# Optimization of Recombinant *E. coli* Fermentation through Biological Manipulation and Engineering Control

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## I. Introduction

In general, no set of hard-and-fast rules exists when high-level expression of a cloned gene is needed because the expression of each gene presents its own unique set of problems (Goeddel, 1990). Nevertheless, some common factors require careful consideration once a particular expression system is chosen to produce a desired protein. Of the various expression systems available, *Escherichia coli, Bacillus subtilis, Saccharomyces cerevisiae,* a number of fungi, mammalian cells, plant cells, and insect cells, I will focus exclusively on *E. coli*.

Many *E. coli* host-vector systems for the production of cloned gene proteins are available and sophisticated expression vectors have been developed to overcome the difficulties encountered when overproduction is desired. Fast growth, availability of low cost cultivation technology, and the vast amount of advanced knowledge related to its genetics and physiology make *E. coli* a valuable host for the production of cloned gene proteins, despite the drawbacks of its limited secretion capacity and lack of certain post-translational modifications of proteins (Balbas and Bolivar, 1990). Many recombinant proteins produced by the *E. coli* host-vector system are shown in Table 1.

Overproduction of the protein encoded by a cloned gene in the *E. coli* expression system is dependent on several factors: genetic factors, environmental factors, and the physiological state of the host cell. All of these factors closely interact with each other and affect the overall productivity of the cloned gene protein (Fig. 1). In order to optimize the production of cloned gene proteins, maximization of the cloned gene expression and optimization of fermentation conditions need to be achieved. Maximizing the expression can be accomplished by designing and constructing an efficient expression vector. Various fermentation parameters, such as medium composition, oxygen transfer rate, pH, temperature, and state and control variables involved in recombinant fermentation processes can be manipulated to find optimal cellular environmental conditions.

## II. Genetic Manipulation of Gene Expression

### 1. Instability of Recombinant DNA

The instability of recombinant DNA is one of the most important problems in the commercial application of many recombinant cells. There are two types of instability problems: structural and segregational. Structural instability is the result of physical changes in the recombinant DNA, such as deletions, insertions, and rearrangements. Segregational instability is caused by uneven distribution of plasmids during cell division (Primrose and Ehrlich, 1981). Both types of instability result in a lower productivity.

**Table 1.** Recombinant proteins produced by *E. coli* expression system (Hodgson, 1993).

| | |
|---|---|
| Immunomodulators | Interferon-alpha |
| | Interferon-gamma |
| | IL-1 |
| | IL-2 |
| | IL-3 |
| | IL-6 |
| | TNF-beta |
| Growth Factors | G-CSF |
| | M-CSF |
| | GM-CSF |
| | Fibroblast growth factor |
| | TGF-alpha |
| | Epidermal growth factor |
| | Platelet-derived growth factor |
| | Connective tissue activator peptide |
| | Angiogenin differentiation-inducing factor |
| Hormones | Insulin-like growth factor 1 |
| | Insulin-like growth factor 2 |
| | Human growth hormone |
| | hGH releasing factor |
| | Somatostatin |
| | Calcitonin |
| | Relaxin |
| | Insulin |
| | Proinsulin |
| | Beta-endorphin |
| Blood proteins | Human serum albumin |
| | Hemoglobin |
| | Factor XIII |
| | t-PA |
| | Prourokinase |
| | Alpha-1-antitrypsin |
| | Apolipoprotein A-I |
| | Apolipoprotein A-IV |
| | Apolipoprotein E |
| | Atrial natriuretic factor |
| | Platelet factor 4 |
| Inhibitors | Elastase inhibitor |
| | Lipocortin |
| Enzymes | Lysozyme |
| | SOD |
| | Renin |
| Vaccines | Whooping cough |

Structural instability is more insidious than segregational instability because the DNA of interest is lost or altered without changing the remainder of the plasmid DNA, including the selectable markers and partition elements. An increased probability of rearrangements is expected when DNA sequences, such as inverted repeats or highly palindromic sequences, exist within the vector and the passenger DNA. To date no means exists to accurately predict whether a particular gene or DNA construction will be stable. Reconstruction of the vector using different strategies and the recA$^-$ strains have been used as alternate approaches to deal with the potential structural instability problem (Balbas and Bolivar, 1990).

