant strains having the mini CipA gene from *C. thermocellum*, we found that *S. cerevisiae* is able to express the scaffolding proteins and reconstitute minicellulosomes possessing saccharification activity on its surface. We also identified deletion mutants that enhanced this activity by deglycosylation. Deletion mutants related to the early processes of N-glycosylation, such as Δalg, were effective at improving cellulosome reconstitution. Furthermore, Δvps3 and Δvps16 showed endosome/vacuole function and exhibited about 3-fold endoglucanase activity when compared with the wild strain.

**KEYWORDS** Cellulosome, *S. Cerevisiae*, Glycosylation, Deletion Mutant, Cohesin

## 1. Introduction

Establishing a production process for renewable energy is an important issue for the future development of a sustainable society. Cellulosic bioethanol, a form of renewable energy, is considered to be necessary to achieve this goal. However, it is important to promote widespread bioethanol production by creating low-cost technologies to overcome the recalcitrant plant structure. Cellulosic bioethanol processing commonly involves many steps; pretreatment of biomass, saccharification, fermentation and distillation. It has been reported by NREL that the enzymatic hydrolysis of corn stover accounts for about 20% of the total processing costs.[1] Thus, it is desirable to devise a microorganism that is able to utilize cellulose and to produce ethanol in order to decrease the costs of the saccharification step.

*Saccharomyces cerevisiae* is a very useful microorganism for ethanol production. However, this yeast has a lower potential for protein secretion than aerobic fungi such as *Trichoderma reesei*. To develop an *S. cerevisiae* that is capable of hydrolyzing and utilizing insoluble cellulose, it is necessary to introduce an effective cellulose degradation system. The cellulosome is a large multienzyme complex for effective degradation of crystalline cellulose or plant cell wall polysaccharides.[2-4] This complex is formed by interactions between multiple cohesin modules in a pivotal noncatalytic "scaffolding protein" and a dockerin module in various enzyme subunits. It is believed that the formation of the enzyme complex contributes to effective cellulose degradation. In fact, effective cellulose degradation by an artificial cellulosome has already been reported in vitro.[5-7] In the present study, we examined the potential of *S. cerevisiae* to produce and display artificial scaffolding proteins consisting of different numbers of cohesins on its cell surface, mimicking the cellulosome.

When prokaryotic proteins are expressed in yeast, there have been problems related to the different secretion systems of prokaryotic and eukaryotic microorganisms. One of these problems is that the amount of secreted prokaryotic protein is low in yeast. Another problem is post-translational modification of expressed protein. Heterologous proteins overexpressed in yeast are modified by hyperglycosylation with mannose,[8] and this affects its functionality and/or protein-protein interactions. If the parts of the cellulosome expressed in yeast are influenced by these events, it will be necessary to resolve these issues in the host cell in order restore the original cellulosome functions.

We therefore used a yeast deletion strain collection disrupted in each of approximately 4800 non-essential genes.[9,10] Synthetic genetic array analyses with this collection or phenotypic analyses have been reported.[11,12] Moreover, genome-wide analyses of

 

the collection have contributed to useful observations for yeast genome wide-data.[13,14] Some genome-wide analyses such as overexpressing yeast cDNA libraries have also been analyzed to enhance heterologous protein production.[16,17] However, genome-wide analyses for gene identification to improve cellulosome activity on the yeast surface using a deletion strain collection have not been accomplished.

In this study, we first aimed to introduce scaffolding proteins to *S. cerevisiae* for artificial cellulosome reconstruction on its surface. Next, we attempted to identify glycosylation mutants to improve cellulosome reconstruction.[18] Finally, we attempted to identify yeast deletion mutants to increase the amount of cellulosomal cellulase production.[19]

## 2. Materials and Methods

### 2. 1 Strain and Media

*S. cerevisae* EBY100 and BY4741 were used as host cells. Yeast EBY100 cells were grown in minimal SD-CAA medium (2% w/v glucose, 6.7 w/v yeast nitrogen base, 5 g/L casamino acid) and expression of scaffolding proteins was induced in SG-CAA medium (2% w/v L galactose, 6.7 w/v yeast nitrogen base, 5 w/v casamino acid). BY4741 cells were grown in SD-CAA and YPD (1% w/v yeast extract, 2% w/v peptone, and 2% w/v glucose) medium.

