Mass Production of Thermostable D-Hydantoinase by Batch Culture of Recombinant *Escherichia coli* with a Constitutive Expression System

Dong-Cheol Lee,[1] Geun-Joong Kim,[1] Yoo-Kyong Cha,[2] Cheo-Young Lee,[2] Hak-Sung Kim[1]

[1]Department of Biological Sciences, Korea Advanced Institute of Science and Technology, 373-1, Kusong-Dong, Yusong-Gu, Taejon 305-701, Korea; telephone: 82-42-869-2616; fax: 82-42-869-2610; e-mail: hskim@sorak.kaist.ac.kr
[2]R&D Section, Tong-Suh Petrochemical Corp. LTD., 154, Bukok-Dong, Nam-Gu, Ulsan 680-110, Korea

Received 5 December 1996; accepted 12 April 1997

Abstract: D-Hydantoinase is an industrial enzyme widely used for the synthesis of optically active D-amino acids. A gene encoding thermostable D-hydantoinase of *Bacillus stearothermophilus* SD-1 has previously been cloned and constitutively expressed by its native promoter in *Escherichia coli* XL1-Blue (Lee et al., 1996b). In this work, we attempted mass production of the D-hydantoinase by batch culture of the recombinant *E. coli* using glycerol as a carbon source. The plasmid content in cells increased in proportion to the culture temperature, which resulted in a two- or three-fold increase of the specific D-hydantoinase activity at 37°C compared with that at 30°C. The plasmid was stably maintained over 80 generations. When glycerol was initially added to a concentration of 100 g/L, the final biomass concentration reached about 50 g-dry cell weight/L in a 50 L-scale fermentation, resulting in the specific enzyme production of 3.8×10^4 unit/g-dry cell weight in a soluble form. Glycerol-using batch cultivation of recombinant *E. coli* was found to be a cost-effective process for the mass production of industrially useful D-hydantoinase. © 1997 John Wiley & Sons, Inc. *Biotechnol Bioeng* **56:** 449–455, 1997.

Keywords: thermostable D-hydantoinase; recombinant *E. coli;* constitutive expression; glycerol; batch cultivation

INTRODUCTION

Currently, D-hydantoinase (E.C. 3.5.2.2.) is employed as an industrial biocatalyst for the synthesis of D-amino acids. Optically pure D-amino acids are widely used in the pharmaceutical field as intermediates for the synthesis of semi-synthetic antibiotics, peptide hormones, pyrethroids, and pesticides (Syldatk et al., 1990). Yamada et al. (1978) developed a chemo-enzymatic process in which a racemic mixture of chemically synthesized D- and L-5-mono substituted hydantoin is asymmetrically hydrolyzed to *N*-carbamoyl-D-amino acid by D-hydantoinase. The resulting *N*-carbamoyl-D-amino acid is further chemically converted to the corresponding D-amino acid under acidic conditions.

The enzyme-catalyzed processes offer several advantages over the chemical counterparts, but the low stability of the enzyme in extreme conditions, such as high temperatures, often limits the development of the enzymatic process. The use of thermostable enzymes has been regarded as a solution to this problem, and isolation of enzymes from thermophiles has recently become a major interest. In the D-hydantoinase-catalyzed process, operation at high temperature results in improved productivity, mainly due to the enhanced solubility of the substrate. The increase in the rate of conversion of L-hydantoins to D-hydantoins is also expected at high temperatures.

We have focused on the screening of thermostable D-hydantoinase-producing microorganisms, and isolated an enzyme from *Bacillus stearothermophilus* SD-1 (Lee et al., 1994). The enzyme has been found to be the most thermostable among the D-hydantoinases reported so far (Lee et al., 1995). The gene encoding the isolated enzyme also has been cloned and constitutively overexpressed by its native promoter in a soluble form in *Escherichia coli* DH5α (Lee et al., 1996b). The recombinant strain showed about a 30-fold increase in specific enzyme production compared with that by *B. stearothermophilus* SD-1. Mukohara et al. (1994) reported the expression in *E. coli* of a thermostable D-hydantoinase gene from *B. stearothermophilus* NS1122A by an inductive promoter, but in this case, the enzyme was produced as an insoluble aggregate. Production of an industrial enzyme in an insoluble form is practically undesirable, because the refolding of the inactive form into the active one requires additional unit operations and the efficiency of the refolding is low. Use of physical and chemical induction systems often leads to low efficiency of expression in a large scale cultivation.

Correspondence to: Hak-Sung Kim
Contract grant sponsor: Tong-Suh Petrochemical Co.

© 1997 John Wiley & Sons, Inc.
CCC 0006-3592/97/040449-07

In order for an industrial enzyme to be practically applicable, the development of a cost effective process for mass production of the enzyme should be given the highest priority. In this work, we attempted mass production of the whole cell enzyme of thermostable D-hydantoinase using a constitutive expression system in recombinant *E. coli.* Culture conditions were investigated and optimized with respect to specific and volumetric production of the enzyme. As an approach to developing a better system for large scale production, we focused on batch cultivation using glycerol as a carbon source. Typical fed-batch culture using glucose was also carried out, and the production cost and volumetric production of D-hydantoinase compared for both culture methods.

