INDUCIBLE PRODUCTION OF RECOMBINANT XYLOSE ISOMERASE BY *ESCHERICHIA COLI* IN FED-BATCH CULTURE

TOMOYASU KAWABE,** TAKAYUKI OHSHIMA, NOBUYUKI UOZUMI,
SHINJI IJIMA AND TAKESHI KOBAYASHI

*Department of Biotechnology, Faculty of Engineering, Nagoya University,
Chikusa-ku, Nagoya 464-01*

Key Words: Biochemical Engineering, *Escherichia coli*, Recombinant DNA, Gene Expression, *Tac* Promoter, Xylose Isomerase

Escherichia coli JM105 harboring an expression plasmid which bears the xylose isomerase gene controlled by *tac* promoter was cultivated under different conditions in order to find an optimal fermentation condition. By shake-flask cultures it was found that low concentration of organic nitrogen sources such as yeast extract or casamino acids was essential for efficient production of xylose isomerase. By fed-batch culture in a jar-fermentor, we obtained very high cell concentration. Glucose did not hamper gene expression as much as glycerol. Excess addition of yeast extract lowered the specific activity of the gene product. Although the specific activity of the enzyme in high-concentration culture was a half that in batch culture, the amount of enzyme produced by unit volume in a representative high-concentration culture was more than 33-fold that by batch culture.

Introduction

Recent advances in genetic engineering have opened a new field for efficient and cost-effective production

of proteins in cultures of recombinant cells. In particular, high-concentration cultivation can contribute to the efficient production of recombinant protein derived from the foreign gene¹³⁾. With these kinds of cultures, several difficulties arose when high-level production of foreign protein is desired. In some cases, overproduction caused stress to the host

* Received May 7, 1992. Correspondence concerning this article should be addressed to T. Kobayashi.

** T. Kawabe is now with Sumitomo Chemical Co., Takarazuka, 665.

cell growth, and thus the choice of expression systems with controlled induction of target protein production seemed to be very important. By using these controlled-expression systems, production could be induced after sufficient cell growth. We proposed that this kind of two-step culture is very effective for production using recombinant microorganisms^{6,7)}.

So far, *Escherichia coli* has been one of the most widely used microorganisms as host, and many expression plasmids with strong and inducible promoters such as *trp*, *lac* and lambda *p_{LP_R}* have been developed. We have studied high-concentration cultivation at jar-fermentor scale with these gene expression systems^{6,7,12,14)} and showed that a two-step culture is very effective for overproduction of the cloned gene product.

In this study, we applied a *tac* promoter³⁾ expression system for xylose isomerase production in a jar-fermentor. Xylose is found in abundance in cellulosic biomass. The derepression of xylose isomerase gene in the recombinant *E. coli* is mediated by the addition of isopropyl- β -D-thiogalactopyranoside (IPTG). We sought suitable cultivation conditions to obtain high concentration of biomass and also to overproduce xylose isomerase for the effective induction of gene expression with IPTG.

1. Experimental

1.1 Plasmid and microorganism

E. coli JM 105 (*thi*, *rpsL*, *endA*, *sbcB15*, *hspR4*, Δ (*lac-proAB*), [*F'*, *proAB*, *lacI^q* Δ DM15, *traD36*]) carrying the plasmid pKKX7 was kindly supplied by C. A. Batt²⁾. *Tac* promoter and *rrnB* terminator were located just upstream and downstream of the xylose isomerase gene (*xylA*); therefore, expression of the gene was controlled under *tac* promoter.

1.2 Cultivation

The recombinant bacterium was grown in fed-batch medium (FB medium¹³⁾) supplemented with 3–5 kg/m³ of glucose, 0.05 kg/m³ of ampicillin and 0.02 kg/m³ thiamine at 37°C for both jar-fermentor (Type MD, Iwashiyama Co., Tokyo) and shake-flask (T-shape flask) cultures. For some shake-flask cultures, Luria broth (Polypepton 10 kg/m³, yeast extract 5 kg/m³, NaCl 5 kg/m³, pH 7.2) and a synthetic Gray-Tatum medium⁴⁾ were used. Unless otherwise mentioned, the synthetic medium for shake-flask culture was supplemented with 2 kg/m³ of either casamino acids or yeast extract. Gene expression was induced by adding 2 mM IPTG at a cell concentration of 0.17 kg-dry cells/m³ in shake-flask cultures.

