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Glutamate production from β-glucan using endoglucanase-secreting *Corynebacterium glutamicum*

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Abstract We demonstrate glutamate production from β-glucan using endoglucanase (EG)-expressing *Corynebacterium glutamicum*. The signal sequence *torA* derived from *Escherichia coli* K12, which belongs to the Tat pathway, was suitable for secreting EG of *Clostridium thermocellum* using *C. glutamicum* as a host. Using the *torA* signal sequence, endoglucanase from *Clostridium cellulovorans* 743B was successfully expressed, and the secreted EG produced 123 mg of reducing sugar from 5 g of β-glucan at 30 °C for 72 h, which is the optimal condition for *C. glutamicum* growth. Subsequently, glutamate fermentation from β-glucan was carried out with the addition of *Aspergillus aculeatus* β-glucosidase produced by recombinant *Aspergillus oryzae*. Using EG-secreting *C. glutamicum*, 178 mg/l of glutamate was produced from 15 g of β-glucan. This is the first report of glutamate fermentation from β-glucan using endoglucanase-secreting *C. glutamicum*.

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Introduction

Corynebacterium glutamicum is a non-pathogenic, non-sporulating, non-motile gram-positive soil bacterium belonging to the order Actinomycetales, which includes *Corynebacteria*, *Nocardia*, *Rhodococci*, and other related microorganisms (George 2001). *C. glutamicum* is an important industrial microorganism for its high production of amino acids such as glutamate and lysine (Hermann 2003; Leuchtenberger et al. 2005; Tateno et al. 2007). Amino acids are widely used in medicine, in fodder, and as supplements; they are also necessary as building blocks of other chemicals (Bourke and Kohn 2003; Fukuoka et al. 2004; US Department of Energy 2004). Engineered *C. glutamicum* is also superior for producing various kinds of organic compounds, such as ethanol as a bio-fuel (Sakai et al. 2007), cadaverine as a component of bio-based nylon (Mimitsuka et al. 2007; Tateno et al. 2009), and some organic acids under oxygen deprivation conditions (Inui et al. 2004; Okino et al. 2008).

Cellulose is a promising renewable carbon source that is composed of glucose units linked together by β-1,4-glycosidic linkages, as it is the most abundant biomass in the world. Since cellulose is unavailable for amino acid production by *C. glutamicum*, a saccharification process is needed prior to fermentation. Enzymatic degradation of crystalline cellulose requires several types of cellulolytic enzymes. Endoglucanase (EG) randomly digests the amorphous region of the cellulose chain. Exocellobiohydrolase (CBH) acts on the resulting reducing and non-reducing ends of crystalline cellulose. By the synergistic action of

both enzymes, cellulose is efficiently degraded to soluble cellooligosaccharides. Finally, β-glucosidase degrades these oligosaccharides to glucose. Since the production cost of these cellulolytic enzymes is very high, a strategy that can reduce the amount of enzyme used needs to be developed.

Expression of heterologous cellulase genes in useful microorganisms is a promising way to achieve cost-effective production processes. For example, direct ethanol fermentation from amorphous cellulose by using a recombinant yeast strain that co-expresses three types of cellulolytic enzymes has been demonstrated (Fujita et al. 2002; 2004). Lactic acid production using EG-expressing lactic acid bacteria has also been reported recently (Okano et al. 2010). Although *C. glutamicum* is superior for producing amino acids from glucose, the production of amino acids from cellulosic material using engineered *C. glutamicum* has not yet been attempted.

In this study, we demonstrated glutamate production from barley β-glucan using an EG-secreting strain of *C. glutamicum* (ATCC 13032). An engineered *C. glutamicum* having a suitable secretion signal sequence and high endoglucanase activity was constructed. Using the endoglucanase-secreting strain, we demonstrated glutamate production from β-glucan in the presence of an added β-glucosidase solution that was produced by recombinant *Aspergillus oryzae*.

