A Cultivation Strategy of Recombinant *Escherichia coli* for Mass Production of Thermostable D-Hydantoinase

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INTRODUCTION

Optically pure D-amino acids are widely used in the pharmaceutical field as intermediates for the synthesis of semisynthetic antibiotics, peptide hormones, pyrethroids, and pesticides. Currently, D-hydantoinase (EC 3.5.2.2) is employed as an industrial biocatalyst for the synthesis of the D-amino acids.[1] We have focused on the screening of thermostable D-hydantoinase-producing microorganisms and have isolated an enzyme from *Bacillus stearothermophilus* SD-1.[2] The enzyme has been found to be most thermostable among the D-hydantoinases reported so far.[3] The gene encoding the enzyme was previously cloned and constitutively overexpressed in *Escherichia coli* XL1-Blue/pHU183 by its native promoter in a soluble form.[4] Mukohara *et al.* also 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.[5]

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 the mass production of the whole cell enzyme of thermostable D-hydantoinase using the recombinant *E. coli*. The harvested cells can be directly employed as a biocatalyst in the enzymatic process.

CULTURE CONDITIONS

Specific production of the enzyme in the recombinant *E. coli* increased three times as the culture temperature was shifted from 30 °C to 37 °C, owing to an increase in the plasmid copy number per cell. Semisynthetic R-medium was used in both batch and fed-batch cultivations in a fermentor. Composition of the R-medium

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was as follows (per liter): glucose or glycerol; KH_2PO_4, 13.5 g; $(NH_4)_2HPO_4$, 4.0 g; $MgSO_4$, 0.7 g; citric acid, 1.7 g; trace metal solution, 10.0 mL. Yeast extract was supplemented to the culture medium at 15 g/L. The plasmid in the recombinant cells was stably maintained over 90 generations.

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 D-hydantoinase was as low as 4~10 units/mg-DCW mainly due to the catabolite repression by glucose regardless of the culture temperature. Thus, we conducted a fed-batch culture using the feeding strategy by 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 the specific and volumetric productions of the D-hydantoinase were about 2.5×10^4 units/g-DCW and 2.0×10^6 units/L, respectively (FIGURE 1). The specific growth rate was about 0.11~0.16 h^{-1}, and the acetate concentration remained as low as 2 g/L during the cultivation.

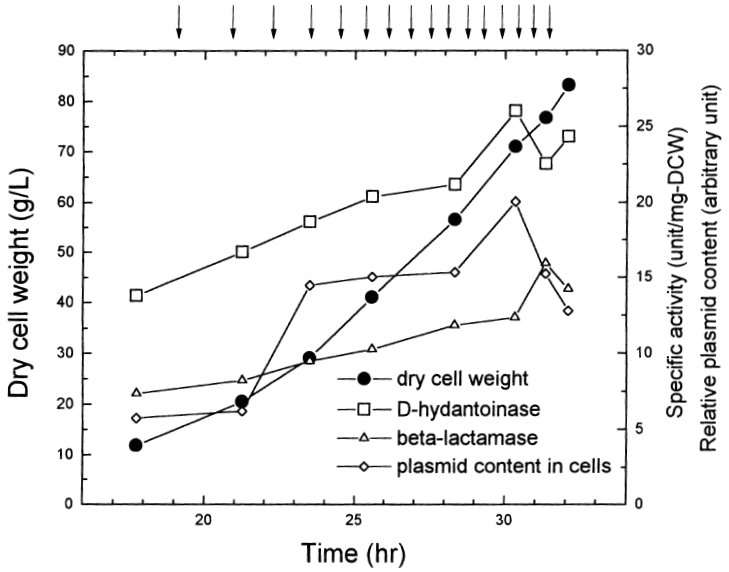

FIGURE 1. Fed-batch cultivation of *E. coli* XL1-Blue/pHU183 using glucose as a carbon source at 30 °C. Glucose and $MgSO_4$ were fed based on the pH-stat method, and the substrate feedings are indicated by arrows. The fed-batch culture was conducted in duplicate, and the data represent mean values.

BATCH CULTURE USING GLYCEROL AS A CARBON SOURCE

When glycerol was used as a carbon source, no significant repression on cell growth and D-hydantoinase production was observed, even at the concentration of 100 g/L. 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. FIGURE 2 shows the typical batch cultivation of *E. coli* XL1-Blue/pHU183 at 37 °C in a 50-L fermentor when the 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, showing a similar pattern to the plasmid content per cell. The 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 units/L, almost similar to that obtained by fed-batch culture using glucose at 30 °C, which was mainly due 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 an agarose gel preparation, and the plasmid-free cells were not detected during the cultivation even without selection pressure.

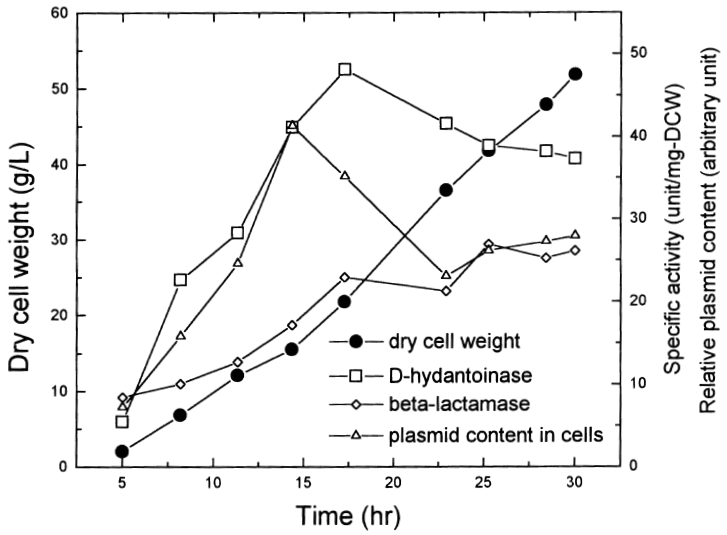

FIGURE 2. Batch cultivation of *E. coli* XL1-Blue/pHU183 using glycerol as a carbon source at 37 °C. Cultivations were carried out in a 50-L fermentor containing 35 L of the R-medium. The batch cultivation was carried out in duplicate, and the data represent mean values.

CONCLUSIONS

In an effort to develop a better process for large-scale production of D-hydantoinase, 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-products such as acetate are less produced when glycerol is used as a carbon source. The growth of *E. coli* XL1-Blue/pHU183 was not repressed even with a high concentration of glycerol, and no catabolite repression was observed. As a result, 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. Furthermore, batch cultivation can be conducted more simply and reliably at an industrial scale. Cost analysis of the entire process for the production of D-amino acids revealed that the media costs for both culture methods are less than 1% of the total production cost. From these analyses, it is thought that glycerol-based batch culture of recombinant *E. coli* XL1-Blue/pHU183 can be employed for industrial production of D-hydantoinase.

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