For jar-fermentor culture, the recombinant bacterium was precultured in a T-shape flask with 0.1 dm³ of FB medium containing thiamine, ampicillin, and glucose as a carbon source. This preculture in logarithmic growth phase was then transferred to the

same medium (0.9 dm³) in the jar-fermentor. Initial glucose concentration was 5 kg/m³. Glucose was fed by the DO stat technique^{13,15)}. In one fermentor culture, acetate in the medium was measured every 20 min by gas chromatography, and a small amount of glucose (about 3 g) was fed so as not to form acetate, as described by Shimizu *et al.*¹⁶⁾. Dissolved oxygen concentration was kept at a level ranging from 0.05 to 5 ppm by adjusting the air velocity and the agitation speed (300–900 rpm). In the later stage of fermentation, pure oxygen gas was also introduced to keep the dissolved oxygen concentration within this range. pH was kept at around 7 by using a pH controller (Oriental Electric Co., Tokyo) with 20% NH₃ solution and 1 N HCl. When necessary, Silicon KM-72 (Shinetsu Polymer Co., Tokyo) was added as an antifoam.

1.3 Analyses

The optical density of the fermentation broth was measured by using a spectrophotometer (UVIDE-310, JASCO, Tokyo) at 660 nm (OD₆₆₀). OD₆₆₀ was converted to dry cell weight by multiplying by a conversion factor of 0.38 kg/m³, which was determined experimentally with this microorganism. Glucose concentration in the culture supernatant was measured by a glucose analyzer (MODEL 27, Yellow Springs Instrument Co., Ohio, U.S.A.). Acetic acid concentration in the culture broth was measured by using a gas chromatograph (Type GC-7A, Shimadzu Co., Kyoto) with a Chromosorb 101 column (Gasukuro Kogyo Co., Tokyo). To determine the xylose isomerase activity, cells were collected by centrifugation (8,000 rpm, 10 min) and suspended in 25 mM Tris-HCl buffer pH 7.5, followed by sonic disruption at 50 W for 5 min on ice (Ohtake Works Co, Tokyo). Cell debris was removed by centrifugation at 10,000 rpm for 10 min. The xylose isomerase reaction was performed by using xylose as the substrate, and the product (xylulose) concentration was then determined by using sorbitol dehydrogenase as described by K. Yamanaka¹⁷⁾. One enzyme unit (1 kat) was defined as the amount of the enzyme to convert 1 mol of xylose per second. The specific activity was defined as the activity per kg of protein. Protein concentration was determined by the method of Lowry *et al.*⁹⁾

To check the plasmid stability, each sample, aseptically withdrawn from the culture, was plated on Luria broth agar after suitable dilution. After overnight incubation at 37°C, 100 colonies were replica plated on a Luria broth agar containing 0.05 kg/m³ ampicillin and the percentage of antibiotic resistant colonies was counted after incubation at 37°C.

2. Results

2.1 Determination of carbon and nitrogen sources for cell growth and xylose isomerase production

Prior to cultivation of the recombinant *E. coli* using a jar-fermentor, preliminary experiments were performed in shake-flasks to determine the procedure for the induction of gene expression. With regard to the specific activity of xylose isomerase, the synthetic media (FB medium or Gray-Tatum medium) gave 4-fold higher value than did complex medium (Luria broth). The two synthetic media showed similar specific activity. We chose FB medium since we have succeeded in high-concentration cultivation of *E. coli* using it^{5,15)}.