Materials and methods

Bacterial strains and media

The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* was grown in LB medium (10 g/l tryptone, 5 g/l yeast extract, and 5 g/l sodium chloride) containing 50 mg/l kanamycin at 37 °C. *C. glutamicum* ATCC 13032 and the recombinant strains were grown in BY medium (10 g/l peptone, 10 g/l meat extract, 5 g/l yeast extract, and 5 g/l sodium chloride) containing 25 mg/l kanamycin at 30 °C (Katsumata et al. 1984). MM-β-glucan medium, which contained 10 g/l $(NH_4)_2SO_4$, 3 g/l K_2HPO_4, 1 g/l KH_2PO_4, 2 g/l urea, 0.3 g/l $MgSO_4{\cdot}7H_2O$, 0.01 g/l $FeSO_4{\cdot}7H_2O$, 0.01 g/l $MnSO_4{\cdot}5H_2O$, 1 mg/l thiamine, 0.03 mg/l biotin, 5 g/l Tween-40, 15 g/l β-D-glucan, and 0.1% (v/v) of BGL solution (final enzyme activity was 0.30 U/ml), was utilized in glutamate fermentation.

Construction of plasmids

All genetic manipulations were done using *Escherichia coli* SCS110, and all polymerase chain reactions (PCRs) were conducted using KOD-plus DNA polymerase (Toyobo, Osaka, Japan). The oligonucleotide primers used for the construction of plasmids are listed in Table 2. The *C.*

Table 1 Strains, genomic DNAs, and plasmids used in this study

Strains or plasmids	Relevant characteristics	Reference or source
Escherichia coli		
SCS110	*rpsL* (Str^r) *thr leu endA thi-l lacY galK galT ara tonA tsx dam dcm supE44Δ*(*lac-proAB*) [*F'traD36 proAB laclqZΔM15*]	STRATAGENE
K12	Wild-type	STRATAGENE
Corynebacterium glutamicum		
ATCC 13032	Wild-type	ATCC
ATCC 14020		ATCC
Clostridium cellulovorans		
(ATCC 35296)	743B	ATCC
Genomic DNA		
Clostridium thermocellum	ATCC 27405	ATCC
Saccharophagus degradans	2-40 (ATCC 43961D-5)	ATCC
Thermobifida fusca	YX (ATCC BAA-629D-5)	ATCC
Zymomonas mobilis	ZM4 (ATCC 31821D-5)	ATCC
Plasmids		
pCC	*E. coli–C. glutamicum* shuttle vector, Km^r	Tateno et al.
pCCS	pCC-containing signal sequence of *cspB*, Km^r	Tateno et al.
pCCC	pCC-containing signal sequence of *cmt2*, Km^r	This study
pCCR	pCC-containing signal sequence of *rpf2*, Km^r	This study
pCCH	pCC-containing signal sequence of *hyaA*, Km^r	This study
pCCT	pCC-containing signal sequence of *torA*, Km^r	This study

Table 2 Primer sequences used in this study

Name	Sequences (5′–3′)
Cth-celA_F	CCCGAGCTCGCAGGTGTGCCTTTTAACACAAAATA
Cth-celA_R	CCGCTCGAGTTA*GACTACAAGGATGACGATGACAAG*ATAAGGTAGGTGGGGTATGCT
ss-cmt2_F	CGCGGATCCATGTCCGTATTTACACGAGCTGG
ss-cmt2_R	CCCGATATCAGCTTGTGCGGTGCCTTGT
ss-rpf2_F	CGCGGATCCATGGCGCCCCATCAGAAGT
ss-rpf2_R	CCCGATATCAGCTGCAGCGGTGACTCC
ss-hyaA_F	CGCGGATCCATGAATAACGAGGAAACATTTTACCAG
ss-hyaA_R	CCCGATATCCGCCCAGGCAATCTTTGGTG
ss-torA_F	CGCGGATCCATGAACAATAACGATCTCTTTCAGGCAT
ss-torA_R	CCCGATATCCGCCGCTTGCGCCGCAGT
ss-cspB_F	CGCGGATCCATGTTTAACAACCGTATCCGC
ss-cspBN10_R	CCCGATATCGAAGCCGTTGGTGGTTACA
ss-cspBN20_R	CCCGATATCTGGCTGTGCGGTGGAACC
Clocel3242_F	CCCGAGCTCTCTACTGCTTTTACAGGTGTACGTGACG
Clocel3242_R	CCGCTCGAGTTA*CTTGTCATCGTCATCCTTGTAGTC*TTTTACTGTGCATTCAGTACCATTCACT
Clocel2821_F	CCCGAGCTCCGTAGTAAAAAATTAATAGCTTGCGTAACA
Clocel2821_R	CCGCTCGAGTTA*CTTGTCATCGTCATCCTTGTAGTC*AGAAAGAAGTTTCTTCTTTAATAAAGCTAAATCTAT
Sde3237_F	CTAGCTAGCATGAAATCAGCAACCACAAATCAATC
Sde3237_R	CCGCTCGAGCTA*CTTGTCATCGTCATCCTTGTAGTC*CCAGCTACCAAATTGCAGGGTGTA
Tfu2712_F	CATGCCATGGGGCACCGCTGACTGGCTGCACA
Tfu2712_R	CTAGCTAGCTTA*CTTGTCATCGTCATCCTTGTAGTC*GGCGGGAGGCGTACCCCAGACCA
ZMO1086_F	CTAGCTAGCATGACCTATAGTCGTCGTTTTATCCTTT
ZMO1086_R	CCGCTCGAGTTA*CTTGTCATCGTCATCCTTGTAGTC*GCGGTTATGCGGTCTTTCTT

