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Efficient ethanol production from starch through development of novel flocculent yeast strains displaying glucoamylase and co-displaying or secreting α-amylase

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Abstract

To develop novel yeasts with high starch-to-ethanol productivity, we constructed two cell-surface-engineered flocculent *Saccharomyces cerevisiae* strains; one co-displaying glucoamylase and α-amylase on the cell surface and the other displaying glucoamylase and secreting α-amylase into the culture medium. With starch as the carbon source, both yeast strains grew faster under aerobic conditions than strains displaying only glucoamylase. In fed-batch fermentation of ethanol, these recombinant yeasts co-expressing sequential amylolytic enzymes also showed higher starch decomposition and ethanol production abilities than yeast cells displaying only glucoamylase, with the concentration of ethanol produced reaching 60 g/l after approximately 100 h fermentation under anaerobic conditions. Both co-display and secretion of α-amylase are thus effective in improving ethanol production from starchy materials in glucoamylase-displaying yeast cells.

Keywords: α-Amylase; Glucoamylase; Cell surface display; Ethanol; Starch; *Saccharomyces cerevisiae*

1. Introduction

Biomass resources, namely starchy and cellulosic materials of plant origin, are the most abundant renewable resources on earth and their utilization for production of energy and chemicals has attracted considerable interest in recent years. In particular, ethanol produced from biomass by fermentation is expected to find use as fuel and in the production of various chemicals.

The yeast *Saccharomyces cerevisiae* is widely used for commercial production of ethanol from starchy materials. Since *S. cerevisiae* lacks the amylolytic enzymes necessary for starch utilization, the currently available production process requires that the raw starch be gelatinated by cooking, liquified by α-amylase treatment and saccharified to glucose by glucoamylase treatment, a multi-step process which

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offers poor economic viability. To simplify the fermentation process by eliminating the separate saccharification step, numerous genetically engineered *S. cerevisiae* strains capable of secreting glucoamylase and/or α-amylase have been developed. However, the starch decomposition abilities of these yeast strains are unsatisfactory because of the limited amount of amylolytic enzymes secreted [1–8].

In our previous studies, we have constructed yeast strains displaying on the cell surface *Rhizopus oryzae* glucoamylase [9–11], an amylolytic enzyme of the exo-type which effectively cleaves both α-1,4-linked and α-1,6-linked glucose from starch [12]. We have also tested glucoamylase-displaying flocculent *S. cerevisiae* YF207 for ethanol production and found it to show high productivity from soluble starch [11] and to retain flocculation ability during ethanol fermentation. However, an insoluble starch fraction accumulates during fed-batch fermentation because of a lack of liquefied enzyme α-amylase.

In the present study, to further improve ethanol productivity from starchy materials, we constructed two recombinant yeast strains co-expressing glucoamylase and α-amylase. Plasmids for cell-surface expression and for secretory expression of *Bacillus stearothermophilus* α-amylase were constructed and co-transformed into the flocculent yeast strain YF207 along with the plasmid for cell-surface expression of *R. oryzae* glucoamylase. The ethanol productivity of these two strains from soluble starch was examined by fed-batch fermentation using a jar fermentor.

2. Experimental

2.1. Strains and media

The *Escherichia coli* strain used for genetic manipulation was NovaBlue [*endA1* hsdR17 (r_k m_k^+) *supE44 thi-1 gyrA96 relA1 lac recA1/F'* {*proAB*$^+$ *lacI*^{q} *ΔM15 Tn10* (*tet*^{r})}] (Novagen Inc., Madison, WI, USA). The flocculent *S. cerevisiae* strain used was YF207 (*MATa ura3-52 trp1 Δ2 his can1-100 FLO8*). *E. coli* was grown in LB medium (1% trypton, 0.5% yeast extract, 1% sodium chloride) containing 100 μg/ml ampicillin. SD medium (0.67% yeast nitrogen base supplemented with appropriate amino acids and nucleotides, 2% glucose) was used for yeast cultivation under selective conditions.