As reported previously^{1,8,10)}, synthesis of exogenous enzyme requires amino acid sources, especially after the induction of gene expression by IPTG. Furthermore, we found that organic nitrogen sources such as yeast extract sometimes stabilized recombinant plasmid^{5,11)}. We therefore studied the effects of yeast extract and casamino acids on recombinant protein production. The specific activities with casamino acids and yeast extract supplemented media were 68.3 mkat/kg-protein and 81.6 mkat/kg-protein respectively after IPTG induction (data not shown). On the other hand, FB medium without organic nitrogen sources gave 50.2 mkat/kg-protein. Therefore, the specific activity of the gene product was increased 1.4–1.6-fold by adding either organic nitrogen source. From this result, we determined to use either of the organic nitrogen sources for fermentor-level cultures.

As carbon sources, we compared glucose with

glycerol for the gene expression from *tac* promoter. The latter is known as a substrate less sensitive to catabolite repression. Although the specific activity was at a similar level in these two substrates, glucose gave better growth than glycerol. Hence, the FB medium with glucose was used in the subsequent studies. A similar effect of carbon sources was also confirmed in high-concentration culture, as shown in **Table 1**.

We cultured the recombinant microorganism using the jar-fermentor in a medium containing yeast extract as organic nitrogen source. **Figure 1** shows the results of batch culture without feeding any nutrients, which resulted in very low cell concentration. Gene expression was induced by adding 2 mM IPTG at a cell concentration of 0.17 kg-dry cells/m³. The cell concentration at the induction was exactly the same as that in shake-flask cultures. The maximum specific activity was 65.5 mkat/kg-protein, which was similar to the level obtained in shake-flask cultures. In this culture the initial glucose and yeast extract concentrations were 2.5 kg/m³ and 3 kg/m³ respectively. Plasmid was unstable after adding the inducer, and 70% of the microorganism lost the plasmid at the end of the culture. When gene expression was induced, there was 2 kg/m³ of glucose and the substrate was not starved at the end of the culture. These results confirmed again that glucose did not hamper gene expression from *tac* promoter.

2.2 Fed-batch culture

1) High cell concentration culture without induction As a control, we cultured the microorganism in FB medium without the induction of gene expression by IPTG (**Fig. 2**). In the later stage of fermentation

Table 1. Summary of fed-batch cultivations under various conditions

Fermentation number	Final cell concentration [kg/m ³]	Maximum specific activity [mkat/kg-protein]	Maximum total activity [kat/m ³]	Maximum acetate concentration [kg/m ³]	Cell concentration at induction [kg/m ³]	IPTG concentration [mM]	Organic nitrogen source ^{a)} [kg/m ³]
<i>E. coli</i> JM 105/pKKX7, Glucose as a carbon source							
1 (batch)	1.1	65.5	0.06	0.7	0.2	2.0	3(YE)
2	74.9	12.8	0.91	19.4	19.0	0.5	16(YE)
3	63.8	24.9	1.25	2.1	19.0	2.0	7(YE)
4	47.5	25.2	1.16	1.5	15.2 + 38.0 ^{b)}	0.2 + 0.4 ^{b)}	9(YE)
5	48.6	32.0	1.21	7.2	19.0	2.0	10(CA)
6	65.0	27.7	2.00	4.7	19.0	4.0	10(CA)
7	30.1	31.5	0.51	5.5	7.6	2.0	16(CA) + 3(YE)
8	26.5	31.2	0.65	9.0	19.0	2.0	22(CA) + 6(YE)
9 (without induction)	75.1	1.9	0.16	3.1	—	—	3(YE)
<i>E. coli</i> JM 105/pKKX7, Glycerol as a carbon source							
10	46.7	14.2	0.68	10.0	19.0	2.0	20(YE)

^{a)} CA and YE represent casamino acids and yeast extract respectively. These nitrogen sources were supplemented intermittently during cultivation.

^{b)} The IPTG was added sequentially.

