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Extracellular Overproduction of β-Cyclodextrin Glucanotransferase in a Recombinant *E. coli* Using Secretive Expression System

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Abstract β-Cyclodextrin glucanotransferase (β-CGTase) was overproduced extracellularly using recombinant *E. coli* by transforming the plasmid pECGT harboring a secretive signal peptide. The β-CGTase gene of alkalophilic *Bacillus firmus* var. *alkalophilus* was inserted into the high expression vector pET20b(+) containing a secretive *pelB* signal peptide, and then transformed into *E. coli* BL21(DE3)pLysS. The optimum culture conditions for the overproduction of β-CGTase were determined to be TB medium containing 0.5% (w/v) soluble starch at post-induction temperature of 25°C. A significant amount of β-CGTase, up to 5.83 U/ml, which was nine times higher than that in the parent strain *B. firmus* var. *alkalophilus*, was overproduced in the extracellular compartment. A pH-stat fed-batch cultivation of the recombinant *E. coli* was also performed to achieve the secretive overproduction of β-CGTase at a high cell density, resulting in production of up to 21.6 U/ml of β-CGTase.

Key words: Recombinant *E. coli*, β-cyclodextrin glucanotransferase, secretive overproduction, *cgt* gene, *pelB* signal peptide, pH-stat fed-batch cultivation

Cyclodextrin glucanotransferase (CGTase, EC 2.4.1.19) is an enzyme that catalyzes the formation of cyclodextrins (CDs) from starch, and can be classified as α-, β-, and γ-type CGTase. The enzyme also catalyzes intermolecular transglycosylation reactions, such as coupling and disproportionation, as well as hydrolysis reactions that form maltooligosaccharides and dextrins from CD or amylose [13].

CGTases are mainly produced from *Bacillus macerans*, *B. circulans*, *B. stearothermophilus*, *B. ohbensis*, alkalophilic *Bacillus* sp., *Klebsiella pneumoniae*, and *Thermoanaerobacter* sp. Alkalophilic *Bacillus* sp. have also received considerable attention as industrial CGTase producers, because of their high activity and stability at wide ranges of pHs and temperatures [1, 13, 14, 20]. Among them, alkalophilic *B. ohbensis* [38], *Bacillus* sp. KC201 [21], and *Bacillus* sp. E1 [39] would appear to be the most promising alkalophilic strains for the production of β-type CGTase.

In our previous studies, an alkalophilic *B. firmus* var. *alkalophilus* was isolated that produces mainly β-CD, and also a small amount of γ-CD without any accumulation of α-CD [4]. A catabolite de-repressed mutant and constitutive mutant were selected to achieve the overproduction of the above β-type CGTase, and the regulatory mechanism of the above strain was also investigated [4, 16]. The CGTase gene (*cgt*), which encodes 674 amino acids with a molecular weight of 75,539 Da, was cloned from *B. firmus* var. *alkalophilus* and found to include three highly conserved regions, namely, two maltose-binding sites and one sequential hydrophobic amino acid region (SHA) in the E-domain of the β-CGTase [26]. The E-domain in the β-CGTase was then modified to investigate the conflicting catalytic functions of β-CGTase [30] using site-directed mutagenesis.

The overproduction of CGTase is a prerequisite for the industrial utilization of CGTase in various reactions. Therefore, to overcome the lower productivity of wild strains, the utilization of recombinant *E. coli* has recently drawn much attention for the industrial production of CGTase. At present, the inducible P_{lac}, P_{tac}, P_{trp}, and P_L promoters have mainly been used for the overproduction of CGTase in recombinant *E. coli* [8, 12, 22, 24, 33]. Thus, about 0.14–0.3 g/l of CGTase can be produced extracellularly from the recombinant *E. coli* encoding the *cgt* gene of *B. circulans* var. *alkalophilus* ATCC 21783 through controlling the *lac* promoter [10].

However, the CGTase mostly accumulates in the intracellular compartment as an inclusion body due to the nonsecretive nature of the expression vector system used for CGTase expression in recombinant *E. coli* [24, 32]. As a result, the activation of the inclusion body requires

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complicated denaturation and refolding processes which are costly, thereby reducing the merits of recombinant *E. coli* as a high workhorse in the production of CGTase [9, 34].

Accordingly, the current study investigated the extracellular overproduction of β-CGTase using recombinant *E. coli* harboring a plasmid with a secretive signal peptide. The *cgt* gene cloned from *B. firmus* var. *alkalophilus* was inserted into the high expression plasmid pET20b(+) including a secretive signal peptide and then transformed into the high-stringency expression host *E. coli* BL21(DE3)pLysS. Thereafter, the effect of the cultivation conditions on the secretive overexpression of β-CGTase in the recombinant *E. coli* was investigated. Localization of the expressed β-CGTase was also examined to confirm the function of the secretive expression system. In addition, a pH-stat fed-batch cultivation was performed to achieve the secretive overproduction of β-CGTase based on a dense cell culture of the recombinant *E. coli*.