### 2. 2 Vector Construction and Protein Expression

Genomic DNA was extracted from *C. thermocellum* ATCC27405. DNA fragments encoding a CBD plus one cohesin(s) were amplified using combinations of the following primers (forward:
5'-acgtaggtaccagcaaatacaccggtatcaggcaatttgaaggttgaattct-3';
reverse: one cohesin,
5'-acgtactcgagatctccaacatttactccaccgtcaaagaactgtgt-3').
A gene for four cohesins with the CBD was constructed by gene synthesis (TOYOBO, Japan). Amplified fragments were inserted into the pYD1 vector. *S. cerevisiae* EBY100 transformed with these recombinant plasmids were grown in minimal SD-CAA at 30ºC to an OD600 of between 2 to 5, transferred to minimal SG-CAA medium for induction of protein expression and incubated for 48 h at 30ºC. All integration vectors for AGA1-AGA2, scaffolding proteins and cellulases had a signal peptide for glucoamylase from *Rhizopus oryzae* and the HOR7 promoter from *S. cerevisiae*, and were cultured in YPD medium for 24 h at 30°C. Cellulase genes were also inserted into 2 μm PRS-436GAP vector and transformants were cultured in SD medium for 24 h or for several days at 30°C.

### 2. 3 Fluorescence Microscopy and FCM Analysis

EBY100 displaying scaffolding proteins were washed with PBS, and were treated with anti-$His_6$ antibody (Ab) and 1 mg/ml BSA, followed by labeling with anti-mouse Ab conjugated with Cy5 and 1 mg/ml BSA. Fluorescence of Cy5 was measured under a fluorescence microscope. For FACS analysis, yeast cells displaying scaffolding proteins were reacted with anti-V5 Ab, followed by labeling with anti-mouse antibody conjugated with Alexa Fluor 488. BY4741 displaying scaffolding proteins by an integration vector were reacted with anti-V5-FITC Ab after harvesting and washing. BY4741 reconstituted mincellulosomes were reacted with anti-$His_6$ Ab. Fluorescence intensities of yeast cells expressing scaffolding proteins were analyzed using a flow cytometer (Beckman Coulter, USA) with an excitation wavelength of 488 nm.

### 2. 4 Reconstitution and Endoglucanase Assay of Minicellulosomes on Yeast Surface

Minicellulosomes were reconstituted on the yeast surface by assembling both CelA synthesized using a cell-free expression system and scaffolding proteins expressed by the pYD-vector. Endoglucanase assay was performed as follows. EBY100 displaying scaffolding proteins were washed with 20 mM Tris-HCl pH 8.0 containing 10 mM $CaCl_2$. After centrifugation, yeast cells were suspended in a solution of 20 mM Tris-HCl (pH 8.0), 0.15 M NaCl, 10 mM $CaCl_2$ and CelA solution from cell-free synthesis (Wako Pure Systems, Japan), and were incubated at 4ºC for 1 h. EBY100 harboring minicellulosome complex on the cell surface were washed with a solution of 20 mM Tris-HCl (pH 8.0), 0.15 M NaCl, 10 mM $CaCl_2$ and 0.05% Tween 20, followed by washing with 50 mM acetate buffer (pH 6.0) containing 10 mM $CaCl_2$. Reaction solution (1% CMC in 50 mM acetate buffer (pH 6.0)

 

containing 10 mM $CaCl_2$) was then added to the cells. Reconstitution of minicellulosome expressed by yeast and endoglucanase assay were performed as follows. BY4741 displaying the scaffolding proteins expressed by integration vector were harvested and washed with PBS. The Supernatant of BY4741 and the glycosylation deletion mutants secreting fusion proteins, *Cel8A* catalytic domain and Cel48 dockerin domain (Cel8Aenz-Cel48Sdoc) were mixed and incubated for 12 hours at 4ºC. Endoglucanase activity was assayed at 60ºC by the TZ-assay method, using CMC as a substrate

