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Direct production of cadaverine from soluble starch using *Corynebacterium glutamicum* coexpressing α-amylase and lysine decarboxylase

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Abstract Here, we demonstrated the one-step production of cadaverine from starch using a *Corynebacterium glutamicum* strain coexpressing *Streptococcus bovis* 148 α-amylase (AmyA) and *Escherichia coli* K-12 lysine decarboxylase (CadA). We constructed the *E. coli–C. glutamicum* shuttle vector, which produces CadA under the control of the high constitutive expression (HCE) promoter, and transformed this vector into *C. glutamicum* CSS secreting AmyA. The engineered *C. glutamicum* expressed both CadA and AmyA, which retained their activity. We performed cadaverine fermentation using 50 g/l soluble starch as the sole carbon source without pyridoxal-5'-phosphate, which is the coenzyme for CadA. *C. glutamicum* coexpressing AmyA and CadA successfully produced cadaverine from soluble starch and the yield of cadaverine was 23.4 mM after 21 h. CadA expression levels under the control of the HCE promoter were assumed to be sufficient to convert L-lysine to cadaverine, as there was no accumulation of L-lysine in the culture medium during fermentation. Thus, we demonstrated that *C. glutamicum* has great potential to produce cadaverine from biomass resources.

Keywords *Corynebacterium glutamicum* · Cadaverine · Lysine decarboxylase · Alpha-amylase · Soluble starch

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Introduction

In order to develop a biobased economy for sustainable economic growth, it is necessary to produce chemicals and fuels from renewable resources, such as biomass (Chotani et al. 2000; Kamm and Kamm 2004; Ohara 2003; Ragauskas et al. 2006). Although production of bio-ethanol from biomass has been widely studied, the production of chemicals such as amino acids and organic acids from biomass would also be of significant importance.

Corynebacterium glutamicum, a Gram-positive bacterium belonging to the order Actinomycetales, is one of the most useful microorganisms for producing chemicals and fuels, as *C. glutamicum* is generally regarded as safe and it is used to produce amino acid industrially (Hermann 2003; Leuchtenberger et al. 2005). Some researchers have reported production of the biodegradable polyester poly-3-hydroxybutyrate (Jo et al. 2006), organic acids such as lactic acid and succinic acid (Inui et al. 2004a; Okino et al. 2005), and fuels such as ethanol (Inui et al. 2004b) using *C. glutamicum*. One of the problems with *C. glutamicum* mediated production, however, is that a saccharification process is needed, as *C. glutamicum* cannot utilize substrates such as starch directly. In our previous study, we constructed *C. glutamicum* CSS, which secretes *Streptococcus bovis* 148 α-amylase (AmyA), in order to

successfully produce L-lysine directly from starch (Tateno et al. 2007b).

In this study, we focused on cadaverine and demonstrated its direct production from starch using engineered *C. glutamicum*. Cadaverine is an attractive chemical, as it can be used as a replacement for the oil-derived hexamethylenediamine as a raw material for polyamide 66 (nylon 66). Cadaverine can be obtained from L-lysine using lysine decarboxylase (Sabo et al. 1974). Mimitsuka et al. (2007) reported the production of cadaverine from glucose as a carbon source using recombinant *C. glutamicum* expressing *Escherichia coli* lysine decarboxylase (CadA), which is known to maintain external pH in order to protect the cells from the acidic conditions (Meng and Bennett 1992; Neely and Olson 1996; Yamamoto et al. 1997; Lemonnier and Lane 1998; Samartzidou and Delcour 1999).

In order to produce cadaverine from starch directly, we introduced *E. coli* K-12 lysine decarboxylase (CadA) into *C. glutamicum* CSS secreting AmyA, thus enabling the production of cadaverine from biomass resources. We investigated the effect of a carbon source on cadaverine production. As a result, effective production of cadaverine directly from starch was achieved using *C. glutamicum* secreting AmyA and expressing CadA.