Materials and Methods

Microorganism and Plasmid

Bacillus firmus var. *alkalophilus* was used as the source for the β-CGTase gene [26]. The pT7Blue-T vector (Novagen, Madison, U.S.A.) was used for the PCR subcloning of the β-CGTase gene. The expression vector was the high expression plasmid pET-20b(+) (Novagen, Madison, U.S.A.) containing a *pelB* signal peptide inducing a periplasmic localization of the target protein. The expression host cell of the CGTase was the high-stringency expression host *E. coli* BL21(DE3)pLysS [F-, *ompT, hsdSB(rB-mB), gal, dcm*, (DE3) pLysS].

Subcloning of β-CGTase Gene

The β-CGTase gene of *B. firmus* var. *alkalophilus* cloned in pZeCD53 [26] was amplified using two oligonucleotide primers, A: 5'-GAATACTCAAGCTATGCAAAGCTTTTT-3' and B: 5'-CAAGCTTCCAAT TAATCATAACCGTAT-3'(*Hin*dIII site underlined), according to the procedure of Griffin and Griffin [6]. Next, the amplified β-CGTase gene was ligated with the pT7Blue-T vector and transformed into *E. coli* TOP10, which was then used as subcloning host according to the protocol of Sambrook *et al.* [31]. The sequence of the subcloned β-CGTase gene was determined using an ABI 377 automatic DNA sequencer (PE Applied Biosystem, CA, U.S.A.).

Construction of Plasmid and Its Transformation

The plasmid containing the subcloned β-CGTase gene was digested with *Hin*dIII, and then ligated with the pET-20b(+) vector containing a *pelB* signal peptide to construct the plasmid pECGT. The pECGT was then transformed into *E. coli* BL21(DE3)pLysS for the expression of β-CGTase. The recombinant *E. coli* harboring the β-CGTase gene was confirmed by observing those colonies with a clear halo on LB agar plates containing 1% (w/v) soluble starch supplemented with 50 μg/ml ampicillin and 34 μg/ml chloramphenicol as the selection markers, and 0.1 mg/ml congo red and 0.01 mg/ml xylene cyanole FF as the detectors of β-CGTase [17].

Cultivation of Recombinant *E. coli*

The recombinant *E. coli* was cultivated in four typical culture media: M9 medium, Luria broth (LB), terrific broth (TB), and super broth (SB), to identify suitable culture media for the overexpression of β-CGTase. The cells were also cultivated in TB medium containing different carbon sources; glucose, fructose, sucrose, glycerol, sorbitol, β-CD, and soluble starch. To examine the post-induction temperature for the secretive overexpression of β-CGTase, the cells were cultivated at 30°C until the OD_{600} reached to 0.8, then IPTG was added up to 0.4 mM for the induction of the T7 promoter, and the cultivation continued at different induction temperatures of 20, 25, 30, and 37°C for 8 h.

pH-Stat Fed-Batch Cultivation of Recombinant *E. coli*

The cells were cultivated in a TB medium containing 0.5% (w/v) soluble starch in a jar fermentor (KF-5*l*, Korea Fermentor Co., Korea) at 1.0 vvm, 250 rpm, and 30°C until the OD_{600} reached to 0.8. IPTG was then added to the culture broth up to 0.4 mM, and the cultivation temperature was shifted to 25°C for secretion of β-CGTase. A fed-batch feeding solution composed of 274 g/l of glucose and 211 g/l of yeast extract was added intermittently for 72 h, using a pH-stat setting at pH 6.85 as the upper limitation for the rise associated with the consumption of the carbon source. NaOH (2 M) was also added between the intermittent feeding of the fed-batch feeding solution below pH 6.75 to control any pH drop caused by acetic acid accumulation.

Measurement of CGTase Activity

The CGTase activity was measured based on the extent of reduction in the color intensity of the phenolphthalein entrapped by the synthesized β-CD [17]. The enzyme solution (0.5 ml) was mixed with an equal volume of 10% (w/v) soluble starch solution, dissolved in 50 mM Tris/maleic acid/NaOH buffer (pH 6.0), and reacted at 50°C for 30 min. Next, phenolphthalein solution composed of 0.02% (w/v) phenolphthalein and 0.053% (w/v) Na_2CO_3 (0.5 ml) and 1% Na_2CO_3 solution (3.5 ml) were added to the mixture to develop the color. The amount of β-CD produced was calculated from a calibration curve of various concentrations of β-CD and absorbances at 550 nm. One unit of enzyme was defined as the amount of enzyme that can form 1 mg of β-CD per min from soluble starch.

Localization of β-CGTase in Recombinant *E. coli*

The β-CGTase expressed was fractionated into extracellular, periplasmic, and intracellular fractions according to the method of Kato *et al.* [18]. The extracellular fraction was obtained after centrifugation of the culture broth. To separate the periplasmic fraction, the fully washed and centrifuged cells were suspended in the same volume of 25% (w/v) sucrose solution containing 1 mM EDTA, and treated with ice-cold water. The residual cells were then resuspended in 0.01 M sodium phosphate buffer (pH 6.2) containing 0.5 mM $CaCl_2$, and sonicated to separate the soluble intracellular fraction. Finally, the residual cell debris containing the inclusion body was resolved in 1% (w/v) SDS to solubilize the insoluble intracellular β-CGTase fraction.