### 2. 5 Enzyme Assay of Cellulase Secreted by Yeast Deletion Mutants

Supernatants of deletion mutants having the PRS436 vector inserted with *Cel8A* were spotted onto CMC plates (0.1%, w/v CMC; 2%, w/v agar; 50 mM sodium acetate (pH 6.0)). Plates were incubated for 24 h at 40ºC and were stained with Congo Red. Endoglucanase activities of deletion mutants were assayed based on halo sizes of the discolored spots. Supernatants were also used for TZ assay at 40ºC in 0.2% (w/v) CMC, 50 mM sodium acetate buffer (pH 5.0).

## 3. Results

### 3. 1 Display of Scaffolding Proteins on Yeast Surface

DNA fragments encoding one and four cohesins with a CBD of *C. thermocellum* CipA (**Fig. 1**(A)) were inserted into the pYD1 CEN/ARS vector that displayed the proteins of interest on the cell surface of *S. cerevisiae* using the AGA1-AGA2 interaction system. Yeast cells transformed with these constructs expressed the scaffolding proteins under the control of the GAL1 promoter. After expression of scaffolding proteins, every transformant was labeled with anti-$His_6$ antibody and anti-mouse IgG conjugated with Cy5. By fluorescence analysis of these recombinant strains, we found that *S. cerevisiae* was able to express the scaffolding proteins (Fig. 1(B)).

For FCM analysis, yeast cells were also stained with anti-V5 antibody and anti-mouse IgG conjugated with Alexa fluor. However, FCM analysis indicated that the expression levels of the proteins decreased, as the copy number of cohesins increased (data not shown). We constructed the complexes from the scaffolding proteins with different numbers of cohesins anchored onto the yeast cell surface and *C. thermocellum* endoglucanase CelA synthesized using a cell-free system, and we then measured the cellulolytic activity of the complexes (Fig. 1(C)). The CMC-hydrolyzing activity of the complex including

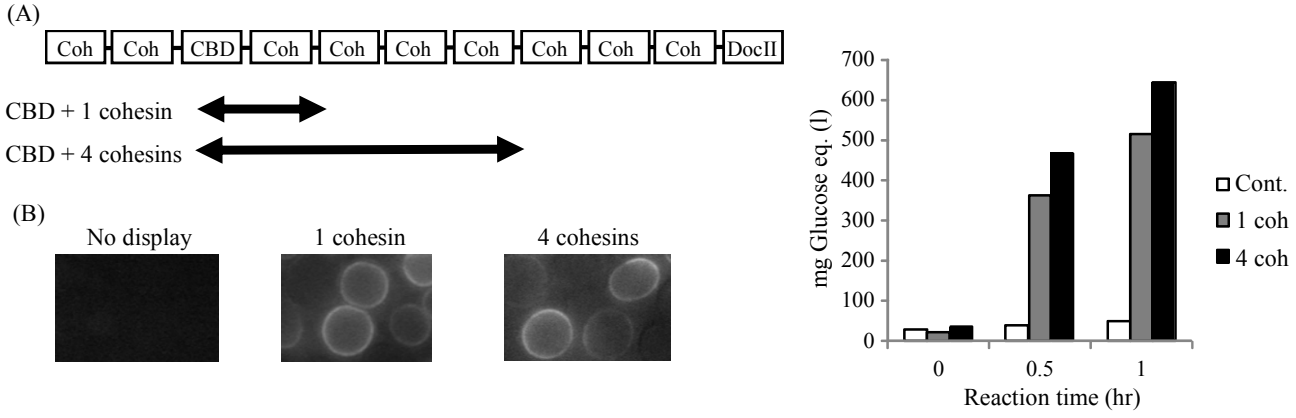


**Fig. 1** (A) Cloning region of mini CipA gene from *C. thermocellum*. Coh, CBD, DocII were abbreviations of type I cohesin, carbohydorate binding domain, type II dockerin. (B) Immunofluorescence labeling of transformants. Yeast cells displayed scaffolding proteins were stained by the protocol described materials and method. (C) CMC hydrolyzing activity of minicellulosome complex on yeast surface. The complex consists of yeast cells displayed scaffolding proteins and *C. thermocellum* CelA.