EXPERIMENTAL METHOD

Strain and Plasmid

Escherichia coli XL1-Blue (*supE*44 *hsdR*17 *recA*1 *endA*1 *gyrA*46 *thi relA*1 *lac*$^-$ F`[*proAB*$^+$ *lac1*^{q} *lacZ*ΔM15 Tn*10*(*tet*^{r})]) strain containing a pUC18-based recombinant plasmid pHU183 with 1.8 kb of the thermostable D-hydantoinase gene from *B. stearothermophilus* SD-1 was employed to overproduce the enzyme. Plasmid construction has been described elsewhere (Lee et al., 1996b).

Inocula Preparation

Cells grown in Luria-Bertani (LB) medium (Difco, Detroit, MI) containing 100 μg/mL ampicillin (Sigma Chemical Co., St. Louis, MO) were maintained as a lyophilized form at −20°C. Inoculum was prepared by streaking LB agar plates containing 100 μg/mL ampicillin with cells obtained from the lyophilized stock. A single colony from the plate was inoculated into a LB liquid medium containing 50 μg/mL ampicillin, and grown overnight at 37°C. 50 mL of this seed culture was added to 1.2 ~ 3 L of the initial batch medium.

Culture Media

LB medium (10 g/L trypton, 5 g/L yeast extract, and 10 g/L NaCl) containing 50 μg/mL ampicillin was used in inocula preparation and optimization of culture conditions. In the experiment to investigate the catabolite repression, cyclic AMP (Sigma Chemical Co.) was added at 5 mM to glucose-containing LB medium as described elsewhere (Busque et al., 1995; Ishizuka et al., 1993; Johnson and Schleif, 1995; Perlman et al., 1969; Yanofsky et al., 1996). Sodium salt of cAMP was dissolved in distilled water, and the resulting solution was aseptically added through sterilized 0.2 μm filter into a 100 mL LB medium.

Semi-synthetic R medium (Lee and Chang, 1993) was used in both batch and fed-batch cultivations in a fermentor. Composition of the R-medium was as follows (per L): glucose or glycerol (stated in Results); KH_2PO_4, 13.5 g; $(NH_4)_2HPO_4$, 4.0 g; $MgSO_4$, 0.7 g; citric acid, 1.7 g; yeast extract, 5 g; trace metal solution, 10.0 mL. The trace metal solution consisted of the following (per L of 5 N HCl solution): $FeSO_4 \cdot 7H_2O$, 10.0 g; $CaCl_2 \cdot 2H_2O$, 2.0 g; $ZnSO_4 \cdot 7H_2O$, 2.2 g; $MnSO_4 \cdot 4H_2O$, 2.0 g; $CuSO_4 \cdot 5H_2O$, 1.0 g; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.1 g; $Na_2B_4O_7 \cdot 10H_2O$, 0.02 g.

Batch Culture

Batch culture was conducted in a 5 L or 50 L fermentor (Korea Fermentor Co. Ltd.). The dissolved oxygen concentration was maintained above 10% of the air saturation level by raising the agitation speed and aeration rate. The dissolved oxygen level was determined with a steam-sterilizable polarographic electrode (Ingold Electrodes, Inc.), and the culture pH was measured with a steam-sterilizable pH probe (Ingold Electrodes, Inc.). Ammonia water (25%) was used as an inorganic nitrogen source and to control the culture pH, and was automatically supplied when the pH dropped below 6.90.

Fed-Batch Culture

Fed-batch culture was carried out in a 2.5 L jar fermentor (Korea Fermentor Co. Ltd.) at 30°C. The feeding solution was composed of 700 g/L glucose and 9.62 g/L $MgSO_4$. The initial volume of the R medium was 1200 mL containing 25 g/L glucose. The seed culture was prepared in 50 mL of the same medium. The substrate feeding strategy was the pH stat method using a setpoint of the high limit at 6.95, which was based on the observation that the pH begins to rise when the principal carbon substrate is depleted (Lee and Chang, 1993; Suzuki et al., 1990). Ammonia water (25%) was used as an inorganic nitrogen source, and to control the culture pH above 6.90. When the culture pH was lowered below 6.90, the pH control unit turned on the base-pump, feeding the ammonia water drop by drop, and the culture pH was recovered and maintained at 6.90. When glucose in the medium was depleted and the culture pH was over 6.95, the pH control unit turned on the acid-pump, and 21 mL of substrate solution was fed at a time. In the fed-batch culture, the concentration of glucose in the medium was maintained below 10 g/L. The dissolved oxygen level was maintained above 10% of the air saturation level by raising the agitation speed and aeration rate.

Enzyme Assay

Whole cell D-hydantoinase activity was determined using the colorimetric method as previously described (Lee et al., 1994). One milliliter of appropriately diluted cell suspension was centrifuged, and the collected cells were resuspended in 1 mL of 0.1 *M* Tris-HCl buffer (pH 8.0) containing 1 m*M* $MnCl_2$ and 1% Triton X-100 (Sigma Chemical Co.). The suspension was incubated at 55°C with shaking

for 1 h, and 5 ~ 10 μL of the treated cell suspension was added to 0.75 mL of 1% hydantoin (Sigma Chemical Co.) solution in 0.1 *M* Tris-HCl (pH 8.0) preincubated at 55°C. The reaction was conducted at 55°C with shaking for 30 min, and reaction was stopped by adding 0.25 mL of 10% *p*-dimethylaminobenzaldehyde (Sigma Chemical Co.) in 6 N HCl. The cell debris was removed by centrifugation, and absorbance of the supernatant was measured at 440 nm to determine the amount of *N*-carbamoyl-glycine produced. One unit of D-hydantoinase activity is defined as the amount of enzyme required to produce 1 μmol of *N*-carbamoyl-glycine per min at 55°C.