2.2. Construction of plasmids

The plasmids pAA12 and pSAA11 were constructed for cell-surface and secretory expression, respectively, of α-amylase from *B. stearothermophilus* (Fig. 1). The plasmid pAA12 was a multi-copy plasmid for expression of the α-amylase/α-agglutinin fusion gene with the secretion signal sequence of *MFα1* under the control of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter. pSAA11 was a multi-copy plasmid for secretory expression of the α-amylase gene with the same secretion signal as pAA12.

The plasmid pAA12 for cell-surface display of α-amylase was constructed as follows: the plasmid

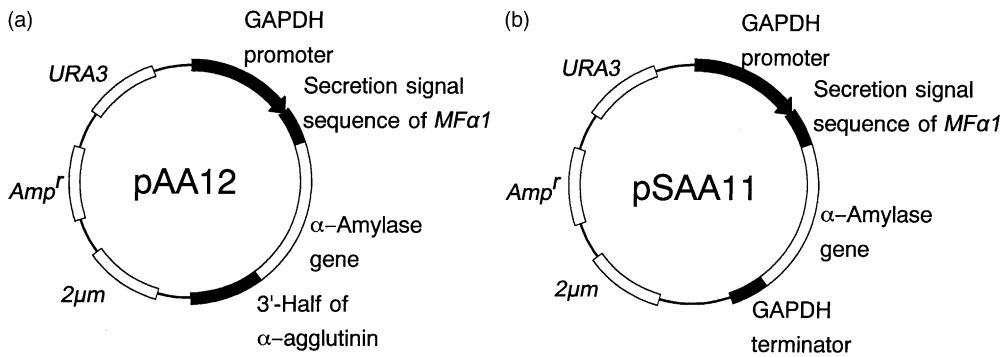

Fig. 1. Construction of the two α-amylase expression plasmids: (a) pAA12 for cell surface expression of α-amylase; and (b) pSAA11 for secretory expression of α-amylase.

pIAAΔ11 [9] was digested with *Xho*I and both the (A) 1.7 and (B) 6.9 kb fragments recovered. Fragment B was self-ligated and digested with *Not*I and *Kpn*I. The resulting (C) 2.1 kb fragment encoding a GAPDH promoter and the 3′-half of the α-agglutinin encoding region was recovered and treated with T4-DNA polymerase. The plasmid pMT34(+3) [13] was digested with *Pvu*II and *Bam*HI and the recovered 6.1 kb fragment treated with T4-DNA polymerase and ligated with fragment C to give the plasmid pUGP12. Fragment A encoding the fusion gene of the prepro secretion signal sequence of *MFα1* and α-amylase from *B. stearothermophilus* was inserted into the *Xho*I site of pUGP12 to give the plasmid pAA12.

The plasmid pSAA11 for secretory production of α-amylase was constructed as follows: a 1.7 kb DNA fragment containing the fusion gene of the prepro secretion signal sequence of *MFα1* and α-amylase from *B. stearothermophilus* was prepared by polymerase chain reaction (primers 5′-ATGCGAGCTCATGAG-ATTTCCTCCAATTTTTACTGCAG-3′ and 5′-ATG-CGAGCTCTCAAGGCCATGCCACCAACCGTGG-TTCGG-3′) with the plasmid pIAA11 [9] and digested with *Sac*I. This fragment was then inserted into the *Sac*I site of pUGP3 [9] to give the plasmid pSAA11.

2.3. Transformation

The plasmids for cell-surface expression of glucoamylase (pGA11) [9] and α-amylase (pAA12) and for secretory expression of α-amylase (pSAA11) were introduced into *S. cerevisiae* via the lithium acetate method using the YEAST MAKER^{TM} transformation system (Clontec Laboratories Inc., Palo Alto, CA). Transformant clones were selected on SD plates without L-tryptophan or uracil and named YF207/pGA11, YF207/pAA12 and YF207/pSAA11. To prepare strains which co-express glucoamylase and α-amylase, the plasmids pGA11 and pAA12 or pSAA11 were introduced stepwise into *S. cerevisiae*. Transformant clones were selected on SD plates without L-tryptophan and uracil and named YF207/[pGA11, pAA12] and YF207/[pGA11, pSAA11].