Materials and methods

Bacterial strains and media

E. coli SCS110 (Invitrogen, Carlsbad, CA, USA) was used for genetic manipulation. *E. coli* NovaBlue (Novagen, Madison, WI, USA) was used to isolate lysine decarboxylase. *E. coli* was grown in Luria–Bertani (LB) medium (10 g/l tryptone, 5 g/l yeast extract, 5 g/l sodium chloride). *C. glutamicum* CSS secreting AmyA (Tateno et al. 2007b) was grown in BY medium (10 g/l peptone, 10 g/l meat extract, 5 g/l yeast extract, 5 g/l sodium chloride) at 30°C (Katsumata et al. 1984). When necessary, LB medium and BY medium containing 50 and 25 mg/l kanamycin, respectively, or BY solid medium containing 10 g/l sucrose was used.

Construction of L-lysine-producing *C. glutamicum*

As a control, L-lysine-producing *C. glutamicum*, which does not produce α-amylase, was constructed. The integration plasmids for the chloramphenicol acetyltransferase gene (*cat*), pTM44-cm, were constructed using the basic plasmid pTM44 (Tateno et al. 2007a) containing the homoserine dehydrogenase gene (*hom*) and the *Bacillus subtilis* levansucrase gene (*sacB*). The *cat* gene was amplified by polymerase chain reaction (PCR) from the plasmid pHLA (Narita et al. 2006) using NgoMIV-cm_F primer (5'-GGAGCCGGCAAATTTAGGAGGCATATCAAATGAACT-3') and cm-NgoMIV_R primer (5'-GGAGCCGGCCAGTCATTAGGCCTATCTGACAATTC-3'). The amplified fragment was digested with *Ngo*MIV and was introduced into the *Ngo*MIV site of *hom* on pTM44. The resulting plasmid was designated pTM44-cm (Fig. 1a).

Transformation of *C. glutamicum* ATCC 13032 was performed by electroporation; two recombination events were performed using kanamycin resistance and *sacB* selection (Tateno et al. 2007a). Integration of *hom* was confirmed using PCR and the auxotrophy of L-homoserine. The recombinant strain integrated using pTM44-cm was designated Cm strain.

Construction and transformation of plasmid for expression of CadA

The gene encoding lysine decarboxylase (*cadA*) was amplified by PCR using *Bam*HI-cadA_F primer (5'-CGCGGATCCATGAACGTTATTGCAATATTGAATCACATGG-3') and cadA-FLAG-*Hin*dIII_R primer (5'-CCCAAGCTTTTACTTGTCATCGTCATCCTTGTAGTCTTTTTTGCTTTCTTCTTTCAATACCTTAACGG-3') with *E. coli* NovaBlue genomic DNA as the template. Amplified *cadA*-FLAG gene was introduced into the *Bam*HI and *Hin*dIII sites of the plasmid pHSG298 (Takara Bio Inc., Otsu, Japan) for cloning. The resulting plasmid was designated pHSG298-cadAF. The *cadA*-FLAG fusion gene was amplified by PCR using *Sac*I-cadA_F primer (5'-CCCGATATCGAGCTCATGAACGTTATTGCAATATTGAATCA-3') and FLAG-*Nhe*I-*Xho*I_R primer (5'-CCCCTCGAGGCTAGCTCACTTGTCATCGTCATCCTTG-3') with pHSG298-cadAF as the template. Amplified *cadA*-FLAG gene was introduced into the *Sac*I and *Xho*I sites of the plasmid pCH (Tateno et al. 2007b). The resulting plasmid was designated pCH-cadAF (Fig. 1b).

Plasmid pCH-cadAF was introduced into *C. glutamicum* Cm and CSS by electroporation, as described previously (Tateno et al. 2007a). *C. glutamicum* Cm and CSS harboring pCH-cadAF were designated Cm-cadAF and CSS-cadAF, respectively. As a control, plasmid pCC, which contains only *cspB* promoter (Tateno et al. 2007a), was introduced into *C. glutamicum* Cm and CSS by electroporation. *C. glutamicum* Cm and CSS harboring pCC were designated Cm-pCC and CSS-pCC, respectively. The strains used are summarized in Table 1.

