BIOREACTOR STUDIES OF HETEROLOGOUS PROTEIN PRODUCTION BY RECOMBINANT YEAST

by

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ABSTRACT

Fundamental engineering studies were carried out on heterologous protein production using a recombinant *Saccharomyces cerevisiae* strain (C468/pGAC9) which expresses *Aspergillus awamori* glucoamylase gene and secretes glucoamylase into the extracellular medium, as a model system. Performance of a conventional aerobic free-suspension culture was compared to a novel immobilized-cell bioreactor bioprocess, for both batch and continuous conditions.

In the YPG nonselective medium, free-suspension batch cultures showed that cell growth was typically diauxic and glucoamylase secretion was growth-associated. Results of continuous cultures confirmed instability of the model recombinant yeast when growing in this medium. Changes in the fraction of plasmid-bearing cells and glucoamylase activity followed exponential decay patterns during continuous culture. The decay rates of both the plasmid-bearing cell fraction and glucoamylase expression increased with increasing dilution rates. Expressed as a function of cell generation, the decay rates were roughly constant over the dilution rates tested. A novel method is proposed to evaluate the instability parameters. The results indicated that the growth rate difference between plasmid-bearing and plasmid-free cells was negligible. Thus the contribution of preferential growth to apparent plasmid instability was negligible. No significant effect of growth rates on the probability of plasmid loss was observed. The importance of metabolic pathways with regard to the recombinant protein formation was analyzed. Production of glucoamylase was shown to be associated with oxidative growths of the recombinant yeast.

With the information from the experimental results and the literature, a mathematical model was formulated to simulate cell growth, plasmid loss and recombinant protein production. The model development was based on three overall metabolic events in the yeast: glucose fermentation, glucose oxidation and ethanol oxidation. Cell growth was expressed as a composite of these events. Contributions to the total specific growth

rate depended on activities of the pacemaker enzyme pools of the individual pathways. The pacemaker enzyme pools were regulated by the specific glucose uptake rate. The effect of substrate concentration on the specific growth rate was described by a modified Monod equation. It was assumed that the recombinant protein formation is only associated with oxidative pathways. Plasmid loss kinetics was formulated based on segregational instability during cell division by assuming a constant probability of plasmid loss. When applied to batch and continuous fermentations, the model successfully predicted the dynamics of cell growth (diauxic growth), glucose consumption (Crabtree effect), ethanol metabolism, glucoamylase production and plasmid instability. Good agreement between model simulations and the experimental data was achieved. Using published experimental data, model agreement was also found for other recombinant yeast strains. The proposed model seems to be generally applicable to the design, operation, control and optimization of recombinant yeast bioprocesses.

The novel immobilized-cell-film airlift bioreactor was based on cotton cloth sheets to immobilize the yeast cells by attachment. Continuous culture experiments were performed in it at different dilution rates in the YPG nonselective medium. The immobilized cell systems gave higher glucoamylase concentration (about 60%) and maintained recombinant protein production for longer periods of time (more than 100%) compared with the corresponding free suspension systems. The more stable glucoamylase production was due to a reduced plasmid loss in the immobilized cell system. By operating the immobilized-cell-film bioreactor in repeated batch mode, further reduction of plasmid instability of the recombinant yeast was obtained. An mathematical model was developed to describe the kinetics of plasmid loss and enzyme production decay based on a biofilm concept. The proposed model successfully described the experimental results and provided useful information for better understanding of the stabilizing mechanisms of the immobilized recombinant cells. It may be concluded that a reduced specific growth rate accompanied by an increased plasmid copy number is the basic explanation for the effective enhanced plasmid stability in the immobilized cell system. In the present case, the attached yeast film could be a dynamic reserve of highly concentrated plasmid-bearing

cells having a higher plasmid copy number and less segregational instability. The immobilized-cell-film airlift bioreactor design has advantages including maintenance of genetic stability, high cell concentration and cloned gene product productivity, suitability for repeated batch or continuous operations for long periods of time, stable reactor operation. Because of its enhanced operational efficiency, it may be useful in the commercial cultivation of recombinant or nonrecombinant yeast cells.

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NOMENCLATURE

a..... growth ratio (dimensionless)

B..... ratio of X^- to X^+ (dimensionless)

C_1..... activity of pacing enzyme pool for glucose fermentation (dimensionless)

C_2..... activity of pacing enzyme pool for glucose oxidation (dimensionless)

C_3..... activity of pacing enzyme pool for ethanol oxidation (dimensionless)

E..... ethanol concentration (g/L)

D..... dilution rate (h^{-1})

F..... fraction of plasmid-bearing cells (dimensionless)

G..... glucose concentration (g/L)

G_0 ..... initial glucose concentration (g/L)

k_a ..... regulation constant for C_1 [(g glucose/g cell h)$^{-1}$]

k_b ..... regulation constant for C_1 (dimensionless)

k_c ..... regulation constant for C_2 [(g glucose/g cell h)$^{-1}$]

k_d ..... repression constant for C_2 (dimensionless)

k_{det}..... cell detachment rate constant from biofilm (h^{-1})

k_f..... first-order plasmid loss constant in free cell system (h^{-1} or generation^{-1})

k_i..... first-order plasmid loss constant in immobilized cell system (h^{-1})

K_o..... saturation constant for oxygen (mg/L)

K_1..... saturation constant for glucose fermentation (g/L)

K_2..... saturation constant for glucose oxidation (g/L)

K_3..... saturation constant for ethanol oxidation (g/L)

p..... probability of plasmid loss (dimensionless)

P..... glucoamylase concentration (units/L)

q_1 ..... specific glucose consumption rate for glucose fermentation (g/g cell h)

q_2 ..... specific glucose consumption rate for glucose oxidation (g/g cell h)

q_G..... total specific glucose consumption rate (g/g cell h)

t ..... culture time (h)

t_{lag} ..... lag time (h)

T..... temperature (°C)

X..... total free cell concentration (g/L)

X^+..... free suspension plasmid-bearing cell concentration (g/L)

X^-..... free suspension plasmid-free cell concentration (g/L)

X_0^+ ..... initial steady-state free plasmid-bearing cell concentration (g/L)

X_0^- ..... initial steady-state free plasmid-bearing cell concentration (g/L)

X_i ..... total biofilm concentration (g/L)

X_i^+..... biofilm plasmid-bearing concentration (g/L)

X_i^-..... biofilm plasmid-free concentration (g/L)

$X_{i,0}^+$ ..... initial steady-state biofilm plasmid-bearing concentration (g/L)

$Y_{P/X}$ ..... growth-associated coefficient of gluccoamylse formation (units/g cell)

$Y_{X/G}^F$ ... cell yield coefficient for glucose fermentation pathway (g cell/g glucose)

$Y_{X/G}^O$ .. cell yield coefficient for glucose oxidation pathway (g cell/g glucose)

$Y_{X/E}$ ... cell yield coefficient for ethanol oxidation pathway (g cell/g ethanol)

$Y_{E/X}$ ... ethanol yield for fermentation pathway based on cell mass (g ethanol/g cell)

Greek Letters

α_2..... growth associated coefficient of glucoamylase for glucose oxidation (units/g cell)

α_3..... growth associated coefficient of glucoamylase for ethanol oxidation (units/g cell)

μ ..... specific growth rate (h^{-1})

μ^+..... specific growth rate of suspended plasmid-bearing cells (h^{-1})

μ^-..... specific growth rate of suspended plasmid-free cells (h^{-1})

μ_i ..... specific growth rate of immobilized cells (h^{-1})

μ_i^+ ..... specific growth rate of immobilized plasmid-bearing cells (h^{-1})

μ_i^- ..... specific growth rate of immobilized plasmid-free cells (h^{-1})

μ_1..... specific growth rate from glucose fermentation (h^{-1})

μ_2..... specific growth rate from glucose oxidation (h^{-1})

μ_3..... specific growth rate from ethanol oxidation (h^{-1})

$\mu_{1,max}$ .. maximum specific growth rate for glucose fermentation (h^{-1})

$\mu_{2,max}$ .. maximum specific growth rate for glucose oxidation (h^{-1})

$\mu_{3,max}$ .. maximum specific growth rate for ethanol oxidation (h^{-1})

γ_f..... first-order enzyme production decay constant in free suspension cell system (h^{-1})

γ_i..... first-order enzyme production decay constant in immobilized cell system (h^{-1})

CHAPTER 1

INTRODUCTION

From its inception only two decades ago, recombinant DNA technology has made spectacular advances. A number of recombinant products are already on the market, and many more are about to reach commercialization. This new technology offers immense possibilities for the manufacture of exotic and valuable substances that were virtually unobtainable previously.

Successful commercial development of gene products requires the coupling of recombinant DNA technology and bioprocess engineering. Recombinant DNA technology enables us to identify, clone, and transform the desired genes, and to have the host cell express the genes efficiently. The task of bioprocess engineering is to transfer DNA technology from laboratory scale to commercial scale. Unfortunately, bioprocess technology lags behind recombinant DNA technology. It is important for biochemical engineers to focus on scale-up problems related to recombinant cell cultivation and to optimize the recombinant fermentation processes.

Foreign DNA is usually introduced into host cells by employing plasmids as carriers. Bacteria and yeast are usually used as the hosts for expression of recombinant products. Ease of handling, high cell growth rate and cell density with these microorganisms are some of the attractive features. Unfortunately, the transformed cells

foreign plasmids. One major challenge in commercial production of recombinant products is to maintain the engineered genetic stability of the recombinant cells. A concerted effort involving geneticists, microbiologists, and biochemical engineers is required to overcome the genetic instability problem.

1.1. Importance of Recombinant *Saccharomyces cerevisiae*

Earlier research on recombinant DNA technology focused mainly on using procaryotes such as *Escherichia coli* as host for expression of recombinant products. It soon became obvious that procaryotes were far from the ideal hosts. Attempts to find a more suitable host led immediately to the well known yeast, *Saccharomyces cerevisiae*, brewer's or baker's yeast. Unlike *E. coli*, *S. cerevisiae* lacks detectable endotoxins and is generally regarded as a safe (GRAS) organism for the production of food and pharmaceutical products. Moreover, the rapid advancements in understanding yeast biology and genetics have made yeast genetic manipulation relatively easy. In addition, yeast grows much faster than other potential eucaryotic hosts such as animal cells; it also is able to glycosylate proteins and to carry out posttraslational foldings and modifications of eucaryotic gene products to ensure their structural integrity and biological activity. Furthermore, yeast bioprocesses had been well established and may be easily adapted to the production of recombinant proteins. Finally, expression in yeast may allow natural extracellular release of the protein because of a secretion system that is similar to that of higher eucaryotes. Biologically active, secreted products are substantially easier to recover

Table 1.1. Examples of Extracellular Recombinant Proteins Expressed in *S. cerevisiae*.

Recombinant Protein	Recombinant Yeast Strain	Reference
Human Epidermal Growth Factor	*S. cerevisiae* (AB103.1/pYαEGF-25)	Coppella et al., 1989
β-endorphin	*S. cerevisiae* (20B12/pYE/αE)	Zsebo et al., 1986
Calcitonin	*S. cerevisiae* (20B12/pYE/αcalc)	Zsebo et al., 1986
Human Atrial Natriuretic Peptide	*S. cerevisiae* (W301-18A/pJC1-5)	Vlasuk et al., 1986
Interleukin-2	*S. cerevisiae* (M2043)	Brake et al., 1984
CTAP-3	*S. cerevisiae* (AB103.1/pαEFG-24)	Mullenbach, 1986
Glucoamylase	*S. cerevisiae* (C468/pGAC9)	Nunberg et al., 1988
Human Erythropoietin	*S. cerevisiae* (RK81/pYE/SCEPO)	Lin, 1987
Pro-Urokinase	*S. cerevisiae* (CGY1891)	Turner et al., 1990
Amylase	*S. cerevisiae* (MC16/pMA230)	Walls and Gainer, 1989
Human Parathyroid Hormone	*S. cerevisiae* (FL200/pαUCXPTH)	Gabrielsen, 1990
Aprotinin	*S. cerevisiae* (WHL292/PS7041)	Barthel et al., 1993
Thermophilic Cellobiohydrolase	*S. cerevisiae* (YTY345/pNB61)	Uozumi et al., 1993
Human Nerve Growth Factor	*S. cerevisiae* (20B-12/pTK10)	Nishizawa et al., 1992
Interleukin-1	*S. cerevisiae* (pLS5-1L-1α)	Livi et al., 1990
α-peptide	*S. cerevisiae* (FY178/pAP1)	Sode et al., 1988a
Somatomedin C	*S. cerevisiae* (BJ1991/p336/1)	Sode et al., 1988b

than the denatured, inclusion body types usually produced by recombinant bacteria (Chisti and Moo-Young, 1994; Rouf et al., 1996). Due to all these advantages, it is not surprising that use of the yeast, *S. cerevisiae*, as a host for expression of recombinant proteins has rapidly accelerated in recent years. *S. cerevisiae* is now an important workhorse in industrial biotechnology and many recombinant proteins, including commercial products, are produced by recombinant *S. cerevisiae*. Table 1.1. provides an overview of some recombinant proteins expressed in *S. cerevisiae* which are secreted into the extracellular medium. Therefore, investigation and development of efficient recombinant yeast bioprocesses are very important.

