Analysis and Optimization of Recombinant Cell Fermentation Processes

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Introduction

Recent progress in recombinant DNA technology has generated a wide spectrum of new applications and products. These include the production of valuable eucaryotic proteins in bacteria, the selective modification of microo rganisms selectively to render new metabolic pathways, and the remodeling of proteins to improve their functional properties. Biological scientists have made rapid developments in the basic techniques of genetic engineering. Meanwhile, biochemical engineers have become increasingly involved with large-scale product manufacture from genetically engineered cells.

As recombinant DNA systems have major implications for bioprocess design and optimization, biochemical engineers are responsivle for molecular design of recombinant expression systems with desired genetic traits, analysis of fundamental characteristics and mechanisms in recombinant cells, and determination of bioreactor types and operation policies.

In connection with development of successful industrial processes based on recombinant DNA technology, a number of factors need to be considered, including genetic control sequences (promoters and transport signal sequences), cloning vehicles, bioreactor types and operating strategies. 〈Figure 1〉 shows a connection between molecular-level control of recombinant DNA systems and reactor dynamics and optimization. In order to provide a systematic guidance for design and optimization, focused experimental and theoretical investigations are required to identify the key process parameters which dictate the overall productivity of the process.

This presentation summarizes our research efforts on experimental analysis of the effects of genetic and environmental parameters on fermentation performance of recombinant cells and theoretical optimization of the process to obtain more favorable operation schemes.

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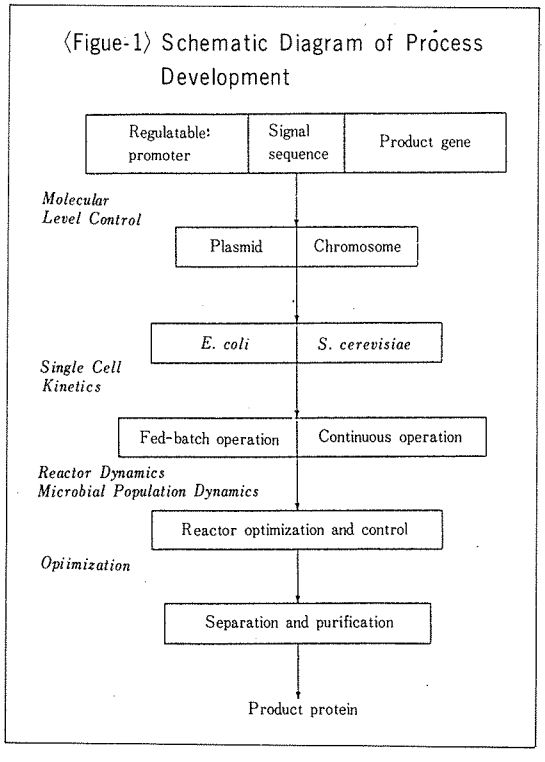
Experimental Characterization

Effects of promoters / Signal Sequences.

Design of expression vectors at the molecular level will be very important in determining the overall yield of cloned-gene protein synthesis. Among the genetic factors considered are plasmid copy number(the number of plasmid molecules), and promoters for the gene expression and signal sequences for protein transport. Previous studies performed with a series of copynumber mutants have suggested the existence of an optimum copy number for maximum productivity. This presentation will focus on regulated promoters and transport signal sequences. A promoter is a DNA site at which RNA polymerase binds and initiates transcription reactions. Promoter strength, defined operationally as the efficiency of transcription initiation, determines the expression level of a cloned-gene protein. Regulated promoters have an advantage over nonregulated promoters in that clonedgene expression can be controlled through manipulation of growth conditions. The use of regulated promoters which permit environmental switching of the clonedgene expression level will improve the product yield by decoupling the cell growth stage and the gene expression phase during the course of fermentations. This approach could be implemented in fed-batch and two-stage continuous fermentor.

〈Figue-1〉 Schematic Diagram of Process Development



〈Table-1〉 Comparison of Expression of the E.coli phoA-lacz Fusion Gene

Strain	Specific β-galactosidace Activity		
	High Pi	Low Pi	Ratio
Chromosome Integration	37.8	4,110	109
Multicopy plasmid	765	8,040	10.5

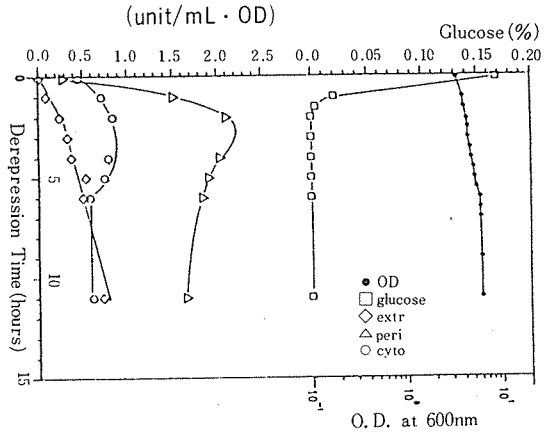
* Growth condition : MOPS-glucose medium at 37℃, PH 7.4

We have studied the gene expression behavior of the E. coli trp promoter cloned on a multicopy plasmid. The E. coli trp promoter is known to be induced by 3 β-indoleac rylic acid (IAA). As summarized in 〈Table 1〉, and increase in IAA concentration to 40 mg / l enhances the gene expression rate of the trp promoter by seven-fold while reducing cell growth rate by half. This is presumably due to the redirection of the limited biosynthetic potential of the host into making plasmid products. Model equations accounting for the experimental results are formulated and incorporated into various operating schemes in an attempt to search for the best operating policy. Theoretical analysis will be discussed later.