2.4. Flow cytometric analysis

Yeast cells were collected by centrifugation at 6,000 × g for 5 min, washed with phosphate buffered saline (PBS; 50 mM phosphate, 150 mM sodium chloride, pH 7.4) and adjusted to $OD_{600} = 10$ with PBS. Next, 100 μl of this cell suspension was centrifuged in a 1.5 ml microtube and the collected cells resuspended with 100 μl of 150 μl/ml goat anti-glucoamylase antibody conjugated with fluorescein-isothiocyanate (FITC) (Rockland Inc., Gilbertsville, PA, USA) in PBS containing 1% BSA and incubated for 1 h under rotation. The cells were then washed with PBS twice and resuspended in 2 ml of PBS. Flow cytometric analysis was performed using FACS calibur (Becton Dickinson, Franklin Lakes, NJ, USA). Event rate was maintained at 500 cells/s and data for 10,000 events collected.

2.5. Fed-batch fermentation

Soluble potato starch (Wako Pure Chemicals Co., Osaka, Japan) was used as the carbon source. The yeast strains co-expressing glucoamylase and α-amylase were precultured in 100 ml of SD medium with 1% casamino acid at 30 °C for 30 h. Thereby, 50 ml of the preculture was used to inoculate 1 l of YPS 4% medium (1% yeast extract, 2% polypepton, 4% soluble starch) with 0.5% glucose and yeast cells were grown at 30 °C under aerobic conditions in a 2 l jar fermentor (BMJ-02PI Biott Corp., Tokyo, Japan). The pH was maintained at pH 5.0 by addition of sulfuric acid and sodium hydroxide and the dissolved oxygen maintained at 2.0 ppm by adjusting agitation speed. When dry cell weight had reached approximately 12 ∼ 15 g/l, cells were harvested by centrifugation for 10 min at 5000 × g. The cell pellet was used to inoculate 1 l of YPS 6% medium (1% yeast extract, 2% polypepton, 6% soluble starch) with 0.5% glucose and fermentation of soluble starch to ethanol was carried out at 30 °C and pH 5.0 under anaerobic conditions with mild agitation (150 rpm) in the 2 l jar fermentor.

When the rate of starch consumption by the yeast cells had decreased, 500 ml of concentrated medium 1 (1 g/l yeast extract, 1 g/l polypepton, 105 g/l soluble starch, 7.5 g/l glucose) was fed into the fermentor and fermentation continued at 30 °C at pH 5.0 under anaerobic conditions. After further decrease, 500 ml of concentrated medium 2 (1 g/l yeast extract, 1 g/l polypepton, 140 g/l soluble starch, 10 g/l glucose) was fed into the fermentor and fermentation continued again

at 30 °C. As a result of these feeds, the total quantity of culture medium reached 2.0 l.

2.6. Enzyme assay

Glucoamylase activity was measured as described previously [11]. One unit of glucoamylase was defined as the amount of enzyme required to release 1 μmol of glucose/min from starch. α-Amylase activity was measured using the 3,5-dinitrosalicylic acid (DNS) method as described previously [14]; α-amylase activity on the cell surface is calculated by subtracting glucoamylase activity from the increased reducing power measured by the DNS method.

2.7. Measurement of starch, glucose and ethanol concentrations, cell growth and flocculation ability

Previously described methods were used to measure starch, glucose and ethanol concentrations [11], yeast-cell growth and flocculation ability [15].

3. Results

3.1. Detection of amylolytic activity of yeast cells on plate

To confirm the expression of α-amylase, plate assays were performed (Fig. 2) using *S. cerevisiae* strains: (A) YF207; (B) YF207/pGA11; (C) YF207/pAA12; (D) YF207/[pGA11, pAA12]; (E) YF207/pSAA11 and (F) YF207/[pGA11, pSAA11]. These strains were inoculated separately onto YPD (1% yeast extract, 2% peptone, 2% glucose) plates containing 0.25% remazol brilliant blue starch, which can detect only α-amylase activity and incubated for 3 days at 30 °C. A large halo was observed around the colonies of strains (E) YF207/pSAA11 and (F) YF207/[pGA11, pSAA11] and a small one around those of strains (C) YF207/pAA12 and (D) YF207/[pGA11, pAA12], indicating cell-surface and secretory expression of α-amylase by the plasmids pAA12 and pSAA11, respectively, in co-expression systems.