1.2. Instability of Recombinant Yeast

In commercial production with recombinant microorganisms, a critical problem is the instability of the recombinant organisms harboring unstable plasmids, where instability is defined as the tendency of the transformed cells to lose their engineered properties due to the loss and/or modification of foreign plasmids. This problem is especially significant with recombinant yeasts because almost all yeast plasmid vectors are hybrid plasmids, or so-called "shuttle vectors", which are relatively unstable. Plasmid instability can be divided into two categories: structural instability and segregational instability (Ryu et al., 1988). Structural instability is often caused by the DNA's deletion, insertion, recombination, or other events, while segregational instability is caused by uneven partition of plasmids during cell division (Primrose et al., 1981; Ryu and Lee, 1988). The genetic instabilities in combination with effects of environmental factors lead recombinant

cells to lose their plasmids. During cell culture, cells lacking the plasmid can appear and a competition in the coexisting mixed culture occurs. Unfortunately, the plasmid-free cells usually have a growth advantage over plasmid-containing cells in a nonselective medium because the presence of the plasmid places additional "stress and burden" on the host cell (metabolic load or metabolic drain) (Seo and Bailey, 1985; Birnaum and Bailey, 1991; Moo-Young et al., 1996). Repeated batch cultures or continuous cultures select for the most competitive cells; hence, in time, the plasmid-free cells overwhelm the original plasmid-bearing population. Consequently, plasmid instability results in a massive loss of productivity of the desired product, thus, being a major hurdle to large scale industrial use of the genetically modified microorganisms.

After hybrid plasmids are introduced into the host yeast cells, interaction between plasmids and the host cells are substantial. This interaction will determine the genetic stability of the recombinants and the expression level of cloned genes. Both genetic factors (make-up of plasmids, copy number of plasmids, expression level, selective markers, and genetic properties of the host cells) and environmental factors (medium formulation, dissolved oxygen, temperature, dilution rate, and bioreactor operation mode) affect the plasmid stability of the recombinant yeast. With increasing understanding of the effects of these factors, it is possible to enhance plasmid stability by manipulating plasmid composition and structure, the genetic and physiological properties of the host cells, and the environmental conditions. The various strategies for enhancing genetic stability in recombinant yeast are summarized in Table 1.2. It would be useful to combine both

Table 1.2. Strategies for Enhancing Genetic Stability in Recombinant Yeast.

Genetic Methods	Environmental Methods
stabilizing genes	addition of antibiotics
efficient partition genes	amino acids deficient media
nature fitness genes	optimum dissolved oxygen tension (DOT)
efficient replicating origins	temperature shifts
optimal copy number	cyclic changes in DOT
regulation of gene expression	cyclic changes in dilution rate
suitable promoter strength	cyclic changes in substrate concentrations
antibiotic resistance makers	fed-batch cultures
copper resistance markers	two-stage continuous cultures
auxotrophic mutants	selective recycled cultures
autoselection systems	immobilization systems
killer toxin-immunity strains	

genetic and environmental methods in overcoming the instability problem. One of the most popular methods is the use of well-defined selective media in combination with the use of selection markers in plasmids and the use of an auxotrophic host mutant. Although this method is usually successful in the laboratory, it has been proven to be unsuitable for large scale culture due to inadequate selection pressure, high cost of material for defined selective media and the difficulty of the gene product recovery related to additions of selective materials (Caunt et al., 1988). Many researchers have tried to maintain plasmid stability in nonselective media by manipulating environmental factors such as temperature (Son et al., 1987), dissolved oxygen tension (Caunt et al., 1989), dilution rate (Caunt et

al., 1990; Impoolsup et al., 1989a) and bioreactor operation modes (Hardjito et al., 1993; Ogden and Davis, 1991; Ryu and Lee, 1988). Currently no effective methods are available for commercial scale culture of recombinant cells in the absence of selection pressure (Kumar and Schugerl, 1990). Investigations of the possible merits of immobilization for these recombinant cells are under way. Numerous benefits with immobilization have been found. Cell immobilization provides a promising alternative for successful commercial exploitation of the engineered organisms.

1.3. Investigations of Immobilized Recombinant Yeast Systems

Immobilization of enzymes and cells by various methods has been widely used to increase biological productivity in bioreactors (Moo-Young, 1988; Overgaard et al., 1989). Immobilized cells have a number of advantages over free cells. High cellular concentration leading to enhanced metabolic activities and productivity, separation of cells from media facilitating downstream treatment, suitability for repeated use for longer periods enhancing process efficiency, and a stabilizing effect prolonging biocatalytic activity are advantages of immobilized systems. Because of these advantages, a number of reports about immobilized recombinant cells have appeared in the literature. Enhanced genetic stability, high cell concentration and expression of recombinant proteins have been demonstrated in various recombinant cell systems in the absence of selection pressure (Kumar and Schugerl, 1990). These reports are summarized in Table 1.3.

Table 1.3. Host Cells, Plasmids, Gene Products and Immobilization Matrices Showing Enhanced Stability.

Host Cells	Plasmid	Gene Products	Matrices	References
E. coli C 600	pBR322	β-Lactamase	Hollow fiber	Inloes et al. (1983)
B. subtilis BS 273	pPCB6	Proinsulin	Alginate	Mosbach et al. (1983)
E. coli BZ 18	pTG201	Dioxygenase	Carrageenan	De Taxis et al. (1986)
E. coli JM 105	pKK223-200	Dioxygenase	Carrageenan	De taxis et al. (1987)
E. coli B	pTG201	Dioxygenase	Carrageenan	Nasri et al. (1987, 1988); Sayadi et al. (1987, 1989)
E. coli HK-8	pCBH4	Hydrogen	Polyacrylamide	Kanayama et al. (1987)
E. coli EC 147	pBR322	Amylase	Silicone Foam	Oriel (1988)
E. coli K-12	pBR322	Human Proinsulin	Agarose	Birnbaum et al. (1988)
S. cerevisiae FY178	pAP1	α-peptide	Alginate	Sode et al. (1988)
S. cerevisiae MC16	pMA230	Amylase	Gelatin	Wall et al. (1989)
S. cerevisiae MC16	p520	Wheat Amylase	Gelatin	Wall et al. (1991)

Investigations of immobilized recombinant systems began with recombinant *E. coli*. Table 1.3 shows that most of the studies were concerned with recombinant *E. coli*. Much less information is available for recombinant yeast systems. Thus, more research is required to confirm the effect of immobilization on these systems. Table 1.3 also shows

that the immobilization methods used are mainly gel entrapment. In case of hydrogel immobilized cells, carbon dioxide evolution will give rise to an internal pressure that may disrupt the matrix. Flexible, porous polymers such as cotton cloth and sponge cloth are favorable from this point of view. Mass transfer limitations and removal of dead cells are additional problems related to many entrapment methods. Selections of suitable immobilization material and methods are very important in the successful development of immobilization systems for recombinant yeast. Although many immobilized yeast systems have been developed for anaerobic ethanol fermentation, they can rarely be applied to aerobic recombinant protein fermentation using recombinant yeast.

The experimental observations indicate higher stability of recombinant cultures under immobilization conditions; however, the reasons behind the higher stability are still not quite clear. At the present time, little is known about immobilized recombinant yeast, especially about the mechanism which facilitate the high degree of plasmid stability in the immobilization system. Much work is needed to investigate any genetic, physiological or morphological changes with immobilized recombinant yeasts, hoping to understand what are the events taking place at the gene and cell levels in or/and on the supporting carriers and to provide reasonable explanation for enhanced stability observed in immobilized systems. Relatively few published reports are available about modeling of recombinant yeast cells. Such modeling can provide valuable information about the nature of recombinant yeast systems and help us design, operate, scale-up, control and optimize bioreactors.

1.4. Immobilized-Cell Bioreactors

Most previous studies relating to immobilization of recombinant cells are concerned mainly with the effect of immobilization on genetic stability. They did not address bioprocess development of immobilized recombinant cell systems. Immobilized cell bioreactor design is a key to immobilization bioprocess development. Three-phase (Gas-liquid-solid) bioreactors are necessary for systems involving immobilized cells. Gaseous reactants (oxygen) must be absorbed into the liquid phase and then transferred to the surface of the immobilized cells. At the same time, dissolved reactants are transported to the surface of the biocatalysts. Finally the biochemical reactions to products occur in the immobilized cells. All such three-phase bioprocesses involve steps of gas-liquid, liquid-solid, intrasolid phase mass transfer and biochemical reactions. The relative importance of these individual steps depends on the type of contact and interaction of the three phases. Therefore the choice of bioreactor is important for optimum performance.

Traditional immobilized cell bioreactors are usually packed-bed or trickle-bed configurations. In these bioreactor systems, overgrowth of the biomass could block the bed which could cause operational instability. The most critical problem in these bioreactor configurations is oxygen transfer limitation. Thus, they may be suitable for anaerobic fermentation such as ethanol production, but they are not suitable for aerobic recombinant fermentation. In recent years, pneumatic type three-phase airlift slurry bioreactors have been receiving increasing attention for immobilized cell culture (Chisti, 1988). However, the density of immobilized cell particles is usually very close to that of

the aqueous fermentation broth. In such systems, liquid-solid mass transfer could be a limiting step due to the low relative velocity between the immobilized cell particles and the fermentation broth. Thus, novel immobilized-cell bioreactors are needed to be developed for successful application with recombinant cells.

1.5. Objectives

In this research, a recombinant *S. cerevisiae* C468/pGAC9 (ATCC 20690), which expresses *Aspergillus awamori* glucoamylase gene under the control of the yeast enolase I (ENO1) promoter and secretes glucoamylase into the extracellular medium, is used as a model system to investigate an immobilized recombinant yeast bioprocess. The C468 strain of *S. cerevisiae* was transformed with the plasmid vector pGAC9 (2 μm-based) which contains a section of the glucoamylase gene from *A. awamori*. The 2μm-based shuttle plasmid has characteristics common to many yeast vectors and therefore serves as a good model system. The objectives of this study are to develop an efficient immobilized cell bioreactor for cultivation of the recombinant yeast cells, to investigate and compare the genetic stability and recombinant protein productivity of free and immobilized yeast cell systems, to examine the mechanisms which facilitate the high degree of plasmid stability in the immobilization system and to develop a mathematical model for describing cell growth, substrate consumption, ethanol metabolism, genetic stability, and recombinant protein production. In order to achieve these objectives, the following tasks were carried out:

1. Study the growth characteristics, the plasmid stability and recombinant protein productivity of this strain in batch and continuous suspension cultures and gain knowledge about the importance of metabolic pathways with regard to the recombinant protein formation.
2. Develop a novel immobilized-cell-film airlift bioreactor for the recombinant yeast culture .
3. Compare genetic stability and protein productivity between the free and immobilized recombinant yeast systems.
4. Model the free and immobilized yeast cell systems based on metabolic pathways, plasmid segregation and stabilizing mechanisms.
5. Seek a general explanation for the improved stability in the immobilized cell systems.

CHAPTER 2

LITERATURE REVIEW

2.1. Yeast Plasmid Vectors

Almost all yeast plasmid vectors are shuttle vectors. They are, in fact, both yeast and *E. coli* vectors, that can replicate in either organism. Figure 2.1. shows the common structure of yeast plasmids. Such vectors usually consist of all or part of an *E. coli* vector, for example pBR322, and a yeast replication system. Because of this arrangement, *E. coli* can be employed as the "preparative" organism for the various manipulations associated with recombinant DNA procedures. Yeast autonomous plasmid vectors are based on one of two replication systems: either the sequences derived from the endogenous yeast 2 μm

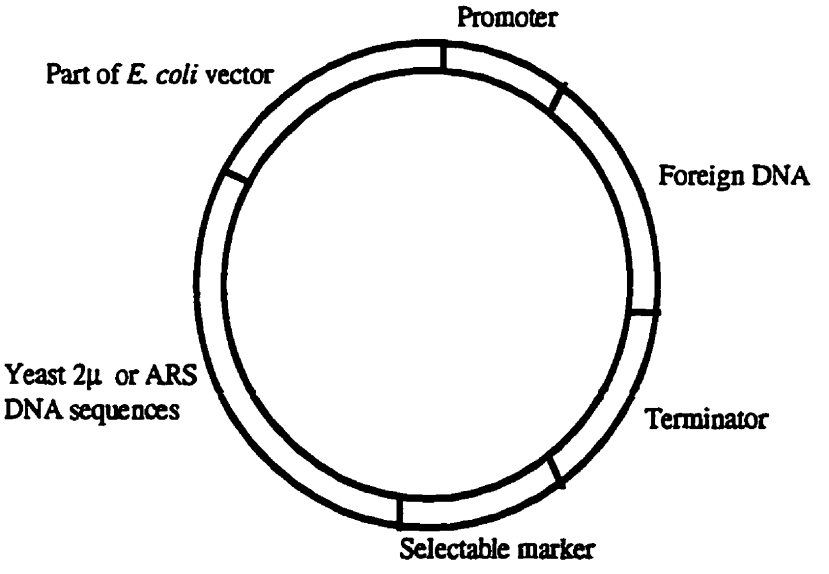


Figure 2.1. Diagram of Hybrid Yeast Plasmids

plasmid are used; or chromosomal DNA fragments that can replicate autonomously are employed (Stinchcomb et al., 1979; Kingsman et al., 1979). The latter type are usually the autonomously replicating sequences (ARSs). Such sequences presumably act as origins of replication in yeast chromosomes. Several different types of yeast vectors have been reported (Panchal, 1987): the principal ones are: (i) yeast integrating plasmids (YIp); (ii) yeast episomal plasmids (YEp); (iii) yeast replicating plasmids (YRp); (iv) yeast centromeric plasmids (YCp); and (v) yeast linear plasmids (YLp). The five groups of plasmids are summarised in Table 2.1.

