

# Production of L-Lysine from starch by *Corynebacterium glutamicum* displaying $\alpha$ -amylase on its cell surface

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**Abstract** We engineered a *Corynebacterium glutamicum* strain displaying  $\alpha$ -amylase from *Streptococcus bovis* 148 (AmyA) on its cell surface to produce amino acids directly from starch. We used PgsA from *Bacillus subtilis* as an anchor protein, and the N-terminus of  $\alpha$ -amylase was fused to the PgsA. The genes of the fusion protein were integrated into the homoserine dehydrogenase gene locus on the chromosome by homologous recombination. L-Lysine fermentation was carried out using *C. glutamicum* displaying AmyA in the growth medium containing 50 g/l soluble starch as the sole carbon source. We performed L-lysine fermentation at various temperatures (30–40°C) and pHs (6.0–7.0), as the optimal temperatures and pHs of AmyA and *C. glutamicum* differ significantly. The highest L-lysine yield was recorded at 30°C and pH 7.0. The amount of soluble starch was reduced to 18.29 g/l, and 6.04 g/l L-lysine was produced in 24 h. The L-lysine yield obtained using soluble starch as the sole carbon source was higher than that using glucose as the sole carbon source after 24 h when the same amount of substrates was added. The results shown in the current study demonstrate that *C. glutamicum* displaying  $\alpha$ -amylase has a potential to directly convert soluble starch to amino acids.

## Introduction

The bacterial cell surface display of heterologous proteins is potentially important in several areas of biotechnological application such as the development of live vaccines and multiple antigen antisera and the production of recombinant proteins (Samuelson et al. 2002). In recent years, many studies have described the fermentation of biomass resources such as starch or lignocellulosic biomass using the cell surface display system of yeasts and bacteria (Katahira et al. 2004; Shigechi et al. 2004; Narita et al. 2006).

*Corynebacterium glutamicum* belongs to the order *Actinomycetales*, which include corynebacteria, mycobacteria, nocardia, rhodococci, and other related microorganisms. The bacterium is widely used for the industrial production of various amino acids. The production of L-glutamic acid is more than 1.5 million tons, and that of L-lysine is more than 600,000 tons. It is expected that the demand for amino acid production will increase in the future (Hermann 2003; Koffas and Stephanopoulos 2005; Leuchtenberger et al. 2005). Genetic modification has been carried out to achieve the efficient production of amino acids, including that of L-lysine, over a few decades (Ohnishi et al. 2002, 2005; Ikeda et al. 2006). *C. glutamicum* has a potential to efficiently produce various organic acids and ethanol. Some organic acids are very important compounds, as some of them, such as lactic acid and succinic acid, can be utilized to synthesize biodegradable plastics (Inui et al. 2004a; Okino et al. 2005). Ethanol, which could also be produced by *C. glutamicum*, can be used as biofuel (Inui et al. 2004b). Therefore, *C. glutamicum* is one of the most attractive and significant microorganisms for producing not only these amino acids but also organic acids and ethanol. As carbon sources for the fermentation to produce L-lysine, sucrose from molasses

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and glucose from starch hydrolysates are widely used. Starchy materials, which include corn, wheat, potato, and cassava, are widely available in nature as carbon sources for cultivating *C. glutamicum* and other microorganisms. However, *C. glutamicum* cannot utilize starch directly. It is required that starch is hydrolyzed before being supplied to *C. glutamicum*. Starch degradation is mainly performed by glucoamylase (EC 3.2.1.3, 1,4-D-glucan glucohydrolase) and  $\alpha$ -amylase (EC 3.2.1.1, 1,4- $\alpha$ -D-glucan-4-glucanhydrolase) (Murai et al. 1999).

Many researchers have studied the *C. glutamicum* cell wall and the proteins associated with it, as these are unique owing to their mycolic acids (Puech et al. 2001; Bayan et al. 2003). However, no *C. glutamicum* cell surface display system has yet been developed, and we developed a cell surface display system of *C. glutamicum* in this study. As the heterologous protein, we selected the  $\alpha$ -amylase (AmyA) of *Streptococcus bovis* 148 to eliminate starch-degrading process. This  $\alpha$ -amylase efficiently hydrolyzes raw corn starch and is easily adsorbed onto raw corn starch (Narita et al. 2004). As the anchor protein, we selected PgsA from *Bacillus subtilis*, as we previously succeeded in expressing a PgsA–AmyA fusion protein in both Gram-negative *Escherichia coli* and Gram-positive lactic acid bacteria (Narita et al. 2005, 2006). *C. glutamicum* ATCC13032 was used as the host strain in this study, as its complete genome sequence has been determined (Ikeda and Nakagawa 2003; Kalinowski et al. 2003). In this study, the simultaneous saccharification and fermentation of starch to amino acids using the recombinant *C. glutamicum* are demonstrated.

