Journal of Microbiology and Biotechnology
Vol. 2, No. 1, 35-40, 1992

Influence of Plasmid Properties on Fermentation Parameters of Recombinant *Escherichia coli*

LEE, IN YOUNG, DONG JIN SEO, AND SUN BOK LEE[1]*

Genetic Engineering Research Institute, KIST,
P.O. Box 17, Taedok Science Town, Taejon 305-606, Korea
[1]Department of Chemical Engineering, Pohang Institute of Science and Technology P.O. Box 125, Pohang 790-600, Korea

Received 30 December 1991 / Accepted 25 January 1992

The influence of the nature of plasmids on fermentation parameters such as cell growth, cell viability, plasmid stability, and product formation has been investigated using *E. coli* M5248 and its recombinant derivatives M5248 [pBR322], M5248[pAS1], and M5248[pNKM21]. At a low temperature (30℃), the cell growth, cell viability, and protein synthesis of the recombinants were nearly identical to those of the host cell. However, at high temperature (42℃), in which transcription from the P_L promoter is derepressed, the recombinant cells showed decreased stability along with lower growth rates and cell viability. The ratio of total protein to cell mass was in the order of *E. coli* M5248>M5248[pBR322]>M5248[pAS1]>M5248[pNKM21]. It was found that transcription from the P_L promoter adversely affect the plasmid maintenance and host cell metabolism even in the absence of the cloned-gene expression. Furthermore, profiles of β-lactamase activity were shown to vary with recombinant strains. *E coli* M5248[pBR322] showed highest β-lactamase activity at 30℃, while at 42℃ β-lactamase activity was significantly reduced irrespective of the strains. The effect of the plasmid properties on plasmid-encoded gene expression has been further examined based on the relationship between β-lactamse activity and plasmid-harboring cell numbers.

The productivity of recombinant organisms depends not only on environmental and operating conditions in a bioreactor system but also on genetic characteristics of recombinants (14). Therefore, recombinant fermentation parameters of cell growth, plasmid stability, and product formation would be determined by both environmental and genetic factors. The environmental factors, which are basically the same process variables used in the conventional fermentation processes, include temperature, pH, medium composition, aeration rate, agitation speed, etc (3, 14). On the other hand, the genetic factors that render characteristics to a given recombinant strain include gene dosage, efficiency of transcription and translation, and stability of recombinant DNA molecules (14, 16). Such genetic parameters of recombinant cells are determined by the genetic background of the host and nature of recombinant plasmid.

Previously, we have investigated the production of recombinant human interleukin-2(rhIL-2) using an rhIL-2 producing recombinant *E. coli* M5248[pNKM21] (2, 9, 10), From these studies, it was found that the productivity of rhIL-2 and the metabolic activity of recombinants were highly affected by the environmental factors such as medium composition and culture temperatures. Optimal fermentation conditions for maximizing desired products by recombinant strains were found to be different even the case where the same gene expression system was employed for the regulation of cloned-gene synthesis (10).

In this paper we have investigated the influence of the properties of recombinant plasmid on fermentation parameters such as cell growth, viability, plasmid stability, and expression of plasmid-encoded gene product using

*Corresponding author
Key words: Recombinant fermentation, properties of plasmids, pBR322, pAS1, pNKM21

E. coli M5248 and its derivatives transformed with the plasmid of pBR322, pAS1, and pNKM21, respectively. The recombinant plasmids used in this work have the same replicon (*ori*) and ampicillin-resistance gene (*bla*) of pBR322. The results shown in this paper may provide a more comprehensive understanding on the host-vector interaction of recombinant microorganisms. Once a fundamental biochemical engineering study is established for many host systems, our in-depth knowledge base can be significantly advanced, and a rational strategy for improving and/or optimizing recombinant fermentation process can be developed.

MATERIALS AND METHODS

Microorganisms and Plasmids

Escherichia coli M5248 (λ*bio*275 cI857 ΔHI) which contains the cI857 gene on its chromosome was used as a host strain. Recombinant microorganisms used in this work are *E. coli* M5248 strains transformed with pBR322, pAS1, or pNKM21 plasmid. As described earlier pNKM21 plasmid (5, 6) was constructed by inserting the coding sequence of human IL-2 gene into pAS1 vector (13). The expression vector pAS1, which contains the P_L promoter of bacteriophage lambda, is a derivative of pBR322. Therefore all the plasmids used in this work have the same replicon and *bla* gene of pBR322 as shown in Fig. 1.

