Journal of Microbiology and Biotechnology
Vol. 7. No. 5, 323-328, 1997

Fed-batch Fermentations of Recombinant *Escherichia coli* to Produce *Bacillus macerans* CGTase

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The recombinant *Escherichia coli* BL21(DE3)pLysE : pTCGT1 was grown to overproduce *Bacillus macerans* cyclodextrin glucanotransferase (CGTase) able to synthesize α-cyclodextrin (CD) with a selectivity of 67%. A number of batch fermentations were performed to test the possibility of using lactose as an inducer of the *E. coli* T7 promoter system. A mixture of isopropyl β-D-thiogalactoside (IPTG) and lactose (1 : 1) gave a maximum CGTase activity of 2.4 U/ml, which was higher than the value obtained with induction by IPTG alone. Fed-batch fermentations involving a glucose-controlled growth period followed by a gene-expression phase with mixtures of IPTG and lactose were employed to achieve high cell density and thereby increase total CGTase activity. Optimized fed-batch fermentation using the modified inducer (IPTG : lactose=1 : 3) and 100 g/l yeast extract solution in the gene-expression phase resulted in a maximum CGTase activity of 62.9 U/ml and a final cell mass of 53.5 g/l, corresponding to a 31-fold increase in CGTase activity and a 29-fold increase in cell mass compared with the control batch fermentation.

Efficient production of value-added materials is greatly influenced by the availability of enzymes. An important compound derived from starch is cyclodextrin (CD) which has a ring structure. Cyclodextrins (CDs) are designated by glucose units which are connected to each other with a α-1,4-D-glycosidic linkage: α-CD for 6 units, β-CD for 7 units and γ-CD for 8 units (14).

Expression of CGTase in recombinant *Escherichia coli* systems has been studied by many research groups looking at different aspects of the process. Park *et al.* (10) produced *Bacillus macerans* α-CGTase in the recombinant *E. coli* BL21(DE3)pLysE:pTCGT1 where CGTase expression was controlled by the T7 promoter. Induction by addition of 0.5 mM IPTG at the late exponential phase gave the highest CGTase activity of 3.8 U/ml. Böck *et al.* (1) combined the structural gene of α-CGTase from *Klebsiella oxytoka* M5al with the *tac* promoter and tandem terminator of the *rrnB* operon. They overproduced α-CGTase by locating the ribosome binding site at -8 base pairs from the translational initiation codon of *E. coli*. Schmid *et al.* (13) inserted the signal peptide of 20 amino acids into the structural gene of β-CGTase of alkalophilic *Bacillus* 1-1. By doing so they could improve the transport efficiency of the cloned CGTase to the *E. coli* periplasm. The recombinant *E. coli* expressed CGTase at a rate 400 times greater than the wild type.

Mass production of foreign proteins in recombinant *E. coli* systems has been developed primarily for production of high value therapeutic proteins. As fermentation processes using recombinant microorganisms are applied to industrial enzymes such as amylase and xylanase, productivity becomes a major concern since the recombinant DNA-based processes have to be more efficient and offer a better return on investment if they are to compete with existing fermentation processes. A number of studies have been carried out with a view to improving the productivity of the recombinant *E. coli* fermentation processes. Fed-batch fermentations are frequently used for acheiving high cell density and high product yields as they can separate a gene-expression phase from a cell-growth state. Furthermore, production of by-products (for example, acetic acid for *E. coli* cultures) can be minimized by controlling growth conditions dur-