3.2. Flow cytometric analysis of cell-surface expression of glucoamylase

Cell-surface expression of glucoamylase was confirmed by flow cytometric analysis (Fig. 3). The peak of cell counts in strains YF207/pGA11, YF207/[pGA-11, pAA12] and YF207/[pGA11, pSAA11] was observed at much higher FITC intensity than in YF207, indicating cell-surface expression of glucoamylase in a co-expression system. The mean FITC intensity of strain YF207/[pGA11, pSAA11] was similar to that of strain YF207/pGA11. Moreover, the mean FITC intensity of strain YF207/[pGA11, pAA12] was only slightly lower than that of YF207/pGA11.

The above results of plate assay and flow cytometric analysis confirmed successful co-expression of glucoamylase and α-amylase in strains YF207/[pGA11, pAA12] and YF207/[pGA11, pSAA11].

3.3. Fed-batch ethanol production from soluble starch using S. cerevisiae strain YF207/[pGA11, pAA12]

In a fed-batch fermentation experiment (Fig. 4), the *S. cerevisiae* strain YF207/[pGA11, pAA12] grew faster in the growth phase than the glucoamylase-displaying yeast YF207/pGA11 reported previously

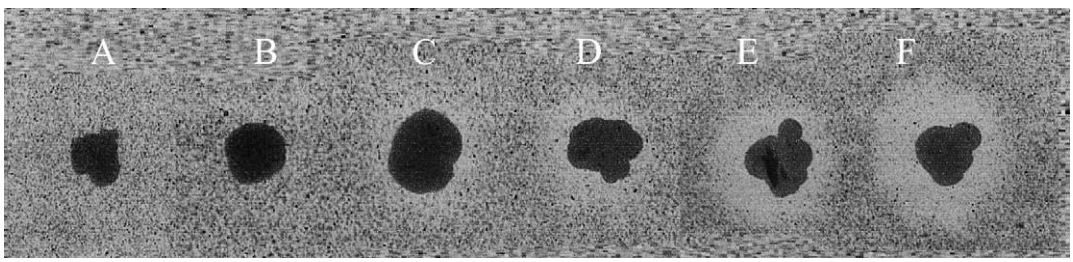

Fig. 2. Plate assays for detection of α-amylase activity of yeast cells. YPD plates containing 0.25% remazol brilliant blue starch were used: (A) YF207; (B) YF207/pGA11; (C) YF207/pAA12; (D) YF207/[pGA11, pAA12]; (E) YF207/pSAA11; (F) YF207/[pGA11, pSAA11].