Table 2.1. Properties of Yeast Vectors (Caunt et al., 1988).

Vector	Transformation frequency	Copy Number per cell	Mitotic stability	Elements of yeast DNA present
YIp	1-10	1	very stable	selectable marker, chromosomal fragment
YEp	10^3-10^5	5-40	slightly unstable	selectable marker, 2 μm origin
YRp	10^3-10^5	3-30	very unstable	selectable marker, ARS chromosomal origin
YCp	10^3-10^4	1	stable	selectable marker, 2 μm or ARS chromosomal origin,centromere
YLp	10^3-10^4	5-30	very unstable	selectable marker, 2 μm or ARS chromosomal origin, telomere

2.2. Factors Affecting Plasmid Stability in Recombinant *S. cerevisiae*

Substantial interactions between plasmid and host cell occur after recombinant plasmids are introduced into host yeast. These interactions determine the stability of plasmids, and the expression extent of cloned genes. Factors that affect these interactions may influence plasmid stability. Compositions and properties of plasmid vectors themselves affect stability, and so do physiological and genetic characteristics of the host, as well as the environmental factors. Those genetic and environmental influences are summarized in Table 2.2, and discussed in detail in the following sections.

Table 2.2. Factors Affecting Plasmid Stability in Recombinant Yeast.

Genetic Factors	Plasmid makeup Plasmid copy number Expression level Selective markers Properties of host cells
Environmental Factors	Medium formulation Dissolved oxygen tension Temperature Dilution rate Bioreactor operation modes

2.2.1. Genetic Factors

2.2.1.1. Plasmid DNA Sequences

Plasmid stability may be enhanced by including stabilising genes in plasmid vectors. The 2 μm DNA-based vectors are the best-analysed vector systems in *S. cerevisiae*. Two replication loci (*REP1* and *REP2*) of the 2 μm are responsible for stability of the native plasmid during replication (Panchal, 1987). Futcher and Cox (1984) have made use of this to construct 2 μm-based yeast plasmid vectors. They showed that the plasmids containing both *REP1* and *REP2* are considerably more stable than those lacking one or both loci. Inclusion of chromosomal centromere regions in vectors was also used to construct stable plasmids. One such plasmid YRp7, containing autonomous replication sequences (ARSs), is fairly unstable in *S. cerevisiae*; however, once a centromere region, CEN, carrying partition function is inserted into YRp7, the plasmid becomes stable (Clarke and Carbon, 1980). Less than 5% of such vectors are lost after 20–40 generations under nonselective conditions. Plasmids can be stabilized by joining with a particular DNA segment that provides the good partition function for *E. coli* (Skogman et al., 1983). When the partition locus of plasmid pSC101 was added to both pBR322 and pACYC184 plasmids, the stabilities of the resulting plasmid increased by at 3- to 10-fold. For the yeast system, the *STB (REP3)* sequence, in conjunction with the *REP1* and *REP2* gene products encoded by the endogenous 2 μm plasmid, ensures a high degree of partition stability (Reynolds et al., 1987). In another study, the insertion of a λ *cos*-containing DNA sequence into one of the pSa plasmids increased the fitness and the growth rate of the host cells carrying this λ *cos* pSa construct (Edlin et al., 1984). While Ogura et al. (1983)

showed that the inclusion of certain particular genes could stabilize plasmid maintenance by coupling host cell division to plasmid proliferation. Although these experiments were done in *E. coli* system, the methods might be applied to *S. cerevisiae* system. However, presence of certain other genes in plasmids can adversely effect plasmid stability. Kuriyama et al. (1992) reported that when *S. cerevisiae* AH22R-2075 harbouring pGLD p31-Rct which contains rHBsAg P31 mutein coding gene and β-isopropylmalate dehydrogenase gene (*LEU2*) was cultivated on a large scale in a leucine-devoid medium, plasmid-free cells appeared at high frequency. The plasmid instability was thought to be caused by the insertion of the *LEU2* gene into endogenous yeast 2 μm DNA as a result of homologous recombination between 2 μm DNA and the expression plasmid (Kuriyama et al., 1992). As these examples prove, the genetic make-up of a plasmid can greatly affect its stability.

2.2.1.2. Plasmid Copy Number

Stability of vectors in *S. cerevisiae* is dependent on the copy number. Since the desired gene is usually inserted into the plasmid vector, the copy number of the plasmid determines the gene dosage in the host cell. In general, plasmids showing a high copy number exhibit greater stability than those with low copy number (Futcher and Cox, 1984). During cell division, efficient partitioning of plasmids between mother and daughter cell will prevent generation of plasmid-free cells. On the other hand, inefficient partitioning will result in plasmid-free cells. Based on this concept, Walmsley et al. (1983) reasoned that a plasmid with a high copy number should generate plasmid-free cells much

less frequently than a plasmid with lower copy number. However, opposing results have been observed. For example, the 2 μm plasmid is more stable than either YEp plasmid or YRp plasmid even though the latter is usually present in higher copy number (Spalding and Tuite, 1989). Spalding and Tuite (1989) further reported that the stability of the YRp plasmid (YRp7M) in a haploid strain was significantly less than that of the YEp plasmid (pMA3a), although the former had an approximately sevenfold higher copy number. This apparent inconsistency occurs because YRp plasmids have no effective plasmid-encoded partition system, and show a strong segregation bias toward the mother cell during mitotic division (Spalding and Tuite, 1989). Thus, a plasmid with high copy number does not necessarily guarantee its stability unless it has an effective partitioning system. The latter depends on the efficiency of the origin of replication. Effective ori regions were found to consist of two fundamental domains: a replication-inducing sequence, and a replication-enhancer sequence. The enhancer sequence is not essential, but ensures optimal stability of the ori-containing vector (Montiel et al., 1984). The efficiency of 2 μm origin is higher than that of *ARS1* origin (Spallding et al, 1989), as was confirmed also by Da Silva and Bailey (1991). The cloned gene product's synthesis was much lower with the *ARS1* plasmid than that with the 2 μm-based plasmid because of the smaller fraction of plasmid-bearing cells (7.5%) when the plasmid's origin of replication was the *ARS1* element (Da Silva and Bailey, 1991). Increased stability with increased copy number has been reported for 2 μm based plasmids (Caunt et al., 1988). Clearly, the effect of copy number on plasmid stability relates to the origins of replication. Stability depends on efficiency of the

origin of replication, and on the copy number. In order to make a plasmid stable, a suitable origin of replication and a optimal copy number should be used.

2.2.1.3. Expression Level

Parker and DiBiasio (1987) used an auxotrophic mutant of *S. cerevisiae* containing a recombinant 2 μm based plasmid to examine the effect of the expression level of the plasmid in continuous culture. The plasmid introduced the ability to synthesize acid phosphatase (AP) that had been deleted from the host. The PHO5 promoter present in the plasmid controlled the transcription of acid phosphatase (expression level) by responding to levels of inorganic phosphate. Expression level of the plasmid was measured via the activity of acid phosphatase. The experimental results showed that as the level of plasmid expression was raised, the plasmid stability declined markedly (Parker and DiBiasio, 1987). This was probably because an increased transcription repressed the replication of the plasmid, increased segregation instability, and overburdened the cell's capability to repair DNA (Parker and DiBiasio, 1987). Furthermore, the known toxicity of large amounts of some foreign proteins toward the host may also contribute to plasmid loss (Ryu and Lee, 1988).

Transcription efficiency can be controlled by the promoter strength and regulatory mechanism in a given host cell. Plasmids with inducible promoters may be used to overcome the adverse effects of cloned gene expression. Such inducible promoters can be switched to control the time and the level of gene expression (Da Silva and Bailey, 1991).

Da Silva and Bailey (1991) used the yeast's galactose regulatory circuit to study the influence of promoter strength on plasmid stability in both batch and continuous culture. The yeast *GAL1*, *GAL10*, and *GAL10-CYC1* promoters, included in plasmids pRY121, pRY123, and pLGSD5 respectively, were employed. Cloned *lacZ* gene expression was regulated by the promoters through the addition of galactose. Higher β-galactosidase production, lower growth rate, and reduced plasmid stability were observed for the strain bearing the plasmid with the strongest *GAL1* promoter (Da Silva and Bailey, 1991). Although a high expression level can be detrimental to plasmid stability and cell growth, in this particular case the reduced plasmid stability and growth rate were more than offset by increased enzyme specific activity, and productivity (Da Silva and Bailey, 1991). In large scale batch or continuous fermentations, plasmid instability is fatal; hence, high expression levels that result in instability are not wanted. Modulation of gene expression at an optimal level demands easily controlled promoter systems. Relationships among expression level, promoter system, and plasmid stability need to be further examined.

2.2.1.4. Selection Markers

Since the yeast 2 μm plasmid confer no overt phenotype on its host cell, it is necessary that a selectable marker is incorporated into the 2 μm vector in order that plasmid-transformed cells can be identified and recovered. In the meantime, presence of a selectable marker allows for stable maintenance of the plasmid in the host cell if a selective pressure is imposed.

The commonly used markers are the genes that can confer resistance to antibiotics. Such genes are incorporated into plasmids and the resulting vectors are transformed into host cells. Antibiotics-containing selective media are now used to eliminate plasmid-free cells while stably retaining the transformed cells. As many antibiotics that are lethal to bacteria are not lethal to yeasts, the use of antibiotics resistance is restricted by the availability of antibiotics. Geneticin G418, an aminoglycoside, has been found to be effective against yeasts. This antibiotic is inactivated by the enzyme aminoglycoside phospho-transferase-3'(I) that is coded by the bacterial transposon Tn601. Yeast transformed with a plasmid carrying Tn601 becomes resistant to G418 at concentrations greater than 150 mg/mL (Jimenez and Davies, 1980). Other selectable markers have been used to confer resistance to antibiotics such as methotrexate (Zhu et al., 1985; Rouf et al., 1996) and chloramphenicol (Hadfield et al., 1986). However, the use of antibiotics is not free of pitfalls. Antibiotics are expensive, and their presence complicates product recovery. Sometimes, for example in wastewater treatment with recombinants (Bryers and Huang, 1995), use of antibiotics may be totally impractical. Another selectable marker is the *CUP1* gene that imparts resistance to high levels of copper. This gene has been cloned and characterized by Butt et al. (1984); it offers a potentially useful alternative to antibiotics.

The genes coding for enzymes in the amino acid biosynthetic pathway are also commonly used for selection markers. Two such markers are *LEU2* or *TRP1*. Host mutants for leucine and tryptophan auxotrophy are easily selected. The plasmid carrying

LEU2 or *TRP1* is cloned into the auxotrophic mutant. Plasmid retention is then necessary for survival of the auxotroph if the medium is devoid of essential leucine or tryptophan. This system requires the use of a defined selective growth medium, a limitation that is overcome in autoselective systems. The latter combines a host mutant that lacks a gene that is essential for survival with a plasmid vector carrying the essential gene. Thus, without the plasmid, the mutant cell dies regardless of the culture medium and conditions. *S. cerevisiae* autoselective strains with mutation in the *URA3, FUR1,* and *URID* genes have been obtained through sequential isolation (Napp and Da Silva, 1993). These mutations effectively block both the pyrimidine biosynthetic and salvage pathways, and, in combination, are lethal to the host. Therefore, a plasmid carrying a *URA3* gene is essential for survival, and nonselective media can be employed without the risk of plasmid loss (Napp and Da Silva, 1993). A more advanced autoselection system was described by Lee and Hassan (1988). In this case, the selection pressure was imposed by utilizing the yeast killer toxin-immunity complementary DNA (cDNA) inserted into a plasmid. Thus, the presence of such a plasmid vector confered not only host immunity but also the selective agent (killer toxin) which was secreted from the cells into the culture medium. The plasmid-carrying cells survived because of the immunity to the killer toxin conferred by the plasmids while the plasmid-free cells perished due to the killer toxin. Lee and Hassan (1988) transformed the plasmid (pYT760-ADH1) containing the yeast killer toxin-immunity cDNA into a leucine-histidine mutant (AH22) and showed that the plasmid was extremely stable (100% killer cells). This system has the potential to operate in any strains which are initially sensitive to killer toxin and in any medium. As autoselection systems

for plasmid maintenance function in growth media of any composition, such systems are ideal for large-scale industrial culture.

2.2.1.5. Genetic properties of host cells

Interactions between the host cell and the plasmids certainly play an important role in maintenance of the plasmid; hence, the properties of the host must also be considered in any discussion of plasmid stability. A certain plasmid transformed into different mutants of a host may display different stability characteristics in different mutants (Yu and Tang, 1991; Kikuchi and Toh-e, 1986). The host mutants may affect plasmid partitioning, replication, or amplification, all of which relate to stability. Some evidence suggests that the plasmids may utilize some form of anchorage to a host cellular structure, hence leading to possible asymmetric inheritance upon cell division (Wu et al., 1987). For the selection system discussed in the above section, in order for the selection to be effective, it is required that host mutants possess very low reversion frequencies. However, it is not easy to obtain a very stable host mutant and much further work is needed in this regard.

The growth cycle of a host greatly affects the processes of replication and transcription of the plasmid, and that relationship is bound to affect plasmid stability. Unfortunately, there is little data on the effects of physiological properties on plasmid stability, and some results are inconsistent. Mead et al. (1986) observed that the rate of plasmid loss was reduced by allowing haploid populations to enter stationary phase periodically. In contrast, for the 2 μm based yeast hybrid plasmids, Kleinman et al. (1987)

reported reduced stability in the stationary phase. Growth rate in the stationary phase is usually minimal, and effects of specific growth rate on plasmid stability have been examined as discussed in later sections of this review.