 

a scaffolding protein with four cohesins was about 20% higher than that of the complex including only one cohesin.

### 3. 2 Identification of Glycosylation Mutants that Enhance Minicellulosome Reconstitution

We attempted to reconstitute the minicellulosome produced by *S. cerevisiae* on its surface. Scaffolding proteins that have one cohesin from *C. themocellum* fused *aga2* were integrated into the yeast genome inserted with *aga1*, and the recombinant strain was designated CtminiCipA. FCM analysis of CtminiCipA indicated positive emissions (**Fig. 2**). The fusion cellulase constructed from the catalytic domain from *C. thermocellum Cel8A* and the dockerin domain from *C. thermocellum Cel48S* was inserted into the PRS436 2 μm vector, and was designated PRSGAP-His-Cel8Aenz-Cel48Sdoc. The culture supernatant of recombinant *S. cerevisiae* transformed by this vector was mixed with CtminiCipA or BY4741. As only CtminiCipA transformed by PRSGAP-His-Cel8Aenz-Cel48Sdoc showed endonuclease activity, it was believed that Cel8Aenz-Cel48Sdoc bound to CtminiCipA on the surface of *S. cerevisiae*.

In order to evaluate the effects of the enhancement of endoglucanase activity by glycosylation mutants as the host, we used a yeast deletion strain collection. A total of 40 deletion strains related to protein glycosylation were transformed by PRSGAP-His-Cel8Aenz-Cel48Sdoc, and these culture supernatants were mixed with CtminiCipA. Endoglucanase activity of minicellulosome reconstituted on yeast surface was assayed by TZ assay (**Fig. 3**). The following mutants related to N-type protein glycosylation were identified. Cax4p synthesizes a pyrophosphate-linked oligosaccharide dolichyl precursor. Alg5p, Alg3p, Alg9p, Alg12p, Alg6p, Alg8p and Die2p elongate oligosaccharides linked to the dolichyl precursor. Ost3p is the subunit of the oligosaccharide transferase complex. It modifies the oligosaccharide to asparagine residues in Ser/Thr-X-Asp as an N-type protein glycosylation consensus sequence. Mutants related to O-type protein glycosylation, Pmt1p, Pmt2p, Pmt3p, Pmt4p and Pmt5p, were also identified. These proteins transfer mannose phosphate-D-mannose from dolichyl to protein serine/threonine residues. OCH1 attaches the mannose to the outer chain of the N-type oligosaccharides in glycoproteins. MNN11 is a subunit of the M-PolII complex. It elongates the polysaccharide mannan chain of glycoproteins into the Golgi body.

Next, we estimated the amount of Cel8Aenz-Cel48Sdoc assembled on the yeast surface (**Fig. 4**). Culture supernatant from 15 mutants that