Fermentation Conditions and Analytical Methods

Composition of medium and fermentaion conditions used in this study are identical to those employed in the previous works (9, 10). Recombinant cells were grown under three different modes of operation: constant-temperature (CT) modes at 30℃ or 42℃, and temperature-shift (TS) mode at 42℃. In the case of TS mode, the culture temperature was changed to 42℃ after 8 h cultivation of recombinant cells at 30℃. Analytical methods are also the same as those described in the companion report (10).

Table 1. Difference in the properties of the strains used in this study

Strains	pBR322 *ori*	pBR322 *bla*	P_L promoter	rhIL-2 gene
E. coli M5248	−	−	−	−
E. coli M5248[pBR322]	+	+	−	−
E. coli M5248[pAS1]	+	+	+	−
E. coli N5248[pNKM21]	+	+	+	+

RESULTS AND DISCUSSION

Summary of Data

In this study, the effect of plasmid properties on fermentation parameters has been investigated using a homologous series of *E. coli* strains, i.e. *E. coli* M5248, M5248[pBR322], M5248[pAS1], and M5248[pNKM 21]. Differences in the properties of the strains are summarized in Table 1 (see also Fig. 1).

The plasmid pAS1 (13) carries the bacteriophage λ P_L promoter, and the P_L-containing λ sequences are inserted between *Hin*dIII and *Bam*HI restriction sites within the Tc^r gene of pBR322 (1). The plasmid pNKM21 (5, 6) is made of pAS1 and a *Bam*HI-*Pst*I restriction fragment derived from cDNA of the human interleukin-2(IL-2) gene. In this plasmid, small parts of pUC8 vector (between *Pvu*II and *Pst*I sites) are incorporated because the IL-2 gene has been initially cloned into the pUC8. In pNKM21, a cysteine residue at position 125 of native IL-2 was changed to a serine residue by site-directed mutagenesis (5).

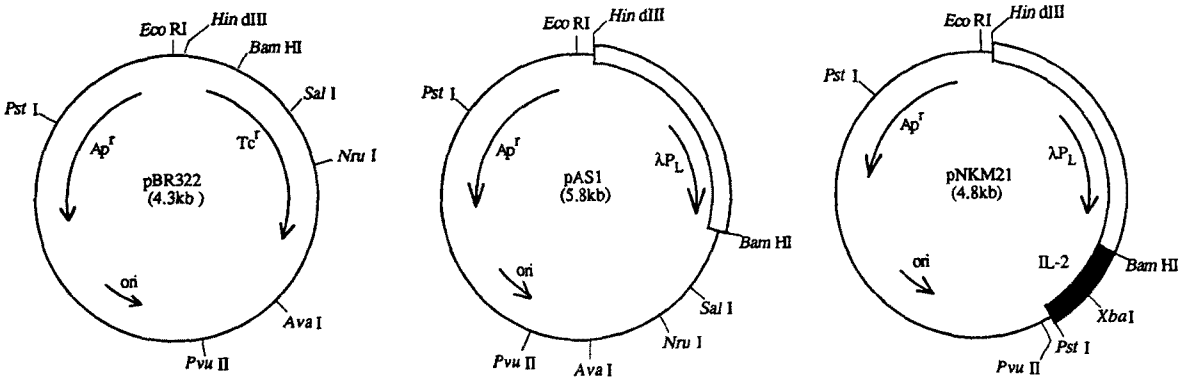

Fig. 1. Schematic diagram of plasmids pBR322, pAS1, and pNKM21.

Batch cultures were carried out under three different culture temperature modes: (1) constant temperature (CT) at 30℃, (2) temperature shift (TS) from 30℃ to 42℃, and (3) constant temperature at 42℃. For each run of fermentation, except *E. coli* M5248, the following parameters have been determined: (i) cell concentraion, (ii) total protein concentration, (iii) the number of viable cells, (iv) the fraction of plasmid-harboring cells, and (v) β-lactamase activity. In the case of *E. coli* M5248 (a host strain of recombinants) the plasmid stability and β-lactamase activity were not determined since this strain does not harbor the plasmid.