Total β-lactamase activity of the cells was determined by the iodometric method (Sargent, 1968; Sawai et al., 1978; Seo and Bailey, 1985). One unit of β-lactamase activity is defined as the amount that hydrolyzes 1 μmol benzylpenicillin per min at 30°C.

Analysis

The biomass concentration was determined by measuring optical density at 600 nm. One absorbance unit (1 OD unit) corresponds to 0.38 ± 0.05 g-dry cell weight/L depending on the growth phase. Glucose was assayed by the dinitrosalicylic acid method (Bailey, 1988). Protein concentration was determined according to Bradford's method (1976) using bovine serum albumin as a standard. Acetate was determined with an enzymatic test kit from Boehringer Mannheim (#148 261).

The segregational stability of the plasmid was determined by subcultivations in 5 mL-tube culture. Cell suspension of the previous culture (10 ~ 50 μL) was inoculated into the next sterile medium. Proportion of plasmid-harboring and plasmid-free cells in population of the culture samples was determined by replica method on LB-agar plate containing ampicillin (Warnes et al., 1991). Plasmid content per cell was determined by the modified method of Moser and Campbell (1983) after the culture samples were diluted to give the same optical density. The plasmid content in each sample was compared by fluorescence intensity on 0.8% agarose gel electrophoresis.

RESULTS

Effect of Culture Temperature and pH

The effect of the culture temperature on the specific growth rate and specific production of D-hydantoinase of *E. coli* XL1-Blue/pHU183 was investigated. As shown in Figure 1a, specific production of enzyme increased three times as the culture temperature was shifted from 30°C to 37°C. The plasmid content per cell and the specific production of β-lactamase expressed on the same plasmid were also observed to be enhanced at 37°C compared with those at 30°C. From these results, the enhanced production of the enzyme

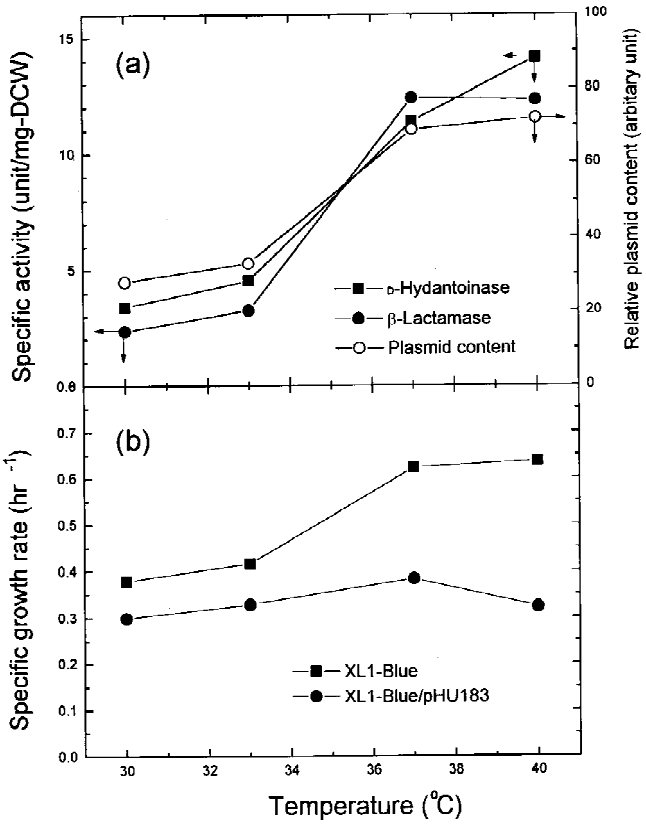

Figure 1. Effect of culture temperature on (a) the production of thermostable D-hydantoinase, and (b) the specific growth rate of *E. coli* XL1-Blue/pHU183. Cells were cultivated in 500 mL flask containing 100mL LB medium with 100 μg/mL ampicillin.

at 37°C seemed to be closely linked with an increase of the plasmid copy number per cell.

The effect of the culture temperature on the specific growth rate was also investigated. Although specific growth rates of both the plasmid-free and plasmid-harboring cells increased with increasing temperature, the temperature dependency of the plasmid harboring cells was much less than that of plasmid free cells (Fig. 1b). The plasmid content in the recombinant cells increased with increasing temperature, producing more enzyme within the cells, which seemed to result in the decrease in the specific growth rate of the host microorganism.

The effect of the culture pH on the production of D-hydantoinase was also examined in the range of 6 ~ 8, but the effect was negligible (data not shown).