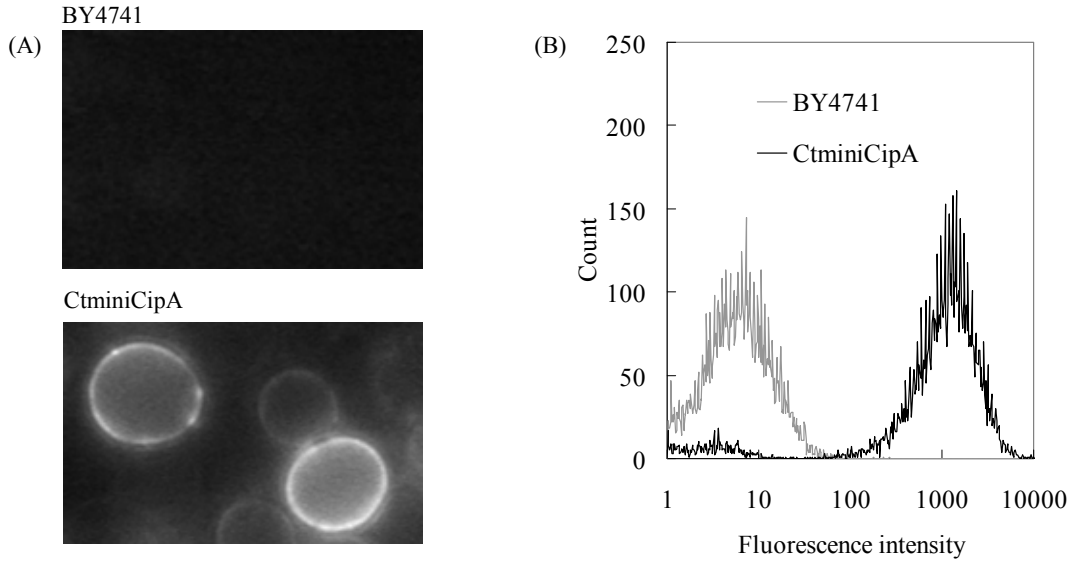


**Fig. 2** (A) Fluorescence images of yeast strains stained with anti-V5-FITC ab. Top, BY4741; bottom, CtminiCipA. (B) Flow cytometric analysis of assembled minicellulosomes. The amount of the CtminiCipA displayed on the yeast cell surface was analyzed by FITC fluorescence using anti-V5-FITC ab. The gray line shows BY4741; the black line shows CtminiCipA.

 

possess PRSGAP-His-Cel8Aenz-Cel48Sdoc was incubated with CtminiCipA. Using FCM analysis, 14 mutants other than the Δcax4 mutant showed increased amounts of dockerin displayed on the surface. These deletion mutants therefore showed enhanced yeast endonuclease.

### 3. 3 Identification of Yeast Mutants that Enhance Cellulosomal Cellulase Production

In order to enhance protein production, more dockerin binding sites for scaffolding proteins are needed as the copy number of cohesins increases.

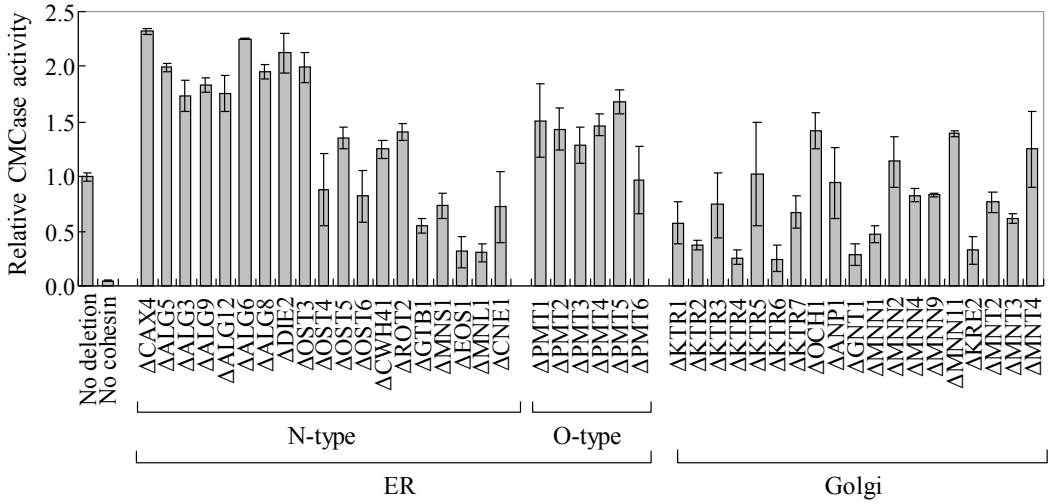


**Fig. 3** Screening for enhancement of the endoglucanase activity of Cel8Aenz using protein glycosylation mutants. CtminiCipA was assembled with the culture supernatant of each protein glycosylation mutant harboring pRS436GAP-His-Cel8Aenz-Cel48Sdoc. “No deletion” indicates CtminiCipA assembled with the culture supernatant of BY4741 harboring pRS436GAP-His-Cel8Aenz-Cel48Sdoc. “No cohesin” indicates BY4741 assembled with the culture supernatant of BY4741 harboring pRS436GAP-His-Cel8Aenz-Cel48Sdoc. “N-type” and “O-type” indicate the protein glycosylation mutants that affect synthesis of the core complex of N-type or O-type glycosylation, respectively. “ER” or “Golgi” indicates the cellular compartment where the disrupted gene was localized (i.e. the endoplasmic reticulum or the Golgi apparatus, respectively).