The experimental data obtained in this work are summerized in Fig. 2. The data for pNKM21 are indentical to those reported in the companion paper (10). In the followings, the effect of plasmid properties on fermentation parameters has been analyzed in a similar way that used in a previous paper (10).

Cell Growth and Protein Synthesis

Since the host cell provides all the facilities and precursors required for the biosynthesis of the product, cellular metabolic activity is one of the most important factors to produce the protein of interest efficiently. Therefore the effect of culture temperature on the cell growth and viability was examined.

When the recombinant microorganisms were cultivated at 30℃, the cell growth and protein synthesis were similar to those of the host cell irrespective of nature of the plasmid. In case that the culture temperature was shifted from 30℃ to 42℃ at the mid-exponential growth phase (8 hrs), cell growth and accordingly protein synthesis were noticeably retarded after a temperature shift. The final cell (protein) concentration of rhIL-2 producing strain (M5248[pNKM21]) was lowest, which implies that rhIL-2 production severely inhibits the cell metabolism. Unexpectively, the final cell (protein) concentration of M5248[pBR322] was also reduced by a temperature shift.

On the other hand, at 42℃ the lag phase of recombinant cells was prolonged as compared to the host cell (*E. coli* M5248). Cell growth was approximately in the order of *E. coli* M5248>M5248[pBR322]>M5248

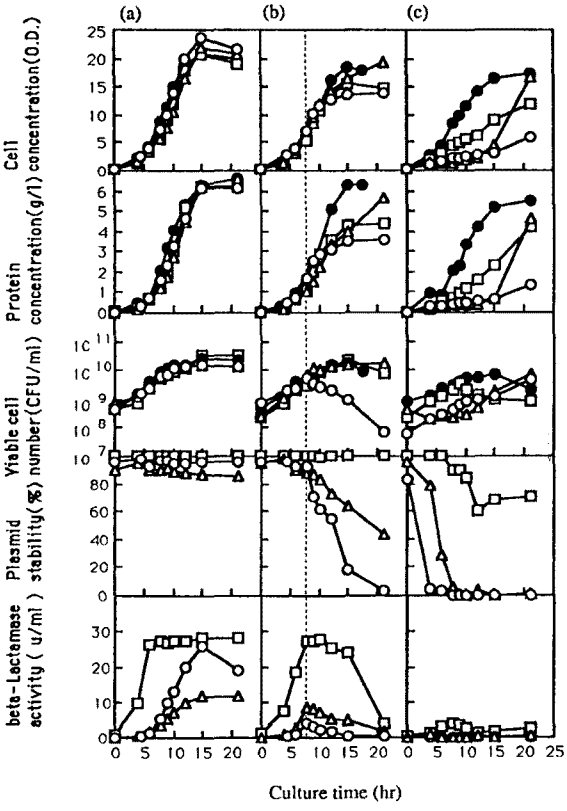

Fig. 2. Effect of culture temperature and plasmid properties on fermentation parameters of *E. coli*.
Culture conditions are: (a) CT mode at 30℃, (b) TS mode at 42℃, (c) CT mode at 42℃. Symbols: (●) M5248, (□) M5248[pBR322], (△) M5248[pAS1],(○) M5248[pNKM21].