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Key words: recombinant *E. coli*, CGTase, α-cyclodextrin, fed-batch

ing the fermentation (6). Typical problems involved in high cell density cultures include a deficiency of substrates or nutrients, inhibition of cell growth by by-products and exhaustion of dissolved oxygen in aerobic cultures. Especially for recombinant *E. coli* fermentations, organic acids such as acetic acid produced in glucose metabolism play an important role in obtaining high cell density (2, 8). It has been shown by many research groups that acetic acid inhibits cell growth and cloned gene expression and thereby decreases product yields (12). Reduction of acetic acid formation is, therefore, a key factor in managing high cell density cultures and achieving high productivity in recombinant *E. coli* fermentations. Some researchers reported that high cell growth rates increased the formation of acetic acid (2, 8). Many feeding strategies for delivering carbon sources have been developed with a view to minimizing the formation of acetic acid: constant feeding, exponential feeding, DO-stat, pH-stat and glucose feeding by monitoring the glucose or acetic acid concentration (6, 17). Overproduction of bulk enzymes using fed-batch fermentations of recombinant *E. coli* was undertaken by a few groups. Interesting experimental results were obtained by Lee (7) who studied the expression of *Bacillus licheniformis* maltogenic amylase in recombinant *E. coli* TG1 : pIJ322. The addition of yeast extract solution in the fed-batch mode resulted in 56.2 g/l of dry cell mass and 85.4 U/ml of amylase activity. Turner *et al.* (16) optimized a specific growth rate for the overproduction of α-amylase controlled by the *lac* promoter. The specific production rate of α-amylase at 0.2 h^{-1} of specific growth rate was 2.8 times higher than that at 0.4 h^{-1}. Sakamoto *et al.* (13) investigated the effect of acetic acid on the expression of the thermophilic protease aqualysin I (AQI) from *Thermus aquaticus* YT-1 with the *tac* promoter. As the accumulation of acetic acid is closely related to the glucose concentration in the culture broth, the glucose concentration was maintained under 0.3 g/l to maintain acetic acid levels of 1 g/l throughout the fermentation and 34 kU/ml of AQI was obtained.

This work was undertaken in order to enhance the product yields of *B. macerans* CGTase in the recombinant *E. coli* system where the expression of the CGTase gene is controlled by the T7 promoter. Specifically, high cell density cultures were achieved with a fed-batch fermentation operation.

MATERIALS AND METHODS

Plasmids and Bacterial Strain

The plasmid pTCGT1 was used as an *E. coli* expression vector of the *cgt* gene coding for CGTase. The plasmid pTCGT1 was composed of the ribosome binding site (SD sequence), signal sequence and structural gene of the *cgt* gene from *B. macerans* cloned into the pET-21(+) system, an *E. coli* expression vector harboring the T7 promoter (5). *E. coli* BL21(DE3)pLysE [F, *omp*T, *r*B$^-$, *m*B$^-$, (DE3), pLysE, Cm^r] was used as a host for expression of the *cgt* gene. Lysogenic bacteriophage DE3 is a derivative which has the immunity region of the phage 21 and carries a DNA fragment containing the *lacI* gene, the *lac*UV5 promoter, the beginning of the *lacZ* gene and the gene for T7 RNA polymerase. A promoter known to direct transcription of the T7 RNA polymerase gene is the *lac*UV5 promoter, which is inducible by isopropyl β-D-thiogalactoside (IPTG). T7 RNA polymerase expressed, in turn, transcribes the *cgt* gene in the plasmid pTCGT1 which is controlled by the T7 promoter. The plasmid pLysE provides T7 lysozyme, which binds specifically to T7 RNA polymerase and inhibits the transcription of a target gene by T7 RNA polymerase produced slightly in the host strain at an early stage [9]. The presence of pLysE increases the tolerance of *E. coli* BL21(DE3) against toxic target plasmids.

Culture Conditions

Riesenberg medium (11) containing glucose and 50 μg/ml of ampicillin was used for cultures of the recombinant *E. coli* BL21(DE3)pLysE : pTCGT1. Medium composition is summarized in Table 1. In fed-batch fermentations, the recombinant strain was grown in a 2.5-liter jar fermentor (KF-5 L, Korea Fermentor Co., Korea) at 28°C with a working volume of 1 liter. pH was controlled between 6.95~7.05 with 28% NH_4OH and 2N HCl. The culture broth was agitated in 1000~1300 rpm and D.O. was maintained above 20%. In the fed-batch mode, the solution for cell growth shown in Table 1 was fed intermittently with a peristatic pump (MasterFlex, U.S.A.) to keep a basal level of glucose concentration in the culture broth.