Effect of Carbon and Nitrogen Sources

Yeast extract was used as a complex nitrogen source. When the initial concentration of yeast extract was 5 g/L in the R medium in 3 L batch cultivation, exponential growth was observed at 30°C. However, when cultivated at 37°C, the growth of cells was suppressed at the optical density of 15 ~ 20 in both the glucose- and glycerol-containing media. Nutrient elements such as vitamins, amino acids, and trace

elements were added to the culture broth, but the exponential growth did not recover. When more yeast extract was added to the concentration of 15 g/L, exponential growth was regained. Thus, yeast extract was supplemented at 15 g/L when the culture temperature was 37°C.

As carbon and energy sources, we examined the effect of glycerol and glucose concentrations on the production of D-hydantoinase by *E. coli* XL1-Blue/pHU183. As seen in Figure 2a, the production of D-hydantoinase was negatively affected when the concentration of glucose increased, whereas the production of β-lactamase expressed on the same plasmid was not negative. The repression of D-hydantoinase by glucose was also observed in the parent strain, *B. stearothermophilus* SD-1 (Lee et al., 1996a). Glucose is known to repress the expression of a gene by the mechanism of catabolite repression. It has been revealed that the catabolite repression can be partially relieved by the addition of exogenous cAMP (Perlman et al., 1969; Gottesman, 1984). As seen in Figure 2b, addition of exogenous cAMP to the culture partially reduced the repressive effect of glucose, which confirmed that expression of D-hydantoinase gene in *E. coli* is repressed by glucose through catabolite repression.

On the other hand, glycerol had no negative effect on the production of D-hydantoinase (Fig. 2c).

Plasmid Stability

Plasmid stability of *E. coli* XL1-Blue/pHU183 was investigated in the R media containing 50 g/L glucose or 50 g/L glycerol at 37°C. As seen in Figure 3, the plasmid was stably maintained over 90 generations in both media. The segregational instability of the plasmid largely depends on two parameters: the probability of generating plasmid-free cells and the ratio of the growth rate between plasmid-free and plasmid-containing cells (Georgiou, 1988). In the case of the pHU183 originated from pUC18, the plasmid copy number was high (>50), and the probability of plasmid-free cells arising became very low, resulting in high plasmid stability. The specific production of D-hydantoinase was observed to be much lower in the glucose-containing medium than in the glycerol-containing one, and this seems to be due to the catabolite repression by glucose. Variation in the specific production of the enzyme with generation was observed in both media. Warnes et al. (1991) also observed a similar result in the continuous cultivation of *E. coli* harboring pUC18-based recombinant plasmid under glucose- or glycerol-limited conditions. They showed that the phenomenon was caused by the variation of plasmid copy number per cell with generation.

Fed-Batch Culture Using Glucose as a Carbon Source

When the glucose concentration was above 50 g/L in a batch cultivation, no further cell growth occurred beyond the optical density of 17 ~ 25, and the specific production of