Expression of CadA and Western blotting

C. glutamicum cells were cultured at 30°C for 24 h in 5 ml of BY medium. After cultivation, cells were centrifuged at

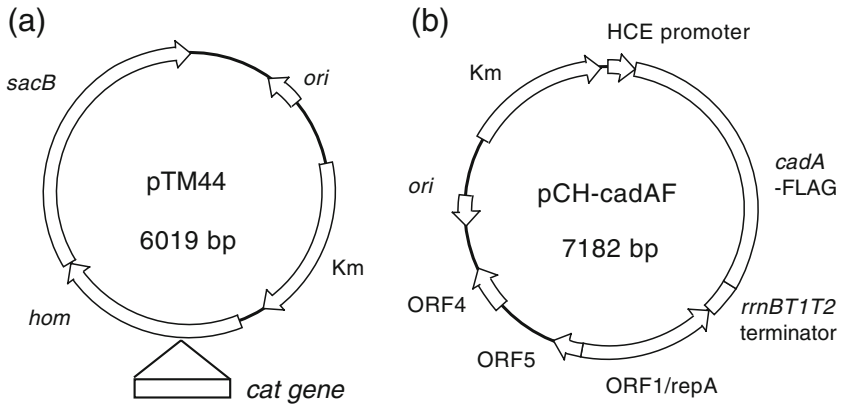

Fig. 1 Construction of plasmids: **a** Integration vector for *cat* gene into *hom* gene; **b** Intracellular expression vector of CadA from *E. coli* under the control of HCE promoter

1,000×*g* for 5 min at room temperature. Cells were washed and resuspended in 700 μl of phosphate-buffered saline (pH 7.2) containing 1% (vol/vol) Protease Inhibitor Cocktail (Nacalai Tesque, Kyoto, Japan). Cells were then disrupted using a Multi Beads Shocker (Yasuikikai, Osaka, Japan) according to a previously described protocol (Tateno et al. 2007b). The insoluble fraction and glass beads were removed by centrifugation at 21,000×*g* for 10 min at 4°C. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer was added to the supernatant, followed by boiling at 100°C for 5 min. Proteins were analyzed by SDS-PAGE using an SDS-polyacrylamide gel (8%: *w*/*v*), after which proteins were electroblotted onto a polyvinylidene difluoride membrane (Millipore Co., Boston, MA, USA) and were allowed to react with the primary mouse anti-FLAG and the secondary goat antimouse immunoglobulin G alkaline-phosphatase-conjugated antibodies (Promega Co., Madison, WI, USA). The membrane was then stained with nitroblue tetrazolium (Promega) and 5-bromo-4-chloro-3-indolylphosphate (Promega) according to the manufacturer's protocol.

Fermentation experiments

Cadaverine fermentation by recombinant *C. glutamicum* was performed in a 2.0-l jar fermentor with a 1.0-l working volume. GMMYE-1 containing 50 g/l glucose as the sole carbon source and SMMYE-1 containing 50 g/l soluble starch (Wako Pure Chemical Industries, Osaka, Japan) as the sole carbon source (Tateno et al. 2007a) were used as fermentation media. GMMYE-1 and SMMYE-1 contained 25 mg/l kanamycin. *C. glutamicum* cells grown on BY solid medium containing 25 mg/l kanamycin at 30°C for 1 day were used to inoculate 5 ml of BY liquid medium containing 25 mg/l kanamycin in a test tube. After incubation at 30°C for 24 h, the culture was transferred into 40 ml of BY medium containing 25 mg/l kanamycin in a 100-ml flask. After incubation in a shaking incubator at 30°C for 24 h, the culture (3% *v*/*v*) was used to inoculate 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 NH_3 was automatically added to the fermentation culture.

Table 1 List of strains and plasmids used in this study

Strains or plasmids	Relevant feature	Reference or source
E. coli		
SCS110	*rpsL* (*Str*^{1}) *thr leu endA thi-l lacY galK galT ara tonA tsx dam dcm supE44* Δ(*lac-proAB*) [F' *traD36 proAB lacI*^{q}ZΔ*M15*]	STRATAGENE
C. glutamicum		
ATCC 13032	Wild-type strain	ATCC
Cm-pCC	Δ*hom*; *C. glutamicum*	This study
CSS-pCC	Δ*hom*; *C. glutamicum* expressing AmyA	Tateno et al. 2007a, b
Cm-cadAF	Δ*hom*; *C. glutamicum* expressing CadA	This study
CSS-cadAF	Δ*hom*; *C. glutamicum* coexpressing AmyA and CadA	This study
Plasmids		
pTM44-cat	*cat* gene integration vector	This study
pCC	No expression (control plasmid)	Tateno et al. 2007a, b
PCH-cadAF	Intracellular expression of *cadA* gene	This study