SDS-PAGE and Other Analytical Methods

The fractionated β-CGTase was separated by SDS-PAGE electrophoresis in 10% polyacrylamide as running gel and 4.5% polyacrylamide as stacking gel of Laemmli buffers, and stained with Coomassie Brilliant Blue R250 [23].

The protein concentration was measured according to Bradford's method [2] using bovine serum albumin as the standard. The cyclodextrins and maltooligosaccarides were analyzed by HPLC (Gilson Medical Electronics Inc., Vilers-le-Bel, France). The cell growth was determined by measuring the absorbance at 600 nm.

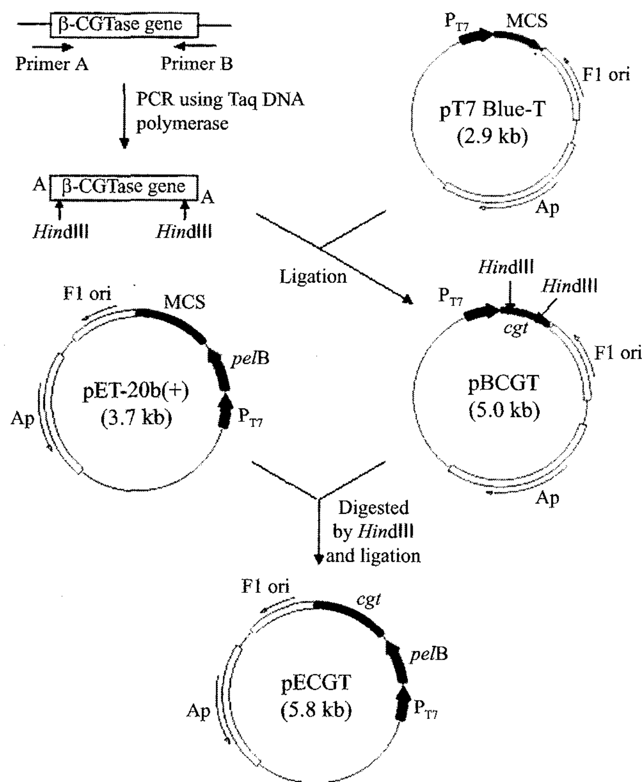

Fig. 1. Construction of secretive plasmid containing the *pelB* secretive signal peptide and the β-CGTase gene of *B. firmus* var. *alkalophilus*.

RESULTS AND DISCUSSION

Construction of Plasmid with Secretive Signal Peptide and Transformation into *E. coli*

The *cgt* gene cloned from the alkalophilic *B. firmus* var. *alkalophilus* was amplified by PCR using the plasmid pZeCD53 [26] as the template. Its size was measured to be 2.1 kb. For subcloning, the amplified *cgt* gene fragment containing the *Hin*dIII restriction site was first inserted into the pT7Blue-T vector to obtain the plasmid pBCGT, which was then transformed into the cloning host *E. coli* TOP 10. The *cgt* gene fragment generated from pBCGT by digesting it with *Hin*dIII was then transferred into the high expression vector pET-20b(+) including a secretive *pelB* signal peptide to obtain the plasmid pECGT. A schematic diagram of the construction of the high expression plasmid pECGT is depicted in Fig. 1. The *cgt* gene cloned in the high expression plasmid pECGT was placed under the control of the powerful T7 promoter and also fused to the *pelB* signal peptide for periplasmic localization.

The β-CGTase gene in the high expression plasmid pECGT with a secretive *pelB* signal peptide was confirmed by *Hin*dIII digestion by gel electrophoresis. The size of the cloned *cgt* gene fragment was measured to be 2.1 kb, and that of the recombined plasmid pECGT was 5.8 kb (data not shown). The constructed plasmid pECGT was transformed into the expression host *E. coli* BL21(DE3)pLysS, which had a low basal expression level. The recombinant clones containing the β-CGTase gene were selected based on observing those colonies with a clear halo on LB agar plates, supplemented with 0.1 mg/ml congo red and 0.01 mg/ml xylene cyanole FF as the detectors of β-CGTase.

Identification of Culture Media for Overexpression of β-CGTase in Recombinant *E. coli* with Secretive Signal Peptide

To select a suitable culture medium for the overexpression of β-CGTase in recombinant *E. coli* with a secretive *pelB* signal peptide, the recombinant *E. coli* was cultivated in 4 *E. coli* typical cultivation media; an M9 medium, Luria broth (LB), terrific broth (TB), and super broth (SB), as shown in Table 1. The β-CGTase was expressed more successfully in the TB and SB media than in the M9 and LB media, and particularly well in the TB medium. It was also noteworthy that the TB and SB media contained relatively large amounts of yeast extract, as high as 2.0% (w/v) compared to the LB medium that only contained 0.5% (w/v) and M9 that had none. However, the effect of yeast extract on the overexpression of β-CGTase in the recombinant *E. coli* other than on cell growth is not yet clear, and requires further investigation. The stimulatory

Table 1. Comparison of culture media for expression of β-CGTase in recombinant *E. coli* harboring a secretive signal peptide.