## Materials and methods

### Bacterial strains and media

*E. coli* SCS110{*rpsL*(Str<sup>r</sup>) *thr leu endA thi-1 lacy galK gal Tara tonA tsx dam dcm supE44 $\Delta$  (*lac-proAB*)[F'*traD36 proAB lacI*<sup>q</sup>Z $\Delta$ M15]}(STRATAGENE) was used as the host for recombinant DNA manipulation. *Corynebacterium glutamicum* ATCC13032 was used to investigate cell surface display and fermentation.*

*E. coli* was grown in Luria–Bertani medium (10 g/l tryptone, 5 g/l yeast extract, 5 g/l sodium chloride) containing 100 mg/l ampicillin. *C. glutamicum* was grown in MMYE medium at 30°C (Ohnishi et al. 2002). When necessary, MMYE solid medium containing 25 mg/l kanamycin or 0.5 g/l L-homoserine and MM solid medium (Ozaki et al. 1985) containing 0.5 g/l L-homoserine and 10 g/l sucrose, which replaced glucose as the carbon source, were used. GMMYE-1 medium was used to grow the inoculum for a 2-l jar fermentor; it consisted of 50 g of glucose, 5 g of

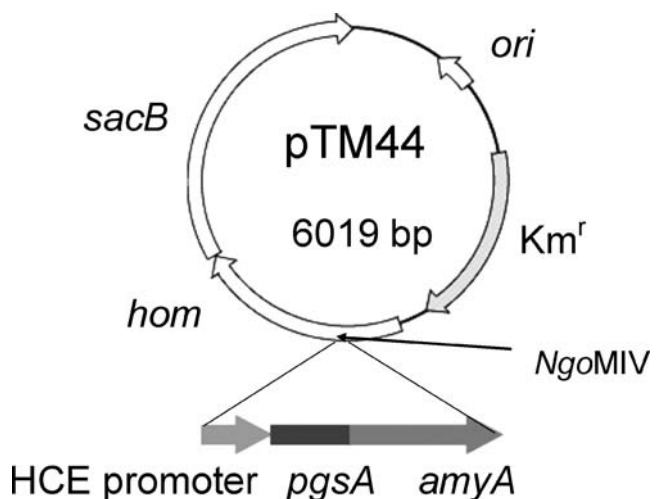
yeast extract, 25 g of NH<sub>4</sub>SO<sub>2</sub>, 1 g of urea, 2.5 g of KH<sub>2</sub>PO<sub>4</sub>, 0.5 g of L-homoserine, 14 mg of nicotinic acid, 7 mg of thiamine HCl, 0.5 mg of D-biotin, 23 mg of  $\beta$ -alanine, 0.75 g of MgSO<sub>4</sub>·7H<sub>2</sub>O, 50 mg of FeSO<sub>4</sub>·7H<sub>2</sub>O, 13 mg of MnSO<sub>4</sub>·5H<sub>2</sub>O, 50 mg of CaCl<sub>2</sub>·2H<sub>2</sub>O, 6.3 mg of CuSO<sub>4</sub>·5H<sub>2</sub>O, 13 mg of ZnSO<sub>4</sub>·7H<sub>2</sub>O, 5 mg of NiCl<sub>2</sub>·6H<sub>2</sub>O, 1.3 mg of CoCl<sub>2</sub>·6H<sub>2</sub>O, and 1.3 mg of (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>·4H<sub>2</sub>O (per liter).

### Construction of plasmids for cell surface display

All polymerase chain reactions (PCR) were carried out using KOD-Plus-DNA polymerase (Toyobo, Osaka, Japan). The integration plasmid, pTM44-HCE-pgsA-amyA, for cell surface display was constructed using the basic plasmid pTM44 (Fig. 1) containing the homoserine dehydrogenase gene (*hom*) and the *Bacillus subtilis* levansucrase gene (*sacB*). The HCE-pgsA-amyA gene was synthesized by a PCR technique using the plasmid pHLA  $\alpha$ AF (Narita et al. 2005) as the template with the oligonucleotides NgoMIV-HCE\_F (GGAGCCGGCGATCTCTCCTTCACAGATTCCCAATCTCTT) and amyA\_R (GGAGCCGGCTATTTTAGCCCATCTTTATTATAGTTTCCA), and the amplified fragment was digested with NgoMIV and introduced into the NgoMIV site of *hom* on pTM44. The resulting plasmid was designated pTM44-HCEpgsAmyA.

The transformation of *C. glutamicum* ATCC13032 was carried out by electroporation. The cells were transformed by electroporation with a 2.5 kV, 200  $\Omega$ , 25  $\mu$ F electric pulse in a 0.2-cm cuvette using a Gene Pulser (Bio-Rad, Richmond, CA).