Manipulating the ploidy of the host cell also has an effect on plasmid stability. For the highly unstable, ARS-based plasmid YRp7M, a significant increase in segregational stability was observed with increasing ploidy, while the relatively stable, 2 μm based plasmid pMA3a showed only a slight increase in stability in strains of higher ploidy (Splalding et al., 1989). Greater plasmid stability in diploids was reported also by Mead et al. (1986). Industrial yeast strains for brewing and baking are usually polyploid.

Attempts to improve plasmid stability have focused mainly on manipulating the plasmid; little attention has been paid to the genetic constitution or the physiological state of the host. Manipulating host cells is certainly an option for improving plasmid stability. Much work remains to be done in this area.

2.2.2. Environmental Factors

Cell's response to the environment originates ultimately at the genetic level; hence, the culture conditions profoundly affect plasmid stability and expression (Table 2.2). Control and manipulation of the environment are particularly relevant to large scale culture where the feasibility of production depends on the cost of providing the requisite environment. Moreover, once a suitably engineered host is selected for production, environmental

manipulation is the sole remaining option for maintaining stability. A discussion of the main environmental influences on plasmid stability follows.

2.2.2.1. Medium Formulations

Metabolic activities of microorganisms are strongly influenced by the composition of culture media. For recombinant cells, medium composition can affect the stability of the plasmid through different metabolic pathways and regulatory systems of the host. In some cases, media that provide selection pressures are essential to maintaining plasmid stability (see Genetic Factors). Generally, minimal media favor stability relative to richer media (Yu and Tang, 1991). Wang and Da Silva (1993) reported on effects of three different media on plasmid stability using recombinant *S. cerevisiae* strain SEY2102/pSEY210, which produced invertase. A minimal medium (SD), a semidefined medium (SDC), and a rich complex medium (YPD) were investigated. Invertase productivity did not improve as the medium was enriched from SDC to YPD. In the complex YPD medium, the plasmid stability dropped from 54% to 34% during a single batch fermentation (Wang and Da Silva, 1993). During long-term sequential batch culture in YPD, the invertase activity decreased by 90%, and the plasmid-containing fraction of the population declined from 56% to 8.8% over 44 generations (Wang and Da Silva, 1993).

2.2.2.2. Dissolved Oxygen Tension (DOT)

A supply of oxygen is essential for most commercially relevant microorganisms. Availability of oxygen may affect growth rate, in addition to having other complex effects

on metabolic pathways. In the fermentation industry, dissolved oxygen concentration is a key parameter used to control microbial growth and fermentation. Effects of dissolved oxygen on microbial metabolism have been extensively described (Ejiofor et al., 1996; Carter and Dawes, 1978; Chisti, 1989); however, there is little information on how oxygen affects the expression and stability of plasmids. Tolentino and San (1988) showed that plasmid stability in recombinant *E. coli* was unaffected by DOT levels. It was observed that the plasmid was stable during a step change in the environment in which oxygen and nitrogen were supplied alternately. In contrast, the concentration of dissolved oxygen affected plasmid stability in a recombinant yeast (Lee and Hassan, 1987). Using a glucose-limited chemostat culture, Lee and Hassan (1987) examined the effects of oxygen tension and dilution rate on the stability and expression of killer toxin plasmid (pADH-10A) in wine yeast (MONtrachet 522). The recombinant yeast was grown in nitrogen-, air-, and pure oxygen-sparged environments. The highest plasmid stability was observed in the air-sparged culture, suggesting the possibility of an optimum dissolved oxygen concentration for greatest stability of the plasmid. Similar results were reported by Caunt et al. (1989) in studies of oxygen limitation on plasmid stability in recombinant yeast grown in a nonselective medium. The yeast strain was YN124/pLG669-z, which produced β-galactosidase. Once the dissolved oxygen level was lowered to below 10% of air saturation, the fraction of plasmid-containing cells declined sharply (Caunt et al., 1989).

Although the yeast *S. cerevisiae* can grow anaerobically, the replication and transcription of a multi-copy plasmid require a large amount of energy. Failure to provide sufficient oxygen will reduce the energy supply, possibly affecting replication and partitioning of the plasmid. As a result, the structural stability and segregational instability may increase. Further work is still needed to understand the reaction mechanism of DOT on plasmid stability, expecting that we are able to maximize the yield of recombinant DNA products and in the mean time to minimize the considerable cost of aeration of large-scale culture.

2.2.2.3. Temperature

In microbial fermentation processes, temperature is also an important factor. Temperature can affect microbial growth rate, biosynthetic pathways and directions, and regulatory systems. Such effects are particularly significant in recombinant fermentations. In one case, the optimal temperature for production of recombinant proteins—interferon and insulin—in *E. coli* was reduced to only 30°C, whereas the optimal growth temperature for the wild host is 37°C (Emerick et al., 1984). Temperature can also affect plasmid stability, but little is known about this aspect, especially in recombinant yeast. Relatively more information is available for recombinant *Bacillus subtilis* and *E. coli* systems. Sayadi et al. (1987) showed that the stability of pTG201 in *E. coli*B was strongly affected by the culture temperature. At 31°C, the plasmid was stable; after 83 generations more than 82% of cells contained plasmid (Sayadi et al., 1987). However, at 37°C, only about 40% of cells retained plasmid after about 87 generations. At a yet higher temperature (42

°C), only about 35% of the population had plasmid after roughly 67 generations. In these studies, the initial percentage of cells carrying pTG201 at 31, 37, and 42°C was, respectively, 97, 85, and 94%. A similar result was observed for the same plasmid in another host strain *E. coli* W3101. Plasmid stability in a recombinant *Bacillus* was shown to decline when temperature rose above 30°C (Son et al., 1987). In continuous culture of *Bacillus subtilis* (pHV1431) without selection pressure, the plasmid was segregationally less stable at 30°C than at 37°C, but no structural instability was observed at either temperature (Craynest et al., 1996).

2.2.2.4. Dilution Rate

Effect of dilution rate on plasmid stability in continuous culture has been extensively studied. Because the dilution rate corresponds to the specific growth rate in continuous culture, the chemostat culture provides an easy way to investigate the relationship between cell growth rate and plasmid stability and the growth characteristics of a mixed cell population. In several studies plasmid stability increased with increasing dilution rate when recombinant *S. cerevisiae* was continuously cultured in selective medium (DiBiasio and Sardonini, 1986; Parker and DiBiasio, 1987; Lee and Hassan, 1987; Lee and Hanssan, 1988; Caunt et al., 1989; Da Silva and Bailey, 1991). When an auxotrophic mutant of *S. cerevisiae* containing a recombinant 2 μm plasmid was grown in selective medium, Parker and DiBiasio (1987) observed that plasmid stability substantially increased at high growth rate. Stability of a plasmid that contained killer toxin cDNA increased as a function of dilution rate in chemostat cultures of *S. cerevisiae*; the plasmid was fully stable once the

dilution rate exceeded a certain value (Lee and Hassan, 1988). These results on the dilution rate effect are remarkably consistent considering the different yeast strains that were tested. The effect of dilution rate seems to relate to the media used. In a nonselective medium, increasing dilution rate was accompanied by increasing segregational instability of plasmids; however, in a selective medium, at high dilution rate the fraction of plasmid-containing cells remained relatively constant for about 100 generations (Hardjito et al., 1993). Impoolsup et al. (1989) examined the stability of 2 μm based yeast plasmid (pLG669-z) during continuous culture with cyclic growth rate changes in a nonselective medium, and showed that plasmid stability fell at high dilution rate. In their experiments, the dilution rate was cycled between a very low growth rate (0.075 h^{-1}) and a higher growth rate (0.25 h^{-1}). During growth at the high dilution rate, the fraction of plasmid-containing cells and the expression fell. After the dilution rate was changed to a low dilution rate, the fraction and expression level rose almost to the initial values (Impoolsup et al., 1989). Higher stability of a yeast plasmid at lower dilution rate in nonselective media was observed also by Caunt et al. (1990). Attempts have been made to explain these effects theoretically at the molecular and cellular levels, as well as through macroscopic models and simulations (Parker and DiBiasio, 1987; Hjortso and Bailey, 1984a; DiBiasio and Sardonini, 1986; Srience et al., 1986; Sdrdonini and DiBiasio, 1987; Stephens and Lyberatos, 1988), but actual mechanisms remain unclear.

The degree of plasmid instability can be determined by the probability of plasmid loss (p) and the growth ratio (α) (Imanaka and Aiba, 1981). The probability of plasmid

loss (p) is defined as the probability of plasmid loss and/or mutation per division of host cells. The growth ratio (a) is defined as the ratio of specific growth rate of plasmid-free cells (μ^-) to that of plasmid-bearing cells (μ^+). The probability of plasmid loss depends largely on genetic factors; whereas the growth ratio is affected by both genetic and environmental factors. The larger the probability of plasmid loss (p) and the growth ratio (a), the less stable is the plasmid. The effect of dilution rate on plasmid stability may can be explained by these two parameters. In chemostat culture, the dilution rate is equal to the specific growth rate of cells which can affect the growth cycle. Dilution rate dependence of specific growth rate was invoked by Parker and DiBiasio (1987) in attempts to explain effects of dilution rate on plasmid stability. Replication and transcription of plasmid were assumed to have the potential to damage the plasmid DNA (Parker and DiBiasio, 1987). In this scenario, plasmid stability would depend on the cell's DNA repair capabilities. Because many of the enzymes required for DNA repair are synthesized periodically, the repair capability is likely to vary with the stage of the growth cycle. Figure 2.1 illustrates the cell cycle of budding yeast as presented by Hjortso and Bailey (1984). Enzymes such as DNA ligase, DNA polymerase I, and nucleases have high levels in the S and G_2 phases. When the growth rate is enhanced through manipulation of the dilution rate, the S and G_2 phases comprise a significantly larger portion of the cell cycle; consequently, the cell's DNA repair capability is high. However, the need for repair remains great at low growth rates when the rates of replication or transcription are large. During slow growth, the long G_1 phase relative to G_2 and M phases could compromise

the cell's ability to complete the necessary repairs, hence increasing the likelihood of loss and mutation.

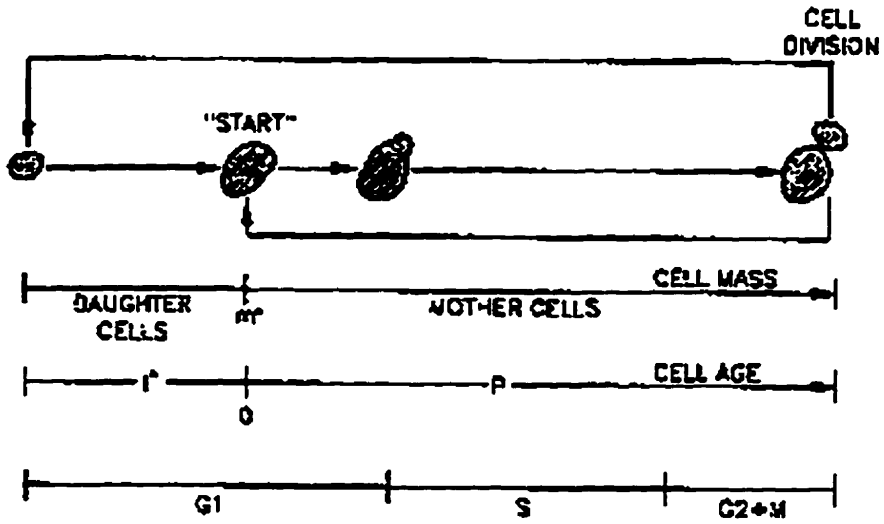


Figure 2.1. Cell Cycle of Budding Yeast (Hjortso and Bailey, 1984).

Although the foregoing reasoning does explain the effect of dilution rate on plasmid stability in selective media, it does not explain that effect in nonselective media. An alternative possible explanation follows. For continuous culture in selective media, increase in dilution rate raises the specific growth rate of plasmid-harboring cells according to the well known Monod equation:

$$\textit{Dilution Rate} = \mu^{+} = \mu_{m}^{+}\frac{S}{K_s + S}\ ; \qquad (2.1)$$

however, plasmid-free cells do not grow, or grow very slowly, because of the selection pressure. Therefore, the growth ratio ($a = \mu^{-}/\mu^{+}$) decreases as the dilution rate is increased, so the plasmid is more stable. In the case of nonselective media, because the growth rate of recombinants is adversely affected by the burden of plasmids and the expression of recombinant DNA (Moo-Young et al., 1996), plasmid-free cells usually have a growth advantage. In general, the growth ratio (a) exceeds unity, ranging over 1.0 to 2.0 (Imanaka and Aiba, 1981). Increasing dilution rate in nonselective media even can further raise the growth ratio (a) because the plasmid-bearing cells are less able to respond to the dilution rate changes. Therefore, it is not surprising that plasmid stability decreases with increasing dilution rate in the nonselective media. In fact, in continuous culture of recombinant cells, it is impossible to achieve steady state in nonselective media if the growth ratio (a) is more than 1. The initial plasmid-bearing cell population is sure to be taken over by the plasmid-free cells eventually.