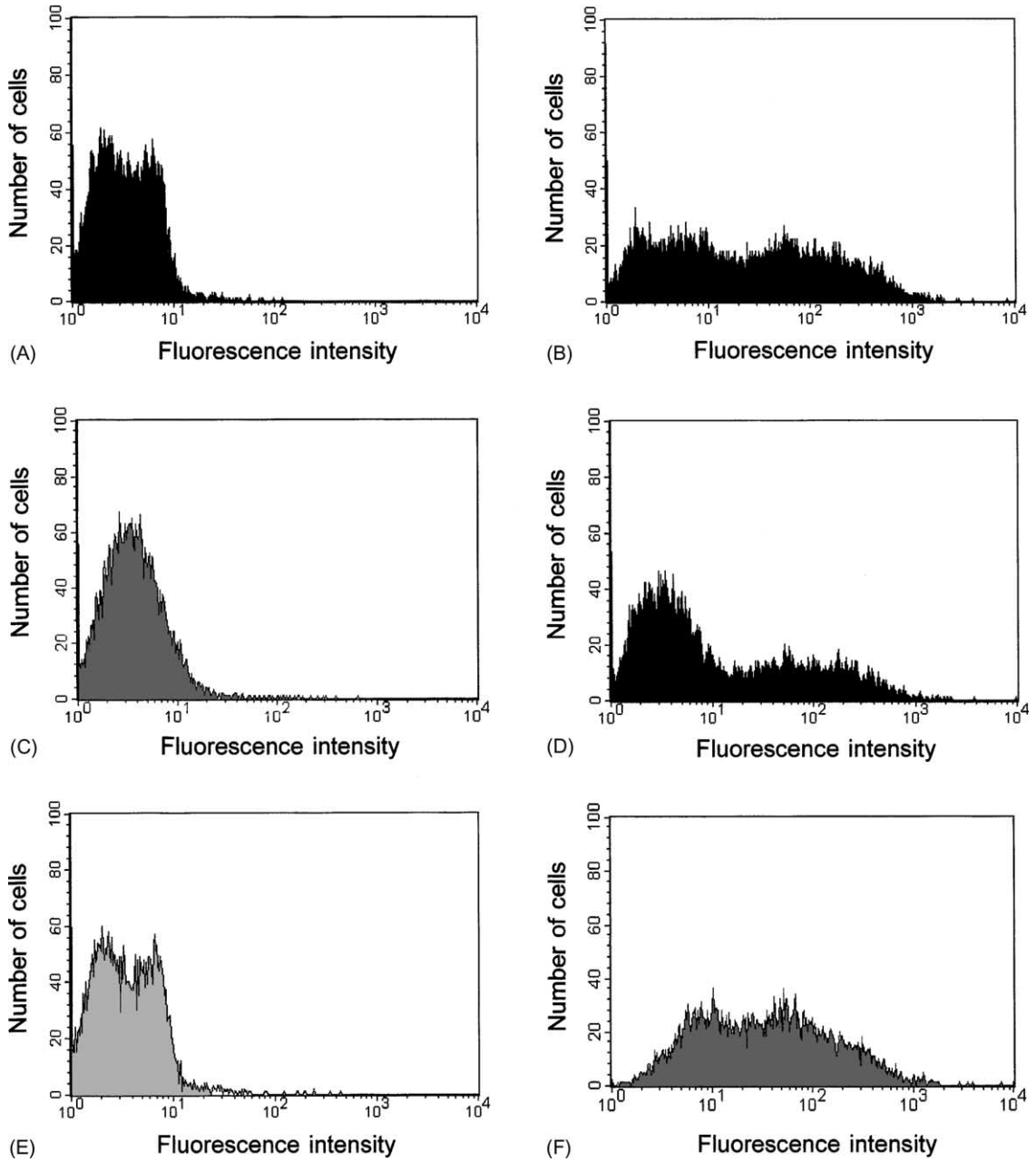

Fig. 3. Flow cytometric analysis. Histogram plots of FITC intensity of YF207, cultivated by using SD medium for 48 h are shown: (A) YF207; (B) YF207/pGA11; (C) YF207/pAA12; (D) YF207/[pGA11, pAA12]; (E) YF207/pSAA11; (F) YF207/[pGA11, pSAA11].