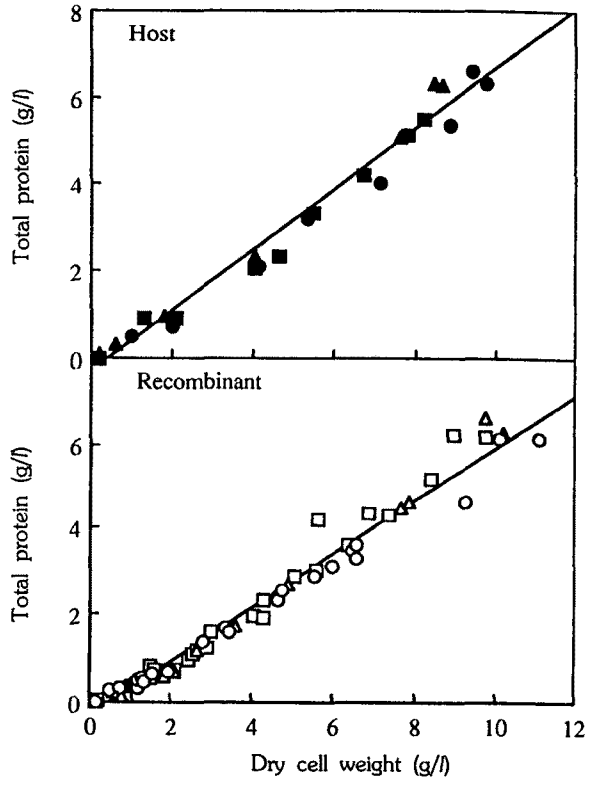

Fig. 3. Relationship between total intracellular protein and dry cell weight.
Host: (●) CT mode at 30℃, (▲) TS mode at 42℃, (■) CT mode at 42℃. Recombinant: (□) M5248[pBR322], (△) M 5248[pAS1], (○) M5248[pNKM21].

[pAS1]>M5248[pNKM21], which illustrates that extra burden on the cell metabolism was increased due to the presence of the P_L promoter and the rhIL-2 gene. There are indications that the growth of recombinant cell is also influenced by the characteristics of the cloned-gene product (11, 12). According to the results of Remaut *et al.* (12), for example, the growth rate decreases sharply after the β-interferon gene is expressed through a temperature shift while a slight increase in growth rate was observed after gene expression in the case of T4 DNA ligase prodution.

In all cases, total intracellular protein concentration was proportional to the cell concentration. In Fig. 3, the data for the host cell and recombinant cells are shown separately. Correlation factor (γ) between dry cell weight and total protein level was 0.988 irrespective of strains. The dependence of protein-to-cell mass ratio on culture temperature was not observed (upper part in Fig. 3), which is consistent with the previous report (10). However, the ratio of total protein to cell mass was slightly different depending on the types of strains. The difference in the protein-to-cell mass ratio between the host cell and recombinants can be seen (Fig. 3). The slopes of individual regression lines for M5248, M5248[pBR 322], M5248[pAS1] are 0.692, 0.665, 0.648, and 0. 565, respectively.

Cell Viability

The data for the number of viable cells during each fermentation are shown in the third panel of Fig. 2. At 30℃, the viable cell number closely follows the cell growth curve. The number of cells, irrespective of the strains, reached $2\text{-}3\times10^{10}$ cells/ml by the end of the fermentation. After a temperature shift, the increase in cell numbers continued but in a slow fashion and ultimately leveled off. Viable cell counts for the experiments of *E. coli* M5248, M5248[pBR322] and M5248[pAS1] gave no appreciable difference. In the case M5248[pNKM21], however, the number of viable cells declined rapidly following induction of the rhIL-2 gene expression. This shows that production of rhIL-2 detrimental to the host cell metabolsim.

Viable cell counts for the CT mode experiment at 42℃ gave very different results than the TS mode experiment. Unlike the low-temperature results, the viable cell number does not follow the cell mass curve. For example, the specific growth rate of the host cell (*E. coli* M5248) calculated from the viable cell counts was much lower than that obtained from the cell mass measurements as shown in Fig. 4. This indicates that even in the absence of the plasmid the cell viability of *E. coli* is reduced at higher culture temperatures. In the case of *E. coli*[pBR322] the number of viable cells reaches a maximum at nine hours and decreases thereafter,