Restriction enzyme sites are underlined, and the FLAG-tag sequence is italicized.

glutamicum–E. coli shuttle vectors with the *cspB* promoter, pCC and pCCS, were constructed in our previous study (Tateno et al. 2007).

To find the appropriate signal sequence for EG expression using *C. glutamicum* as a host, the following plasmids were constructed. The signal sequences of *cmt2* and *rpf2* were amplified by PCR from *C. glutamicum* ATCC 14020 using the primer pairs ss-cmt2_F and ss-cmt2_R, and ss-rpf2_F and ss-rpf2_R, respectively. The signal sequences of *hyaA* and *torA* were amplified by PCR from *E. coli* K12 using the primer pairs ss-hyaA_F and ss-hyaA_R, and ss-torA_F and ss-torA_R, respectively. These amplified fragments were digested with *Bam*HI and *Eco*RV, and digested fragments were introduced into the same sites of pCC. The resultant plasmids were named as pCCC (pCC containing cmt2 signal sequence), pCCR (pCC containing rpf2 signal sequence), pCCH (pCC containing hyaA signal sequence), and pCCT (pCC containing torA signal sequence). We also prepared modified *cspB* signal sequences (Peyret et al. 1993). The *cspB* signal sequence containing several amino acids of N-terminal mature CspB was amplified by PCR using the following primer pairs: ss-cspB_F and ss-cspBN10_R, or ss-cspB_F and ss-cspBN20_R, respectively. The amplified fragments were digested with *Bam*HI and *Eco*RV and inserted into the same sites of pCC. The resultant plasmids were named as pCCS-N10 (pCC containing *cspB* signal sequence and 10 amino acids from *cspB* N terminus) and pCCS-N20 (pCC containing cspB signal sequence and 20 amino acids from *cspB* N terminus).

An EG gene, celA from *Clostridium thermocellum*, was amplified by PCR from genomic DNA of *C. thermocellum* ATCC 27405 using primer pairs Cth-celA_F and Cth-celA_R. The NCBI accession is YP_001036436.1, and Gene ID is 4810536. The amplified fragment was digested with SacI and XhoI and introduced into pCCS, pCCC, pCCR, pCCH, pCCT, pCCS-N10, and pCCS-N20. The resultant plasmids were named pCCS-Cth-celA, pCCC-Cth-celA, pCCR-Cth-celA, pCCH-Cth-celA, pCCT-Cth-celA, pCCS-N10-Cth-celA, and pCCS-N20-Cth-celA, respectively.

Other putative EG genes were amplified by PCR from various kinds of genomic DNAs. We chose five kinds of putative EGs: Clocel3242 from *Clostridium cellulovorans* 743B (NCBI accession YP_003844692.1 and Gene ID 9610136), Clocel2821 from *C. cellulovorans* 743B (NCBI accession YP_003844281.1 and Gene ID 9609710), Sde3237 from *Saccharophagus degradans* 2-40 (NCBI

accession YP_528706.1 and Gene ID 3965710), Tfu2712 from *Thermobifida fusca* YX (NCBI accession YP_290768.1 and Gene ID 3580691), and ZMO1086 from *Zymomonas mobilis* ZM4 (NCBI accession YP_162821.1 and Gene ID 3188168). A FLAG-tag sequence was introduced at the C terminus of each EG gene and their original signal sequences were removed. The amplified Clocel3242 and Clocel 2821 fragments were digested with *Sac*I and *Xho*I. The amplified Sde3237 and ZMO1086 fragments were digested with *Nhe*I and *Xho*I. The amplified Tfu2712 fragments were digested with *Nco*I and *Nhe*I. Each digested fragment was inserted into the same respective sites of pCCT. The resultant plasmids were named as pCCT-Clocel3242, pCCT-Clocel2821, pCCT-Sde3237, pCCT-ZMO1086, and pCCT-Tfu2712, respectively. All PCR-amplified DNA fragments were confirmed by DNA sequencing analysis using ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