Segregational instability, which is usually worse under the conditions of gene expression than the repressed condition for a cloned gene, can be alleviated by inserting certain genes into vectors: (1) genes for selection (antibiotic resistant genes), (2) survival function in combination with a host that requires the corresponding gene product for survival (*ssb, valS, dapD*) (Nilsson and Skogman, 1986; Porter et al., 1990; Degryse, 1991), or (3) partitioning systems (*par*) (Zurita et al., 1984; Skogman et al., 1983; Gerdes, 1988; Wood et al., 1990). The lambda vector system has also been used for stable gene expression (Padukone et al., 1990; Park et al., 1991).

The addition of antibiotics during fermentation is usually expensive and inefficient in large scale fermentors. In addition, the final product can become contaminated by the added antibiotics. The expression of the plasmid-encoded survival function should not interfere with normal cellular functions. Also, the metabolite or product should not diffuse into culture media or accumulate in the cytoplasm. In that case, the daughter cells may take up or inherit the product while losing the plasmid and still survive (Balbas and Bolivar, 1990). The incorporation of a cis-acting partitio-

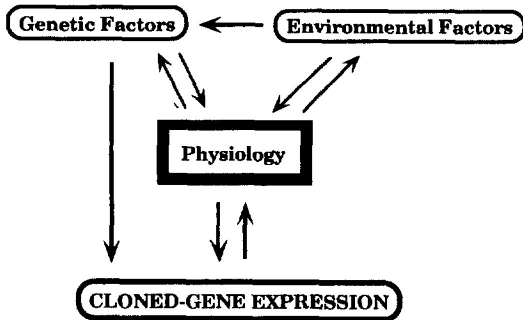


**Fig. 1.** Schematic diagram of interrelationship between the factors involved in the expression of a cloned gene.

ning element has shown that the utility of this approach is restricted: the partition function was not as good as expected when the plasmids with the partitioning gene actively expressed cloned genes (Nilsson and Skogman, 1986; Skogman et al., 1983; Lee and Edlin, 1985). Unless a multistage fermentation system is used, the lambda vector system also has its own limitations, such as contamination of products with phages and discontinuity of the process. Since each strategy to avoid the instability problem of recombinant DNA has limitations, it is advisable to choose an appropriate alternative method depending on the situation, or to use the methods which combine the principles of bioreactor design and operating system such as a two-stage continuous culture.

2. Gene Dosage

In principal, the productivity of a cloned gene protein is expected to be proportional to gene dosage or plasmid copy number (Uhlin and Nordstrom, 1977). However, productivity does not increase beyond a certain level when the gene dosage is too high since the primary metabolism of the host organism is severely affected and the rate-limiting step is not dependent on the number of gene copies at higher copy numbers (Zabriskie and Arcuri, 1986). The method of optimally controlling the plasmid copy number becomes important in certain cases (Ryu and Lee, 1987). The plasmid copy number is largely determined by the genetic characteristics of the plasmid and growth conditions. In some cases, the plasmid copy number is controlled by deregulating the plasmid replication system (Uhlin et al., 1979) or by using a dual origin plasmid (Yarranton et al., 1984).

3. Gene Expression

A. Transcription

Transcription efficiency depends, to a great extent, on the promoter strength, which is determined by the promoter sequence and the specific sequences in the vicinity of the promoter. The general features of the promoters recognized by $E\sigma^{70}$, the major RNA polymerase in *E. coli*, have been described (Gralla, 1990). For maximum productivity, a strong, easily controlled promoter is highly desirable because of the following reasons: (1) usually the plasmids containing uncontrollable, strong promoters are highly unstable; (2) some cloned gene products are very toxic to *E. coli*; (3) the degradation or instability of gene products can be minimized when the products are made at a higher rate with controllable promoters rather than when the protein is synthesized throughout the whole fermentation time (Ryu and Lee, 1987). The most utilized promoters in *E. coli* expression systems are *trp*, *lac*, $P_L$, and *tac*. The T7 RNA polymerase expression system is also used in combination with one of the controllable promoters (Studier et al., 1990; Tabor and Richardson, 1985; Studier and Moffatt, 1986).