Fig. 1. Induction of gene expression from *tac* promoter by IPTG in batch culture

Fig. 2. Fed-batch culture of recombinant *E. coli* without induction by IPTG

Thin arrow shows the time when 3 kg/m³ of yeast extract was added

(10 h), 3 kg/m³ of yeast extract was supplied. In Fig. 2, glucose concentrations measured every hour are also shown. They were between 0–5 kg/m³, and no evident acetate accumulation from glucose was detected. In this culture, the microorganism grew very well and the final cell concentration was 75.1 kg-dry cells/m³. The final activity and specific activity were 1.9 mkat/m³ and 0.16 mkat/kg-protein respectively. In this culture, plasmid was almost stable and 3.1 kg/m³ of acetate was finally accumulated.

2) Inducible production of xylose isomerase Next, we tried to induce gene expression at a similarly high cell concentration. Since organic nitrogen source

Fig. 3. Induction of gene expression from *tac* promoter by IPTG

A: Total 7 kg/m³ of yeast extract was supplied. Thin arrows show the time when 1 or 2 kg/m³ of yeast extract was added
B: Total 16 kg/m³ of yeast extract was supplied. Thin arrows show the time when 4 kg/m³ of yeast extract was added

facilitated recombinant protein production in shake-flask culture, we cultured the microorganism in FB medium containing yeast extract. As shown in **Fig. 3A**, production of the enzyme was induced by the addition of 2 mM of IPTG at a cell concentration of 19 kg-dry cells/m³. The final cell concentration became 63.8 kg-dry cells/m³ and the maximum activity and specific activity were 1.25 kat/m³ and 24.9 mkat/kg-protein respectively. The specific activity was 40% of that obtained with the batch culture shown in Fig. 1. In this culture, a total of 7 kg/m³ of yeast extract was added after induction. **Figure 3B** shows the results for a similar culture, to which total 16 kg/m³ of yeast extract was added. In this culture, cells grew very well and the final cell concentration was 74.9 kg-dry cells/m³. On the other hand, the enzyme specific activity and the activity were reduced to 12.8 mkat/kg-protein and 0.91 kat/m³ respectively. These results suggested that higher concentration of yeast extract inhibited gene expression from *tac* promoter. Plasmid was lost after induction, and less than 40% of the microorganism contained the plasmid in both cultures shown in Figs. 3A and B (data not shown). In this host vector system, IPTG seemed to destabilize the plasmid. In fact, when IPTG was added in the early logarithmic-growth phase (1.9 or 7.6 kg-dry cells/m³), evident plasmid loss and decrease in specific activity in the latter stage of culture were observed (data not shown).

Figure 4 shows that gene expression was induced at a cell concentration of $19 \text{ kg-dry cells/m}^3$ by adding 2 mM IPTG and a total of 10 kg/m^3 of casamino acids. The final cell concentration was $48.6 \text{ kg-dry cells/m}^3$. The enzyme activity and specific activity were 1.21 kat/m^3 and $32.0 \text{ mkat/kg-protein}$ respectively. At 10 h , the specific activity was $4.5 \text{ mkat/kg-protein}$. Therefore, the specific activity increased almost 7-fold by induction. This value of specific activity was a little higher than that obtained with yeast extract (Fig. 3A). In this culture, the percentage of plasmid-bearing cells dropped gradually after IPTG addition, and 6 kg/m^3 of acetate was accumulated at the end of the culture.

To obtain higher enzyme production, the concentration of IPTG was increased to 4 mM with addition of a total of 10 kg of casamino acids (data not shown). The final cell concentration was $65.0 \text{ kg-dry cells/m}^3$ and the maximum activity and specific activity were 2.00 kat/m^3 and $27.7 \text{ mkat/kg-protein}$ respectively. In this culture, increased concentration of IPTG did not affect the specific activity of the gene product. Plasmid was also lost after induction. The specific activities obtained in these two cultures were almost half that obtained in the batch culture shown in Fig. 1.

3) Effects of various metabolites In high-concentration cultures of *E. coli*, acetate is usually formed from excess glucose and inhibits cell growth and sometimes enzyme production. Therefore, we studied the effect of acetate, especially on the specific activity of gene product, so as to know the reason why the specific activity of the gene product in high-cell concentration culture was low. In our studies in shake-flask culture, 5 kg/m^3 of acetate slightly affected the cell growth, but up to 10 kg/m^3 of acetate had no effect at all on the specific activity of the gene product. In addition to acetate, we could detect low concentrations of formate and lactate in the culture (maximum $3\text{--}4 \text{ kg/m}^3$). However, up to 4 kg/m^3 of these organic acids affected neither cell growth nor specific enzyme activity.