The 12 plasmids described above were introduced into *C. glutamicum* ATCC 13032. The transformation was conducted by electroporation with a 2.5-kV, 200-Ω, 25-μF electric pulse in a 0.2-cm cuvette using a Gene Pulser (Bio-Rad, Richmond, CA, USA). The resultant *C. glutamicum* strains were named as *C. glutamicum*-pCCS-Cth-celA, *C. glutamicum*-pCCC-Cth-celA, *C. glutamicum*-pCCR-Cth-celA, *C. glutamicum*-pCCH-Cth-celA, *C. glutamicum*-pCCT-Cth-celA, *C. glutamicum*-pCCS-N10-Cth-celA and *C. glutamicum*-pCCS-N20-Cth-celA, *C. glutamicum*-pCCT-Clocel3242, *C. glutamicum*-pCCT-Clocel2841, *C. glutamicum*-pCCT-Sde3237, *C. glutamicum*-pCCT-ZMO1086, and *C. glutamicum*-pCCT-Tfu2712, respectively.

Endoglucanase assay

First, we tested endoglucanase activity using *E. coli* SCS110 as a host. All plasmids were introduced into *E. coli* SCS110 and EG activity was evaluated by a halo assay using carboxy methyl cellulose (CMC) plates (20). LB agar plates containing 0.75% CMC (Nacalai Tesque, Kyoto, Japan) (w/v), 0.03% trypan blue (Nacalai Tesque), and 50 mg/l kanamycin (LB-CMC plate) were used. *E. coli*-harboring plasmids were incubated on LB-CMC plates at 37 °C for 24 h and their EG activities were detected by halo formation. We also tested EG activity using *C. glutamicum* ATCC 13032 as a host. The transformants of *C. glutamicum* were incubated on BY plates containing 0.75% CMC, 0.03% trypan blue, and 25 g/l kanamycin (BY-CMC plate) at 30 °C for 48 h.

The activity of secreted EG was evaluated using the amount of total reducing sugars derived from barley β-D-glucan (Sigma, St. Louis, MO, USA). All transformants were precultivated in 5 ml of BY medium containing 25 mg/l kanamycin in a test tube. *C. glutamicum*-harboring pCC was used as a negative control. After precultivation at 30 °C for 24 h, all culture medium was inoculated into 20 ml of BY medium containing 25 mg/l kanamycin in a 200-ml flask. After cultivation at 30 °C for 24 h, the supernatant was collected by centrifugation at 12,000 rpm for 5 min at 4 °C. Then 1.8 ml of supernatant was mixed with 2 ml of 1% (w/v) β-D-glucan and 0.2 ml of 1 M citrate buffer (pH 6.0). In the case of *C. glutamicum*-pCCS-Cth-celA, *C. glutamicum*-pCCC-Cth-celA, *C. glutamicum*-pCCR-Cth-celA, *C. glutamicum*-pCCH-Cth-celA, *C. glutamicum*-pCCT-Cth-celA, *C. glutamicum*-pCCS-N10-Cth-celA, and *C. glutamicum*-pCCS-N20-Cth-celA, the mixtures were incubated for 48 h at 60 °C, which was the optimal temperature for CelA, and others were incubated for 72 h at 30 °C. After incubation, the amount of reducing sugar was determined by the Somogyi–Nelson method (Somogyi 1952).

Glutamate production from glucan

Glutamate fermentation from barley β-glucan was performed as follows.