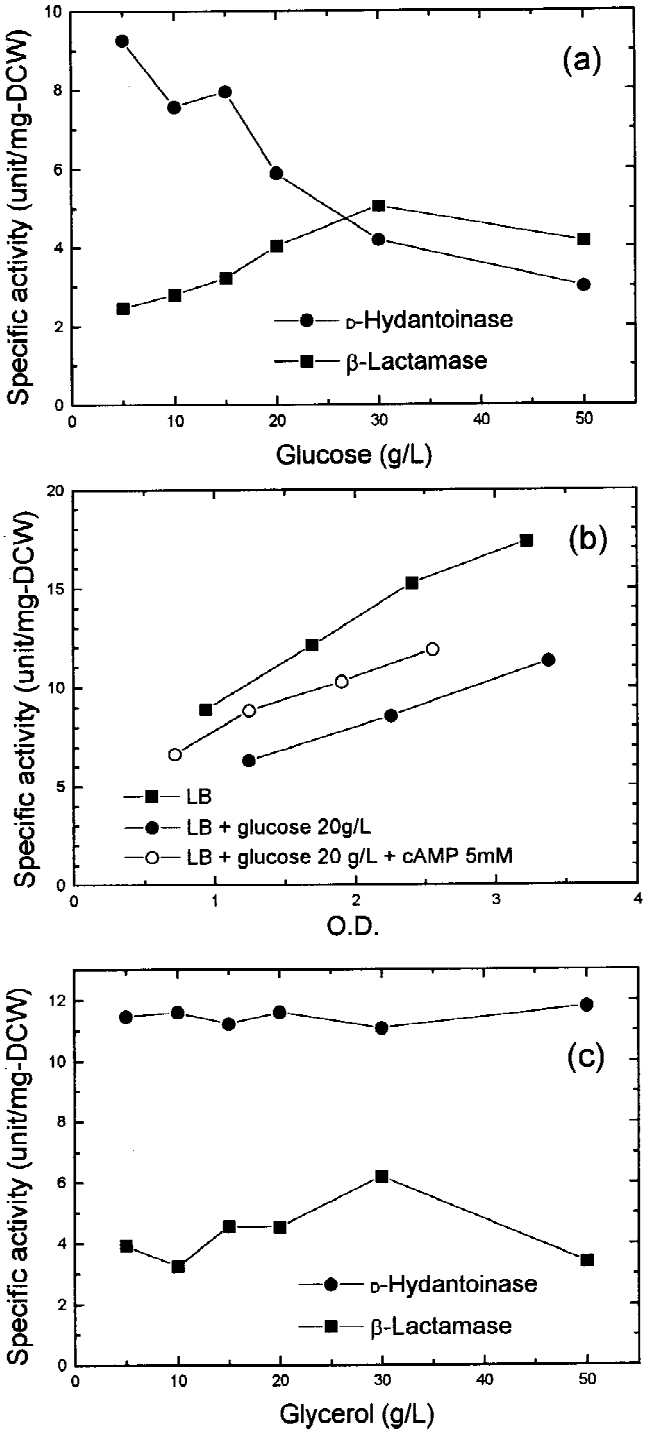

Figure 2. (a) Effect of glucose on the production of thermostable D-hydantoinase and β-lactamase by *E. coli* XL1-Blue/pHU183. Cells were cultivated in the R medium containing glucose at 30°C, and the D-hydantoinase activity was assayed when OD reached about 5. (b) Effect of exogenous cyclic AMP on the production of D-hydantoinase by *E. coli* XL1-Blue/pHU183. Cyclic AMP was initially added at 5 m*M* to the glucose-containing LB-medium. (c) Effect of glycerol on the production of thermostable D-hydantoinase and β-lactamase by *E. coli* XL1-Blue/pHU183.

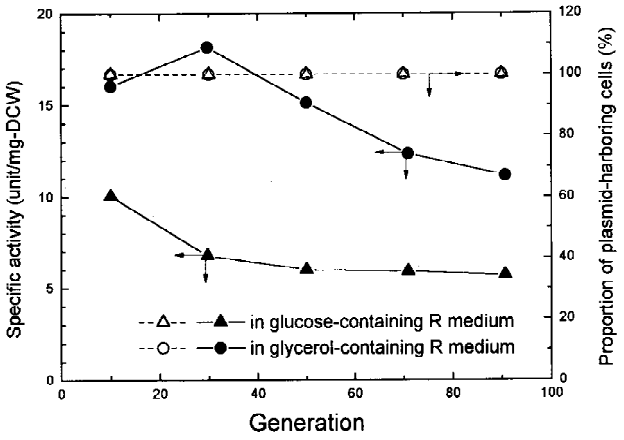

Figure 3. Effect of glucose and glycerol on the plasmid stability and the production of thermostable D-hydantoinase by *E. coli* XL1-Blue/pHU183 in successive subcultivations in the R medium without selective pressure at 37°C. The concentration of each carbon source was 50 g/L.

D-hydantoinase was as low as 4 ~ 10 units/mg-DCW mainly due to catabolite repression regardless of the culture temperature. Thus, we conducted the fed-batch culture using the pH-stat for the mass production of the enzyme. In the typical fed-batch culture at 30°C, the final cell concentration reached about 83 g-DCW/L, and specific and volumetric productions of the D-hydantoinase were about 2.5×10^4 units/g-DCW and 2.0×10^6 unit/L, respectively (Fig. 4). The specific growth rate was about 0.11 ~ 0.16 h^{-1}, and the acetate concentration remained as low as 2 g/L during cultivation. The critical specific growth rate which leads to the formation of acetate was reported to be 0.2 and 0.35 h^{-1} for complex and defined media, respectively (Riesenberg, 1991). Therefore, the low level of acetate could be attributed to the fact that the relatively low specific growth rate was maintained during the cultivation owing to the constitutive expression of D-hydantoinase. Fed-batch culture at 37°C was also carried out, but reproducible data were not obtained mainly due to the appearance and outgrowth of plasmid-free cells after feeding of substrate started (data not shown).

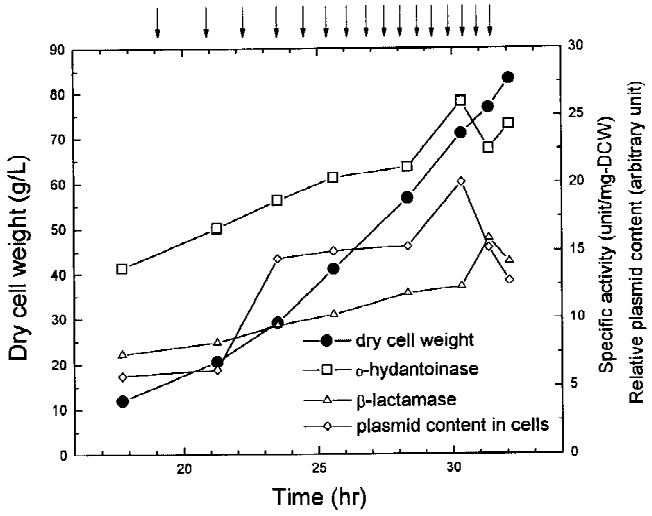

Figure 4. Fed-batch cultivation of *E. coli* XL1-Blue/pHU183 using glucose as a carbon source at 30°C. Glucose and $MgSO_4$ was fed based on the pH stat method, and the substrate feedings are indicated by arrows. The fed-batch culture was conducted in duplicate, and the data represent the mean value.