Two recombination events were performed using kanamycin resistance and *sacB* selection (Ohnishi et al. 2002). The integration of *hom* was confirmed using a PCR



**Fig. 1** Expression plasmid for display of  $\alpha$ -amylase on *C. glutamicum* ATCC13032 cell surface. *sacB* Levansucrase, *hom* homoserine dehydrogenase

technique and the auxotrophy of L-homoserine. The recombinant strain was designated  $\Delta hom::HPA$ .

#### Preparation of cell surface fractions of *C. glutamicum*

The cell surface fraction was extracted as described by Hansmeier et al. (2004) with minor modifications. *C. glutamicum* cells grown for 15 h were collected by centrifugation at  $5,000\times g$  and  $4^{\circ}\text{C}$  for 5 min and washed with 50 mM Tris–HCl buffer (pH 6.8) three times. The cell pellet was resuspended in 50 mM Tris–HCl buffer (pH 6.8) containing 2% (w/v) SDS to an optical density of 10 at 600 nm ( $OD_{600}$ ). Subsequently, the cell suspension was heated at  $100^{\circ}\text{C}$  for 5 min and centrifuged at  $5,000\times g$  and  $4^{\circ}\text{C}$  for 5 min. To the supernatant, which contained only cell-wall-associated proteins (Kacem et al. 2004),  $2\times$  sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer was added. Before loading the samples to the gel, all samples were heated at  $100^{\circ}\text{C}$  for 5 min.

#### Western blot analysis of the proteins

Proteins were analyzed by SDS-PAGE using an SDS-polyacrylamide gel (8%; w/v). As a molecular weight marker, dual-color, prestained Precision Plus protein standards (Bio-Rad Laboratories, Richmond, CA) was used. The proteins were electroblotted onto a polyvinylidene difluoride membrane (Millipore, Boston, MA) and allowed to react with the primary rabbit anti-AmyA and the secondary goat anti-rabbit IgG alkaline phosphatase-conjugated antibody (Promega, Madison, WI). The membrane was then stained with nitroblue tetrazolium (NBT; Promega) and 5-bromo-4-chloro-3-indolylphosphate (BCIP; Promega) according to the manufacturer's protocol. The staining solution was prepared by sequentially adding 180  $\mu\text{l}$  of NBT and 50  $\mu\text{l}$  of BCIP to 30 ml of alkaline phosphate buffer [100 mM Tris–HCl (pH 9.0) containing 150 mM NaCl and 1 mM  $\text{MgCl}_2$ ].

#### Immunofluorescence microscopy

Immunostaining was performed as follows: *C. glutamicum* cells grown for 15 h were centrifuged at  $3,500\times g$  for 5 min and washed with phosphate-buffered saline (PBS; pH 7.2). The antibodies were preincubated with wild-type cells as a control to prevent nonspecific binding. After resuspending the cells in PBS containing 10 g/l bovine serum albumin to an  $OD_{600}$  value of 1.0, the cells were incubated with the pretreated antibody at room temperature for 1.5 h. As the primary antibody, 1  $\mu\text{l}$  of rabbit immunoglobulin G (IgG) against AmyA was diluted in 300  $\mu\text{l}$  PBS. After washing the cells with PBS, the cells were resuspended in the

solution containing the secondary antibody, goat anti-rabbit IgG (H + L) antibody conjugated with Alexa Fluor 488 (1  $\mu\text{l}$  of antibody solution in 300  $\mu\text{l}$  PBS) and allowed to react with the cells at room temperature for 1.5 h. The cells were washed with PBS before their observation using a microscope. Immunofluorescence microscopy was performed using a BZ-8000 inverted fluorescence and phase contrast microscope (Keyence, Osaka, Japan) equipped with a 12-V, 100-W halogen lamp for transmitted light illumination, a 120-W mercury arc lamp for fluorescence illumination, a green fluorescent protein filter set (excitation wavelength range, 480 to 530 nm; absorption wavelength, 510 nm), a Nikon PlanApo  $20\times$  objective lens, and a cooled CCD camera. The images obtained were analyzed with the BZ analyzer program (version 2.0; Keyence).

#### Flow-cytometric analysis of the immunostained cells

A flow-cytometric analysis of the cells was performed using a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA) equipped with a 15-mW, 488-nm, air-cooled argon ion laser and a cell-sorting catcher tube. The performance of the instrument was monitored using CaliBRITE Beads (Becton Dickinson Immunocytometry Systems). The cells grown in the GMMYE-1 medium at  $30^{\circ}\text{C}$  for 15 h were collected by centrifugation at  $3,500\times g$  for 5 min at  $4^{\circ}\text{C}$  and washed with PBS (pH 7.2). Then, the cells were immunostained as described previously. Immunostained cell samples were diluted to approximately  $10^6$  cells per milliliter and analyzed at a low flow rate corresponding to 150 to 300 cells per second. A band-pass filter of 530 nm (515 to 545 nm) was used to collect the cells emitting green fluorescence (FL1). Data were analyzed with the CELL-Quest program (version 3.3; Becton Dickinson Immunocytometry Systems), with 10,000 counts analyzed in each experiment. Counts were made in triplicate for each experiment.