C. glutamicum-pCCT-Clocel3242 was precultivated in 5 ml of BY medium containing 25 μg/ml of kanamycin at 30 °C for 20 h. The preculture (0.2 ml) was transferred to 20 ml of BY medium containing kanamycin. After 24 h cultivation, 2 ml of the culture was inoculated into 20 ml of MM-β-glucan medium. The β-glucosidase solution was prepared using recombinant strains of *A. oryzae* (Adachi et al. 2008). To assist the β-glucan degradation, 300 μl of preculture supernatant was added. The fermentation was carried out at 30 °C, and OD_{600} and the glutamate concentration of culture supernatants were monitored. Glutamate concentration was measured by the amino acid analyzer system Prominence (Shimadzu) using a Shim-pack Amino-Li column (0.5 μm, 100 mm×6.0 mm I.D.)

Results

Signal peptide optimization for endoglucanase expression using *C. glutamicum*

Several secretion signal sequences were selected as candidates for EG expression using *C. glutamicum* as a host. One set of signal sequences, *cspB*, *cmt2*, and *rpf2*, belong to the Sec pathway, while the other sequences, *hyaA* and *torA*, belong to the Tat pathway. We also prepared slightly modified *cspB* signal sequences that included 10 or 20 amino acids derived from N-terminal mature *cspB*. These signal sequences were fused to the N terminus of EG from *C. thermocellum* (Cth-celA) and expressed using *C. glutamicum*.

The transformants of *C. glutamicum* strains, *C. glutamicum*-pCCS-Cth-celA, *C. glutamicum*-pCCC-Cth-celA, *C. glutamicum*-pCCR-Cth-celA, *C. glutamicum*-pCCH-Cth-celA, *C. glutamicum*-pCCT-Cth-celA, *C. glutamicum*-pCCS-N10-Cth-celA, and *C. glutamicum*-pCCS-N20-Cth-celA, were cultivated in BY medium. The culture supernatant was incubated with β-glucan as a substrate at 60°C and the EG activity was evaluated by the amount of produced reducing sugar. After a 72-h reaction, the *torA* signal sequence fused to EG showed the highest endoglucanase activity and produced 890 mg/l of reducing sugar from β-glucan (Table 3). Modified *cspB* sequences fused to EG produced about half the amount of reducing sugar compared to that of *torA*. Other signal sequences fused to EG showed no activity in the culture supernatant. Therefore, the *torA* signal sequence was used in following experiments.

Screening for EG having high activity under *C. glutamicum* growing conditions

At first we simply checked the EG activities using *E. coli* as a host. Halo assays on LB plates containing 0.75% CMC were used to detect EG activity. Five kinds of EGs showed clear halos (data not shown). These EG genes are as follows: from *C. cellulovorans* (Clocel3242, Clocel2841), *C. thermocellum* (Cth-celA), *S. degradans* (Sde3237), *T. fusca* (Tfu2712), and *Z. mobilis* (ZmO1086). Plasmids carrying EG genes were introduced into *C. glutamicum* ATCC 13032.

The EG activities of EG-expressing *C. glutamicum* were similarly evaluated by Halo assays described above, and all six recombinants of *C. glutamicum* showed EG activity halos on CMC plates (data not shown). Then the enzyme activity of the culture supernatant was evaluated by the amount of produced reducing sugar using β-glucan as a substrate at 30 °C, which is the optimal temperature for *C. glutamicum* growth. As shown in Fig. 1, Clocel3242 (EG from *C. cellulovorans*) showed the highest EG activity, and 123 mg/l of reducing sugar was released from 5 g/l of β-glucan at 30 °C after 72 h. The activities of CelA and Sde3237 were about half of the activity of Closel3242. ZMO1086, Clocel2842, and Tfu2712 produced only a small amount of the reducing sugar.

Table 3 The amount of reducing sugar after 48 h incubation with β-glucan and culture supernatant at 60 °C

Transformants	Reducing sugar (mg/l)
C. glutamicum-pCC	0
C. glutamicum-pCCH-Cth-celA	50±21
C. glutamicum-pCCS-N10-Cth-celA	296±41
C. glutamicum-pCCS-N20-Cth-celA	344±14
C. glutamicum-pCCT-Cth-celA	890±35

C. glutamicum strains harboring pCCH-Cth-celA, pCCS-N10-Cth-celA, pCCS-N20-Cth-celA, pCCT-Cth-celA, or pCC were cultured in BY medium for 24h at 30 °C. The culture supernatant was mixed with 0.5% of β-glucan in 0.05 M citrate buffer (pH 6.0) and after the incubation for 48 h at 60 °C, the amount of reducing sugar derived from β-glucan was measured by Somogyi–Nelson method