Batch Culture Using Glycerol as a Carbon Source

When glycerol was used as a carbon source, no significant repression of cell growth was observed even at the concentration of 100 g/L (Table I). Final biomass concentration reached about 50 g/L when the glycerol concentration was 100 g/L regardless of the culture temperature. As the culture temperature was elevated from 30°C to 37°C, the specific production of D-hydantoinase was almost doubled, whereas the specific growth rate was lowered by half (See also Fig. 1b).

Figure 5 shows the typical batch cultivation of *E. coli* XL1-Blue/pHU183 at 37°C in a 50 L fermentor when glycerol concentration was 100 g/L as the sole carbon source. The final cell concentration was about 50 g/L in 30 h, and the specific activity of D-hydantoinase increased along with cell growth up to 17 h, and then slightly decreased (See also Fig. 3), showing a similar pattern to the plasmid content per cell. Specific production of D-hydantoinase reached 3.8×10^4 units/g-DCW, and the content of the soluble enzyme was estimated to be about 30% of the total intracellular proteins in *E. coli.* Volumetric production of the enzyme was 1.9×10^6 unit/L, almost similar to that obtained by fed-batch culture using glucose at 30°C, which was mainly owing to the almost doubled specific production level of the enzyme at 37°C. Structural instability of the plasmid was not observed when the plasmid DNA from culture samples was investigated on agarose gel preparation, and the plasmid-free cells were not detected during the cultivation even without selection pressure. We also tested the glycerol concentration above 100 g/L, but no improvement was observed due to the prolonged cultivation time.

Table I. Effect of glycerol concentration on the production of thermostable D-hydantoinase by recombinant *E. coli* XL1-Blue/pHU183 at different temperatures.[a]

Concentration (g/l)	50		100	
Culture temp. (°C)	30	37	30	37
Final biomass (g/L)	25	24	49	52
Specific growth rate (h^{-1})	0.26	0.09	0.20	0.11
Specific production of D-hydantoinase ($\times 10^3$ unit/g-DCW)	22	43	19	41
Volumetric production of D-hydantoinase ($\times 10^6$ unit/L-broth)	0.55	1.0	0.95	2.1

[a]Cells were cultivated in a 5 L jar fermentor containing 3 L of R-medium. Specific growth rate of each culture is the mean value over the culture time. Each cultivation was conducted in duplicate, and the data represent the mean value.

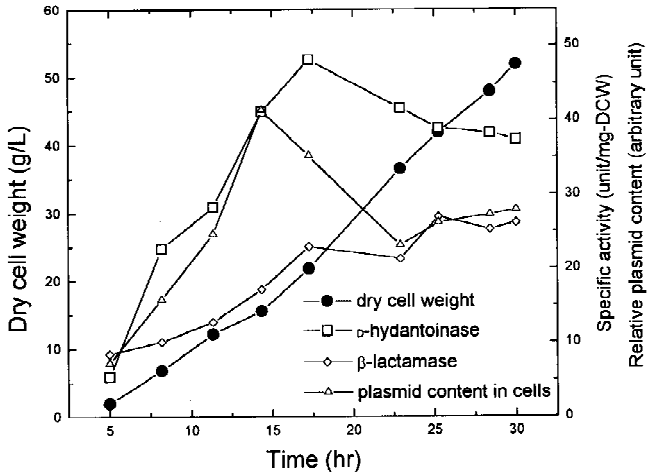

Figure 5. Batch cultivation of *E. coli* XL1-Blue/pHU183 using glycerol as a carbon source at 37°C. Cultivations were carried out in a 50-L fermentor containing 35 L of the R medium. The batch cultivation was carried out in duplicate, and the data represent the mean value.

DISCUSSION

In order for an industrially useful enzyme to be practically applied, cost-effective production of the enzyme is a prerequisite. Much attention has been paid to the overproduction of pharmaceutical proteins by recombinant microorganisms, but only a limited number of examples have been reported concerning the production of industrial enzymes (Hodgson, 1993; Lee, 1996). We demonstrated in this work that whole cell enzyme of thermostable D-hydantoinase, one of the industrially useful enzymes, could be successfully mass-produced by glycerol-based batch cultivation of recombinant *E. coli.* The harvested recombinant *E. coli* cells can be directly employed as a biocatalyst in the enzymatic process.

As a carbon source, glucose or glycerol is usually used for the cultivation of recombinant *E. coli* (Jensen and Carlsen, 1990; Korz et al., 1995; Warnes et al., 1991). Glucose has been most frequently used mainly because of its cheap price and high biomass yield. But, the growth of *E. coli* is known to be inhibited when the glucose concentration is above 50 g/L (Riesenberg, 1991). In addition, *E. coli* cultures in the presence of glucose produce acetate which has a detrimental effect on the cell growth and the production of recombinant proteins (El-Mansi and Holms, 1989; Luli and Strohl, 1990). The expression in *E. coli* XL1-Blue/pHU183 of the D-hydantoinase gene by its native promoter originated from *B. stearothermophilus* SD-1 was found to be under catabolite repression by glucose. Thus, when glucose is used as a carbon source, fed-batch cultivation is suggested.