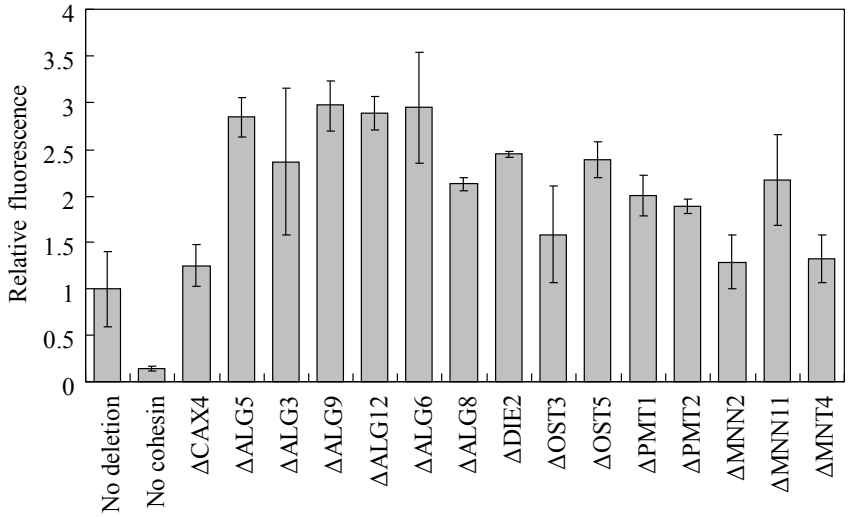


**Fig. 4** Cytometric analysis of the minicellulosomes assembled with candidate glycosylation mutants. CtminiCipA was assembled with the culture supernatant of each protein glycosylation mutant harboring pRS436GAP-His-Cel8Aenz-Cel48Sdoc. “No deletion” indicates CtminiCipA assembled with the culture supernatant of BY4741 harboring pRS436GAP-His-Cel8Aenz-Cel48Sdoc.

 

The cellulase gene *C. thermocellum Cel8A* was inserted into the PRS436 2 μm vector, and this was designated PRSGAP-CelA. This vector was used for transformation of the 5197 yeast deletion mutant. Transformants were grown in SD-medium and culture supernatant was subjected to CMC plate assay. A total of 96 deletion strains showed increased endoglucanase activity; however, 13 deletion strains were not available because the deletion position on genomic PCR check was found to be incorrect. Next, 83 mutants were transformed with an integration vector having *Cel8A* under the HOR7 promoter. These transformants were grown in YPD medium, and culture supernatant was subjected to CMC plate assay. Finally, 55 strains that showed enhanced endoglucanase activity were identified (**Fig. 5**(A)), and were categorized into 8 groups based on function (**Table 1**). Moreover, we selected 10 stains as having the highest endoglucanase activity. In particular, Δvps3 and Δvps16 showed endosome/vacuole function with about 3-fold higher endoglucanase activity when compared with the wild strain (Fig. 5(B)).

## 4. Discussion

The present results suggest that *S. cerevisiae* is able to produce cellulosomal parts, scaffolding proteins and cellulase, and reconstitute the artificial cellulosome on its surface. This is the first time that an artificial cellulosome derived from *C. thermocellum* was found to be displayed on its surface (US Patent No. 8361752, issued Jan. 29, 2013). *C. cellulovorans* and various cellulosome-expressing yeast have been reported previously.[20,21] Moreover, we identified deletion mutants that showed better glycosylation and production of cellulosomal cellulase in yeast. With regard to glycosylation mutants, N-type glycosylation deletion mutants such as Δalg showed increased cellulosomal cellulase binding to scaffolding protein. It is known that hyperglycosylation of heterologous proteins in the Golgi body occurs after N-glycosylation in ER,[20] and that alg genes function in the early processes of N-type glycosylation.[22,23] Suppression of N-type glycosylation in its early stage is thought to contribute to reduced hyperglycosylation. CelA having the dockerin domain is about 10 kDa, on the other hand, the molecular weight of hyperglycosylation in the heterologous protein reached a few hundred kDa.