#### $\alpha$ -Amylase activity measurement

$\alpha$ -Amylase activity was measured with an  $\alpha$ -amylase measurement kit (Kikkoman, Tokyo, Japan) using 2-chloro-4-nitrophenyl 6<sup>5</sup>-azido-6<sup>5</sup>-deoxy- $\beta$ -maltopentaoside (N3-G5- $\beta$ -CNP) as the substrate. The assay mixture contained 400  $\mu\text{l}$  of reaction solution and 40  $\mu\text{l}$  of sample solution. To prepare a sample solution, *C. glutamicum* cells collected from the culture were washed and resuspended in PBS (pH 7.2) by vigorous mixing. The suspension was diluted in PBS before the  $\alpha$ -amylase activity assay. The assay mixture was incubated at  $37^{\circ}\text{C}$  for 10 min, and the enzymatic reaction was terminated by adding 800  $\mu\text{l}$  of a reaction stop solution.  $\alpha$ -Amylase activity was determined

according to the manufacturer's instruction by measuring the absorbance of the liberated 2-chloro-4-nitrophenol (CNP) at 400 nm. One unit (U) of activity was defined as the amount of enzyme required to release 1  $\mu\text{mol}$  of CNP from N3-G5- $\beta$ -CNP per minute at 37°C.

#### Fermentation experiment

L-Lysine fermentation by recombinant *C. glutamicum* was performed in a 2.0-l jar fermentor containing a 1.0-l working volume. The fermentor containing the GMMYE-1 medium (without glucose) and soluble starch solution were heat-sterilized (121°C, 15 min) separately. After heat sterilization, the soluble starch solution was added to the fermentor to a final soluble starch concentration of 50 g/l. This fermentation medium containing soluble starch instead of glucose was labeled SMMYE-1. *C. glutamicum* cells grown on the MMYE-homoserine solid medium at 30°C for 1 day were used to inoculate 5 ml of GMMYE liquid medium in a test tube. After incubation at 30°C for 24 h, the culture was transferred into 40 ml of GMMYE-1 medium in a 100-ml flask. After incubation in a shaking incubator at 30°C for 24 h, the culture (1%; v/v) was used to inoculate a fermentor medium, and fermentation was performed at an agitation speed of 650 rpm and an aeration rate of 2.0 l/min at 30°C. To maintain the medium pH at 7.0, 5 N  $\text{NH}_3$  was automatically added to the fermentation culture.

Cell growth was monitored by measuring the absorbance of the culture at 600 nm. A colorimetric method based on the phenol-sulfuric acid reaction (Dubois et al. 1956) was used to determine the amount of total sugars corresponding to the starch and starch hydrolysis products. Starch degradation was analyzed by thin-layer chromatography (TLC) according to Seibold et al. (2006).

L-Lysine concentration was determined by a reverse-phase high performance liquid chromatography (HPLC; GL Science, Osaka, Japan) fitted with an MP710 micro-flow pump, a column [Inertsil ODS-3 (5  $\mu\text{l}$ , 1.5  $\times$  150 mm)], and an UV detector (MU701). The samples for HPLC were prepared as follows: Each sample was centrifuged at 10,000  $\times g$  at 4°C for 5 min. The supernatant was gently mixed with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) and centrifuged at 15,000 rpm for 5 min. The supernatant was added into a 1.5-ml centrifuge tube containing an equal volume of chloroform, mixed gently, and centrifuged at 15,000 rpm for 5 min. The supernatant (25  $\mu\text{l}$ ) was mixed with 25  $\mu\text{l}$  of 1,4-butandiamine (2.0 g/l), 400  $\mu\text{l}$  of  $\text{NaHCO}_3$  (0.075 M), and 150  $\mu\text{l}$  of 1-fluoro-2,4-dinitrobenzene (0.2 M), and incubated at 37°C for 1 h. The reaction mixtures were centrifuged at 10,000  $\times g$  for 10 min, and 50  $\mu\text{l}$  of the sample supernatant was diluted with 950  $\mu\text{l}$  of acetonitrile. After filtering 1 ml of the resulting

solution using a Millex-LH filter unit (0.45  $\mu\text{m}$  pore size, Millipore, Boston, MA), 2  $\mu\text{l}$  of the sample solution was injected to the HPLC system. The mobile phase used was 0.1% (w/w) formate and acetonitrile [4:6 (v/v)]. The flow rate and column temperature during the HPLC analysis were 150  $\mu\text{l}/\text{min}$  and 40°C, respectively. Quantification of the L-lysine was performed by measuring absorbance at 360 nm on an UV detector.

To examine the effect of temperature, the fermentation experiment was performed at various temperatures (30, 34, 37, and 40°C) at pH 7.0; to examine that of pH, the experiment was carried out at various pHs (6.0, 6.5, and 7.0) at 30°C.