In an effort to develop a better process for large scale production, we focused on the batch cultivation of recombinant *E. coli* XL1-Blue/pHU183 by using glycerol as a carbon source instead of glucose. Glycerol is more expensive than glucose and gives a lower growth rate. On the other hand, inhibitory by-product such as acetate is produced less when glycerol is used as a carbon source (Holms, 1986). The growth of *E. coli* XL1-Blue/pHU183 was not repressed even with a high concentration of glycerol, and no catabolite repression was observed. When 100 g/L glycerol was used, the final biomass concentration reached 50 g/L at 37°C in a 50 L-scale fermentation. This value is one of the highest among the results in batch cultivations of recombinant *E. coli* reported so far. The volumetric production of the D-hydantoinase was comparable to the result of fed-batch culture using glucose. Plasmid pHU183 was stably maintained during cultivation even without selective antibiotics at both 37°C and 30°C. Cultivation without antibiotics is required for industrial application from an environmental point of view because the use of antibiotics might raise a serious problem in large-scale cultivations. From these results, a batch culture using glycerol at high concentrations can be a reliable alternative to fed-batch culture using glucose for mass production of thermostable D-hydantoinase.

In batch cultivation, as the culture temperature was shifted from 30°C to 37°C, the specific production of D-hydantoinase increased about two-fold, but the specific growth rate decreased almost by half, probably owing to the enhanced plasmid content and recombinant protein synthesis per cell at the elevated temperature. Cultivation of recombinant cells at lower temperatures has often been attempted to reduce the growth rate, by which the formation of inhibitory by-products such as acetate and the cellular oxygen demand can also be decreased (Lee, 1996). Additionally, the formation of inclusion bodies can be avoided for some proteins (Kane and Hartley, 1988). On the other hand, in the constitutive expression system of *E. coli* XL1-Blue/pHU183 used in this work, the growth rate decreased with the increasing culture temperature, and the specific production of foreign proteins was considerably enhanced. It was unnecessary to supply oxygen-enriched air or pure oxygen even at 37°C. Thus, the cultivation at 37°C was more efficient in this case. Cultivation at high culture temperature can be economical in a large scale process for the production of industrial enzymes considering the amount of cooling water needed.

In both culture methods, the D-hydantoinase was slowly accumulated inside the cell with cell growth due to the constitutive expression of the enzyme gene, thereby the formation of inclusion bodies was avoided even at high specific production level of the enzyme. It is also unnecessary to use an expensive inducer such as IPTG. Production of pharmaceutical proteins by recombinant *E. coli* usually employs the inductive expression system for overproduction of the target proteins, and the overexpression of foreign proteins for a short period of time frequently leads to the formation of inclusion bodies. It has also been reported that expression of the thermostable D-hydantoinase gene by inductive promoter led to the production of the enzyme as an insoluble aggregate in *E. coli* (Mukohara et al., 1994). The production of industrial enzymes as an insoluble form is

obviously not practical, because additional unit operations are required for refolding; furthermore, the efficiency is low. From a practical standpoint, a simple constitutive expression system seems to be more suitable for the production of industrial enzymes by recombinant *E. coli.*

Cost analysis of the entire process for the production of D-amino acids revealed that the media cost for both culture methods are less than 1% of total production cost (Tong-Suh Petrochemical Co., Korea. 1996. Personal communications.). From these analyses, it is thought that glycerol-based batch culture of recombinant *E. coli* XL1-Blue/pHU183 can be employed for mass production of D-hydantoinase. In addition, batch cultivation can be conducted more simply and reliably at an industrial scale.

The approach attempted in this work may make a significant contribution to the mass production of industrial enzymes by recombinant *E. coli.*

We gratefully acknowledge Dr. S. Y. Lee at the Department of Chemical Engineering in KAIST for his helpful comments. We also thank Dr. J. Y. Lee for helping with the manuscript preparation.

References

Bailey, M. J. 1988. A note on the use of dinitrosalicylic acid for determining the products of enzymatic reactions. Appl. Microbiol. Biotechnol. **29**: 494–496.

Bradford, M. M. 1976. A rapid and sensitivity method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**: 248–254.

Busque, P., Letellier, A., Harel, J., Dubreuil, J. D. 1995. Production of *Escherichia coli* STb enterotoxin is subject to catabolite repression. Microbiology **141**: 1621–1627.

El-Mansi, E. M. T., Holms, W. H. 1989. Control of carbon flux to acetate excretion during growth of *Escherichia coli* in batch and continuous cultures. J. Gen. Microbiol. **135**: 2875–2883.

Georgiou, G. 1988. Optimizing the production of recombinant proteins in microorganisms. AIChE. J. **34**: 1233–1248.

Gottesman, S. 1984. Bacterial regulation: Global regulatory networks. Annu. Rev. Genet. **18**: 415–441.

Hodgson, J. 1993. Expression systems: A user's guide. Bio/Technology. **11**: 887–893.

Holms, W. H. 1986. The central metabolic pathways of *Escherichia coli:* Relationship between flux and control at a branch point, efficiency of conversion to biomass, and excretion of acetate. Curr. Top. Cell Regul. **28**: 69–105.

Ishizuka, H., Hanamura, A., Kunimura, T., Aiba, H. 1993. A lowered concentration of cAMP receptor protein caused by glucose is an important determinant for catabolite repression in *Escherichia coli.* Mol. Microbiol. **10**: 341–350.