Media	Cell growth (A_{600})	CGTase activity (U/ml)
M9	1.18	0.25
SB	3.34	3.17
TB	3.02	4.29
LB	2.10	0.37

The recombinant *E. coli* was cultivated in each media at 30°C until A_{600} of 0.8, then cultivated at 25°C for 8 h after induction with 0.4 mM IPTG. One unit of enzyme was defined as the amount of β-CGTase producing 1 mg β-CD per min.

effect of yeast extract on the overexpression of α-amylase in recombinant *B. stearothermophilus* [36] and *B. subtilis* [19] has been also reported.

To identify suitable carbon sources for the overexpression of β-CGTase, the recombinant *E. coli* was cultivated in a TB medium while substituting the carbon sources with glucose, fructose, glycerol, sorbitol, sucrose, β-CD, and soluble starch. As shown in Table 2, the β-CGTase was expressed more successfully in the recombinant *E. coli* with unconventional carbon sources unfit for rapid utilization by *E. coli*, such as soluble starch, β-CD, glycerol, and sorbitol. Meanwhile, a comparatively lower level of β-CGTase was expressed with readily utilizable carbon sources, such as glucose, sucrose, and fructose, thereby suggesting potential catabolite repression by rapidly utilizable carbon sources [32]. Finally, soluble starch was identified as the most suitable carbon source for the expression of the β-CGTase gene in the recombinant *E. coli* with a secretive signal peptide.

Since β-CGTase exhibited amylase-like activity, it can hydrolyze soluble starch into a form that is readily utilizable by *E. coli*. It can be used as a carbon source, but it also reduced the catabolite repression quickly to utilizable carbon sources. When varying the soluble starch concentration from 0 to 5% (w/v), maximum cell growth and β-CGTase production were achieved with soluble starch concentration of 0.5% (w/v) (data not shown).

Table 2. Comparison of carbon sources for expression of β-CGTase in recombinant *E. coli* harboring a secretive signal peptide.

Carbon sources	Cell growth (A_{600})	CGTase activity (U/ml)
Glucose	4.14	2.68
Fluctose	4.00	4.48
Sucrose	3.11	3.15
Glycerol	3.25	5.09
Sorbitol	4.40	5.30
β-CD	4.00	5.65
Soluble starch	3.20	7.48
None	3.10	3.48

The recombinant *E. coli* was cultivated in a TB medium containing various carbon sources (0.5%, w/v) at 30°C until an A_{600} of 0.8, then cultivated at 25°C for 8 h after induction with 0.4 mM IPTG.

Effect of Post-Induction Temperatures on Secretive Overexpression of β-CGTase

The post-induction temperature has been found to significantly influence the secretion of a target protein in recombinant *E. coli* [3, 28]. Therefore, to determine the most suitable post-induction temperature for the secretive overexpression of β-CGTase in the recombinant *E. coli* harboring a secretive signal peptide, the cells were cultivated at different induction temperatures of 20, 25, 30, and 37°C up to 24 h after the induction of the T7 promoter by adding 0.4 mM IPTG.

Figure 2 compares the cell growth and extracellular β-CGTase produced by the recombinant *E. coli* cultivated at different post-induction temperatures. The cell growth decreased as the post-induction temperature decreased. However, the secretion of the expressed β-CGTase was significantly enhanced at lower post-induction temperatures. The highest secretion of the expressed β-CGTase was exhibited at the relatively low temperature of 25°C.