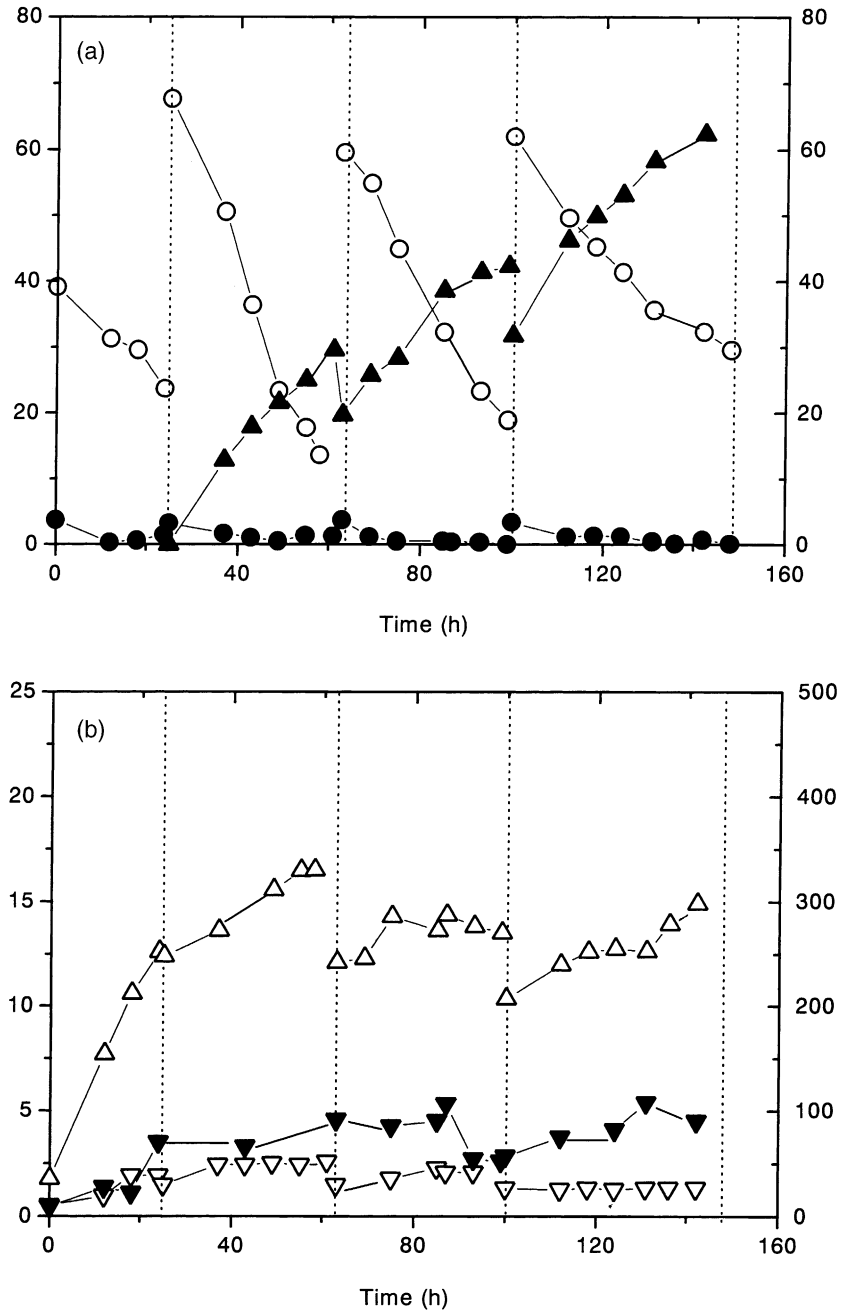

Fig. 4. Fed-batch fermentation of starch to ethanol by YF207/[pGA11, pAA12]. YF207/[pGA11, pAA12] cells were grown under aerobic conditions (2.0 ppm), harvested and used for fed-batch fermentation under anaerobic conditions. To the left of the solid line in the figure is the growth phase and to the right the ethanol fermentation phase. Dotted lines show concentrated starch feeding times. Symbols: (△) dry cell weight, (g/l); (▽) glucoamylase activity in cell pellet, (U/l); (▼) α-amylase activity in cell pellet, (U/l); (○) starch concentration, (g/l); (●) glucose concentration, (g/l); (▲) ethanol concentration, (g/l).