hom homoserine dehydrogenase gene

Supernatant was used to determine α-amylase activity with an α-amylase measurement kit (Kikkoman, Tokyo, Japan; Tateno et al. 2007b). The activity of CadA was measured according to the protocols described in Mimitsuka et al. (2007). Protein concentration was measured with a BCA Protein Assay Kit (Takara Bio Inc.). Glucose concentration was determined using a Wako Glucose CII-Test kit (Wako Pure Chemical Industries, Osaka, Japan). 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 starch and starch hydrolysis products. Cadaverine and L-lysine concentrations were determined by reverse-phase high-performance liquid chromatography (GL Science Co., Osaka, Japan; Tateno et al. 2007a).

Results

Expression of CadA in *C. glutamicum*

In order to test the expression of CadA in *C. glutamicum*, the intracellular fractions of *C. glutamicum* cells were analyzed by Western blot analysis after 24 h of cultivation. In the case of Cm-cadAF and CSS-cadAF, a clear band corresponding to CadA (82 kDa) was observed (Fig. 2, lanes 3 and 4). Expression of cadA in CSS-pCC was not observed (Fig. 2, lane 2). Furthermore, CadA activity was confirmed using L-lysine as a substrate with the intracellular fractions of Cm-cadAF and CSS-cadAF. The CadA activity of Cm-cadAF and CSS-cadAF was almost the same, but CadA activity was not detected in CSS-pCC (data not shown). These results indicate the successful intracellular expression of CadA in Cm-cadAF and CSS-cadAF.

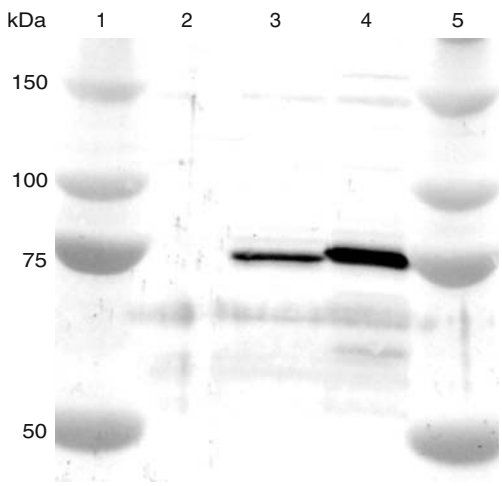

Fig. 2 Western blot analysis of CadA protein. *Lanes 1* and *5*: Marker proteins with size indicated; *lanes 2*, *3*, and *4*: intracellular fractions of CSS-pCC, Cm-cadAF, and CSS-cadAF, respectively

Fermentation experiments with glucose as sole carbon source

We then evaluated cadaverine production using glucose as the sole carbon source with CSS-pCC, Cm-cadAF, and CSS-cadAF.

Figure 3a–c shows the results for cell growth, α-amylase activity, and sugar consumption, respectively. Cell growth of CSS-pCC and CSS-cadAF was slower when compared with Cm-cadAF. Cm-cadAF showed almost the same growth as cells harboring the control plasmid (data not shown). The Cm-cadAF strain consumed all of the glucose within 18 h. On the other hand, CSS-pCC and CSS-cadAF had consumed 40.0 and 23.4 g/l after 24 h, respectively. The CSS-pCC and CSS-cadAF strains consumed less glucose when compared with Cm-cadAF. These results showed that AmyA expression, as well as AmyA and CadA coexpression, in *C. glutamicum* led to a reduction in cell growth and glucose consumption. Figure 3d, e shows the results for L-lysine and cadaverine production, respectively. The CSS-pCC strain produced 33.3 mM L-lysine. The Cm-cadAF strain produced 49.4 mM cadaverine from glucose, and only 4.2 mM L-lysine remained after 24 h. Surprisingly, CSS-cadAF did not produce L-lysine and produced only 3.2 mM cadaverine after 24 h, despite expressing both AmyA and CadA.

Fermentation experiments with soluble starch as the sole carbon source

In order to evaluate cadaverine production with soluble starch as the sole carbon source, CSS-pCC and CSS-cadAF were used.