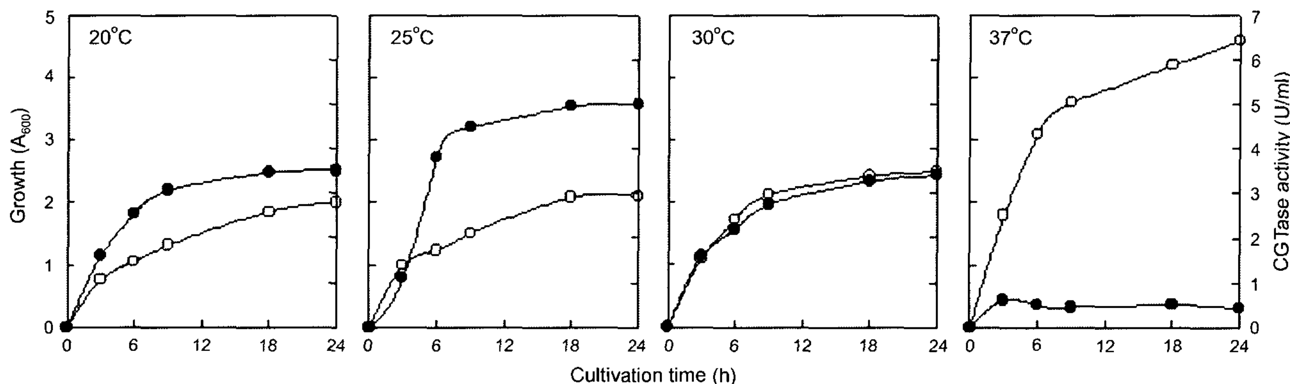

Fig. 2. Effect of post-induction temperatures on expression of β-CGTase in recombinant *E. coli* with a secretive signal peptide.
The cells were cultivated in a TB medium containing 0.5% (w/v) soluble starch at 30°C until an A_{600} of 0.8, then induced with 0.4 mM IPTG and further cultivated at different temperatures for 24 h. Symbols: ○, cell growth; ●, CGTase activity.

Table 3. Localization of expressed β-CGTase in recombinant *E. coli.*

Activity	Cell fraction			
	Extracellular	Periplasmic	Intracellular	Inclusion body
CGTase activity (U/ml)	5.30	0.56	0.30	0.10
Fraction of total activity (%)	84.7	8.95	4.79	1.60

The highly depressed secretion of β-CGTase in the recombinant *E. coli* at a high temperature of 37°C can be explained by the de-repression of the *lacUV5* promoter controlling the T7 RNA polymerase transcription, thereby causing plasmid instability and the formation of an inclusion body. In addition, post-induction at a low temperature is important in the secretion of a target protein, because it increases the heterologous protein production by decreasing any deleterious effects, such as inclusion body formation, product degradation, and stress responses [15, 37].

Localization of Overexpressed β-CGTase in Recombinant *E. coli*

β-CGTase was overexpressed in the recombinant *E. coli* under the T7 promoter, as well as being fused to the *pelB* signal peptide for periplasmic localization. The expressed β-CGTase in the recombinant *E. coli* with a secretive signal peptide was localized to confirm the efficacy of the secretive expression system after fractionation into 4 fractions; extracellular, periplasmic, intracellular, and the inclusion body. Table 3 compares the activities of the localized β-CGTase in each cellular fraction. Most of the β-CGTase was located in the extracellular fraction, whereas a small amount of β-CGTase was detected in both the periplasmic and intracellular fractions. Up to 84.7% of the total expressed β-CGTase was secreted into the extracellular fraction, indicating successful secretion of the expressed β-CGTase by the *pelB* signal peptide.

The localization of the β-CGTase was further confirmed by SDS-PAGE of each fraction of the β-CGTase produced by the recombinant *E. coli* harboring pECGT with secretive signal peptide. As shown in Fig. 3, the overexpressed β-CGTase was successfully secreted into the extracellular region in the recombinant *E. coli* with a secretive signal peptide.

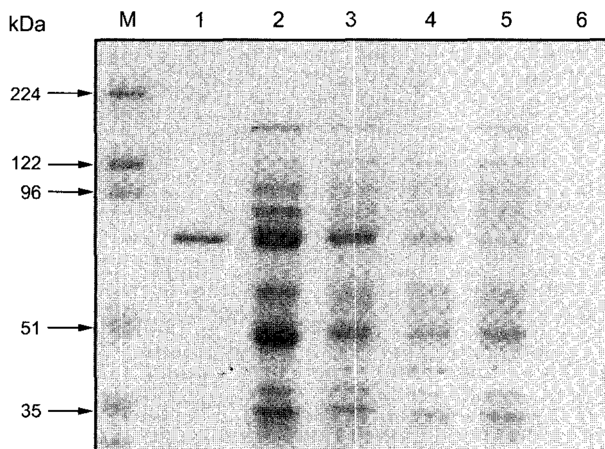

Fig. 3. SDS-PAGE of localized β-CGTase expressed by recombinant *E. coli.*
Lane M, molecular size markers (low-range marker; Bio-Rad, U.S.A.); lane 1, purified β-CGTase; lane 2, total cellular protein in recombinant *E. coli*; lane 3, extracellular fraction; lane 4, periplasmic fraction; lane 5, soluble intracellular fraction; and lane 6, inclusion body fraction.

Production Pattern of β-CGTase in Recombinant *E. coli* with Secretive Signal Peptide

Figure 4 illustrates the cell growth, plasmid stability, and expression patterns of the total and fractionated β-CGTases during the cultivation of the recombinant *E. coli* with a secretive signal peptide. The expression of β-CGTase increased along with the cell growth for 16 h and then remained at a constant level thereafter. A certain amount of the expressed β-CGTase was localized in the periplasmic and intracellular regions at the induction times up to 12 h. However, the β-CGTase accumulated in the intracellular fraction was transferred to the periplasm by the *pelB*