Ideally, to avoid instability, lower growth rates, or lethality, specific transcription from such promoters should be tightly repressed until the onset of induction by adding inducers or by depleting or inactivating repressors. This condition is not easily achieved when a multicopy plasmid is employed as an expression system because some transcriptions escape regulation (Balbas and Bolivar, 1990). To overcome this problem, a novel approach was made: A strong promoter was oriented away from the gene to be expressed. When the

culture reached a desired optical density, this promoter was inverted and turned on by adding the λInt product and a short heat pulse, so the possibility of basal transcription could be eliminated (Podhajska et al., 1985; Hasan and Szybalski, 1987).

To prevent the leaky transcription in the T7 RNA polymerase expression system, a *lac* operator sequence was inserted just downstream of the start site of a T7 promoter. The *lac* repressor acted both to repress transcription of the T7 RNA polymerase gene by *E. coli* RNA polymerase and to block transcription of the target gene by any T7 RNA polymerase made from the basal transcription. This double repression significantly reduced the basal expression of the target gene in uninduced cells but seemed to have little effect on the level of expression on induction (Studier et al., 1990).

B. Translation

Translation efficiency depends on translation initiation and elongation rates, although the formation of initiation complex is believed to be rate limiting in translation (Hershey, 1987). The important factors determining the translation initiation event are an initiation codon, a Shine-Dalgarno (SD) sequence or ribosome binding site (RBS), the spacing between these two regions, the nucleotide sequences of the spacing region, and the mRNA secondary structure (Stormo, 1986). An example that the actual coding sequence can have a great effect on translation efficiency was provided: a certain mutation in the third position of the fourth codon resulted in a more than 30-fold increased expression level of the human γ-interferon gene in *E. coli* (de Boer and Hui, 1990). Other approaches to increase translation initiation efficiency include a two-cistron expression system (Schoner et al., 1990) and a specialized ribosome system (de Boer and Hui, 1990).

Elongation of a nascent polypeptide chain does not proceed at a constant rate because various regions of the mRNA act as a regulatory mechanism for gene expression (Yanofsky, 1981) and the pattern of nonrandom codon usage appears to vary with the levels of gene expression (de Boer and Karstelein, 1987). *E. coli* does not necessarily use the same codons for each amino acid as other organisms do and the presence of the rare codons in cloned genes might be expected to reduce translation efficiency. However, the relevance of codon usage, and therefore of tRNA availability, in high-level expression systems has not yet been clearly demonstrated (Robinson et al., 1984; Holm, 1986; Ernst and Kawashima, 1988). Bonekamp et al. (1989) claimed that translation rates of individual codons were not affected by tRNA abundance or codon usage. On the other hand, the stimulatory effect by resynthesized and optimized codons was reported by Wong et al. (1988).

4. Stability of mRNA

Not much information exists on the enzymes and other factors involved in the mRNA stability, and few examples of mRNA stabilization for the purpose of protein overproduction have been given (Balbor and Bolivar, 1990). It has been known that two 3'→5' exonucleases, RNaseII and polynucleotide phosphorylase, would be primarily responsible for the major degradation of mRNA (Donovan and Kushner, 1986; Deutscher, 1985; Brawerman, 1987). The insertion of ρ-independent terminator or one or more REP sequences is thought to increase the half-life of mRNA because the highly structured mRNA 3' ends are expected to act as a barrier for the 3'→5' exonucleolytic degradation of the transcript (Merino et al., 1987; Newbury et al., 1987). For this reason, some bacteriophage-derived stabilizing sequences and the phage effector trans-acting function may also be included in the expression system (Gorski et al, 1985). The use of the g10-L (T7 bacteriophage gene 10 leader) has remarkably increased translation efficiency, often one hundred fold or more (Olins and Rangwala, 1990). This stimulating effect

seems to be due to a combination of increased mRNA stability with a potent enhancement of translation initiation (Olins and Rangwala, 1989). As another way of dealing with the potential mRNA instability problems, mutants for the known major ribonucleases have been examined, but the variety of findings suggest that no single RNase is solely responsible for the degradation of every RNA (Kennell, 1986).