Figure 4a–c shows the results for cell growth, α-amylase activity, and sugar consumption, respectively. CSS-pCC and CSS-cadAF showed similar cell growth and reached almost the same maximum OD_{600} value. CSS-pCC and CSS-cadAF consumed 40.6 and 35.6 g/l soluble starch after 24 h, respectively. The sugar consumption of both strains was also similar. Notable differences between cell growth and sugar consumption were not observed between AmyA-expressing and AmyA–CadA-coexpressing *C. glutamicum* strains. Figure 4d, e shows the L-lysine and cadaverine concentrations, respectively. CSS-pCC produced 32.7 mM L-lysine. The L-lysine productivity of CSS-pCC from soluble starch was almost equal to that from glucose. CSS-cadAF strain produced 22.9 mM cadaverine directly from soluble starch after 24 h and did not accumulate L-lysine.

Discussion

The aim of this study is the direct production of cadaverine from starch using *C. glutamicum* coexpressing AmyA and

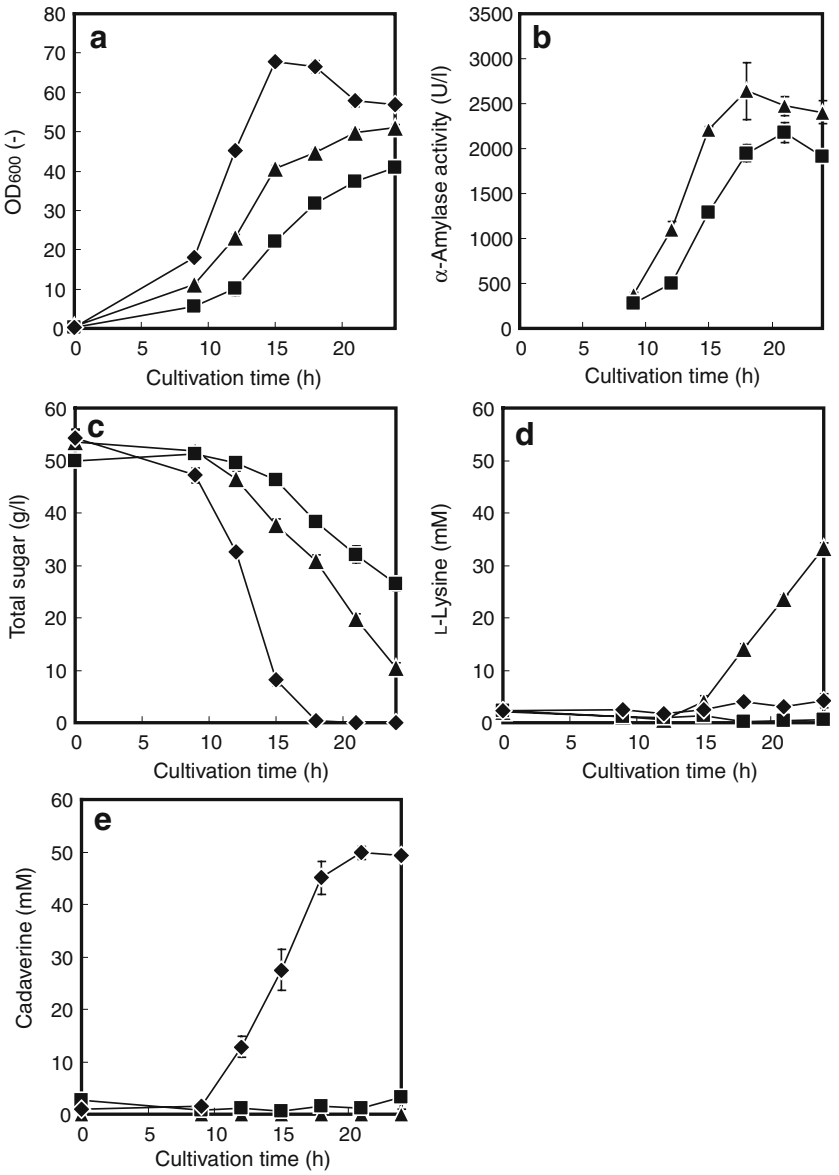

Fig. 3 Results of cadaverine fermentation experiments with glucose as the sole carbon source using CSS-pCC (*triangles*), Cm-cadAF (*diamonds*), and CSS-cadAF (*squares*). Changes in cell growth by measurement of OD_{600} (**a**), α-amylase activity (**b**), total sugar concentration (**c**), L-lysine concentration (**d**), and cadaverine concentration (**e**) are shown in the figure. Data points represent means and standard deviation of three independent experiments