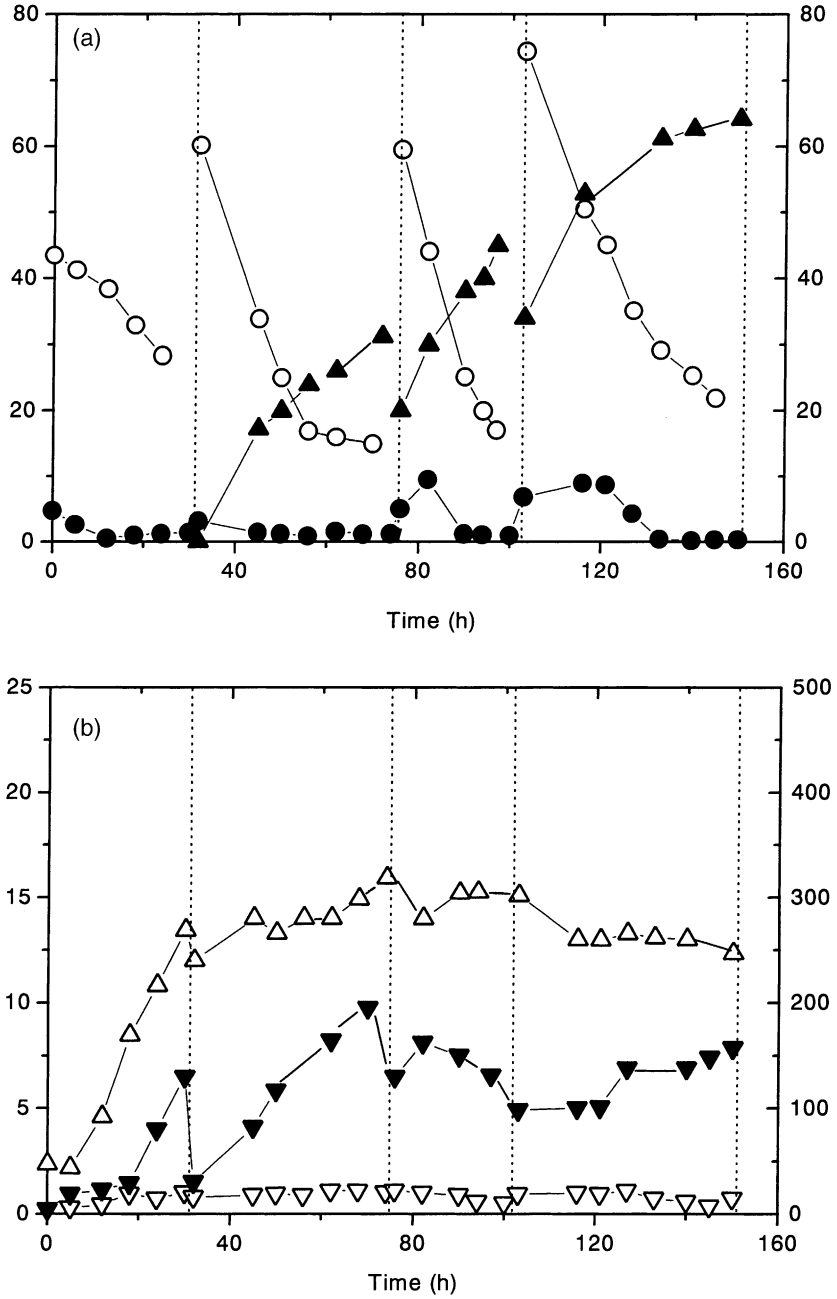

Fig. 5. Fed-batch fermentation of starch to ethanol by YF207/[pGA11, pSAA11]. YF207/[pGA11, pSAA11] cells were grown under aerobic conditions (2.0 ppm), harvested and used for fed-batch fermentation under anaerobic conditions. To the left of the solid line in the figure is the growth phase and to the right the ethanol fermentation phase. Dotted lines show concentrated starch feeding times. Symbols as for Fig. 4.

[11] and the activities of the glucoamylase and α-amylase displayed on the cell surface were maintained during the ethanol fermentation phase. The amount of starch accumulation in the fed-batch operation was smaller than with YF207/pGA11 [11] and the concentration of ethanol produced reached 60 g/l in approximately 100 h fermentation. In addition, the flocculation ability of the glucoamylase- and α-amylase-displaying yeast did not change (0.75 ∼ 0.8) during the fed-batch operation, maintaining almost the same value as in strains YF207 and YF207/pGA11.

3.4. Fed-batch ethanol production from soluble starch using S. cerevisiae strain YF207/[pGA11, pSAA11]

In a fed-batch fermentation experiment (Fig. 5), strain YF207/[pGA11, pSAA11] grew faster in the growth phase than the glucoamylase-displaying yeast YF207/pGA11 reported previously [11], the activities of the glucoamylase displayed on the cell surface were maintained and the secreted α-amylase was accumulated during the ethanol fermentation phase. This recombinant yeast, which displays glucoamylase on the cell surface and secretes α-amylase, also showed reduced amounts of starch accumulation during the fed-batch operation compared with strain YF207/pGA11. The concentration of ethanol produced reached 60 g/l after 100 h fermentation, similar to the value in strain YF207/[pGA11, pAA12], but glucose concentration in the medium was slightly higher. This is probably because strain YF207/[pGA11, pSAA11] secretes α-amylase, which decomposes starch in the culture medium. The flocculation ability of the glucoamylase-displaying and α-amylase-secreting yeast did not change (0.75 ∼ 0.8) during the fed-batch operation, maintaining almost the same value as in strains YF207 and YF207/pGA11.