Determination of CGTase Activity

To prepare soluble CGTase, the culture broth was centrifuged at 5,000 rpm and 4°C for 5 min. The cell pellet was resuspended in an equal volume of 50 mM phosphate buffer (pH 6.0). After sonication of the suspension

Table 1. Composition of Riesenberg medium and growth solution for fed-batch fermentations (11).

Component	Concentration	Growth solution
KH_2PO_4	13.3 g/l	
$(NH_4)_2HPO_4$	4.0 g/l	
Citric acid	1.7 g/l	
$MgSO_4 \cdot 7H_2O$	1.2 g/l	7.48 g/l
Glucose	10.0 g/l	0.6 kg/l
Thiamine HCl	4.5 mg/l	
Trace metal solution	10.0 ml/l	

Trace metal solution: 6 g/l Fe(III) citrate, 1.5 g/l $MnCl_2 \cdot H_2O$, 0.8 g/l Zn $(CH_3COO)_2 \cdot 2H_2O$, 0.3 g/l H_3BO_4, 0.25 g/l $Na_2MoO_4 \cdot 2H_2O$, 0.25 g/l $CoCl_2$, 0.15 g/l $CuCl_2 \cdot 2H_2O$, 0.84 g/l EDTA disodium slat $\cdot 2H_2O$.

at 40% output for 4 min with an ultrasonic processor (50-watt Model, Cole-Parmer, U.S.A.), the crude CGTase solution was obtained by centrifugation of the cell-distrupted suspension at 12,000 rpm and 4°C for 30 min. Reaction products from soluble starch reacted with CGTase were analyzed with HPLC (4). A reaction mixture was made in a total volume of 0.8 ml containing 5% soluble starch in 50 mM phosphate buffer (pH 6.0). The reaction was started by adding 200 µl of the diluted enzyme solution to the prewarmed reaction mixture at 50°C. After 5 min, the reaction was stopped by adding 1 ml of acetonitrile followed by filtering the mixture with a 0.2 µm filter paper. HPLC (HPLC Pump 64, Knauer Ltd., Germany) equipped with an RI detector was employed for analysis. The column used was a 0.5 µm Hi-bar prepacked column Licrosorb NH_2 (Merck, Germany.). Column temperature was maintained at 80°C. The flow rate of the solvent (CH_3CN : H_2O [65 : 35, v/v]) was 1.0 ml/min. One unit of CGTase activity was defined as that producing 1 µmol of α-cyclodextrin under the reaction conditions.

Determination of Concentrations of Glucose and Lactose

Glucose concentrations were measured by using either a glucose analyzer (Yellow Spring Instruments 1500 Sidekick, U.S.A.) or a glucose kit (Sigma Chemical Co., U.S.A.). Lactose concentrations were determined by measuring reducing sugar concentrations with DNS solution (1 g/l NaOH; 1 g/l 3,5,-dinitrosalicyric acid; 300 g/l sodium potassium tartrate [Rochelle salt]).

RESULTS AND DISCUSSION

Utilization of Lactose for Induction

IPTG is normally utilized to induce the T7 promoter system in small-scale cultures. On a large-scale, however, the high cost of IPTG makes IPTG-induction systems unfeasible. A series of batch cultures were run to test the possibility of using lactose as an inducer for the T7 promoter system without sacrificing product yields. The recombinant *E. coli* BL21(DE3)pLysE : pTCGT1 was grown batchwise in the Riesenberg medium at 28°C and pH 7.0 (Fig. 1). Various molar ratios of lactose to IPTG were investigated: 0, 50, 75 and 100%. Induction was made by 0.5 mM of IPTG or mixed solutions at the late exponential phase, since these conditions were found to be the optimal induction timing and inducer concentration in our previous study (10). A 1 : 1 mixture of lactose and IPTG resulted in the highest CGTase activity of 2.4 U/ml, which is even higher than that obtained from the control experiment where IPTG alone was used as an inducer. This study suggests that lactose could be used as a supplementary inducer for the T7 promoter system.