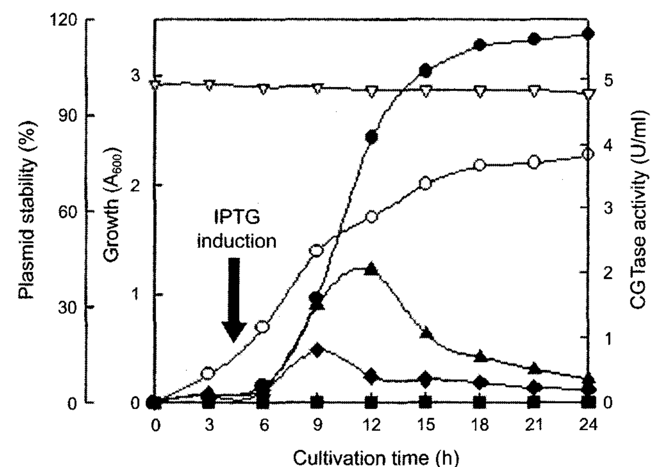

Fig. 4. Cell growth, plasmid stability, and expression pattern of β-CGTase gene during cultivation of recombinant *E. coli* with secretive signal peptide.
The cells were cultivated in a TB medium containing 0.5% (w/v) soluble starch at 30°C until an A_{600} of 0.8, then further cultivated at 25°C for 24 h after induction with 0.4 mM IPTG. Symbols: ○, cell growth; ▽, plasmid stability; ●, extracelluar CGTase; ▲, periplasmic CGTase; ◆, intracellular CGTase; ■, inclusion body CGTase.

signal peptide, and most of the β-CGTase was secreted extracellularly thereafter, leaving only a small amount of β-CGTase in the periplasmic and intracellular fractions.

The mechanism for the enhanced secretion can also be explained by the distinct structural characteristic of the host strain *E. coli* BL21(DE3)pLysS, which carries the plasmid pLysS that is coded for the T7 lysozyme, which cleaves a specific bond in the peptidoglycan layer of the cell wall, enabling more effective secretion of the β-CGTase through the cell wall [11]. Furthermore, the structure of the host strain is also deficient in the *lon* protease and *ompT* outer membrane protease, thereby preventing any degradation of the β-CGTase [7, 25].

Most of the cells still maintained the plasmids at the end of the cultivation without any segregational loss, structural instability, or other factors causing a reduction in the plasmid copy number. As a result, the recombinant *E. coli* extracellularly produced 5.83 U/ml of β-CGTase into the culture media, corresponding to around nine times the amount of the enzyme produced by the parent strain, *B. firmus* var. *alkalophilus* [5].

β-CGTase Overproduction in Recombinant *E. coli* Using pH-Stat Fed-Batch Cultivation

A pH-stat fed-batch cultivation of the recombinant *E. coli* was also performed to achieve the secretive overproduction of β-CGTase. Fed-batch feeding media composed of 274 g/l of glucose and 211 g/l of yeast extract were added intermittently by the pH-stat method, while setting pH 6.85 as the upper limit to maintain a low glucose concentration to reduce the catabolite repression of glucose. Figure 5 illustrates the time profiles for the dry cell weight, β-CGTase secreted, and glucose concentration during the pH-stat fed-batch cultivation of the recombinant *E. coli*, along with the supply of the feeding medium.

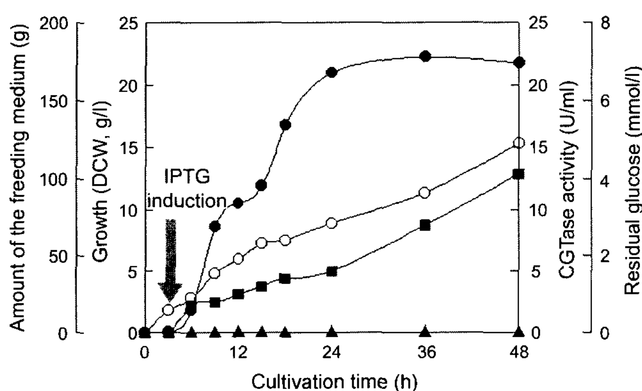

Fig. 5. pH-Stat fed-batch cultivation of recombinant *E. coli* harboring pECGT with a secretive signal peptide for overexpression of β-CGTase.

Feeding media composed of 274 g/l of glucose and 211 g/l of yeast extract and the cells were cultivated in a TB medium containing 0.5% (w/v) soluble starch at 30°C until an A_{600} of 0.8, then further cultivated at 25°C for 48 h after induction with 0.4 mM IPTG. Symbols: ○, cell growth; ●, extracellular CGTase; ■, amount of the feeding medium; ▲, residual glucose.

A high cell density of 15.0 g/l was achieved after 48 h. The extracellular β-CGTase produced was up to 21.6 U/ml, corresponding to a value of 3.7 times higher than that produced by the batch cultivation of the recombinant *E. coli*. The glucose concentration was successfully maintained at low level through intermittent addition of the feeding solution using pH-stat fed-batch cultivation to reduce the catabolite repression.