The other genetic component to be considered is a transport signal sequence which directs

cloned proteins out of the cytoplasm of host cells. Secretion minimizes many problems associated with the overproduction of foreign proteins in microorganisms. These include product degradation by proteases, difficulty in product separation, and formation of inclusion bodies which, although resistant to protease attack, requires an unfolding and refolding process for biological activity. In order to obtain secretion, positioning of a signal sequence of 20-25 amino acids upstream of the product gene is necessary, even though such a signal sequence is not sufficient for secretion in general.

〈Figue-2〉 Specific Invertase Activity (unit/mL · OD)



We have studied protein transport in S. cerevisiae(better known as baker's yeast) using two plasmid systems as a model : one containing the SUC2 structural gene fused with the MFα1(α-factor) signal sequence, the other containing the entire SUC2 gene(coding for invertase). The yeast SUC2 segment provides a regulated promoter and a transport signal sequence. The MFα1 and SUC2 signal sequences were able to transport, respectively, 83% and 77% of cloned invertase out of the cytoplasm to the periplasm or the fermentation broth. Kinetic analysis done with the SUC2 gene-containing plasmid demonstrated that during derepression of the SUC2 promoter, expression and transport of invertase out of the cytoplasm occurred at a faster rate than passage across the cell wall, resulting in a rapid buildup of invertase activity in the periplasmic space 〈Figure 2〉.

Research is currently in progress to character ize with flow cytometry the influence of expression and transport on metabolic states of the recombinant cells.

Effects of Cloning Vectors

The usual way of constructing recombinant expression systems is to insert a product gene together with necessary control sequences into a multicopy plasmid molecule. Multiple copies of a product gene yield a high level of the product protein and also cause allocation of the host cell's limited biosynthetic machinery to plasmid-directed synthesis, resulting in reduced growth rate and plasmid instability. Integration of a product gene into host cell's chromosome was proposed as an alternative solution to such problems. Even though this method limits the copy number to one or two copies per cell, it can increase plasmid stability as the fusion gene propagates in the same manner as the chromosome.

We attempted to quantitatively analyze and compare the expression behavior and segregati onal stability of the E. coli phoA-lacZ fusion gene cloned on a multicopy plasmid and integrated into the host cell's chromosome(Table 2). When the fusion gene was integrated to the chromosome of a lacZ-host cell, specific β-galactosidase activity was enhanced by 100-fold by changing initial phosphate concentration from 1 mM to 0.1mM. For a recombinant cell containing the fusion gene on a multicopy plas-

mid, however, only a ten times increase in specific β-galatosidase activity was achieved, mainly due to high activity of the product protein in repressed conditions. The plasmid-bearing strain produced only two times more β-galactosidase than the chromosome-integrated strain, indicating that the phoA-directed β-galact osidase synthesis is limited by other factors rather than the number of gene copies.

〈Table-2〉 Specific growth rate and Gene expression rate of recombinant E. coli containing the cloned trp promoter

IAA (μg/ml)	Specific Growth Rate (1/hr)	Gene Expression Rate (units/1/hr/OD)
0	1.08	49.5
20	0.91	326
40	0.53	367
60	0.35	284

* Growth conditions : M9 medium with 2g/l glucose and 5g/l casamino acids at 37℃
* Determined during exponential phase.

Comparison of cloning site effects has important implications for the design of expression vectors. This study indicates that chromosome integration of a fusion gene has compensating advantages over cloning in a multicopy plasmid in terms of more tight control of fusion gene expression levels. Leaky expression observed in the plasmid-bearing strain caused reduced specific growth rate.

Theoretical Optimization

Maximizing the productivity of recombinant DNA systems requires special atention to the trade-off between cloned-gene expression and the growth and biosynthetic activity of host cells. Theoretical analysis based on Pontryagin's maximum principle implies that the optimal operating strategy in general consists of an initial high growth rate stage followed by a high cloned-gene expression rate stage. As mentioned above, separation of cell growth and gene expression can be achieved by using regulated promoters which allows environmental control of a cloned-gene expression level.

We have mathematically determined optimal operating conditions in a fed-batch and two-stage continuous fermentor. The model system used is recombinant E coli containing the trp promoter cloned on a multicopy plasmid. The theoretical results revealed the existence of an optimal switching time from cell growth to product formation in a fed-batch mode and of the best combination of dilution rates in a two-stage continuous fermentation system. The methodology developed in this work can provide a framework for determination of an optimal operating scheme for novel recombinant DNA systems.

Concluding Remarks

The central theme of this presentation is a fundamental engineering study of recombinant cell fermentation processes in an attempt to provide systematic guidance for commercial applicatio of recombinant DNA technology. Integration of experimental characterization, mathematical modeling, and theoretical otimization will (1) expand our understanding and knowledgy of recombinant DNA systems at genetic and reactor levels, (2) provide a rational design policy of expression systems and fermentation processes, (3) create an infrastructure needed to train biochemical engineers for effective collaboration with biological scientists in an interdisciplinary environment, and (4) builda solid research and education base of biotechnology in academia.