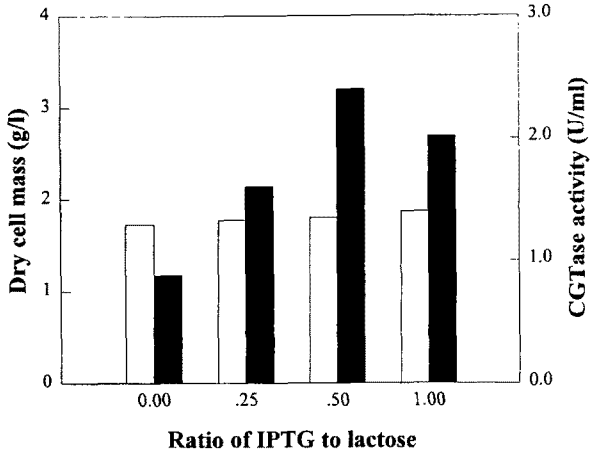

Fig. 1. Effects of IPTG ratio to lactose on dry cell mass (□) and CGTase activity (■) determined at 4 h of induction for recombinant *E. coli* BL21(DE3)pLysE : pTCGT1 at 28°C.

Fed-batch Cultures

Fed-batch cultures were performed to study the expression patterns of the CGTase gene controlled by the T7 promoter. The concentration of an inducer was fixed at 0.29 mmol/g dry cell in all fed-batch fermentations as determined from the experimental results of batch fermentations. IPTG or a mixed inducer solution (IPTG : lactose = 1 : 1) was added at about 40 g/l of dry cell mass. Profiles of cell mass and CGTase activity induced by IPTG only are shown in Fig. 2. The fermentation was run batchwise for the first 10 h and switched to a fed-batch mode by feeding the growth solution in Table 1 to increase cell mass. Cell mass increased dramatically from 4 g/l to 34 g/l during the first fed-batch period of 7

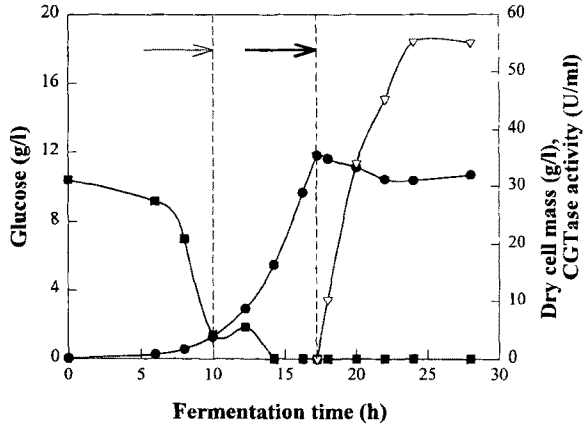

Fig. 2. Profiles of fed-batch fermentation of recombinant *E. coli* BL21(DE3)pLysTCGT1 at 28°C and pH 7.0.
The thin arrow indicates the start of feeding the growth solution and the thick arrow indicates the addition of IPTG in pH-stat. ●—●, dry cell mass; ■—■, Glucose; ▽—▽, CGTase activity.

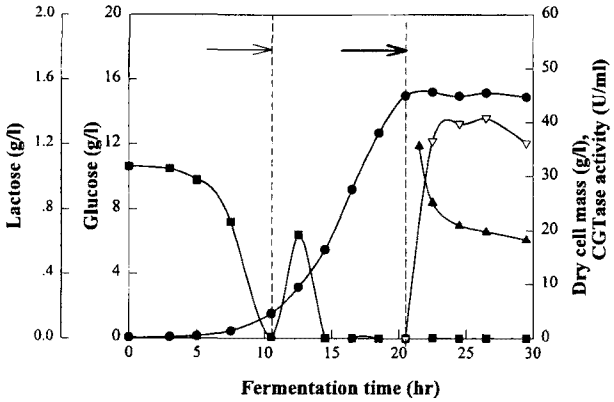

Fig. 3. Profiles of fed-batch fermentation of recombinant *E. coli* BL21(DE3)pLysE : pTCGT1 using a IPTG and lactose mixture as an inducer.