5. Stability of Protein

In many cases, degradation of a cloned gene protein is responsible for the failure of high-level expression. But what determines the instability of a protein is not known, even though there are many examples of unstable proteins synthesized in *E. coli* (Buell et al., 1985; Furman et al., 1987; Kishimoto et al., 1986; Taniguchi et al., 1980; Sakaguchi et al., 1988). There are several approaches that can minimize protein degradation: (1) fusion proteins, (2) secretion, (3) inclusion body formation, and (4) protease mutants.

The in-frame fusion of stable carrier proteins to the C or N terminus of a labile protein can protect it from proteolysis (Schulz et al., 1987; Hammarberg et al., 1989; Butt et al., 1989). It has been suggested that the carboxy-terminal amino acids may play a role in determining the extent of proteolysis (Parsell, 1990).

Secretion of the proteins which are sensitive to degradation into a growth medium or periplasm of *E. coli* can be another way of avoiding protein degradation (Wong et al., 1988; Chang et al., 1987). To do this, leader peptides from various periplasmic or outer membrane proteins of *E. coli* are fused to the protein of interest and the fusion protein is then targeted for secretion. However, the transport of the fusion protein into the periplasm or growth medium can not be controlled, but results are rather empirical. The proper processing of the fusion protein by signal peptidase is also unpredictable (Boggosian et al., 1991).

Proteins produced in an aggregated form are generally resistant to the normal proteolysis (Kitano et al., 1987; Kleid et al., 1981). The formation of inclusion bodies is desirable only when the polypeptides can be renatured efficiently, although that is not usually the case for large and structurally complex proteins (Georgiou and Bowden, 1991).

Protease mutants can be used to limit the protein degradation. Several protease mutants have been characterized (Maurizi et al., 1985; Baneyx and Georgiou, 1990; Strauch et al., 1989), but most of the studies on mutations in proteases and their effects on the protein degradation have involved the *lon* gene encoding protease La, a major ATP-dependent protease (Goldberg and Goff, 1986; Gottesman, 1989). In many instances, the *lon* mutations have proved useful in stabilizing cloned gene proteins (Buell et al., 1985; Obukowicz et al., 1988; Sambucetti et al., 1986). One drawback with the *lon* mutants is the pleiotrophy, that is, UV sensitivity and a mucoid phenotype. One genetic method of avoiding the pleiotrophy is to introduce a second mutation into the *lon* mutants. Such mutations in *gal* and *cps* have been used to inactivate the structural or regulatory genes necessary for capsule synthesis (Trisler and Gottesman, 1984). Inactivation of *sulA* by mutation should eliminate the lethal filamentation phenotype that comes out in response to DNA damage (Gottesman, 1990). Cells defective in sigma-32, the product of the *htpR* gene, are generally defective in proteolysis (Baker et al., 1984; Goff et al., 1984) and have been used to increase the accumulation of cloned gene proteins (Buell et al., 1985). Cells carrying both the *lon* and *htpR* mutations, in some cases appear to prevent the degradation of foreign proteins better than either one alone (Gottesman, 1990). Moreover, mutations in *clp*, a second ATP-dependent protease, may allow an improvement in the ability of *lon*$^-$ mutant hosts to stabilize foreign proteins (Gottesman, 1990).

Numerous examples in the literature have been

reported of cloned gene proteins accumulating to relatively low levels intracellularly in *E. coli*. In many instances when proteolysis seems to be a problem, however, the rate of protein synthesis can actually be the limiting factor. Furthermore, each cloned gene protein has its own unique set of properties governing its susceptibility to proteolysis in *E. coli*. Thus, more fully assessing the utility of protease mutants on a case-by-case basis following optimization of transcription and translation is necessary (Bogosian et al., 1991).

## III. Environmental Manipulation of Gene Expression

In the following sections, how environmental factors can be manipulated for maximal expression will be reviewed. Manipulating gene expression can be performed by adjusting conditions or by operating a novel bioreactor. In recombinant fermentation processes, basically the same process variables or parameters that are used in the traditional fermentation processes can be considered. However, more accurate control strategies and techniques are required because recombinant cells are more sensitive to such environmental conditions as dissolved oxygen, medium composition, and temperature.