Glutamate fermentation from β-glucan

Encouraged by these findings, we proceeded to carry out glutamate fermentation from barley β-glucan using EG Clocel3242-secreting *C. glutamicum*. To assist the degradation of β-glucan, a β-glucosidase solution produced by recombinant *A. oryzae* that expresses *A. aculeatus* BGL1 was added. *C. glutamicum*-harboring pCC was used as a control. As shown in Fig. 2a, following the addition of the β-glucosidase solution, the OD_{600} of EG Clocel3242-secreting *C. glutamicum* was increased after fermentation, while that of pCC-harboring *C. glutamicum* did not increase at all. The amount of produced glutamate is shown in Fig. 2b. After 72 h fermentation, EG Clocel3242-secreting *C. glutamicum* successfully produced 178 mg/l of glutamate from 15 g/l of β-glucan, which was 5-fold higher than that of pCC-harboring *C. glutamicum*. In the absence of BGL solution, no glutamate was produced from glucan using Clocel3242-secreting *C. glutamicum* (Fig. 2b). This result demonstrated successful production of glutamate from β-glucan using EG-secreting *C. glutamicum*

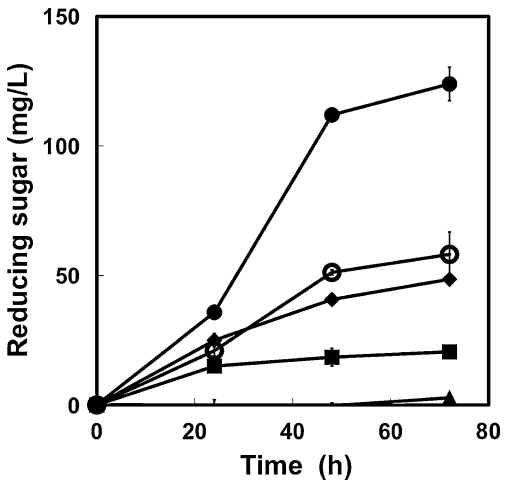

Fig. 1 The amount of reducing sugar after incubation with β-glucan and *C. glutamicum* strains harboring endoglucanase expression vectors. *Symbols* show *C. glutamicum* strains harboring pCC (*triangles*), pCCT-Cth-celA (*open circles*), pCCT-Clocel3242 (*closed circles*), pCCT-ZMO1086 (*squares*), and pCCT-Sde3237 (*diamonds*). The culture supernatant was mixed with 0.5% of β-glucan, and the amount of reducing sugar was measured after 48 h incubation at 30 °C. Data points represent the mean and standard error of three independent experiments

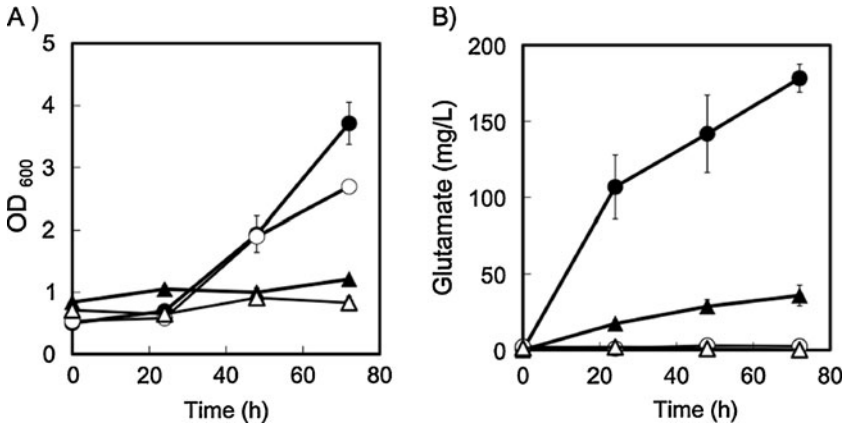

Fig. 2 Fermentation from β-glucan using *C. glutamicum* harboring pCCT-Clocel3242 (*circles*) or pCC (*triangles*) in the presence (*closed symbols*) or in the absence (*open symbols*) of β-glucosidase solution produced by recombinant *A. oryzae*. **a** OD_{600}. **b** Glutamate concentration. Data points represent the mean and standard error of three independent experiments

supplemented with a β-glucosidase solution, and the data support synergism between EG and BGL1 activities.