2.2.2.5. Bioreactor Operation Modes

Fermentation processes are usually operated as batch, fed-batch, or continuous culture. The modes of operation can influence the plasmid stability and the productivity of expressed products. In batch culture, plasmids are relatively stable because the culture periods are generally short, hence, the number of generations since inoculation is small. Because of long growth periods, continuous culture of recombinant cells suffers from

plasmid instability particularly in nonselective media (DiBiasio and Sardonini, 1986; Impoolsup et al., 1989; Mosrati et al., 1993). A two-stage continuous culture system based on inducible operators has been devised in attempts to overcome the instability problem (Ryu and Seigel, 1986; Lee and Ryu, 1988; Sayadi et al., 1987; Fu et al., 1993). In this scheme, a repressor protein binds to the operator of the plasmid to block transcription until needed. When necessary, an inducer is added, to bind to the repressor, and prevent its interaction with the operator, thus allowing transcription to begin. In the first stage of the two-stage system, the cells are grown in the repressed state (no foreign protein is synthesized) or under selective pressure in order to prevent plasmid loss, and obtain a high fraction of plasmid-containing cells. The cells are then continuously transferred to the second stage, where the inducer is added to enable expression. Another possibility for maintaining plasmid-harboring cells in continuous culture is the use of selective recycle (Ogden and Davis, 1991). In such a process, plasmid-bearing cells leaving the reactor are selectively concentrated and subsequently recycled back to the reactor, resulting in increased plasmid-bearing cell concentration and product synthesis. Feasibility of selective recycle depends on the ability to easily separate the plasmid-containing cells from the mixed population; therefore, the phenotype of the plasmid-bearing cells must include a property that can be the basis of separation. Such a property may be cell size, density, or flocculation behavior.

Another promising stabilizing option is cycling of operational variables. Using a theoretical analysis, Stephens and Lyberatos (1988) showed that cycling of substrate

concentration could lead to coexistence of plasmid-containing and plasmid-free cells in continuous culture. In an experimental study with *E. coli* K21 strain being grown in nonselective continuous culture, Weber and San (1988) observed that when the dilution rates were kept constant, the fraction of plasmid-bearing cells decreased after a period of time. Eventually, the plasmid-free cells displaced the recombinants. However, when the cells were exposed to forced oscillations in the dilution rate, the reactor culture was able to maintain a mixed population of plasmid-free and plasmid-containing cells for longer periods. For recombinant yeast, Caunt et al. (1989, 1990) showed that cycling of dissolved oxygen with a frequency of a few minutes could enhance plasmid stability. Whereas Impoolsup et al. (1989) observed that cyclic variations in dilution rate had a stabilizing effect on the 2 μm based yeast plasmid. Stabilizing effects of growth rate (dilution rate) cycling may be simulated in fed-batch fermentations by controlled intermittent feeding (Impoolsup et al., 1989). Plasmid stability of recombinant *S. cerevisiae* YN124/pLG669-z in fed-batch culture was examined by Hardjito et al. (1993). The fraction of plasmid-containing cells could be maintained constant over the length of one fed-batch culture, suggesting that fed-batch processes were better suited to producing recombinant proteins. Although the effects of cyclic environmental changes on plasmid stability are clear, the reasons for those effects are not fully understood.

Newer bioreaction strategies are being developed continually to improve productivity. One such example is the airlift bioreactor (Chisti, 1989) that is particularly capable of microenvironmental cycling within a mechanically robust, simple, and scalable

device. Little information exists on the use of airlift devices in large scale culture of recombinants, even though airlift bioreactors are thoroughly proven in the fermentation industry (Chisti, 1989). Although further studies are needed, immobilized cell bioreactors seem to be very promising for the production of recombinant products as discussed in the following sections.

2.3. Plasmid Stability in Immobilized Cells

2.3.1. Effect of Immobilization

E. coli. was the first immobilized recombinant organism studied. Inloes et al. (1983) reported that a significantly higher cell concentration and consequently higher productivity of plasmid coded gene product can be obtained in a hollow fiber membrane bioreactor. Similarly, Dhulster et al.(1984) immobilized recombinant *E. coli* BZ18/pTG 201 cells in Kappa-carrageenan gel beads and obtained high cell densities in the cavities of gel. They pointed out the potential of gel immobilization system and that the immobilization might have an effect on plasmid stability. This effect was investigated by De Taxis du Poet et al. (1986). Plasmid (pTG201)-harboring *E. coli* were immobilized in K-carrageenan beads, and plasmid maintenance was studied for both free and immobilized cells in a chemostat. Gel immobilization was seen to have a stabilizing effect on plasmid stability; but once the cells were released from the beads, their plasmid-loss frequency was the same as for free cells. A theoretical analysis based on compartmentalization resulting from the immobilized growth was proposed (De Taxis du Poet et al., 1986); but the plasmid stability predicted by this model was inferior to that actually observed. This suggested

that factors other than immobilization may have contributed to the observed enhancement of stability. Later, De Taxis Du Poet et al. (1987) extended this analysis to recombinant *E. coli* JM105 containing plasmid pKK223-200. Once again, immobilization enhanced plasmid stability. Increased plasmid stability was associated with the modified plasmid copy number, media used, oxygen limitation, as well as immobilization (De Taxis Du Poet et al., 1987). It was observed that the plasmid copy number of both plasmid-bearing cells in beads and those released from beads rose. Compartmentalization of cell growth, and growth gradients due to diffusional limitations in the immobilization matrix were said to explain the increased plasmid stability in immobilized cells. The stabilizing effect of immobilization has been observed also with *Bacillus subtilis* (pHV1431) (Craynest et al., 1996).

Nasri et al. (1987) examined the stability of pTG201 during continuous culture of three genetically different *E. coli* hosts. No selection pressures were employed, but the plasmid was stably maintained in all strains during immobilized culture. In contrast, the plasmid in all strains showed various degrees of instability in continuous suspension culture. Nasri et al. (1987) concluded that increasing plasmid stability was due neither to plasmid-transfer between immobilized cells nor to an increase of the plasmid copy number in the immobilized cells. Two likely explanations for plasmid stability were offered: one was the absence of competition between plasmid-free and plasmid-containing cells in the immobilized system; the second considered gel beads as a reservoir of plasmid-carrying cells. In further studies, the stability of three different plasmid vectors was examined in *E.*

coli during immobilized continuous cultures (Nasri et al., 1988). In all cases, the loss of plasmids could be prevented by immobilizing plasmid-bearing cells in carrageenan beads.

Work by Sayadi et al. (1987) showed that immobilization of cells increased the stability of pTG201 considerably, even under conditions of high expression of the cloned product. A two-stage continuous immobilized cell system was described for maintaining high plasmid stability. In studies employing different environmental growth conditions, Sayadi et al. (1989) reported that decreasing specific growth rate increased the plasmid (pTG201) copy number and the cloned enzyme's activity, but the stability decreased. Even under glucose, nitrogen, or phosphate limitation, immobilization enhanced the stability of plasmid (Sayadi et al., 1989). However, with magnesium limited culture, the plasmids were relatively unstable, and the viable cell count declined during immobilized continuous culture (Sayadi et al., 1989). These observations were not explained.

Detailed studies of plasmid stability in *E. coli* immobilized in κ-carrageenan gel beads were reported by Berry et al. (1988). Effects of factors such as inoculum size, gel bead volume, and gel concentration were examined. Plasmid (pTG201) stability increased with increasing inoculum size in the gel. Larger inoculum reduced the number of cell divisions required to fill the cavities in the immobilization matrix; hence, the culture time was reduced, and the plasmid copy number was nearly equal to the initial value and remained high. In addition, because of the large inoculum, only a few cavities were contaminated by plasmid-free cells, so there was little competition between plasmid-

bearing and plasmid-free cells. Gel bead volume in the reactor, and the κ-carrageenan concentration in the gel apparently did not affect plasmid stability. The plasmid was extremely stable for the three bead volumes and three gel concentrations tested. Effects of agitation rate on plasmid stability in immobilized and free continuous cultures of recombinant *E. coli* were examined by Huang et al. (1990). For free cells, the plasmid stability declined generally more rapidly when highly agitated; however, the gel immobilized recombinant cells displayed increased plasmid stability even when intensely agitated. The immobilization matrix must have limited the exposure of the cells to the agitated environment.

All the researchers mentioned above used gel entrapment to immobilize recombinant cells. In a few studies, recombinant cells have been immobilized as biofilms. Inloes et al (1983) showed the maintenance of a plasmid-containing strain of *E. coli* in the absence of antibiotic selection pressure when immobilized in a hollow fiber membrane. Huang et al. (1993) reported on plasmid stability in suspension, and biofilm-immobilized cultures without selection pressures. In contrast to other data, the average probability of plasmid loss for suspended *E. coli* DH5a/pMJR1750 population was lower than that of biofilm-bound cells. No explanation was given, but biofilm immobilized cells probably did not experience the same confining environment as would occur in an entrapment matrix.

Information on immobilized culture of recombinant yeast is relatively sparse. Sode et al. (1988b) reported the continuous production of α-peptide using immobilized yeast *S.*

cerevisiae. Yeast FY178 cells, which secreted α-peptide, were immobilized in Ca-alginate gel and a fermentation was performed in nonselective media in a column bioreactor. Experiments showed that both the plasmid stability and α-peptide productivity were enhanced by immobilization. Walls and Gainer (1989) described another immobilized *S. cerevisiae* system. The yeast used was the Mc16 strain *S. cerevisiae* containing the plasmid vector pMA230 (2 μm based); the plasmid encoded for the amylase. The enzyme was secreted into the culture medium. They used the beads of gelatin as the carrier and covalently coupled the yeast cells to the carrier surface using glutaraldehyde. The beads were suspended in a fluidized bed bioreactor for continuous culture using a selective minimal medium. The immobilized cell system was examined at a dilution rate below washout to see if the attached population retained the plasmid while the free cell population gradually lost it. After 50 hours of continuous culture, the free cell population began to show plasmid loss, but the attached cells remained stable. Furthermore, the plasmid instability in free cells varied with the dilution rate, greater plasmid loss was seen at lower dilution rates. For the attached cell population, no such variation was observed. However, no sound explanations for these results were offered. Later, they extended their work by using the same yeast strain but a different plasmid p520, which expressed a wheat amylase (Walls and Gainer, 1991). The same immobilization method was used, but a nonselective complex medium was employed. In this case, continuous suspension culture was unstable and rapidly lost the plasmid. The plasmid stability improved greatly upon immobilization, and a near constant fraction of plasmid-containing cells was maintained during continuous culture. Relative to free suspension, immobilization improved protein

productivity irrespective of the mode of operation of the reactor (Walls and Gainer, 1991). Several immobilization related effects—enhanced plasmid stability, increased cell concentration, and operation at high dilution rate—contributed to enhanced productivity.

In view of the many experimental observations, immobilization can be concluded to have a near universal stabilizing effect on recombinant cells. It is thought that immobilization can reduce the probability of plasmid-loss by reducing or eliminating plasmid structural and segregational instabilities. Several hypotheses have been to proposed to explain this effect, but the specific mechanisms remain unclear.

2.3.2. Possible Stabilizing Mechanisms

Stabilization that accompanies immobilization may have several possible sources, including one or more of the following:

1) Immobilization may maintain or increase plasmid copy number (Sayadi et al., 1987).
2) Compartmentalization in the immobilizing gel may separate the plasmid-bearing cells from plasmid-free cells, thereby eliminating their growth competition (De Taxis Du Poet et al., 1986).
3) Diffusional nutrient limitations in the gel may cause a growth rate gradient in the matrix, and hence morphological and physiological changes in immobilized cells (De Taxis Du Poet et al., 1987).
4) The close proximity of immobilized cells could promote transfer of plasmid DNA between two populations by either conjugation or transformation (Huang et al., 1993).

5) Slower growth rate of cells may limit opportubities for plasmid loss (Flickinger and Rouse, 1993).

These suggestions regarding possible mechanisms are largely speculative. A sound demonstration of the mechanism of stabilization at the molecular or cellular levels remains to be accomplished. In fact, enhanced plasmid retention upon immobilization may not have a single explanation; multiple interactions are more likely to be responsible.

2.4. Immobilization Methods and Immobilized Cell Bioreactors

Choice and design of immobilized cell bioreactors depends, to large extent, on cell immobilization materials and methods. Before discussing immobilized cell bioreactors, it is necessary to give a brief review of yeast cell immobilization.

Research in alcohol fermentation using immobilized yeast cells has been carried out for many years. Many materials and methods have been developed to immobilize yeast cells. One commonly used method is gel entrapment. Different types of gel matrices were used to entrap yeast cells, including calcium alginate (Kierstan and Bucke, 1977), agar (Kuu and Polack, 1983), carrageenan (Wada et al., 1980) and polyacrylamide (Couderc and Baratti, 1980). There are two major drawbacks relating to gel entrapment methods. One is the instability problem of gel particles. The growth of cells and the evolution of carbon dioxide will weaken and disrupt the gel matrices. The other is the severe intraparticle mass transfer limitations, which causes cells inside the gel to be practically

inactive and useless. The problem is intensified in the case of aerobic fermentations due to oxygen transfer rate limitation..

Yeast cells can also be immobilized by adsorption of cells onto solid supports, in which only external surface area is used for cell retention. Such supports include glass, ion-exchange resin, wood chips, P.V.C. chips and ceramic materials (Moo-Young et al., 1980; Lamptey, 1983). Because cells only reside on outer surface of the supports, the internal mass transfer limitations can be overcome. The life time of the support materials seems to be unlimited due to their inert property and strong mechanic strength. However, cell leakage is substantial in these systems since the binding forces involved are mainly due to Van der Waals' forces (Rutter and Vincent, 1980) and hydrogen bonds. The interactions between the microbial cells and the supports can be influenced by temperature, pH, hydrodynamic forces and ionic strength of the medium. Generally, lower cell concentration is achieved by this method. In order to increase cell concentration, some soft, highly porous materials such as polymer foam, cotton cloth and sponge have been developed to immobilize yeast cells (Joshi and Yamazaki, 1984; Philipps, 1992; Roostaazad, 1993). Unlike solid carries, both external and internal surfaces are available for cell retention in these materials. Combined with their low cost, easy availability and long life time, they could be attractive materials for immobilization.