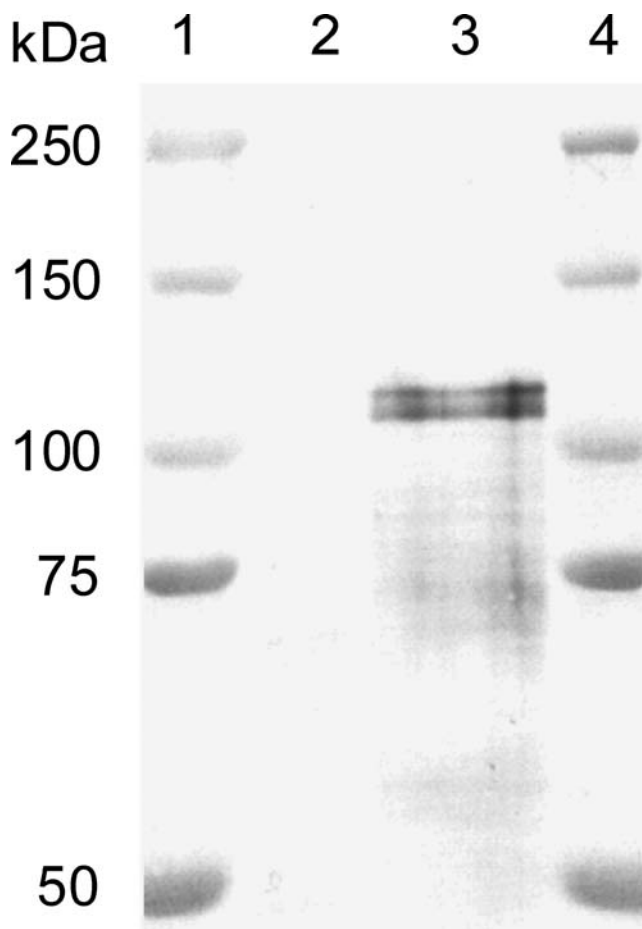
## Results

### Expression of PgsA-AmyA fusion protein on cell surface

To directly ferment starch to L-lysine, we engineered *C. glutamicum* ATCC13032 cells displaying  $\alpha$ -amylase on the cell surface. The expressed fusion protein was analyzed by Western blot analysis of the cell wall fraction of  $\Delta hom::$ HPA using the rabbit anti-AmyA antibody. The result is shown in Fig. 2. The molecular sizes of the PgsA and AmyA proteins were approximately 43 and 78 kDa, respectively, and that of the PgsA-AmyA fusion protein, therefore, was predicted to be approximately 121 kDa. A clear band was observed at the estimated molecular size, indicating the successful expression of the fusion protein in the cell wall fraction.

### Immunofluorescence microscopy and flow-cytometric analysis

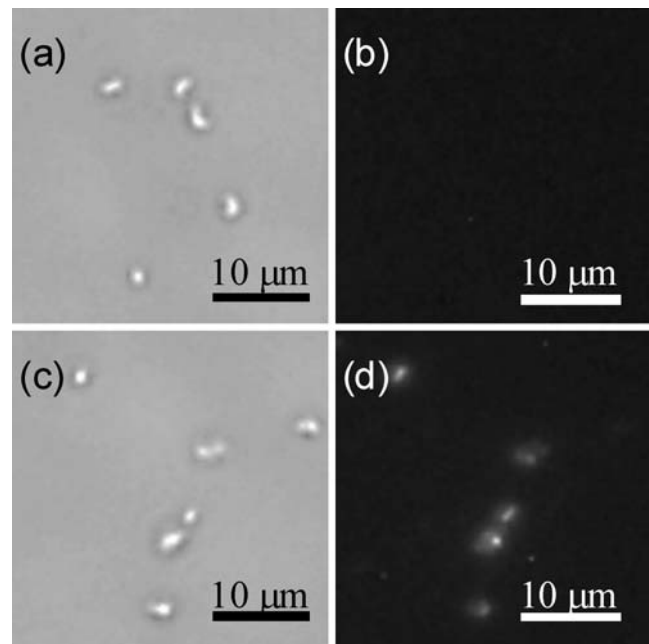
To confirm the display of AmyA on the cell surface, the immunofluorescence labeling of AmyA was carried out using the rabbit anti-AmyA antibody as the primary antibody and Alexa Fluor 488-conjugated goat anti-rabbit IgG as the secondary antibody. As shown in Fig. 3, the green fluorescence of the immunostained PgsA-AmyA fusion protein was observed in the  $\Delta hom::$ HPA cells, but not in the wild-type cells, indicating that AmyA was displayed on the cell surface of  $\Delta hom::$ HPA. Flow cytometry (FCM) was also performed to confirm the display of AmyA on the cell surface (Fig. 4). AmyA was stained with the primary and secondary antibodies, and the wild-type cells were used as the control for FCM. The  $\Delta hom::$ HPA cells showed significantly higher fluorescence signal intensity than the control cells. This result agreed with that obtained from the immunofluorescence labeling experiment shown in Fig. 3, and both results indicate the successful cell surface display of AmyA.