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REFERENCES

1. Bae, K.-M., S.-K. Kim, I.-S. Kong, and H.-K. Jun. 2001. Purification and properties of cyclodextrin glucanotransferase synthesizing 2-*O*-α-D-glucopyranosyl L-ascorbic acid from *Paenibacillus* sp. JB-13. *J. Microbiol. Biotechnol.* **11:** 242–250.
2. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248–254.
3. Chalmers, J. J., E. K. Kim, J. N. Telford, E. Y. Wong, W. C. Tacon, M. L. Shuler, and D. B. Wilson. 1990. Effects of temperature on *Escherichia coli* overproducing β-lactamase or human growth factor. *Appl. Environ. Microbiol.* **56:** 104–109.
4. Do, E. J., J. B. Park, and Y. H. Lee. 1993. Screening of new alkalophilic strain for overproduction of cyclodextrin glucanotransferase. *Kor. J. Appl. Microbiol. Biotechnol.* **21:** 119–124.
5. Do, E. J., H. D. Shin, C. Kim, and Y. H. Lee. 1993. Selection and characterization of catabolite repression resistant mutant of *Bacillus firmus* var. *alkalophilus* producing cyclodextrin glucanotransferase. *J. Microbiol. Biotechnol.* **3:** 78–85.
6. Griffin, H. G. and A. M. Griffin. 1994. *PCR Technology - Current Innovations*, pp. 5–100. CRC Press, Boca Raton, U.S.A.
7. Grodberg, J. and J. J. Dunn. 1988. *OmpT* encodes the *Escherichia coli* outer membrane protease that cleaves T7 RNA polymerase during purification. *J. Bacteriol.* **170:** 1245–1253.
8. Georganata, G., T. Kaneko, T. Kudo, and K. Horikoshi. 1991. Expression of the CGTase gene of alkalophilus *Bacillus* sp. No.38-2 in various hosts. *Starch* **48:** 361–363.
9. Hashimoto, Y., T. Ono, M. Goto, and T. A. Hatton. 1998. Protein refolding by reversed micelles utilizing solid-liquid extraction technique. *Biotechnol. Bioeng.* **57:** 620–623.
10. Hellman, J., W. Monika, K. Mati, K. Timo, and M. Pekka. 1990. Effects of modifications at the C-terminus of cyclomaltodextrin glucanotransferase from *Bacillus circulans*