The scaffolding proteins having cohesin domains bind with the dockerin domains of various enzymes.[24] Steric obstruction between cohesin and dockerin by hyperglycosylation may inhibit the reconstitution of cellulosome. These mutants will be useful for reducing hyperglycosylation of the cohesin domain.

The Δvps3 or Δvps16 mutants exhibiting endosome/vacuole function showed enhanced production of cellulosomal cellulase. These deletion mutants have been shown to have enhanced endoglucanase activity when compared with *C. cellulolyticum* endoglucanase 5A and 9M,[18] but the production efficiency of scaffolding proteins displayed on the yeast surface were not evaluated. As the β-glucosidase activity from *A. aculeatus* displayed on the yeast surface was enhanced by these deletion mutants,[18] the amount of exogenous and yeast-anchored protein will also increase.

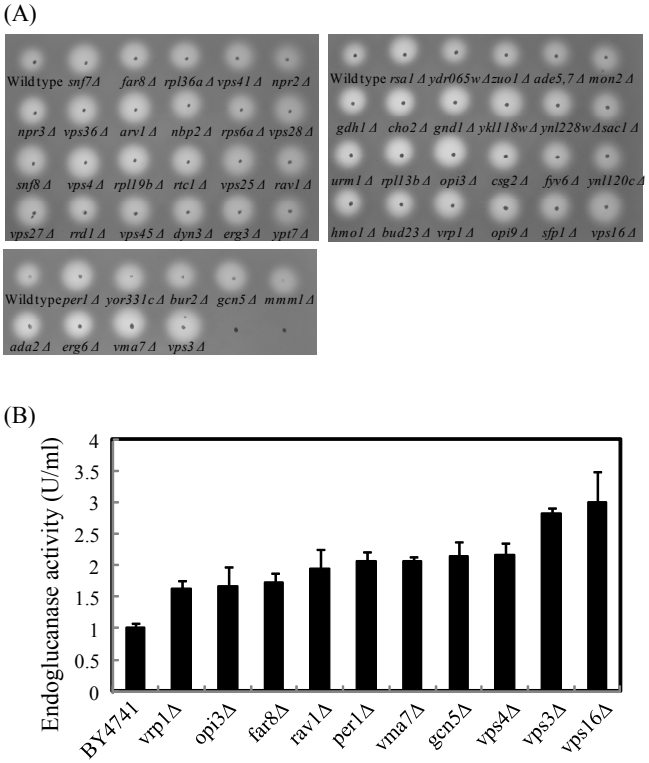


**Fig. 5** Deletion strains that exhibit enhanced endoglucanase activity of the Ctcel8A enzyme. (A) Ctcel8A gene DNA fragment was integrated into the chromosome of haploid deletion strains. Transformants were grown in YPD medium. Supernatants of the cultures were diluted, and then aliquots were spotted onto a CMC plate. (B) Enzyme activity of Ctcel8A. Ctcel8A transformants were grown YPD medium, and then supernatants of the cultures were subjected to activity measurements with CMC.

 

Based on the above results, deletion mutants will be useful for improving the properties as of the host cells; however, multiple gene deletions may have an undesirable influence on the host cells. It is well known that the exogenous proteins expressed in *S. cerevisiae* were glycosylated on the asparagine of Ser/Thr-X-Asp by N-type glycosylation. To suppress these negative effects, protein engineering may be a more practical method. The amino acid substitution of asparagine with other amino acids in dockerin contributes to reduced hyperglycosylation.