Discussion

The goal of this study was to achieve direct amino acid production from cellulosic materials, the most abundant biomass in the world, using *C. glutamicum* as a host. In order to simplify the manufacturing process and to reduce the production cost of amino acids from cellulose, simultaneous saccharification and fermentation of cellulose has been attempted. Although a cellulase-expressing *Corynebacterium* was reported previously (Paradis et al. 1987; Adham et al. 2001), there has been no report demonstrating direct glutamate production from cellulosic materials using endoglucanase-expressing *C. glutamicum*.

We chose endoglucanase from *C. thermocellum* (Cth-celA) to elucidate a suitable signal sequence for secretion using *C. glutamicum* as a host. Heterologous expression of EG (Cth-celA) using lactic acid bacteria as a host has been previously reported (Okano et al. 2010). However, in the case of *C. glutamicum*, EG activity was not detected in the supernatant when the *cspB* signal sequence, which is suitable for amylase secretion, was used to express EG Cth-celA. Western blotting analysis using FLAG-tag showed that Cth-celA was located in the intracellular, not extracellular, fraction (data not shown). This suggested a failure of secretion, and hence we focused on optimization of the secretion signal sequence.

Most bacteria possess two types of protein export pathways, the secretory (Sec) pathway and the twin-arginine translocation (Tat) pathway (Settles and Martienssen. 1998; Berks et al. 2000). *C. glutamicum* possesses both the Sec and Tat pathways (Kikuchi et al. 2009). Heterologous protein expression has been achieved using *C. glutamicum* through both pathways (Date et al. 2003; Kikuchi et al. 2006, 2009). Here, we selected Sec pathway-dependent signal sequences (*cspB*, *cmt2*, and *rpf2*) and Tat pathway-dependent signal sequences (*hyaA* and *torA*). We also employed two types of modified signal sequence of *cspB*. Although EG activity was not detected when *cmt2* or *rpf2* signal sequence was used, the use of modified *cspB* signal sequences (CspB-N10 and CspB-N20) improved the secreted EG activity (Table 3). Alternatively, when using the *torA* signal sequence, EG activity significantly improved, resulting in about 4-fold higher reducing sugar production compared to that using modified *cspB* signals. These results showed that the *torA* signal sequence, which is included in the Tat pathway, is suitable for expression of secreted EG using *C. glutamicum* as a host. In our previous report, the *cspB* signal sequence allowed a high level of expression of secreted α-amylase (Tateno et al. 2007), which may indicate that the appropriate signal sequence depends on the target protein.

To achieve more efficient degradation of cellulose, we evaluated several kinds of EG activity and glutamate production at 30 °C (Fig. 1), which is an appropriate condition for *C. glutamicum* growth. EG Clocel3242, an endoglucanase derived from *C. cellulovorans*, showed the highest activity at 30 °C, and three times the amount of reducing sugar was produced compared to that of Cth-celA.

In an effort to test further applications of EG-secreting *C. glutamicum*, we performed glutamate fermentation with barley β-glucan (Fig. 2). Glutamate fermentation was successfully achieved only when both EG-secreting *C. glutamicum* and a β-glucosidase solution were used, and 178 mg/l of glutamate was produced under growth conditions for *C. glutamicum*. Although the optimal temperature of EG activity is higher than the optimal temperature for *C. glutamicum* growth (around 30 °C), our result showed efficient β-glucan degradation and glutamate fermentation under suitable conditions for *C. glutamicum*. This successful result also can be explained by the synergistic action of both endoglucanase and BGL1 produced by recombinant *A. oryzae*. Although the yield of glutamate from 15g/l of β-glucan (178 mg/l) using Clocel3242-secreting *Corynebacterium* was not enough

compared to that from glucose (Yao et al. 2009), increasing both cellulase activities (EG and BGL) leads to a more efficient degradation of β-glucan and improved glutamate yield.

In conclusion, we have achieved for the first time glutamate fermentation from cellulosic materials using EG-secreting *C. glutamicum*. As a next step, it is necessary to express several kinds of BGL using *C. glutamicum* as a host. Further studies are needed to co-express BGL and EG in *C. glutamicum* for efficient glutamate fermentation from cellulose.

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