var. *alkalophilus* on catalytic activity. *Biotech. Appl. Biochem.* **12:** 387–396.
11. Inouye, M., N. Arnheim, and R. Steruglanz. 1973. Bacteriophage T7 lysozyme is an N-acetylmuramyl-L-alanine amidase. *J. Biol. Chem.* **248:** 7247–7252.
12. Jin, H.-H., N. S. Han, D.-H. Kweon, Y.-C. Park, and J.-H. Seo. 2001. Effects of environmental factors on *in vivo* folding of *Bacillus macerans* cyclodextrin glycosyltransferase in recombinant *Escherichia coli. J. Microbiol. Biotechnol.* **11:** 92–96.
13. Kobayashi, S. 1996. Cyclodextrin producing enzyme (CGTase), pp. 23–41. *In* K. H. Park, J. F. Robyt, and Y. D. Choi (eds.), *Enzymes for Carbohydyrate Engineering*, Elsevier Science B.V., Amsterdam, The Netherlands.
14. Kaneko, T., K. B. Song, T. Hamamoto, T. Kudo, and K. Horikoshi. 1989. Construction of chimeric series of *Bacillus* cyclodextrin glucanotransferase and analysis of the thermal stabilities and pH optima of the enzymes. *J. Gen. Microbiol.* **135:** 3447–3457.
15. Kim, C. I., M. D. Kim, Y. C. Park, N. S. Han, and J. H. Seo. 2000. Refolding of *Bacillus macerans* cyclodextrin glucanotransferase expression as inclusion bodies in recombinant *Escherichia coli. J. Microbiol. Biotechnol.* **10:** 632–637.
16. Kim, C., H. D. Shin, and Y. H. Lee. 1995. Selection of the constitutive mutant of *Bacillus firmus* var. *alkalophilus* and its characteristics of cyclodextrin glucanotransferase production. *J. Microbiol. Biotechnol.* **5:** 61–67.
17. Kaneko, T., T. Kato, N. Nakamura, and K. Horikoshi. 1987. Spectrophotometric determination of cyclization activity of β-cyclodextrin-forming cyclodextrin glucanotransferase. *J. Jpn. Soc. Starch Sci.* **34:** 45–48.
18. Kato, C., K. Toshiaki, W. Kazuho, and K. Horikoshi. 1993. Extracellular production of *Bacillus* penicillinase by *Escherichia coli* carrying pEAP2. *Eur. J. Appl. Microbiol. Biotechnol.* **18:** 339–343.
19. Kim, D. O., K. M. Park, Y. B. Kim, and J. H. Seo. 1996. A parametric study production of maltotetraose-producing amylase in recombinant *Bacillus subtilis* LKS88. *Foods Biotechnol.* **5:** 322.
20. Kimura, K., T. Takano, and K. Yamane. 1987. Molecular cloning of β-cyclodextrin synthetase gene from an alkalophilic *Bacillus* and its expression in *Escherichia coli* and *Bacillus subtilis*. *Appl. Microbiol. Biotechnol.* **26:** 149–153.
21. Kitamoto, N., T. Kimura, Y. Kito, and K. Omiya. 1992. Cloning and sequencing of the gene encoding cyclodextrin glucanotransferase from *Bacillus* sp. KC201. *J. Ferment. Bioeng.* **74:** 345–351.
22. Kimura, K., I. Yasumasa, K. Shinsuke, T. Toshiya, and K. Yamane. 1990. Expression of the β-cyclodextrin glucanotransferase gene of an alkalophilic *Bacillus* sp. #1011 in *Escherichia coli* cells and characterization of the synthesized enzyme. *Agric. Biol. Chem.* **54:** 641–648.
23. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680–685.
24. Nitschke, L., K. Heeger, H. Bender, and G. E. Schulz. 1990. Molecular cloning nucleotide sequence and expression in *Escherichia coli* of the β-cyclodextrin glycosyltransferase gene from *Bacillus circulans* strain No. 8. *Appl. Microbiol. Biotechnol.* **33:** 542–546.
25. Phillips, T. A., R. A. Van Bogelen, and F. C. 1984. Neidhardt. *Lon* gene product of *Escherichia coli* is a heat-shock protein. *J. Bacteriol.* **159:** 283–287.
26. Park, T. H., H. D. Shin, and Y. H. Lee. 1999. Characterization of β-cyclodextrin glucanotransferase gene of *Bacillus firmus* var. *alkalophilus* and its expression in *E. coli*. *J. Microbiol. Biotechnol.* **9:** 811–819.
27. Park, Y. C., C. S. Kim, N. S. Han, and J. H. Seo. 1995. Expression of cyclodextrin glucanotransferase from *Bacillus macerans* in recombinant *Escherichia coli. Foods Biotechnol.* **4:** 290–295.
28. Piatak, M., J. A. Lane, W. Laird, M. J. Bjorn, A. Wang, and M. Williams. 1998. Expression of soluble and fully functional ricin A chain in *Escherichia coli* is temperature-sensitive. *J. Biol. Chem.* **263:** 4837–4843.
29. Shin, H. D., C. Kim, and Y. H. Lee. 1998. Purification and characterization of β-cyclodextrin glucanotransferase excreted by *Bacillus firmus* var. *alkalophilus*. *Kor. J. Appl. Microbiol. Biotechnol.* **26:** 323–330.
30. Shin, H. D., T. H. Park, and Y. H. Lee. 2000. Site-directed mutagenesis and functional analysis of maltose-binding site of β-cyclodextrin glucanotransferase gene of *Bacillus firmus* var. *alkalophilus*. *Biotechnol. Lett.* **22:** 115–121.
31. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, U.S.A.
32. Saito, N. and K. Yamamoto. 1975. Regulatory factors affecting α-amylase production in *Bacillus licheniformis*. *J. Bacteriol.* **121:** 848.
33. Sin, K. A., A. Nakamura, H. Masaki, and T. Uozumi. 1993. Extracellular production of *Bacillus ohbensis* cyclodextrin glucatransferase by *B. subtilis*. *Biosci. Biotech. Biotechnol.* **33:** 346–347.
34. Tasi, A. M., M. J. Betenbaugh, and J. Shiloach. 1995. The kinetics of RCC1 inclusion body formation in *Escherichia coli*. *Biotechnol. Bioeng.* **48:** 715–718.
35. Tomoko, Y., K. Shiraki, S. Fujiwara, M. Takagi, K. Fukui, and T. Imanaka. 1999. *In vitro* heat effect on functional and conformational changes of cyclodextrin glucanotransferase from *hyperthermophilic archaea*. *Biochem. Biophys. Res. Commun.* **265:** 57–61.
36. Welker, N. E. and L. L. Campbell. 1963. Effect of carbon sources on formation of α-amylase by *Bacillus stearothermophilus*. *J. Bacteriol.* **86:** 681.
37. Wetzel, R. and B. A. Chrunyk. 1994. Inclusion body formation by interleukin-1β depends on the thermal sensitivity of a folding intermediate. *FEBS Lett.* **350:** 245–248.
38. Yagi, Y., M. Sato, and T. Ishikura. 1986. Comparative studies of CGTase from *Bacillus obhensis*, *Bacillus macerans* and *Bacillus circulans* and production of cyclodextrin using those CGTase. *J. Jpn. Soc. Starch Sci.* **33:** 144–151.
39. Yong, J. S., J. N. Choi, S. S. Park, C. S. Park, K. H. Park, and Y. D. Choi. 1996. Secretion of heterologous cyclodextrin glycosyltransferase of *Bacillus* sp. E1 from *Escherichia coli*. *Biotechnol. Lett.* **18:** 1223–1228.