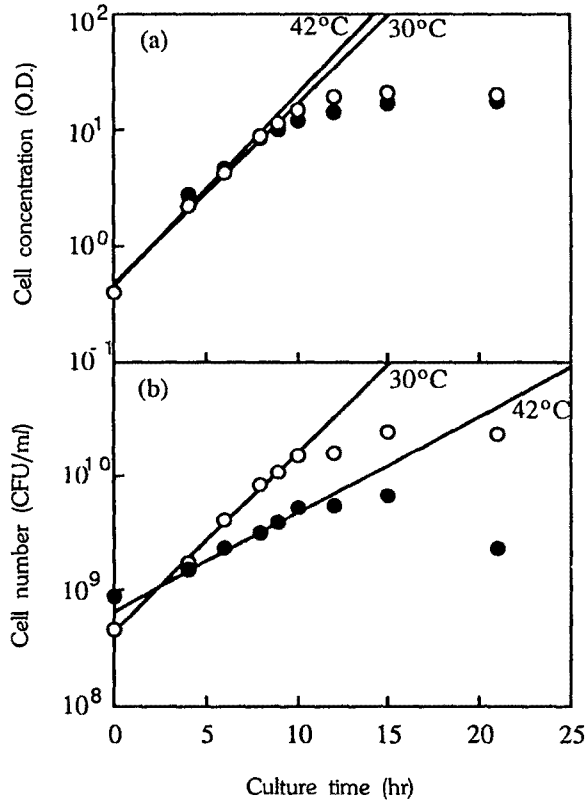

Fig. 4. Effect of culture temperature on cell mass and viable cell numbers.

Culture conditions are: (○) CT mode at 30℃, (●) CT mode at 42℃. Specific growth rates (h^{-1}) are: (a) 0.363(30℃), 0.383 (42℃), (b) 0.358(30℃), 0.173(42℃).

although the cell concentration continues to increase. The patterns of viable cell counts for M5248[pAS1] and M5248[pNKM21] are significantly different from those observed in the temperature-shift experiment. However, a similarity exists when comparing cell viability for the two recombinant cells. The number of viable cells remained at lower level until most of plasmids are lost and then the increase in viable cell numbers continued for the remainder of the fermentation. This result is reasonable because the increase in viable cell numbers at later times in the fermentation is caused by the growth of plasmid-free cells.

Plasmid Stability

The fourth panel of Fig. 2 shows the plasmid stability of recombinant microorganisms at different culture conditions. At 30℃ all the recombinant cells remained relatively stable. In the case of temperature-shift mode of operation, however, the plasmid stability was found to be significantly dependent on the properties of the plasmid.

The stability of *E. coli* M5248[pBR322] was unaffec-

ted by a temperature shift from 30℃ to 42℃. In the case of M5248[pAS1], on the other hand, the stability was considerably decreased upon turning on the P_L promoter. This reflects that transcription from the P_L promoter, even in the absence of the cloned gene, significantly influences the stability of plasmid. When a strong promoter is used, the transcription of the replicon contained in the same vector system is often affected adversely as a result of transcription in opposite directions from strong promoters (4, 15). These findings might support our result that excessive transcription from the P_L promoter of the pAS1 plasmid interferes with plasmid maintenance. Reduction of plasmid stability is even more profound in the case of M5248[pNKM21]. It appeares that production of rhIL-2 is detrimental to both cell growth and plasmid maintenance.

When the recombinants were cultivated under CT mode at 42℃ the fraction of plasmid harboring cell populations was more significantly reduced as compared to the case of TS mode of operation. Within 8 h of cultivation at 42℃, more than 90% of pAS1 plasmid was lost and pNKM21 was lost even more rapidly than pAS1. The stability of *E. coli* M5248[pBR322] was also reduced to about 60~70%, in contrast to the TS mode of operation, and the appearance of small transparent colonies was observed during prolonged cultivation at 42℃.

In Fig. 5 the number of plasmid-harboring cells in the culture determined by multiplying the number of viable cells by the plasmid-harboring cell fraction is shown for each fermentation. As described in a previous paper(10), only plasmid-harboring cell populations can produce the gene product encoded in the plasmid. The changes in plasmid-harboring cell populations with the properties of the plasmid are distinct at a higher temperature.

β-Lactamase Activity

Since all the recombinant microorganisms used in this work contained the same β-lactamase (*bla*) gene derived from pBR322, the β-lactamase of the recombinant strains was measured and compared to determine the effects of the plasmid properties on plasmid-encoded gene expression (Fig. 2).