In alcohol fermentation using immobilized yeast cells, packed-bed immobilized cell bioreactors are usually employed (Corieu, et al., 1976; Kierstan and Bucke, 1977; Wada

et al., 1980; Mcghee et al., 1982; and Lampty, 1983). Immobilized yeast cells were packed into a column to form a packed bed reactor, which was run in repeated batch or continuous mode. Much higher ethanol productivity could be obtained in immobilized systems. However, in the packed-bed reactors, serious problems such as substrate channelling, dead zone, bed blocking due to overgrowth of cell mass and CO_2 build-up were unavoidable. These problems resulted in the instability in the reactor's performance. In addition, it is very difficult to operate aerobically due to aeration difficulty and oxygen transfer limitation. Consequently, this reactor configuration is not suitable for aerobic fermentations.

In order to overcome the problems encountered in packed-bed reactors, several other forms of immobilized cell bioreactors have been proposed. Zhang et al (1989) reported four different types of reactor configurations used in continuous beer fermentation by immobilized yeast. They were fluidized-bed reactor with recycling, agitated screen frame reactor, multi-screened plate reactor and multi-plate reactor with individual overflow pipe and exhaust gas outlet. Reactor stability and mass transfer efficiency were greatly improved in these reactors, which were successfully used for beer fermentation (Zhang et al., 1989). A novel airlift-driven packed-bed bioreactor was proposed for aerobic fermentation of the yeast *Saccharomyces diastaticus* by Roostaazad (1993). High cell loading, enhanced liquid-solid mass transfer, and high enzyme productivity were observed.

Aerobic fermentation using immobilized cells can be realized in three-phase airlift reactors. The airlift reactors provide good hydrodynamics and mass transfer characteristics at low capital and operating costs. Undoubtedly, very efficient biological processes can be achieved in three-phase airlift bioreactors. In recent years, many bench and pilot scale applications of the three-phase airlift bioreactors have been studied over a variety of immobilized cells. The most active area is the wastewater treatment using immobilized heterogeneous microorganisms (Fan et al., 1987; and Heijnen et al., 1990). The following sections present a brief review of some of the more recent studies involving immobilized cell airlift bioreactors.

Keshavarz et al. (1990) used immobilized *Penicillium chrysogemm* P2 as a model system to demonstrate the feasibility of penicillin production in a three phase airlift reactor. *Penicillium chrysogemm* P2 was immobilized on celite and the obtained immobilized cell slurry was introduced into a 250-320 L working volume draft tube airlift reactor. Air flow rate was varied between 0.2 to 1.0 v.v.m. to maintain bulk liquid circulation and the dissolved oxygen tension (DOT) above 50% air saturation. Both batch and continuous operations were carried out. They showed that operating a large-scale three phase airlift reactor for production of penicillin by immobilized cells was feasible. Immobilized cells were able to maintain biosynthetic activity for long time. After 500 hours of fermentation, penicillin was still being synthesized, but an increase in free biomass concentration was observed at later stages of operation.

Guo et al. (1990) reported production of α-amylase by immobilized *Bacillus subtilis* in an airlift bioreactor. Cells were entrapped into carrageenan gel and approximate 4 mm immobilized cell particles formed. The results showed that the productivity of α-amylase by immobilized cells was increased significantly. Kinetic analysis indicated that the specific growth rate of imobilized cells decreased but the specific enzyme production rate was four-fold higher than that of free cells.

El-Sayed et al. (1990) investigated production of dextransucrase by alginate bead immobilized *Leuconotoc mesrnteroides* cells in both bubble column and airlift reactors. They demonstrated that in the airlift reactor enzymatic activity produced by the immobilized cells was about 34% greater than that in the bubble column, which was attributed to the better mass transfer characteristics of the airlift bioreactor. In another study, Hamada et al. (1990) reported continuous production of 4-ethylguaiacol (4-EG) by immobilized *Candida versatilis* in a draft tube airlift reactor. The effects of pH, temperature, aeration, and residence time on the production of 4-EG were examined. They concluded that immobilization combined with airlift reactor configuration was effective for producing 4-EG by *C. versatilis*.

Citric acid production with immobilized *Yarrowa lipolytica* yeast was also attempted in a continuous airlift bioreactor (Rymowicz et al., 1993). In order to make the alginate bead resistant to possible disruption by hydrodynamic forces, the beads were hardened by cross-linking with glutaraldehyde. The effect of the height-to-diameter ratio

(ranging from 1 to 7) on citric acid productivity was examined. They observed that the highest productivity was obtained at the height-to-diameter ratio of 3.

Almost all previous studies involve three-phase slurry airlift reactor with gel entrapped immobilized cells. For hydrogel immobilized cells, the disruption problem still exists in the airlift reactor. As discussed above, the use of soft and highly porous natural or synthetic materials may overcome this. However, slurry three-phase airlift reactors are not suitable with these materials due to their low density. Even after loading with biomass, they still have a density equal to or less than that of the continuous liquid phase. While the particles circulating through the airlift reactor, they tend to block the downcomer if the particle loading increases to certain level, causing the instability of reactor operation (preliminary experiment result). Besides, liquid-solid mass transfer limitation may occur in the reactor due to low liquid-solid slip velocity. Many of these studies were conducted on a small scale, and concerned mainly with the feasibility and fermentation kinetics rather than bioprocess development and transport phenomena. Little information is available for airlift design, operation, and scale-up during actual fermentation processes. Therefore, much work is urgently needed in these aspects.

2.5. Modelling of Recombinant Yeast

Interest in modelling of plasmid instability kinetics in recombinant cells has increased with our better understanding of the mechanism of plasmid instability. In turn, such modelling can provide valuable information about the nature of plasmid instability and give a greater

understanding of this complex phenomenon. With proper models it may be possible to predict in advance whether a plasmid-host combination will be unstable and if so, to what extent. This information is potentially useful in determining optimum bioreactor operating conditions, which are necessary to maximize the production of gene products in recombinant cell cultures. Therefore, a large number of models describing the plasmid stability in recombinant cells, both structured and unstructured, have been developed (Lee and Bailey, 1984; Seo and Bailey, 1985; Cooper et al., 1987; Wittrup and Bailey, 1988; Bentley and Kompala, 1989; Kim and Shuler, 1990a; Laffend and Shuler, 1994; Mosrati et al., 1993). For modelling the plasmid instability, investigators usually developed their models based on two important kinetic parameters: the probability of plasmid-loss (p) due to structural instability and/or segregational instability and the growth ratio (a) reflecting the specific growth rate difference between plasmid-bearing and plasmid-free cells.

Imanaka and Aiba (1981) considered that a mixed culture of plasmid-harbouring (H) and plasmid-free(F) cells could be represented by the following reaction mechanism:

$$\begin{aligned} H &\xrightarrow{\mu^{\cdot}} (2-p)H + F \quad (F = pH) \\ F &\xrightarrow{\mu^{-}} 2N \end{aligned} \tag{2.2}$$

Imanaka and Aiba (1981) further assumed: (i) cells that lose plasmids cannot regain them; (ii) host cell growth is exponential; (iii) the probability of losing the plasmid per cell division is constant; (iv) a constant growth ratio; and (v) zero initial concentration of plasmid-free cells. Based on these assumptions, they first proposed a mathematical model for describing plasmid loss in batch culture. According to the model, the plasmid-carrying

cell fraction decreases monotonically with generation number (n) according to the following equation:

$$H_n = \frac{1-a-p}{1-a-p\,2^{n(a+p-1)}} \qquad (2.3)$$

where $a = \mu^-/\mu^+$. From Eq. (2.3) it is obvious that in batch system with any $a > 1.0$ and $p > 0$, $H_\infty = 0$. The Imanaka and Aiba (1981) model is unstructured, but elegantly simple. In principle, any recombinant organism satisfying the underlying assumptions should conform to the model. Unfortunately, the p and a usually vary during the culture period, and the assumed exponential growth is not exactly followed.

Pioneering developments in structured modeling of mixed recombinant culture came from Bailey's and Shuler's groups working with recombinant *E. coli* (Lee and Bailey, 1984; Seo and Bailey, 1985; Ataai and Shuler, 1986; Kim and Shuler, 1990a; Kim and Shuler, 1990b). The plasmid stability and the expression of a cloned-gene product has been described by a mathematical model based upon the molecular mechanism of plasmid replication, partition and transcription. These studies laid the foundation for further mathematical modelling and pointed the way for future experimental work. Later, at a single-cell level, using population balance models, Hjortso and Bailey mathematically described the plasmid stability in yeast *S. cerevisiae* with selection pressure (Hjortso and Bailey, 1984a) and without selection pressure (Hjortso and Bailey, 1984b). Two assumptions were examined for plasmid partitioning: (i) random and independent distribution of plasmids between the mother and the daughter cells at partition; and (ii) a greater or equal probability of a plasmid residing in the mother cell after division than in

the daughter cells. Two models were developed based on the two mechanisms for plasmid replication. In the first model, the cells replicated plasmids such that the total number of plasmids was the same for all dividing cells in the population. In the second, the cells produced plasmids at the same rate, irrespective of the initial copy number. The results obtained from those models were compared with the corresponding results from a nonstructured model, and pronounced differences were found at low growth rates. These models were able to relate observable bulk properties of growth to kinetic events at the cell level. In later work, a general single-cell model for plasmid propagation in recombinant yeast was developed (Wittrup and Bailey, 1988). This model included the plasmid burden effects on host cell growth rate, and it could be combined with bioreactor performance equations to simulate a production process.

A macroscopic population dynamics model for plasmid stability in continuous culture of recombinant *S. cerevisiae* with selection pressure was described by Sardonini and DiBiasio (1987). Several assumptions were made, including: (1) plasmid-free cell could propagate to some degree in the selective media, which could be explained by a metabolite being excreted into the media by the plasmid-carrying strain. This metabolite supported the growth of plasmid-free cells; (2) plasmid loss occurs at constant probability; (3) the growth rate of plasmid-carrying cells is limited by a single substrate described by a Monod equation; (4) growth rate of the plasmid-free cells is limited by this substrate as well as an additional metabolite described using a dual Monod form; (5) continuous culture with a constant reactor volume and sterile feed. The Sardonini and DiBiasio

(1987) model allows for plasmid instability due to growth of plasmid-free cells in spite of selection pressure, and that due to plasmid loss. Moreover, the model directly relates to measurable macroscopic parameters. The results predicted by this model agree with experimental data. Furthermore, the model shows that in chemostat mixed culture without selection pressure, coexistence is impossible if $\mu^- > \mu^+$ and $p > 0$. If this occurs, the original plasmid-bearing population will be eventually replaced by plasmid-free cells.

However, the models about recombinant yeast discussed above dealt only with the kinetics of plasmid instability. These models did not assimilate the information regarding the metabolic pathways in yeast *S. cerevisiae*. They were unable to simulate the dynamics of cell growth, substrate consumption and gene product formation. In recent years, only a few studies regarding modelling of recombinant yeast addressed all of these aspects.

By utilizing the available knowledge on the behaviour of recombinant *S. cerevisiae*, a model for cell growth, heterogeneous protein production and plasmid segregation was formulated to examine specific experimental data by Coppella and Dhurjati (1990). Three catabolic pathways of yeast: glucose fermentation, glucose oxidation and ethanol oxidation were considered in their model. Three simplified enzyme pools were assumed to be responsible for regulating the above catabolic pathways. These enzyme pools could be either induced or depressed by glucose concentration depending their functionality. The three catabolic pathways could be simultaneously activated. By combining catabolic pathways, stoichiometry, mass balance, plasmid segregation and

recombinant protein synthesis, a mathematical model was developed. The model required 43 equations and 48 parameters. Although this model successfully described the observed results for the fermentation of recombinant *S. cerevisiae* strain AB103.2/pYαEGF-25 in both batch and fed-batch bioreactors, its complexity poses some practical difficulties (eg., parameter estimations) for engineering application.

Hardjito et al. (1992) developed an unstructured model to describe the growth and product formation of a recombinant *S. cerevisiae* expressing β-galactosidase. They thought that glucose transported into the cell was catabolized by both fermentative and oxidative pathways. But they used only one Monod equation to express the glucose uptake process. In addition, it was assumed that ethanol accumulated in the medium could be further utilized by the cells but the ethanol utilization rate was inhibited by available glucose. Furthermore, they assumed that β-galactosidase formation also followed Monod kinetics. Under these assumptions, a model with 12 equations and 12 parameters was formulated. With the parameters determined using a nonlinear regression technique, the model showed good agreement with the experimental data. The simplicity and accuracy were claimed to be the advantage of the model. But it provides us little insight into the complex biological system.

2.6. Concluding Remarks

The above sections have described the plasmid stability in recombinant *S.cereviae* at the molecular, cellular and engineering levels. The genetic and environmental factors which

affect plasmid stability have been outlined. Scientists in genetics and microbiology have done much work at the gene and cell levels in order to construct high stability plasmids for maximizing gene expression. Biochemical engineers are more interested in maintaining plasmid stability and optimizing gene product production by manipulating process environmental conditions. Many environmental approaches have been proposed to maintain genetic stability of recombinants in a bioreactor, but many of them were unsuccessful in the absence of selection pressure (Kumar and Schugerl, 1990). As reviewed in Section 2.3, immobilization offers a promising way for recombinant cell culture. However, relatively little knowledge and engineering experience are available in the immobilized recombinant yeast systems. In order to commercially exploit the immobilization technique for cultivation of recombinant yeasts, much work is required to understand the nature of the recombinants, the stabilizing mechanisms of immobilization and to develop better immobilization methods and immobilization bioreactor with a good potential to be used in recombinant cell bioprocesses for the production of extracellular proteins.