### 1. Growth Rate Control

It has been known that plasmid content, instability, and gene expression efficiency are influenced by growth rate (Engberg and Nordstrom, 1975; Seo and Bailey, 1985; Noack et al., 1981; Reinikainen and Virkajarvi, 1989; Lee et al., 1988). The copy number of a plasmid is determined primarily by the genetic makeup of the plasmid but is also strongly influenced by the genetics and physiology of the host cell. Since the plasmid content within a certain range will affect the productivity of a cloned gene protein, the plasmid copy number should be maintained at a certain level that will give maximal productivity and minimal interference with the host cell physiology. It is expected that the optimal plasmid copy number will be different depending on cloned genes and/or promoters, even if the same parental plasmid is used for each construction. Different plasmids can show different degrees of stability and the same plasmid can exhibit different degrees of stability in different hosts (Zabriskie and Arcuri, 1986).

The kinetics of plasmid stability are also influenced by the specific growth rate of the host cell (Lee et al., 1988). In a batch system,

$$\frac{dX^+}{dt} = \mu^+ X^+ (1-\theta) \quad (1.1)$$

$$\frac{dX^-}{dt} = \mu^+ X^- \theta + \mu^- X \quad (1.2)$$

where $\theta$ is the relative segregation rate (the ratio of the specific rate of plasmid-free cell generation by segregation to the specific growth rate of plasmid-harboring cells), X is the cell concentration, $\mu$ is the specific growth rate, and t is time. Plasmid-harboring and plasmid-free cells are represented by + and −, respectively.

In a two-stage continuous system, for the first stage:

$$\frac{dX_1^+}{dt} = -D_1 X_1^+ + \mu_1^+ X_1^+(1-\theta_1) \quad (1.3)$$

$$\frac{dX_1^-}{dt} = -D_1 X_1^- + \mu_1^+ X_1^+ \theta_1 + \mu_1^- X_1^- \quad (1.4)$$

and for the second stage:

$$\frac{dX_2^+}{dt} = -D_2 X_2^+ + D_{12} X_1^+ + \mu_2^+ X_2^+ (1-\theta_2) \quad (1.5)$$

$$\frac{dX_2^-}{dt} = -D_2 X_2^- + D_{12} X_1^- + \mu_2^+ X_2^+ \theta_2 + \mu_2^- X_2^- \quad (1.6)$$

where $D_1$ and $D_2$ represent the dilution rate in the first and second stages, respectively, and $D_{12}$ denotes the dilution rate for the stream coming

from the first stage to the second stage. All the kinetic equations demonstrate that the population of plasmid-harboring cells changes depending not only on segregational instability but also on the specific growth rates. It was found that decreasing specific growth rate decreased plasmid stability (Brownlie et al., 1990; Sayadi et al., 1989). As a consequence of all the factors mentioned above, the specific growth rate of recombinant cells affects the productivity of cloned gene proteins (Curless et al., 1990; Kim and Ryu, 1991).

2. Culture Conditions

Considerable improvements in the productivity of a cloned gene protein can be obtained by manipulating culture conditions. Several researchers have published studies about the effects of environmental conditions on the expression level of a cloned gene (Jensen and Carlsen, 1990; Galindo et al., 1990; Kapralek et al., 1991; Ryan et al., 1989). Prochymosin could be obtained in the amount that can be up to 48% of the total protein when different carbon/energy sources were arranged to find an optimal fermentation condition (Kapralek, 1991). Notably the levels of protein production are different depending on the way the inocula are grown: sub-culture in a minimal medium tends to increase the production of a cloned gene protein, but sub-culture in a rich medium leads to a decrease in production (Galindo, 1990). Oxygen limitation has also been shown to affect the stability of recombinant cells (Hopkins, 1987), and in one study a maximum expression of a cloned gene was obtained at an aeration rate less than the maximum rate examined (Ryan, 1989).

An inexpensive and convenient substrate to obtain a high cell concentration is a simple carbohydrate, such as glucose (Rinas et al., 1989). But under aerobic growth conditions with high glucose concentrations or high growth rates, acetate or other metabolic by-products are formed (Doelle et al., 1977; 1982). The physiological background for acid production has been found to be a result of unbalanced rates of cell metabolism (Anderson and von Meyenberg, 1980; Holms, 1986). The acetate accumulation results in growth inhibition and reduced product yields during fermentation. Several attempts have been made to avoid or alleviate the acetate accumulation: dialysis culture (Landwall and Holms, 1977), controlled glucose feeding (Konstantinov et al., 1990), and excessive feeding of metal ions (Reiling et al., 1985).