**Fig. 2** Result of the Western blot analysis of PgsA–AmyA fusion protein. *Lanes 1 and 4* Marker proteins, dual-color prestained Precision Plus Protein standards (Bio-Rad Laboratories, Richmond, CA), with sizes indicated; *lanes 2 and 3* cell wall fractions of wild-type *C. glutamicum* ATCC13032 and  $\Delta hom::HPA$ , respectively. Cells were collected by centrifuging the fermentation culture incubated at 30°C and pH 7.0 for 15 h. The samples were separated by SDS-PAGE using an 8% (w/v) gel and stained with the primary rabbit anti-AmyA antibody followed by staining with the secondary goat anti-rabbit IgG conjugated with alkaline phosphatase

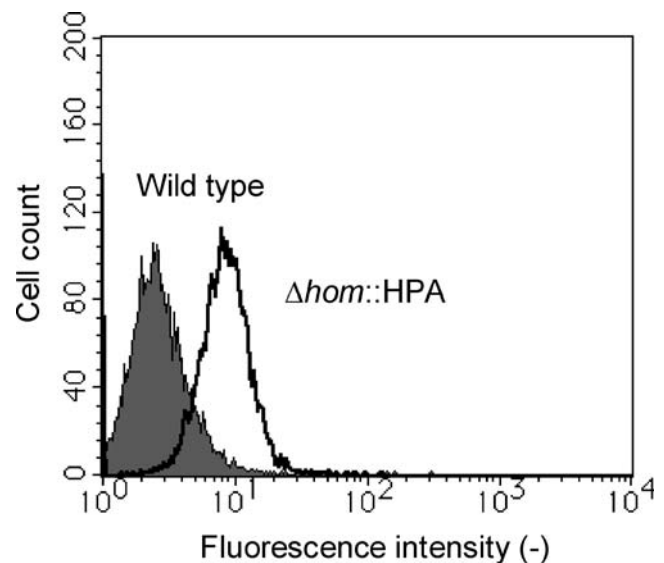
#### L-Lysine fermentation using AmyA-displaying *C. glutamicum*

The *C. glutamicum* strain displaying AmyA,  $\Delta hom::HPA$ , was used for the direct simultaneous saccharification and fermentation to produce L-lysine from soluble starch. AmyA displaying *C. glutamicum* cells were grown in the SMMYE-1 medium containing 50 g/l soluble starch as the sole carbon source. To determine the effects of temperature and pH on the L-lysine fermentation, L-lysine fermentation by recombinant *C. glutamicum* was performed at various temperatures and pHs (Table 1). The pH of the fermentation medium was maintained at 7.0 by automatically adding 5.0 N  $NH_3$ , which also acted as the nitrogen source. The growth rate was significantly lower at 40°C and at pH 6.0



**Fig. 3** Images obtained from immunofluorescence labeling of wild-type *C. glutamicum* and  $\Delta hom::HPA$ . Nomarski differential interference micrographs of wild-type (a) and  $\Delta hom::HPA$  (c). Immunofluorescence micrographs of wild-type (b) and  $\Delta hom::HPA$  (d). The wild-type and  $\Delta hom::HPA$  were collected by centrifuging the fermentation culture incubated at 30°C and pH 7.0 for 15 h. Cells were labeled with the rabbit anti-AmyA antibody before staining with the goat anti-rabbit IgG conjugated with Alexa Fluor 488

after 24 h. No notable difference in L-lysine productivity was recorded at the temperatures tested except at 40°C, at which the yield was significantly low compared to those at



**Fig. 4** Results of flow cytometric analysis of wild-type *C. glutamicum* and  $\Delta hom::HPA$ . The wild-type and  $\Delta hom::HPA$  were collected by centrifuging the fermentation culture incubated at 30°C and pH 7.0 for 15 h. Cells were labeled with the rabbit anti-AmyA antibody before staining with goat anti-rabbit IgG conjugated with Alexa Fluor 488. For each experiment, 10,000 cells were analyzed

**Table 1** Summary of cell growth and L-lysine production by  $\Delta hom::HPA$  at various temperatures and pHs after 24 h of cultivation to show the effects of temperature and pH on L-lysine fermentation

	Glucose 30°C–pH7.0	Effect of temperature <sup>a</sup>				Effect of pH <sup>b</sup>		
		30	34	37	40	6.0	6.5	7.0
OD <sub>600</sub>	48.40	40.73	42.30	44.50	11.90	8.90	34.20	40.73
Consumed sugar (g)	42.45	31.97	35.77	34.16	7.97	5.81	23.75	31.97
L-Lysine (g)	4.29	6.04	5.38	5.11	0.19	0.41	4.09	6.04
Yield (%) <sup>c</sup>	10.11	18.89	15.04	14.96	2.38	7.06	17.22	18.89