4. Discussion

For the efficient decomposition of starch, sequential reactions with glucoamylase and α-amylase are effective. A previous study showed that a yeast strain co-displaying glucoamylase and α-amylase grew faster with starch as a sole carbon source than did strains displaying only glucoamylase [10]. In the present study, we investigated the ethanol productivity from starch of the two yeast strains co-expressing glucoamylase and α-amylase. For cell-surface display and secretory expression of amylolytic enzymes, multi-copy type plasmids were used, as previous studies had shown these to remain stable within yeast cells during long-term fermentation of ethanol from starch [11] and to have higher expression levels of amylolytic enzymes than integration type plasmids [9–11].

As shown in Fig. 4, the yeast strain YF207/[pGA11, pAA12], which co-displays glucoamylase and α-amylase on the cell surface, showed higher ability to degrade starch and produce ethanol than strain YF207/pGA11 [11]. α-Amylase is an endoglucanase that hydrolyzes the linkages of starch in a random fashion and produces oligosaccharides. Therefore, a cooperative and sequential reaction probably resulted from the increased concentration of molecules with non-reducing ends produced from starch by α-amylase and these in turn served as substrate molecules for glucoamylase, thereby increasing the rate of free glucose formation. This co-display system of the sequential amylolytic enzymes enabled the yeast to improve its starch decomposition and ethanol production abilities, as a result of which ethanol concentration reached 60 g/l in approximately 100 h fed-batch fermentation (Fig. 4). This ethanol concentration is higher than that produced by strain YF207/pGA11, which displays only glucoamylase (approximately 50 g/l for 120 h fermentation) [11].

The yeast strain YF207/[pGA11, pAA12] maintained both glucoamylase and α-amylase activity on the cell surface during fed-batch fermentation. The glucoamylase activity of strain YF207/[pGA11, pAA12] was almost the same as that of strain YF207/pGA11, which displays only glucoamylase on the cell surface [11]. The FACS analysis shown in Fig. 3 corroborates this finding. This probably demonstrates the additivity of the amount of cell-surface-displayed glucoamylase and α-amylase, although the space of the yeast-cell surface is limited.

The flocculation ability of yeast strain YF207/[pGA11, pAA12] did not change during fed-batch fermentation and was almost the same as for yeast strains YF207 and YF207/pGA11. This finding suggests that co-display of two enzymes on the cell surface does not influence the flocculation ability of yeast cells. These cell-surface-engineered flocculent yeast strains

are thus considered to offer advantages in industrial production of ethanol from starchy materials.

In addition, the ethanol concentration produced by YF207/[pGA11, pSAA11], which displays glucoamylase on the cell surface and secretes α-amylase, also reached 60 g/l during fed-batch fermentation (Fig. 5). Since cell-surface-displayed glucoamylase directly decomposed soluble starch to form glucose, YF207/[pGA11, pSAA11] produced ethanol without time lag. Moreover, since α-amylase was secreted and accumulated in the culture broth of YF207/[pGA11, pSAA11] during the fed-batch operation (Fig. 5), the starch was efficiently decomposed by the cooperative action of secreted and surface-displayed amylolytic enzymes.

In the present study, we examined ethanol fermentation from soluble starch by cell-surface-engineered yeast strains. Both co-display and secretion of α-amylase were concluded to be effective in improving ethanol productivity from starchy materials in glucoamylase-displaying yeast cells. Since direct production from raw starch would significantly reduce ethanol production costs, there is a demand for further improvement of cell-surface-engineered yeast strains through use of amylolytic enzymes which hydrolyze raw starch efficiently.

Acknowledgements

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