CadA. We previously reported the direct production of L-lysine from starch using *C. glutamicum* CSS, which secretes AmyA. Therefore, we introduced the *E. coli* lysine decarboxylase *cadA* gene into *C. glutamicum* CSS to produce cadaverine from starch.

We constructed *C. glutamicum* Cm harboring pCH-cadAF (Cm-cadAF strain). Figure 3d, e shows that Cm-cadAF could efficiently convert L-lysine to cadaverine without accumulating intracellular L-lysine. Our results for cadaverine production (49.4 mM) from 50 g/l of glucose using Cm-cadAF were approximately two-fold higher than in previous studies (Mimitsuka et al. 2007) using pyridoxal-5'-phosphate, which suggests that CadA activity using pCH-cadAF under the control of HCE promoter is sufficient to convert L-lysine to cadaverine. CadA requires pyridoxal-5'-phosphate as a coenzyme (Sabo et al. 1974; Sabo and Fischer 1974), similar to other important enzymes such as alanine racemase (Oikawa et al. 2006), threonine dehydrogenase (Möckel et al. 1992), cystathionine γ-synthase, and *o*-acetylhomoserine sulfhydrylase (Lee 2005) and purified CadA activity without pyridoxal-5'-phosphate was approximately one tenth compared with pyridoxal-5'-phosphate in vitro (Sabo and Fischer 1974). Nevertheless, we achieved the efficient production of cadaverine using Cm-cadAF without the addition of such coenzymes to the culture medium. It is advantageous to perform fermentation without pyridoxal-5'-phosphate with regard to costs.

Glucose is the most preferable carbon source for almost all microorganisms. Interestingly, our results show that cell growth of CSS-pCC and CSS-cadAF using soluble starch as the sole carbon source was much better than when