The thin arrow indicates the initiation of fed-batch mode and the thick arrow indicates the induction by a 1 : 1 mixture of IPTG and lactose in pH-stat. ●—●, dry cell mass; ■—■, Glucose; ▲—▲, lactose; ▽—▽, CGTase activity.

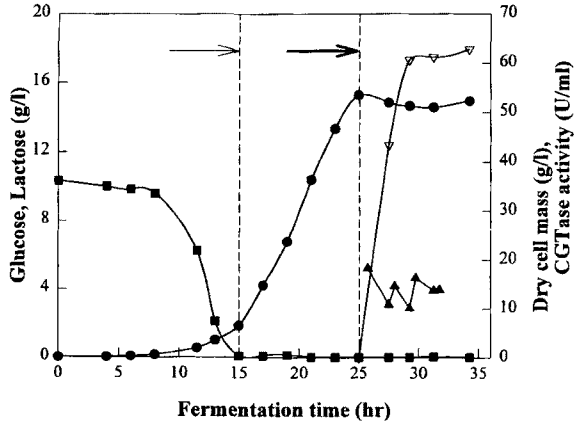

Fig. 4. Profiles of fed-batch fermentation of recombinant *E. coli* BL21(DE3)pLysE : pTCGT1 with feeding of 100 g/l yeast extract solution induced by a 1 : 3 mixture of IPTG and lactose.

The thin arrow indicates the initiation of fed-batch mode and the thick arrow indicates the feeding of 1 : 3 mixture of IPTG and lactose and a yeast extract solution. ●—●, dry cell mass; ■—■, Glucose; ▲—▲, lactose; ▽—▽, CGTase activity.

h. Glucose was maintained at a low level enough to minimize acetic acid formation (data not shown). The second fed-batch operation was initiated by adding 2.3 g of IPTG to induce the expression of CGTase. No cell growth was observed in the induction period, in fact cell mass was slightly reduced by dilution. CGTase activity, however, was enhanced sharply: 34.0 U/ml at 2 h of induction and 55.0 U/ml at 5 h. The experimental results obtained with a mixed inducer solution of lactose and IPTG (1 : 1) were qualitatively much the same as that of the previous fermentation depicted in Fig. 3. An increase in cell mass was, however, achieved during the first fed-batch mode. By feeding 50 ml of a mixture of 32 g/l IPTG and 44 g/l lactose for 30 min, the maximum CGTase activity was obtained 40.9 U/ml at 6 h of induction, corresponding to 74% of the result achieved with IPTG induction.

Even though final dry cell mass was higher, CGTase activity was reduced slightly compared with the IPTG-induced fermentation. Such a reduced level of CGTase activity was probably due to the low concentration of lactose utilized as a carbon source and an inducer during the induction period (3). The lactose concentration in the culture broth was gradually decreased from 1.2 to 0.6 g/l during 9 h of induction without any increase in cell mass, suggesting that the lactose concentration should be kept high to maintain a high gene-expression level.

In order to improve the productivity of CGTase expression in fed-batch fermentations, the feeding of an organic nitrogen source was attempted. Fig. 4 shows the results of another fed-batch culture where 100 g/l of yeast extract solution was fed continuously for 1 h on induction of the T7 promoter with a mixture of IPTG and lactose (IPTG : lactose = 1 : 3). In order to maintain the gene-expression level, a concentrated lactose solution was added to the culture broth every 2 h. The two step fed-batch fermentation resulted in a maximum CGTase activity of 62.9 U/ml and a dry cell mass of 53.5 g/l at 9 h of induction. CGTase activity was kept constant after 4 h of induction as CGTase was accumulated as a form of inclusion body in the recombinant *E. coli* cells (10). The total amount of CGTase expressed increased with induction time even beyond 4 h of induction.