Jensen, E. B., Carlsen, S. 1990. Production of recombinant human growth hormone in *Escherichia coli:* Expression of different precursors and physiological effects of glucose, acetate, and salts. Biotechnol. Bioeng. **36**: 1–11.

Johnson, C. M., Schleif, R. F. 1995. In vivo induction kinetics of the arabinose promoters in *Escherichia coli.* J. Bacteriol. **177**: 3438–3442.

Kane, J. F., Hartley, D. L. 1988. Formation of recombinant protein inclusion bodies in *Escherichia coli.* Trends Biotechnol. **6**: 95–101.

Korz, D. J., Rinas, U., Hellmuth, K., Sanders, E. A., Deckwer, W. D. 1995. Simple fed-batch technique for high cell density cultivation of *Escherichia coli.* J. Biotechnol. **39**: 59–65.

Lee, D. C., Lee, S. G., Kim, H. S. 1996a. Production of D-*p*-hydroxyphenylglylycine from D,L-5-(4-hydroxyphenyl)hydantoin using immobilized thermostable D-hydantoinase from *Bacillus stearothermophilus* SD-1. Enzyme Microb. Technol. **18**: 35–40.

Lee, D. C., Lee, S. G., Hong, S. P., Sung, M. H., Kim, H. S. 1996b. Cloning and overexpression of thermostable D-hydantoinase from thermophile in *E. coli* and its application to the synthesis of optically active D-amino acids. Ann. N.Y. Acad. Sci. **799**: 401–405.

Lee, S. G., Lee, D. C., Hong, S. P., Sung, M. H., Kim, H. S. 1995. Thermostable D-hydantoinase from thermophilic *Bacillus stearothermophilus* SD-1: Characteristics of purified enzyme. Appl. Microbiol. Biotechnol. **43**: 270–276.

Lee, S. G., Lee, D. C., Sung, M. H., Kim, H. S. 1994. Isolation of thermostable D-hydantoinase-producing thermophilic *Bacillus sp.* SD-1. Biotechnol. Lett. **16**: 461–466.

Lee, S. Y. 1996. High cell-density culture of *Escherichia coli.* Trends Biotechnol. **14**: 98–105.

Lee, S. Y., Chang, H. N. 1993. High cell density cultivation of *Escherichia coli* W using sucrose as a carbon source. Biotechnol. Lett. **15**: 971–974.

Luli, G. W., Strohl, W. R. 1990. Comparison of growth, acetate production, and acetate inhibition of *Escherichia coli* strains in batch and fed-batch fermentations. Appl. Environ. Microbiol. **56**: 1004–1011.

Moser, D. R., Campbell, J. L. 1983. Characterization and complementation of pMB1 copy number mutant: Effect of RNA I gene dosage on plasmid copy number and incompatibility. J. Bacteriol. **154**: 809–818.

Mukohara, Y., Ishikawa, T., Watabe, K., Nakamura, H. 1994. A thermostable hydantoinase of *Bacillus stearothermophilus* NS1122A: Cloning, sequencing, and high expression of the enzyme gene, and some properties of the expressed enzyme. Biosci. Biotechnol. Biochem. **58**: 1621–1626.

Perlman, R. L., de Crombrugghe, B., Pastan, I. 1969. Cyclic AMP regulates catabolite and transient repression in *E. coli*. Nature. **223**: 810–812.

Riesenberg, D. 1991. High cell density cultivation of *Escherichia coli.* Curr. Opin. Biotechnol. **2**: 380–384.

Sargent, M. G. 1968. Rapid fixed-time assay for penicillinase. J. Bacteriol. **95**: 1493–1494.

Sawai, T., Takahashi, I., Yamagishi, S. 1978. Iodometric assay method for beta-lactamase with various beta-lactam antibiotics as substrates. Antimicrob. Agents. Chemother. **13**: 910–913.

Seo, J. H., Bailey, J. E. 1985. Effects of recombinant plasmid content on growth properties and cloned gene product formation in *Escherichia coli.* Biotechnol. Bioeng. **27**: 1668–1674.

Suzuki, T., Yamane, T., Shimizu, S. 1990. Phenomenological background and some preliminary trials of automated substrate supply in pH-stat model fed-batch culture using a setpoint of high limit. J. Ferment. Bioeng. **69**: 292–297.

Syldatk, C., Läufer, A., Müller, R., Höke, H. 1990. Production of optically pure D- and L-α-amino acids by bioconversion of D,L-5-monosubstituted hydantoin derivatives. Adv. Biochem. Eng./Biotechnol. **41**: 29–75.

Warnes, A., Stephenson, J. R., Fooks, A. R., Melling, J., Brown, M. R. W. 1991. Expression of recombinant protein A from the *lac* promoter in *Escherichia coli* JM83 is not subject to catabolite repression when grown under specific conditions of continuous culture. Biotechnol. Bioeng. **38**: 1050–1058.

Yamada, H., Takahashi, S., Kii, Y., Kumagai, H. 1978. Distribution of hydantoin hydrolyzing activity in microorganisms. J. Ferment. Technol. **56**: 484–491.

Yanofsky, C., Horn, V., Nakamura, Y. 1996. Loss or overproduction of polypeptide release factor 3 influences expression of the tryptophanase operon of *Escherichia coli.* J. Bacteriol. **178**: 3755–3762.