In the jar-fermentor culture shown in Fig. 3A, acetate accumulated to around 20 kg/m^3 during the culture. Thus, we tried to prevent acetate formation so as to improve the specific activity. In **Fig. 5**, glucose was fed in such a way as not to form acetate, as described by Shimizu *et al.*¹⁶⁾, and a total of 9 kg/m^3 of yeast extract was added. To induce gene expression, 0.2 and 0.4 mM IPTG was added sequentially. By this method of addition, plasmid became stable and 1.16 kat/m^3 of enzyme was produced. The maximum specific activity was $25.2 \text{ mkat/kg-protein}$. This value was similar to that for the culture shown in Fig. 3A and thus was not improved. These results suggested that acetate is not a major factor in

Fig. 4. Induction of gene expression from *tac* promoter by IPTG in medium containing casamino acids (Total 10 kg/m^3)

Thin arrows show the time when 2 kg/m^3 of casamino acids was added

Fig. 5. Induction of gene expression from *tac* promoter at low acetate concentration

Thin arrows show the time when 1 or 2 kg/m^3 of yeast extract was added

limiting the specific activity.

To look for other factors in the broth which reduce the specific activity in high-concentration cultivation, gene expression was also induced in spent medium supplemented with glucose and 2 kg/m^3 of either yeast extract or casamino acids by shake-flask culture. For preparation of the spent medium, the microorganism was grown in FB medium supplemented with 6 kg/m^3 of casamino acids. When cell concentration became $19 \text{ kg-dry cells/m}^3$, the microorganisms were removed by centrifugation and the supernatant was sterilized by filtration. The specific

activities of the cultures using spent medium were of a similar level to that with fresh FB medium containing 2 kg/m³ of either casamino acids or yeast extract. This indicated that metabolites in the broth do not hamper the gene expression.

Discussion

In fed-batch culture using a jar-fermentor we could obtain a high cell concentration, more than 60 kg-dry cells/m³. Therefore, the total amounts of the gene product (corresponding to the enzyme activity) obtained by the fermentor culture after induction were more than 33-fold that obtained by the batch culture. However, the specific activities, which corresponded to the extent of gene expression, obtained with the high-concentration cultures were only about half that with the batch culture shown in Fig. 1. We have cultured *E. coli* bearing *trp* and lambda *p_{LP}* promoters expression system at high cell concentration. However, the specific activities in high-concentration cultures were almost the same as those in shake-flask cultures. The kind of problem just described did not occur with those systems.

To seek the reason for this lower specific activity, we studied several culture conditions. *Tac* promoter is a composite promoter of *trp* and *lac* control units. Gene expression from *tac* promoter is repressed by *lac* repressor but not by *trp* repressor nor glucose. However, the choice of sugar substrate sometimes had unexpected effects on gene expression in high-concentration cultures. For instance, gene expression from *trp* promoter was unexpectedly repressed by glucose¹⁴⁾. Therefore, we studied the effects of glucose but did not detect any inhibitory effects on gene expression from *tac* promoter.

We also surmise that growth inhibition by acetate or plasmid loss by IPTG caused this problem. To make these points clear, we added glucose so as not to form acetate (Fig. 5). However, the specific activity of the gene product was lower, being comparable with that of the other fed-batch cultures. We also tried to keep plasmid stable by adding a low concentration of IPTG little by little (Fig. 5). By this manner of addition, plasmid loss was partly prevented but we could not get higher specific activity of the gene product. In the present study, glucose was controlled very roughly so as not to limit cell growth. However, the above results indicate that substrate concentration did not have profound effects on production except for the culture shown in Fig. 3A, in which a high concentration of acetate was accumulated.