For the experiments under CT mode at 30℃, the cell growth, the cell viability and plasmid stability were similar irrespective of strains. It was therefore expected that β-lactamase would be formed at a similar level in these recombinant strains. However, profiles of β-lactamase activity were found to be changed significantly depending on the recombinant strains. *E. coli* M5248[pBR322] showed highest β-lactamase activity, followed by M5248 [pNKM21] and M5248[pAS1] in that order. This result was different from our expectation, and formation of β-lactamase was likely to be influenced by the nature of the plasmid.

For the temperature-shift experiment, β-lactamase activity of M5248 [pAS1] and M5248 [pNKM21] at 42℃ was significantly reduced presumably due to the P_L-promoted transcription and hence the decrease in the number of plasmid harboring cells. After a temperature shift to 42℃, β-lactamase activity of M5248[pBR322] was also decreased. This may result from the thermal inactivation of β-lactamase or the inhibition of β-lactamase synthesis at high temperatures. In order to discriminate these two possibilities, changes in β-lactamase activity during incubation for one day at 42℃ were examined using β-lactamase enzyme partially purified from *E. coli* M5248[pBR322] which was cultivated at 30℃. In view that no activity loss of β-lactamase was observed (data not shown), diminition of β-lactamase activity at 42℃ is likely to be due to the inhibition of the enzyme formation at this temperature. There is a report supporting

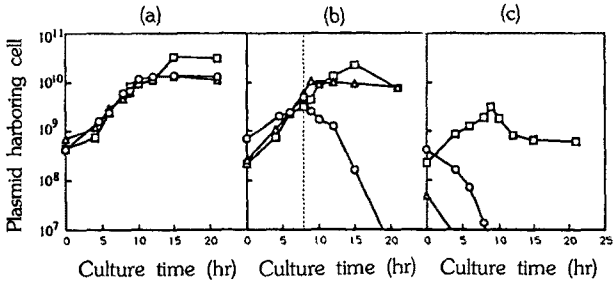

Fig. 5. Effect of culture temperature on total number of plasmid-harboring cells in the culture.
Culture conditions are: (a) CT mode at 30℃, (b) TS mode at 42℃, (c) CT mode at 42℃, Symbols: (□) M5248[pBR322], (△) M5248[pAS1], (○) M5248[pNKM21].

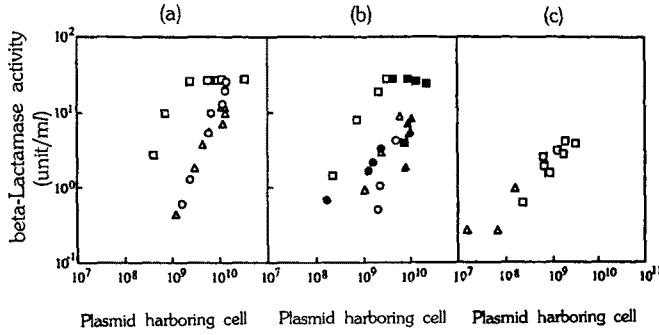

Fig. 6. Relationship between β-lactamase activity and the number of plasmid-harboring cell.
See Fig. 5 for the legend. Closed symbols in (b) represent the data after a temperatue shift.

this possibility. Recently, Kuriki has reported that the synthesis of β-lactamase in *E. coli* K-12[pBR322] is repressed with a temperature shift-up from 30 ℃ to 42℃ (7). He has also found, from *in vitro* experiments, that a factor required for efficient synthesis of pBR322-encoded β-lactamase is inactivated at higher temperatures above 40℃ (8).

In order to further examine the *bla* gene expression efficiency of each recombinants, the relationship between β-lactamase activity and plasmid-harboring cell numbers has been analyzed. Fig. 6(a) shows that at 30℃ the apparent gene expression efficiency—determined using a linear regression analysis of the β-lactamase activity versus plasmid-harboring cells—is nearly identical for both M5248[pAS1] and M5248[pNKM21] (cf. β-lactamase data in Fig. 2). In Fig. 6(b), the data for temperature-shift experiments are shown. After a temperature shift-up (closed symbols), dependences of β-lactamase activity on the number of plasmid-harboring cells appear to be different from those at 30℃ (open symbols). Furthermore, a decrease in gene expression of pBR322-encoded β-lactamase at 42℃ is apparent when comparing Fig. 6(c) with Fig. 6(a).