Despite the previous efforts, up to now the modelling of recombinant yeast systems is very much in its infancy because our understanding about the genetics, biochemistry and physiology of the recombinant yeast cells is still inadequate. It is the intent of this work to develop a simple robust working model describing the behaviour of a recombinant yeast, which is suitable for engineering application. Furthermore, the unexplored area of modelling immobilized recombinant yeast systems requires special

attention. We expect that these modelling efforts will aid in the development and commercialization of recombinant yeast bioprocesses.

CHAPTER 3

MATERIALS AND METHODS

3.1. Recombinant *S. cerevisiae* and Culture Media

3.1.1. Microorganism and Plasmid

The recombinant *S. cerevisiae* strain used is C468/pGAC9 (ATCC 20690), which secretes glucoamylase into the extracellular medium (Nunberg et al., 1988). The strain contains the hybrid plasmid pGAC9. The plasmid pGAC9 contains a portion of the yeast 2μ plasmid (2μ micron circle), a DNA fragment from the *E. coli* plasmid pBR322, a DNA fragment (LEU2 gene) from *S. cerevisiae* that encodes the LEU2 gene product (leucine), and a section of a glucoamylase gene from *Aspergillus awamori*, under control of the yeast enolase I promoter and terminator. A plasmid map for pGAC9 is illustrated in Appendix A. The yeast strain C468 (host cell) is haploid *S. cerevisiae* with auxotrophic markers for leucine and histidine and carries a mutation (mal) blocking the utilization of maltose as carbon source. Therefore, the host cell C468 is complementary to the leucine prototrophy by inserting the selectable marker (LEU2) into the expression plasmid and the presence of the glucoamylase gene on the plasmid allows the host cell to grow on maltose. This recombinant yeast was selected for its ability to excrete an enzyme, and the 2μ-based shuttle vector, pGAC9, has features common to many yeast vectors and thus serves as a good model organism.

3.1.2. Composition of Culture Media

The culture media employed in this study are briefly summarized as follows:

I. YNB selective minimal medium: 6.7 g/L yeast nitrogen base (YNB, w/o amino acids), 20 g/L glucose and 40 mg/L histidine (Nunberg et al., 1988).

II. Maltose selective minimal medium: 6.7 g/L yeast nitrogen base (YNB, w/o amino acids), 20 g/L maltose, 40 mg/L histidine and 40 mg/L lysine.

III. YPG nonselective complex medium: 5 g/L yeast extract, 10 g/L peptone, and 20 g/L glucose (Barnett et al., 1983).

IV. YNB selective solid agar medium: YNB selective minimal medium plus 20 g/L agar.

V. Maltose selective solid agar medium: Maltose selective minimal medium plus 20 g/L agar.

VI. YPG nonselective solid agar medium: YPG nonselective complex medium plus 20 g/L agar

3.2. Strain Screening under Double Selection Pressure

The original strain obtained from ATCC was maintained on the slants of YNB selective solid agar medium (medium IV). This YNB selective medium does not contain leucine. Since the host cell strain C468 is *S. cerevisiae* with autotrophic marker for leucine, it can not grow without leucine supplementation. The plasmid pGAC9 contains a section of LEU2 gene that encodes the enzyme for leucine synthesis. Only the plasmid bearing cells can grow on the YNB selective medium. However, the YNB selective medium confers only one selection pressure. Under this selection pressure, the glucoamylase gene can be

still deleted from the plasmid. Such plasmid-bearing cells can grow in YNB selective medium but are not able to produce glucoamylase. Therefore, after several transfers on the agar slants containing YNB selective medium, recombinant cells will gradually lose their ability to produce glucoamylase. In order to overcome this problem, maltose selective solid agar medium (medium V) was used to select and maintain this recombinant strain. Since the host cell strain C468 carries a mutation blocking the utilization of maltose as carbon source, the presence of the plasmid which contains the glucoamylase gene is necessary for the host cells to grow on maltose. The maltose selective medium imposes double selection pressure (lack of leucine and the use maltose as carbon source). Only cells that contain plasmids with both LEU2 and glucoamylase genes are able to grow on the maltose selective medium independently. In the screening process, cells from the original strain were diluted in sterile water to obtain individual colonies and then the diluted cell solution was spread onto agar plates containing maltose selective medium to obtain individual colonies. Each colony formed on the above agar plates contained both LEU2 and glucoamylase genes. Such screened colonies are referred to as the selected strain. This screening process was carried out every three months and the selected recombinant cells were maintained on the maltose selective solid agar medium in order to keep the biological characteristics invariant.

3.3. Analytical Methods

3.3.1. Cell Concentration

Free cell concentration was measured by dry weight and optical density methods. The dry weight cell concentration was determined by filtering 20 ml of a sample through 0.2-μm filter paper, which was washed with deionized water twice and then dried at 100 °C for 24 hours. The optical density (*OD*) was measured at 600 nm using a Turner (Model 330) spectrophotometer. The correlation between dry cell weight cell concentration (X) and optical density (*OD*) was then determined as $X = 1.4038 \cdot OD + 0.1726$. Immobilized cell mass was determined from total weight of the dried cotton material containing cells, less the weight of the cotton material alone.

3.3.2. Glucose Measurement

Glucose concentrations were determined using glucose kit (Procedure No. 510) from Sigma (St. Louis, Mo). The Sigma procedure is based upon the following coupled enzymatic reactions:

$$Glucose + 2H_2O + O_2 \xrightarrow{Glucose\ Oxidase} Gluconic\ Acid + 2H_2O_2$$

$$H_2O_2 + o\text{-}Dianisidine\ [Colorless] \xrightarrow{Peroxidase} Oxidized\ o\text{-}Dianisidine\ [Brown]$$

The intensity of the brown color measured at 425-475 nm is proportional to the original glucose concentration. The measurement procedure is outlined as follows according to the manual:

1) Reagent solution preparation was carried out as follows. Enzyme Solution was prepared by adding contents of 1 capsule of PGO enzymes, No. 510-6, to 100ml distilled water in an amber bottle. Invert bottle several times with gentle shaking to dissolve. Color Reagent Solution was prepared by reconstituting one vial of o-Dianisidine Dihydrochloride, Catalogue No. 510-50, with 20 ml water. Combined Enzyme-Color Reagent Solution was prepared by combining 100 ml Enzyme Solution and 1.6 ml Color Reagent Solution. The solutions were mixed by inverting several times or with mild shaking. 1000 mg/L Glucose Standard Solution was supplied ready to use.
2) Label the testing tubes as follows: BLANK, STANDARD, TEST 1, TEST 2, etc.
3) To BLANK tube add 0.5 ml water. To STANDARD tube add 0.5 ml of a 20-fold dilution of 1 g/L Glucose Standard Solution. To TEST tubes add 0.5 ml of a suitable diluted samples (not greater than 100 mg g/L).
4) To each tube add 5.0 ml of Combined Enzyme-Color Reagent Solution and mix each tube thoroughly.
5) Incubate all tubes at 37 °C for 30 minutes in water bath.
6) At the end of incubation period, remove all tubes from water bath, read absorbance [A] of STANDARD and TESTS at 450 nm, using BLANK as reference.
7) Calculate TEST values as follows:

$$Glucose\ [mg / L] = \frac{A_{test}}{A_{standard}} \times 50 \qquad (3.1)$$

3.3.3. Ethanol Analysis

Ethanol concentration were measured by a Hewlett Packard 5880A Series gas chromatograph. The chromatograph was linked to a HP 5880A level 3 integrator and equipped with an FID detector (Avondale, PA). The column was a 1.5 foot 1/8" telfon tubing filled with 8-100 mesh PORAPAK-T packing (WATERS, Framingham, MA). Temperatures of the oven, injection port and the detector were 100, 200 and 200 °C respectively. Helium was used as the carrier gas at a flow rate of 20 ml/min and the gas stream to the FID detector was a mixture of air and hydrogen.

3.3.4. Glucoamylase Assay

To assay glucoamylase, an aliquot of 0.2 ml enzyme solution and 1.8 ml 2% starch (SIGMA Chemical Co., St. Louis, MO; catalogue no. S-2630) in 0.1 M citrate buffer were mixed and allowed to react at 37 °C for 30 minutes. The reaction was stopped by boiling the reaction mixture at 100 °C for 10 minutes. Glucose produced during the reaction was assayed by the SIGMA glucose kit as described in section 3.3.2. One unit of glucoamylase activity is defined as the release of 1 μmol glucose per minute from soluble starch in 0.1 M citrate buffer, pH 5.0 at 37 °C (Nunberg et al., 1988).

3.3.5. Determination of Fraction of Plasmid-Bearing Cells

Samples from actively growing cultures were diluted (1×10^{-5} - 1×10^{-6}) and 100 μl of this solution were spread onto both YNB selective and YPG nonselective agar plates simultaneously, and then incubated at 30 °C for 2 days. All cells can grow on YPG

nonselective agar plates, but only the plasmid-bearing cells can grow on YNB selective agar plates. The proportion of plasmid-bearing cells were found by counting the colonies on both types of plates (100-250 colonies per plate).

3.3.6. Determination of Maximum Specific Growth Rates

The maximum specific growth rates of plasmid-free and plasmid-bearing were determined using the wash-out method:

$$\frac{dX}{dt} = (\mu_{max} - D)X$$
$$Ln(\frac{X}{X_o}) = (\mu_{max} - D)t \qquad (3.2)$$

At one fixed dilution rate ($D > \mu_{max}$), μ_{max} can be found from semilogarithmic plots of cell concentration versus time. Batch exponential growth was also used to determine the maximum specific growth rates.

3.4. Free Suspension Culture

The objectives of free suspension culture experiments were as follows:

1) To gain knowledge about the behaviour of the model recombinant yeast (growth characteristics, plasmid stability, recombinant protein productivity, etc.).
2) To investigate the effects of fermentation conditions, growth rates and growth media on plasmid stability and recombinant protein production.
3) To examine the importance of the metabolic pathways in yeast with regard to recombinant protein formation.
4) To obtain the parameters required for mathematical modelling.

5) To make a comparison between the airlift and stirred-tank bioreactors and investigate the possibility of application of airlift bioreactor for recombinant yeast fermentation.

6) To offer a basis for comparison of immobilization system.

3.4.1. Batch Fermentation

The recombinant yeast was maintained at 4 ℃ on slants of maltose selective solid agar medium (medium V). Inoculum was grown in this YNB selective solid agar medium (medium IV) in shake flasks at 30°C and 200 rpm for 36 hours and the inoculum size was 5% of the bioreactor working volume for all batch culture experiments. A non-selective complex medium (medium III), the yeast extract-peptone-glucose (YPG) medium (Barnett et al., 1983), was used in batch fermentations. The batch culture was carried out in an airlift bioreactor at 30 ℃ and an aeration rate of 1 VVM (volume per volume of medium per minute). A split column airlift bioreactor with an inner diameter of 6.5 cm and aspect ratio of 7 was used as a culture vessel. A 6.5×28.5 cm baffle was set in the middle of the column to form the riser and downcomer sections. The working liquid volume in the reactor was 1200 mL. The clearance between the sparger and the bottom of baffle was 6.5 cm. The distance between the liquid surface and the top of the baffle was 6.5 cm. The pH and dissolved oxygen were monitored by the Ingold pH and DO probes during the fermentation process. Fermentation profiles were obtained by analyzing cell mass, glucose, ethanol and glucoamylase concentrations and fraction of plasmid-bearing cells. Batch fermentations were also performed in a stirred-tank bioreactor (New Brunswick, Model 19) at 30 ℃. The bioreactor had a 1.5 L

working liquid volume. The aeration rates were kept constant at 0.6 to 1.5 L/min (0.4 to 1.0 VVM).

3.4.2. Continuous Fermentation

Continuous culture operation was used to investigate plasmid stability and recombinant protein productivity because it gave rise to a relatively well-defined, reproducible environment and a state of balanced growth. As the dilution rate corresponds to the specific growth rate in continuous culture, it also offers a convenient way to examine the effect of growth rate on plasmid stability, which is still a controversial issue.

The inoculum preparation was the same as in batch fermentation and the inoculum size was 10% of the bioreactor working volume for all continuous culture experiments. A YPG non-selective complex medium (medium III) or YNB selective minimum medium (medium I) was used in continuous culture. The continuous cultures were carried out in both airlift and stirred-tank bioreactors at 30 °C and an aeration rate of 1 VVM. The bioreactors used were the same as described in batch fermentation.

In order to achieve the objectives of this part of the research, the following experiments were performed:

1) Continuous suspension fermentations using nonselective medium (medium III) at different dilution rates (D = 0.05, 0.10, 0.20, 0.25 h^{-1}). Cell, glucose, ethanol and

glucoamylase concentrations and fraction of plasmid-bearing cells were followed during chemostat cultures.

2) Continuous suspension fermentations using different glucose feeding concentrations (5, 10, 20 g/L) at a fixed dilution rate (D = 0.10 h^{-1}) to investigate glucose effect and obtain quantitative knowledge about the metabolic pathways of the recombinant yeast.
3) Continuous suspension fermentations using YNB selective medium (medium I) at a fixed dilution rate (D = 0.10 h^{-1}) to examine the effect of selection pressure.