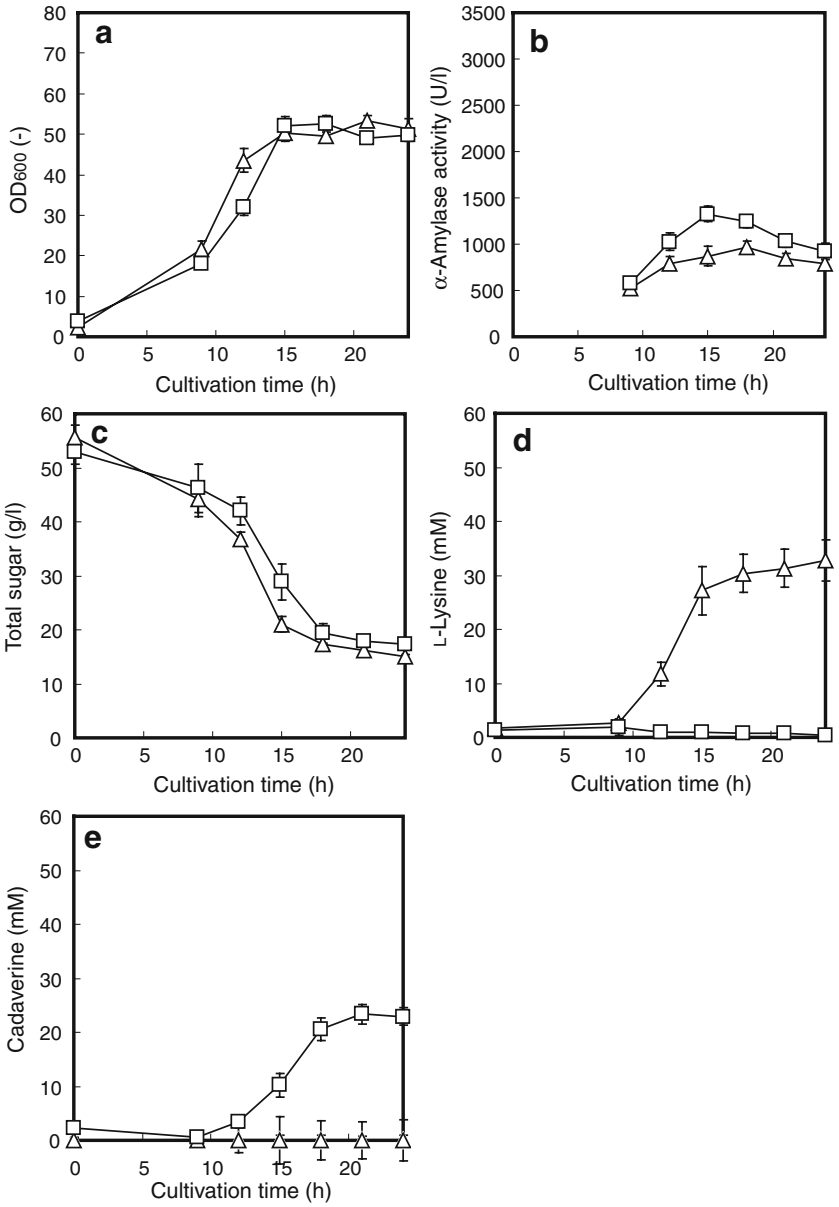

Fig. 4 Results of cadaverine fermentation experiments with soluble starch as the sole carbon source using CSS-pCC (*triangles*) and CSS-cadAF (*squares*). Changes in cell growth by measurement of OD_{600} (**a**), α-amylase activity (**b**), total sugar concentration (**c**), L-lysine concentration (**d**), and cadaverine concentration (**e**) are shown in the figure. Data points represent means and standard deviation of three independent experiments

glucose was used as the sole carbon source. Figure 4e also shows that CSS-cadAF was able to produce cadaverine from soluble starch as the sole carbon source, although it did not produce cadaverine from glucose. When maltose was supplied as the sole carbon source, the maximum OD_{600} value for CSS-cadAF reached 49.3 and CSS-cadAF produced 34.3 mM cadaverine (data not shown). Therefore, *C. glutamicum* strain CSS-pCC and CSS-cadAF are apparently adversely affected due to AmyA expression with glucose, as the cell growth of CSS-pCC and CSS-cadAF was lower than that of Cm-CadAF (Fig. 3a). These effects might be explained by the reduction of stress resistance caused by AmyA expression. *C. glutamicum* can produce trehalose, which has a role in protecting the cells from hyperosmotic stress (Tzvetkov et al. 2003; Wolf et al. 2003), using three pathways (OtsAB, TreYZ, TreS). The OtsAB pathway produces trehalose from UDP-glucose and glucose-6-phosphate. The TreYZ pathway produces trehalose from oligo–polymaltodextrins–glycogen by maltooligosyltrehalose synthase (TreY) and maltooligosyltrehalose trehalohydrolase (TreZ). The TreS pathway produces trehalose from maltose by trehalose synthase (TreS). As soluble starch degraded by AmyA contains TreY or TreS substrates such as maltose, CSS-pCC and CSS-cadAF may obtain resistance to the stresses caused by AmyA expression. Wolf et al. (2003) also reported that an intracellular trehalose content in *C. glutamicum* using maltose was higher than using glucose. Therefore, the results using maltose also indicate that CSS-cadAF is able to tolerate the stresses caused by AmyA expression via the TreS pathway.

In fact, fluorescent-probe-based real-time PCR analysis using CSS-cadAF culture after 15 h revealed that the *treY* gene expression levels using soluble starch was 1.4-fold higher than using glucose and maltose, and the *treZ* gene expression levels using maltose and soluble starch were 3.3-fold and 4.0-fold higher than using glucose, respectively, and the *tre*S gene expression level using maltose was 1.2-fold higher than using glucose (data not shown).

In conclusion, we demonstrated a direct fermentation system from starch using *C. glutamicum* coexpressing AmyA and CadA. Our results indicate that starch is a more suitable substrate for *C. glutamicum* expressing AmyA than glucose in some cases. Furthermore, the application of AmyA and CadA coexpression to the industrial L-lysine-producing *C. glutamicum* strain may enable the production of cadaverine directly from starchy materials on an industrial scale.

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