As mentioned above, low concentration of organic nitrogen sources was essential for production. However, excess yeast extract in turn seems to show inhibitory effects on specific activity. Table 1

summarizes the results of all fermentor-scale cultures. In fact, 16 kg/m³ of yeast extract gave a specific activity of 12.8 mkat/kg-protein (fermentation No. 2), but 7 or 9 kg/m³ of yeast extract (fermentation No. 3, 4) gave a value of around 25 mkat/kg-protein. This result suggests that higher concentration of yeast extract lowered the specific activity of the gene product. On the other hand, casamino acids did not show such inhibitory effect in the range of 10–22 kg/m³ (Table 1, fermentation No. 5–8), and gave a specific activity of 28–32 mkat/kg-protein. Therefore, selection of suitable nitrogen sources and optimal concentration of nitrogen source may be very important. We are studying this point as well as identifying the inhibitory substances in yeast extract.

Conclusion

In this paper, we cultured a recombinant *E. coli* bearing a *tac* gene expression system at a high concentration, and the productivity of the recombinant protein was very high compared to that of a conventional batch culture. However, the specific activity of the gene product was almost a half of that with batch culture. We found that organic nitrogen sources were essential for production but that a component in yeast extract hampered the extent of gene expression. For further improvement of productivity, identification of the inhibitory substance is necessary.

Acknowledgment

This work was supported in part by a Grant-in-Aid (No. 04453125) for Scientific Research from the Ministry of Education, Science and Culture of Japan.

Literature Cited

- 1) Anderson, K. B., E. Grulke and P. Gerhardt: *Bio/Technology*, **2**, 891 (1984).
- 2) Batt, C. A., E. O'Neill, S. R. J. Ko and A. J. Sinskey: *Biotechnology Progress*, **2**, 140 (1986).
- 3) De Boer, H. A., L. J. Comstock and M. Wasser: *Proc. Natl. Acad. Sci. U.S.A.*, **80**, 21 (1983).
- 4) Gray, C. H. and E. L. Tatum: *Proc. Natl. Acad. Sci. U.S.A.*, **30**, 404 (1944).
- 5) Iijima, S., K. Kai, S. Mizutani and T. Kobayashi: *J. Chem. Technol. Biotechnol.*, **36**, 539 (1986).
- 6) Iijima, S., S. Kawai, S. Mizutani, M. Taniguchi and T. Kobayashi: *Appl. Microbiol. Biotechnol.*, **26**, 542 (1987).
- 7) Kawai, S., S. Mizutani, S. Iijima and T. Kobayashi: *J. Ferment. Technol.*, **64**, 503 (1986).
- 8) Landwall, P. and H. W. Doelle: *J. Gen. Microbiol.*, **17**, 23 (1977).
- 9) Lowry, O. H., N. J. Rowebrogh, A. L. Farr and R. J. Randall: *J. Biol. Chem.*, **193**, 265 (1951).
- 10) Luli, G. W. and W. R. Strohl: *Appl. Environ. Microbiol.*, **56**, 1004 (1990).
- 11) Matsui, T., H. Sato, S. Sato, S. Mukataka and J. Takahashi: *Agric. Biol. Chem.*, **54**, 619 (1990).
- 12) Mizutani, S., S. Iijima and T. Kobayashi: *J. Chem. Eng.*

- Japan*, **19**, 111 (1986).
- 13) Mizutani, S., H. Mori, S. Shimizu, K. Sakaguchi and T. Kobayashi: *Biotechnol. Bioeng.*, **28**, 204 (1986).
- 14) Mizutani, S., H. Naito, S. Iijima and T. Kobayashi: *J. Chem. Eng. Japan*, **21**, 658 (1988).
- 15) Mori, H., T. Yano, T. Kobayashi and S. Shimizu: *J. Chem. Eng. Japan*, **12**, 313 (1979).
- 16) Shimizu, N., S. Fukuzono, K. Fujimori, N. Nishimura and Y. Odawara: *J. Ferment. Technol.*, **66**, 187 (1988).
- 17) Yamanaka, K.: *Agric. Biol. Chem.*, **33**, 834 (1969).