The maximum achieved data for the batch and the three fed-batch fermentations are summarized in Table 2. The two step fed-batch fermentations using the IPTG and lactose mixture (IPTG : lactose = 1 : 3) and the yeast extract solution gave a 29-fold enhancement in dry cell mass and a 31-fold enhancement in CGTase activity compared with the batch fermentation. The specific CGTase activity was obtained 1.20 kU/g cell which was a 1.1-fold increase compared with the batch fermentation, while the productivity of CGTase expression in the two step fed-batch fermentation was 17.4 U/ml/h at 4 h of induction, much better than the 15.1 U/ml/h of the first fed-batch fermentation in which IPTG alone was used as an inducer. These results clearly show that induction using a mixture of IPTG and lactose along with feeding a yeast extract solution on induction increased both final cell mass and the productivity of CGTase expression. Lee (7) also reported that an improved product yield of *Bacillus licheniformis* maltogenic amylase in recombinant *E. coli* was obtained by adding a yeast extract solution at the late stage of fed-batch fermentaions. The reason for such

Table 2. Summary of maximum experimental results for four different fermentations.

Culture type	Inducer	Max. dry cell mass (g/l)	Max. CGTase activity (U/ml)	Max. specific CGTase activity (kU/g cell)	Max. productivity of CGTase (U/ml/h)
Batch	IPTG	1.87	2.02	1.08	0.75
Fed-batch	IPTG	35.4	55.2	1.76	15.1
	Mixed inducer 1[1]	45.7	40.9	0.90	18.3
Two step fed-batch[2]	Mixed inducer 2[3]	53.5	62.9	1.20	17.4

[1] IPTG : lactose = 1 : 1. [2] Feeding of 100 g/l of yeast solution in the induction period. [3] IPTG : lactose = 1 : 3.

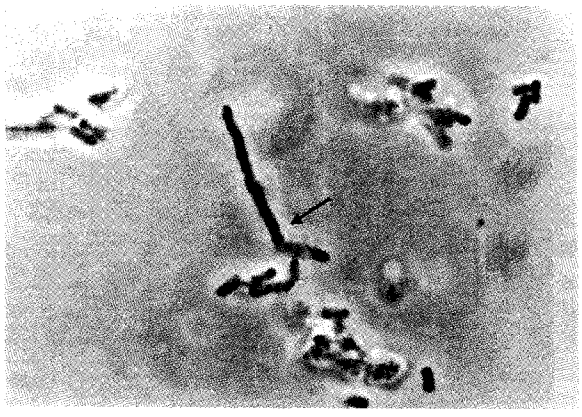

Fig. 5. A microscopic picture of recombinant *E. coli* BL21(DE3)pLysE : pTCGT1 of 4 h of IPTG induction, which was grown in Riesenberg medium at 28°C and pH 7.0.
The arrow indicates inclusion bodies of CGTase.

an increase was not clear, although, the feeding of the yeast extract solution somehow seemed to maintain cell viability even during the late fed-batch period.

A micrograph of the recombinant *E. coli* BL21(DE3) pLysE containing the plasmid pTCGT1 using phase contrast micrograpy (60 Hz, Model optiphoto-2, Nikon, Japan) clearly showed the inclusion body formation (Fig. 5). Inclusion bodies had a gall-like shape and were produced in the whole cell as well as at both ends. Inclusion body formation caused cell elongation and sometimes cell disruption. Chalmers *et al.* (3) found that inclusion body formation was related with the intrinsic properties of the proteins expressed. Extensive cell lysis was also observed with *tac*-mediated induction of the human epidermal growth factor, a foreign protein to *E. coli*, whose gene was fused to the *Omp*A signal sequence. On the other hand, the synthesis of β-lactamase, a native *E. coli* periplasmic protein, with its own signal sequence by the same promoter did not cause significant lysis.

Acknowledgement

This work was supported by the Korea Science and Engineering Foundation through the Research Center for New-Biomaterials in Agriculture, Seoul National University.

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(Received April 28, 1997)