The above approaches will be useful for deducing the original saccharification potential of the artificial cellulosomes displayed on the yeast surface. Synergistic actions among different enzymes in the cellulosome complex are also important. In this experiment, the effects of only endoglucanase on scaffolding proteins were evaluated. It will be

**Table 1** The enhanced genes of Ctcel8A enzymes activities.

| Standard name | Systematic name | Description |
|---|---|---|
| **Endosome/Vacuole function** | | |
| *MON2* | YNL297C | Peripheral membrane protein with a role in endocytosis and vacuole integrity |
| *RAV1* | YJR033C | Subunit of the RAVE complex (Rav1p, Rav2p, Skp1p) |
| *SNF7* | YLR025W | One of four subunits of the ESCRT-III complex |
| *SNF8* | YPL002C | Component of the ESCRT-II complex |
| *VMA7* | YGR020C | Subunit F of the eight-subunit V1 peripheral membrane domain of V-ATPase |
| *VPS3* | YDR495C | Component of the CORVET-tethering complex |
| *VPS4* | YPR173C | AAA-ATPase involved in multivesicular body (MVB) protein sorting |
| *VPS16* | YPL045W | A subunit of the class C *VPS* complex |
| *VPS25* | YJR102C | Component of the ESCRT-II complex |
| *VPS27* | YNR006W | Endosomal protein that forms a complex with Hse1p |
| *VPS28* | YPL065W | Component of the ESCRT-I complex |
| *VPS36* | YLR417W | Component of the ESCRT-II complex |
| *VPS41* | YDR080W | A subunit of the HOPS complex |
| *VPS45* | YGL095C | Protein of the Sec1p/Munc-18 family |
| *YDR065W* | YDR065W | Protein of unknown function, required for vacuolar acidification |
| *YKL118W* | YKL118W | Dubious open reading frame; partially overlaps with VPH2 |
| *YOR331C* | YOR331C | Dubious open reading frame; partially overlaps with VMA4 |
| *YPT7* | YML001W | GTPase required for the homotypic fusion event in vacuole inheritance |
| **ER/golgi function** | | |
| *ARV1* | YLR242C | Protein functioning in transport of glycosylphosphatidylinositol |
| *CSG2* | YBR036C | Required for mannosylation of inositolphosphorylceramide |
| *FAR8* | YMR029C | Protein involved in G1 cell cycle arrest in response to pheromones |
| *PER1* | YCR044C | Protein required for GPI-phospholipase A2 activity that remodels the GPI anchor |
| *SAC1* | YKL212W | Phosphatidylinositol phosphate (PtdInsP) phosphatase |
| **Phospholipid synthesis** | | |
| *CHO2* | YGR157W | Phosphatidylethanolamine methyltransferase |
| *ERG3* | YLR056W | C-5 sterol desaturase |
| *ERG6* | YML008C | Delta(24)-sterol C-methyltransferase |
| *OPI3* | YJR073C | Phospholipid methyltransferase (methylene-fatty-acyl-phospholipid synthase) |
| **Transcription** | | |
| *ADA2* | YDR448W | Transcription coactivator of the ADA and SAGA transcriptional adaptor |
| *GCN5* | YGR252W | Histone acetyltransferase of the ADA and SAGA |
| *HMO1* | YDR174W | Chromatin-associated high mobility group (HMG) family member |
| *SFP1* | YLR403W | Transcription factor that controls the expression of many ribosome biogenesis genes |
| **Translation** | | |
| *BUD23* | YCR047C | Methyltransferase, methylates residue G1575 of 18S rRNA |
| *RPL13B* | YMR142C | Protein component of the large (60S) ribosomal subunit |
| *RPL19B* | YBL027W | Protein component of the large (60S) ribosomal subunit |
| *RPL36A* | YMR194W | N-Terminally acetylated protein component of the large (60S) ribosomal subunit |
| *RPS6A* | YPL090C | Protein component of the small (40S) ribosomal subunit |

 

necessary to investigate the optimal combinations of enzymes that indicate strong synergistic effects on the surface of yeast cells displaying artificial scaffolding proteins.

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