Acknowledgement

We thank to Ki-Joo Kim and Min Gon Kim for their help in analyzing experimental data. This work was supported by grants from the Ministry of Science and Technology and the Korea Science and Engineering Foundation.

REFERENCES

1. **Bolivar, F., R.L. Rodriguez, P.J. Greene, M.C. Betlach, H.L. Heynecker, and H.W. Boyer.** 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. *Gene* **2**: 95-113.
2. **Chung, B.H., D.J. Seo. Y.H. Park, S.B. Lee, and M.H. Han.** 1991. Production of recombinant human interleukin-2 by *Escherichia coli* with computer controlled temperature induction. *Biotechnol. Techniq.* **5**: 163-168.
3. **Fiechter, A.** 1984. Physical and chemical parameters of microbial growth. *Adv. Biochem. Eng. Biotechnol.* **30**: 7-60.
4. **Gentz, R., A. Langer, A.C.Y. Chang, S.N. Cohen, and H. Bujard.** 1981. Cloning and analysis of strong promoters is made possible by the downstream placement of a RNA termination signal. *Proc. Natl. Acad. Sci. USA* **78**: 4936-4940.
5. **Kang, S, J. Kwak, J.B. Kwon, S.W. Kim, I.Y. Lee, S.B. Lee, H.-Y. Yun, K.-S. Hahm, and D.S. Na.** 1989. Production of Ser^{125}-interleukin-2 by site directed mutagenesis. *Korean Biochem. J.* **22**: 306-311.
6. **Kang, S., S.W. Kim, H.J. Ha, D.S. Na, S. Park, J.Y. Kim, and M.H. Han.** 1988. Overproduction of human interleukin-2 in *E. coli*. *Korean Biochem. J.* **21**: 38-45.
7. **Kuriki, Y.** 1987. Response to temperature shifts of expression of the *amp* gene on pBR in *Escherichia coli* K-12. *J. Bacteriol.* **169**: 2294-2297.
8. **Kuriki, Y.** 1987. Requirement of a heat-labile factor(s) for in vitro expression of the *amp* gene of pBR322. *J. Bacteriol.* **169**: 5856-5858.
9. **Lee, I.Y., M.K. Kim, D.S. Na, K.-S. Hahm, M.H. Han, and S.B. Lee.** 1988. Optimization of fermentation conditions for production of recombinant human interleukin-2 in *E. coli*. *Kor. J. Appl. Microbiol. Bioeng.* **16**: 327-333.
10. **Lee, I.Y., M.K. Kim, and S.B. Lee.** 1992. Effect of induction temperature on the P_L promoter controlled production of recombinant human interleukin-2 in *Escherichia coli*. *J. Microbiol. Biotechnol.* submitted for publication.
11. **Remaut, E., P. Stanssens, and W. Fiers.** 1981. Plasmid vectors for high-efficiency expression controlled by the P_L promoter of coliphage lambda. *Gene* **15**: 81-93.
12. **Remaut, E., P. Stanssens, and W. Fiers.** 1983. Inducible high level synthesis of mature human fibroblast interferon in *Escherichia coli*. *Nucleic Acids Res.* **11**: 677-688.
13. **Rosenberg, M., Y.-S. Ho, and A. Shatzman,** 1983. The use of pKC30 and its derivatives for controlled expression of genes. *Methods Enzymol.* **101**: 123-138.
14. **Ryu, D.D.Y. and S.B. Lee.** 1988. Development and optimization of recombinant fermentation processes. p. 95-124. *In* S. Aiba(ed.), Horizons of Biochemical Engineering, University of Tokyo Press.
15. **Stueber, D. and H. Bujard.** 1982. Transcription from efficient promoters can interfere with plasmid replication and diminish expression of plasmid specified genes. *EMBO J.* **1**: 1399-1404.
16. **Zabriskie, D.W. and E.J. Arcuri.** 1986. Factors influencing productivity of fermentations employing recombinant microorganisms. *Enzyme Microb. Technol.* **8**: 706-717.