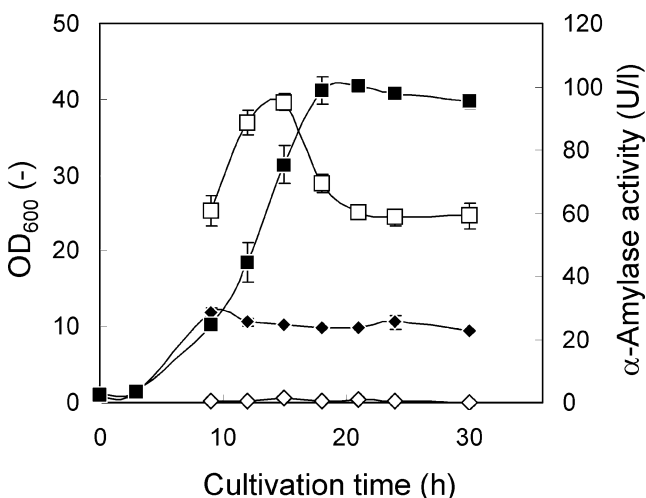
<sup>a</sup> Culture pH was set at 7.0.

<sup>b</sup> The culture was incubated at 30°C.

<sup>c</sup> Yield is expressed as gram of L-Lysine produced per gram of total sugar consumed.

the other temperatures. As culture pH was decreased, the growth rate and the maximum OD<sub>600</sub> value decreased. The maximum OD<sub>600</sub> value at pH 6.0 was only 8.9 after 24 h, which is one fifth that at pH 7.0. The highest L-lysine production was recorded at 30°C at pH 7.0. Although the amount of remaining sugar in the culture after 24 h was higher in the culture containing soluble starch, the amount of L-lysine produced and the L-lysine yield were higher using soluble starch as the sole carbon source than using glucose as the sole carbon source.

Figure 5 shows the  $\alpha$ -amylase activity and cell growth of the AmyA-displaying and wild-type *C. glutamicum* cells during the batch fermentation at 30°C at pH 7.0.  $\alpha$ -Amylase activity (U/l) indicates the activity of AmyA displayed on the cell surface per liter of culture. The recombinant strain  $\Delta hom::HPA$  showed a high  $\alpha$ -amylase activity (95.2 U/l) at 15 h, whereas no activity was detected in the wild-type cells.  $\alpha$ -Amylase activity was detected in



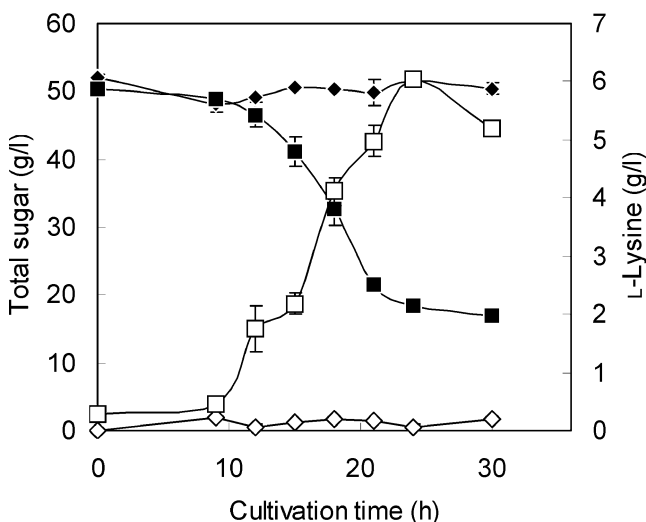
**Fig. 5** Results of L-lysine fermentation experiments with soluble starch as the sole carbon source using wild-type *C. glutamicum* (diamond) and  $\Delta hom::HPA$  (square) at 30°C at pH 7.0. Changes in OD<sub>600</sub> values (closed symbols) and  $\alpha$ -amylase activity (open symbols) are shown in the figure. The data points represent the mean value and standard deviation of three independent experiments

the supernatant during prolonged fermentation. The active AmyA-displaying cells had a higher density than the wild-type cells.

Figure 6 shows the changes in L-lysine concentration and starch concentration by the  $\Delta hom::HPA$  and wild-type cells in batch fermentation more than 30 h at 30°C at pH 7.0. L-Lysine production was almost completed within 24 h of fermentation and, L-lysine concentration reached approximately 6.04 g/l. TLC analysis showed that 18.29 g/l unconsumed sugar did not include glucose, maltose maltotriose, or maltoheptaose, but included high-molecular-weight sugar (data not shown).