Studies on the effect of yeast extract on fermentation performance have shown that a higher productivity was obtained with a complex medium containing yeast extract than with a minimal medium (Tsai et al., 1987). When metabolic roles of peptone and yeast extract were investigated using recombinant *E. coli* overexpressing the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene, it was found that yeast extract helped the accumulated acetate to be assimilated and peptone stabilized the GAPDH activity (Nancib et al., 1991).

MacDonald and Neway (1990) examined the effects of medium quality on the expression of human interleukin-2 (IL2) at high cell density. They replaced spent medium by perfusion with fresh medium and consequently improved IL-2 expression. Their results suggest that the buildup of waste product(s) or nutrient exhaustion before induction does not limit IL-2 production, but IL-2 accumulation is limited at least in part by the buildup of toxic waste products, such as acetate, during induction. In addition, later IL-2 accumulation appears also to have been limited by factors intrinsic to the organism or the protein itself.

3. High Cell Density Culture

In order to achieve high volumetric productivity that is one of the goals for recombinant fermentations, high cell density fermentation process can be employed as one of the bioprocessing strategies. For the high cell density culture, it is important to maintain optimum amounts of nutrients

and minimum concentrations of toxic wastes. A fed-batch culture is one of the best processes to keep substrates in a bioreactor at an appropriate level and to minimize production of toxic wastes (MacDonald and Neway, 1990; Yano et al., 1991; Shimizu et al., 1988).

During the expression of a cloned gene, recombinant *E. coli* may undergo significant physiological changes which have not been well understood. A better understanding of these physiological changes would provide valuable information on how the recombinant cells respond to such a reallocation of energy, precursors, and protein synthesizing machinery. Thus, better models for controlling nutrient feeding during overexpression of a cloned gene can be developed to increase both biomass and product concentration (MacDonald and Neway, 1990). Since, unfortunately, such information has not been available until just recently, substrate feeding rates in the fed-batch culture have been controlled by such methods as pH-stat, DO-stat, monitoring of a limiting substrate or inhibitory substance, or controlling of growth rates (Nishio et al., 1977; Yano et al., 1991; Konstantinov et al., 1990; 1991; Shimizu et al., 1988; Chim-Anage et al., 1991). Cell concentrations obtained for genetically-engineered strains producing a recombinant proteins are generally over 40 gDCW/*l* (Zabriskie and Arcuri, 1986) and sometimes greater than 60 gDCW/*l* (Strandberg and Enfors, 1991; Riesenberg et al., 1990), although non-recombinant *E. coli* strains could be cultivated to concentrations over 100 gDCW/*l*.

4. Cell Immobilization

To overcome the difficulties of cultivating recombinant cells, immobilization of recombinant cells was developed (Dhulster et al., 1984; Mosbash et al., 1983). One potential advantage of cell immobilization is to obtain high cell concentrations and consequently reduce the reactor size and capital investment cost (Luong and Tseng, 1984).

Recombinant *E. coli* cells immobilized in carageenan gel beads were compared with free recombinant cells in a chemostat culture without selective pressure (De Taxis du Poet et al., 1986). The plasmid encoding catechol 2,3-dioxygenase (pTG201) was maintained at a stable level during 80 generations in the case of immobilized cells and the immobilized system showed a better production of catechol 2,3-dioxygenase than did the cell free system. It was speculated that special macro- and microenvironments generated by immobilizing cells in carageenan beads could provide physiological changes that would be favorable to plasmid stabilization. The plasmid pTG201 was transformed into three different hosts and plasmid stability was tested with free and immobilized cells in continuous culture systems (Nasri et al., 1987a). They found that the plasmid pTG201 was stably maintained with immobilized cells in the three strains while various degrees of stability were observed with the free cells. And the plasmid-free cells could not dominate the culture when coimmobilized and grown in competition with the plasmid-harboring cells. The mechanism by which immobilization increases the plasmid stability appears to be based on the mechanical properties of the gel bead system that allow only a limited number of cell divisions (10-16) to occur in each clone of cells before the clone escapes from the gel bead (Nasri et al., 1987b).

This recombinant cell immobilization technique was coupled to a two-stage continuous culture system to overcome the deleterious effects of high levels of the expression of a cloned gene (Sayadi et al., 1987; 1989). For the two-stage continuous culture system with immobilized cells, immobilized recombinant *E. coli* cells were produced in the first stage (repressed condition) and cells released from gel beads were continuously transferred to the second stage (derepressed condition). Using this system, after 230 generations no loss of the plasmid pTG201 was observed under the repressed condition and 3% of the recombinant

cells lost plasmid in the derepressed condition. Thus, both the good plasmid stability and high cell concentration, which can be obtained by a two-stage continuous culture system with immobilized recombinant cells lead to an increase in the overall productivity of cloned gene products in the second stage (Sayadi et al., 1987).

### 5. Bioreactor Configuration

Improvement in productivity of a cloned gene protein can also be achieved by a bioreactor configuration. One such bioreactor system for recombinant fermentation process is a two-stage continuous culture system. The idea of the two-stage continuous culture process, which was first proposed by Siegel and Ryu (1985), is to separate the growth stage from the production stage, thus increasing plasmid stability in the growth stage and maximize the productivity in the production stage. It was shown that a high level of gene product concentration could be maintained in the second stage of the two-stage continuous culture system (Lee et al., 1988). A two-stage continuous culture system with cell recycle has also been theoretically analyzed (Park et al., 1991). It was suggested that the productivity of inducible intracellular proteins could be increased in a two-stage continuous process by implementing a cell recycling system, which results in a longer induction time and higher cell concentrations than processes without the recycle.

Recently, a more practical application of the two-stage continuous process has been reported. A two-stage, cyclic fed-batch fermentation process was used to produce recombinant human lymphokine (Curless et al., 1991). The first stage is operated under repressed conditions in a fed-batch mode with a portion of the cells in the first reactor being transferred to the second reactor, in which a cloned gene is induced and the cells grown in a fed-batch mode. When the desired cell density is obtained in the second stage, a whole cycle is repeated after harvesting the cells in the second stage. The volumetric productivity of the two-stage, cyclic fed-batch process was twice that of a single-stage, fed-batch fermentation process and 30 cycles were completed with a consistent yield of human lymphokine and cell density in each cycle (Curless et al., 1991).

Another type of bioreactor configuration for recombinant fermentation is a selective recycle bioreactor. In a recycling process, both cell concentrations and volumetric productivity are increased only when the recycled cells are productive. For a selective recycle system, the concentration process must preferentially separate the desired (plasmid-bearing) strains from the undesired (plasmid-free) strains. If recombinant cells are selectively chosen through recycling, the reactor will be enriched with productive recombinant cells. This allows for maintenance of recombinant cells in the bioreactor and for continuous overproduction of a cloned gene protein at high rates (Ogden et al., 1991). It has been demonstrated that selective recycle maintained unstable recombinant cells in a continuous reactor (Henry et al., 1990a; 1990b; Ogden et al., 1991). They took advantage of bacterial flocculation to separate plasmid-harboring cells from plasmid-free cells. The plasmid-harboring cells which overproduce pilin protein are flocculent, but plasmid-free cells are non-flocculent. By installing an inclined settler, flocculent recombinant cells were successfully separated from nonflocculent cells on the basis of differences in their sedimentation velocities. However, this selective recycling system may have the same problems found with the cell immobilization since the plasmid-harboring cells sometimes become nonviable when a cloned gene protein is overproduced. One solution might be to use a two-stage continuous system equipped with an inclined settler.

## IV. Conclusion

Optimizing protein production in recombinant

*E. coli* strains involves manipulation of genetic and environmental factors. In designing a production system, attention must be paid to gene expression efficiency, culture conditions and bioreactor configuration. Although not much emphasis was given to the physiology of host strains in this review, an understanding of the relationship between the physiology of host cell growth and the overproduction of a cloned gene protein is of primary importance to the improvement of the recombinant fermentation processes. Sometimes it is desirable to make use of gene fusion systems, e.g. protein A, polypeptide, gutathione-S-transferase, or pneumococcal murein hydrolase fusion, to facilitate protein purification.

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