## Discussion

In this study, we successfully developed *C. glutamicum* displaying  $\alpha$ -amylase on its cell surface, and this recombi-



**Fig. 6** Results of L-lysine fermentation experiments with soluble starch as the sole carbon source using wild-type *C. glutamicum* (diamond) and  $\Delta hom::HPA$  (square) at 30°C at pH 7.0. Changes in total sugar (closed symbols) and L-lysine (open symbols) concentrations are shown in the figure. The data points represent the mean value and standard deviation of three independent experiments

nant *C. glutamicum* was shown to grow using soluble starch as the sole carbon source and to produce L-lysine directly from starch.

The cell surface of *C. glutamicum* has been extensively studied, as *C. glutamicum* has a unique cell wall, being composed of peptidoglycan, arabinogalactan, and a monolayer of mycoloyl residues. Therefore, the cell wall of *C. glutamicum* is similar to that of Gram-negative microorganisms (Costa-Riu et al. 2003). In this study, we have developed a cell surface display system in *C. glutamicum* using a PgsA anchor, a transmembrane anchor. The PgsA–AmyA fusion protein was demonstrated to be successfully displayed on the cell surface by immunofluorescence labeling (Figs. 3 and 4), showing the possibility that PgsA is able to be utilized as an anchor protein in various bacteria, for example, *E. coli*, lactic acid bacteria, and *C. glutamicum* (Narita et al. 2005, 2006). The AmyA activity of the cells was 2.21 U/g wet cell after 15 h in the fermentation experiment at 30°C at pH 7.0. AmyA activity in the supernatant during prolonged fermentation was detected. However, released AmyA into the supernatant is assumed not to be the disadvantage, as it could also degrade soluble starch.

Although AmyA was successfully displayed on *C. glutamicum* cells, the differences in optimum temperature and pH between the activity of enzyme [50°C and pH 5.5 (Sato et al. 1993)] and the growth of *C. glutamicum* [30°C and pH 7.0 (Ohnishi et al. 2003)] were assumed to influence fermentation performance. By performing the fermentation experiments at various temperatures and pHs (Table 1), it was confirmed that the optimum temperature and pH of *C. glutamicum* (30°C and pH 7.0) were also optimum for the fermentation of starch using *C. glutamicum* displaying AmyA. The yield and production of L-lysine using soluble starch as the sole carbon source were 18.89% and 6.04 g/l at 30°C and pH 7.0, respectively. These L-lysine production and yield were higher than those in the glucose-supplied culture at 24 h. When glucose is used as the sole carbon source, glucose is consumed for growth rather than for L-lysine production during the exponential growth phase. Furthermore, glucose is supplied to the recombinant *C. glutamicum* more slowly using soluble starch than using glucose as the sole carbon source, as soluble starch must be degraded by AmyA for its uptake. The decrease in the rate of glucose uptake might affect metabolic flux. These might be the reasons for the more efficient production of L-lysine using soluble starch than using glucose as the sole carbon source.

The recombinant *C. glutamicum* was shown to be able to produce L-lysine between 30 and 37°C. Its ability to ferment at high temperatures results in a reduction in cooling cost, as a large amount of cooling water is necessary to maintain temperature (Ohnishi et al. 2003).

As shown by the comparative fermentation experiments (30°C, pH 7.0) using the wild-type and recombinant *C. glutamicum* strains (Figs. 5 and 6), the OD<sub>600</sub> value of  $\Delta hom::HPA$  was shown to be 4.2 times higher than that of the wild-type utilizing soluble starch as the carbon source. The wild-type was presumed to have grown slightly using glucose produced by the thermal decomposition of soluble starch. As L-lysine concentration increased as total sugar concentration decreased, L-lysine was clearly produced by the simultaneous saccharification and fermentation of soluble starch by  $\Delta hom::HPA$ . Although the fermentation conditions for this system were optimized in terms of pH and temperature, the conditions are yet to be optimized in terms of the AmyA activity. This might be the reason for the remaining unconsumed sugar in the culture after 30 h of incubation. Another possible reason for the remaining unconsumed sugar could be explained by the inability of  $\alpha$ -amylase to hydrolyze  $\alpha$ -1,6-glycosidic bonds in starch. From the TLC analysis result, AmyA was able to degrade soluble starch, but not high-molecular-weight sugar. When glucoamylase (Oriental Yeast) was added to the culture supernatant after 24 h of incubation, around 90% of the unconsumed sugar (33.9% of the total sugar added to the culture) was converted to glucose. This result indicates that the yield could be further increased by the coexpression of glucoamylase with AmyA.

The results of this study indicate that an efficient direct conversion of starch to L-lysine can be achieved by displaying AmyA on *C. glutamicum*. AmyA can also be reused easily by recovering the cells displaying AmyA (Narita et al. 2006). This is the first report on a cell surface display system of *C. glutamicum* and on bioconversion using *C. glutamicum* expressing a heterologous protein on its cell surface. Using such a cell surface display system, the enzyme used could easily be recycled; hence, this novel system using *C. glutamicum* can be applied in a wide variety of processes.

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