3.4.3. Repeated-Batch Fermentation

Repeated batch fermentation experiments were designed to investigate plasmid stability and recombinant protein productivity in this reactor operation mode. A 120 ml inoculum, prepared in shake flasks in YNB selective medium, was used to inoculate the airlift bioreactor filled with 1080 ml of nonselective YPG medium (1.2 L total working liquid volume). After inoculation, the fermentation proceeded in the batch mode for 24 hours. The culture fluid was pumped out from the bioreactor and about 120 ml was retained as the inoculum for the next batch fermentation. The next batch fermentation was initiated by pumping the 1080 ml of fresh YPG nonselective medium into the bioreactor. This procedure was repeated every 24 hours. The repeated-batch fermentation was performed for 2 weeks. During this period, cell, glucose, ethanol and glucoamylase concentrations and fraction of plasmid-bearing cells were measured.

3.5. Immobilized Cell Culture

3.5.1. Structure of Immobilized-Cell-Film Airlift Bioreactor

In view of the limitations of the other types of immobilized-cell bioreactors, a novel immobilized-cell-film airlift bioreactor was devised. The immobilized-cell-film airlift bioreactor and cage for supporting cotton cloth are shown in Fig. 3.1. The same split column airlift bioreactor as in the free suspension culture was used as a culture vessel. Its inner diameter is 6.5 cm and its aspect ratio is 7.0. A 6.5×28.5 cm baffle was set in the middle of the column to form the riser and downcomer. Two cotton cloth cages of half column (diameter = 6.0 cm, height = 26 cm) were placed on both sides of the baffle. The cotton cloth was fitted onto the 2mm stainless steel wire mesh in the cages to form double-layer cloth films. The distance between the cloth films was 7.0 mm. The total cloth area in the bioreactor available for cell attachment was 1566.5 cm^2. The liquid volume in the bioreactor was 1100 ml. The clearance between the sparger and the bottom of baffle was 6.5 cm. The distance between the liquid surface and the top of the baffle was 6.5 cm. Dissolved oxygen (DO) and pH were monitored during the fermentation process by using appropriate electrodes. Temperature was controlled at 30 °C by circulating heated water from a water bath through a jacket around the bioreactor. When the filtered sterile air was injected into the bioreactor through the sparger, steady liquid circulation was achieved throughout the reactor due to the bulk density difference between riser and downcomer, providing good mixing and mass transfer. In the case of free suspension culture, the cages were taken out from the bioreactor.

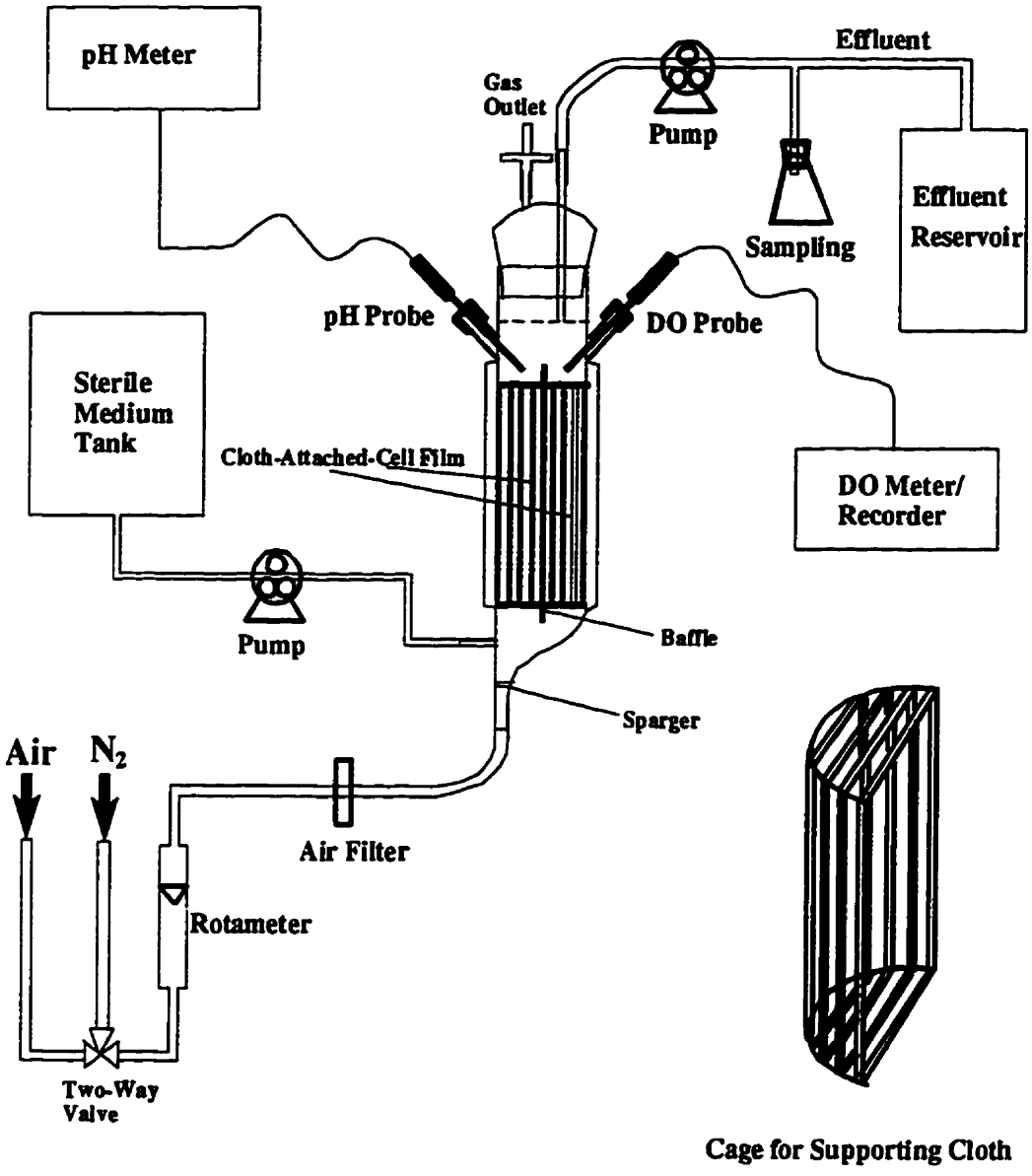


Figure 3.1. Immobilized-Cell-Film Airlift Bioreactor and Setup for Continuous Culture

3.5.2. Cell Immobilization

Yeast attachment on cloth was initiated according to Joshi and Yamazaki (1984). Inoculum prepared in shake flasks in YNB selective medium was used to inoculate the above reactor; the inoculum size was 110 ml. The culture was grown in batch mode for 12 hours at 1 v.v.m. air supply and 30 °C. Then the culture was completely drained and the fresh medium was pumped into the bioreactor. After 2 hours, the medium was drained again and cells were allowed to grow on the cotton cloth for 10 hours. The immobilized-cell bioreactor was then ready for continuous culture.

3.5.3. Continuous Fermentation with Immobilized Cells

The objectives of the continuous fermentation experiments with immobilized cell system are:

1) To investigate the effects of immobilization on plasmid stability and recombinant protein productivity.
2) To examine the effect of dilution rates and glucose effect in immobilization system.
3) To test the efficiency and feasibility of the proposed immobilized-cell-film airlift bioreactor.

After the immobilization procedures, the YPG nonselective medium was continuously introduced into the bioreactor by the inlet pump, and at the same time the effluent was pumped out from the top of the reactor; continuous culture was initiated immediately to give a certain dilution rate. Samples were taken periodically from the

effluent for the measurements of cell, glucose, ethanol and glucoamylase concentrations and plasmid stability. The following continuous fermentation experiments were carried out to realize the objectives of this part of the research:

1) Continuous cultures in immobilized-cell-film airlift bioreactor using YPG complex medium at dilution rates of 0.10, 0.20, 0.30 h^{-1}.
2) Investigate the glucose effect in immobilized cell system by varying the inlet glucose concentration at a fixed dilution rate.

3.5.4. Repeated Batch Fermentation with Immobilized Cells

By using yeast film attached on cotton cloth, repeated batch fermentation experiments were performed in the proposed immobilized-cell bioreactor to further examine the effects of immobilization. The immobilized-cell-film bioreactor was easy to be operated in a repeated-batch mode. After immobilization procedures, batch fermentation was initiated by pumping the nonselective YPG medium into the bioreactor. After 24 hours, the culture was completely harvested and the fresh medium was then pumped to the bioreactor to start the next batch fermentation. This procedure was repeated in every 24 hours. The repeated-batch fermentation was performed for 2-3 weeks. During this period, cell, glucose, ethanol and glucoamylase concentrations and fraction of plasmid-bearing cells were measured.

3.6. Experimental Error and Reproducibility

In this research, the main fermentation variables were concentrations of glucose, cell, ethanol and glucoamylase as well as fraction of plasmid-bearing cells. They were measured via the methods described in chapter 3. All the measurements were in triplicate and the mean values were used (outlier points were deleted). Maximum variance in measurements of glucose, cell mass, ethanol and glucoamylase did not exceed +/- 10% of the average measured value. Relatively larger deviation occurred in measuring the fraction of plasmid-bearing cells by plate count method. Maximum deviation associated with measurements of fraction of plasmid-bearing cells could be as large as +/- 15% of the reported values. Based on +/- 10% experimental error, generally only 2 significant figures were warranted. However, in some cases more than 2 significant figures were taken in the thesis because the data were generated by computer.

Most of the experiments reported in the previous chapters were performed twice to three times in order to check reproducibility. Typical reproducibility checks in batch suspension fermentation, continuous suspension fermentation, continuous and repeated-batch fermentations with the immobilized cells are shown in Figure B.1, B.2, B.3 and B.4 respectively. These figures show good agreement between two experimental runs in terms of trends as well as values. Considering the measurement variance discussed in the previous section, good reproducibility may be generalized to all the experimental data obtained in this research. Furthermore, the conclusions in this study were made based on the results from different bioreactor operations or different strains. For example, by using

selected strain higher enzyme productivity was obtained in shake flasks, airlift, stirred-tank and immobilized cell bioreactors; enhanced enzyme production and plasmid stability were observed in the immobilized cell systems for both the original and selected strains. Therefore, the conclusions made in this research have a strong basis and the overall accuracy for the study is considered satisfactory.

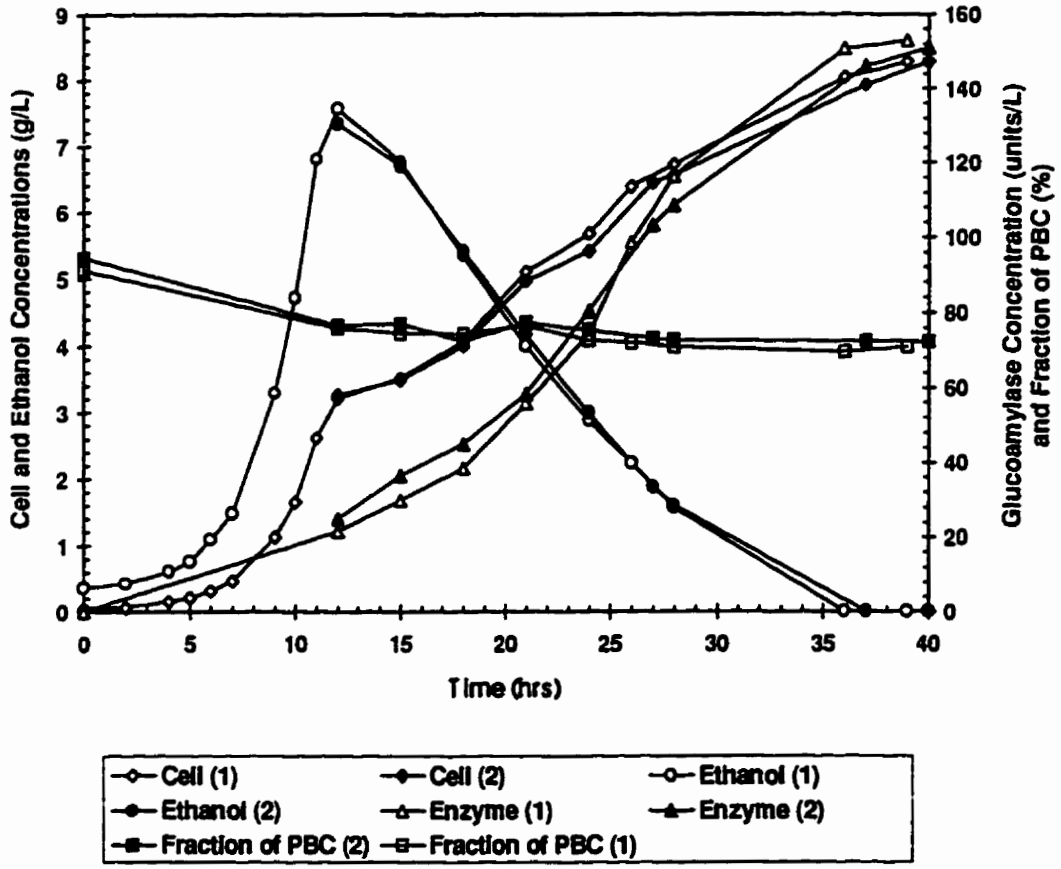


Figure 3.2. Reproducibility Check in Free Suspension Batch Fermentation (Temperature = 30 °C, Aeration Rate = 1.0 VVM).

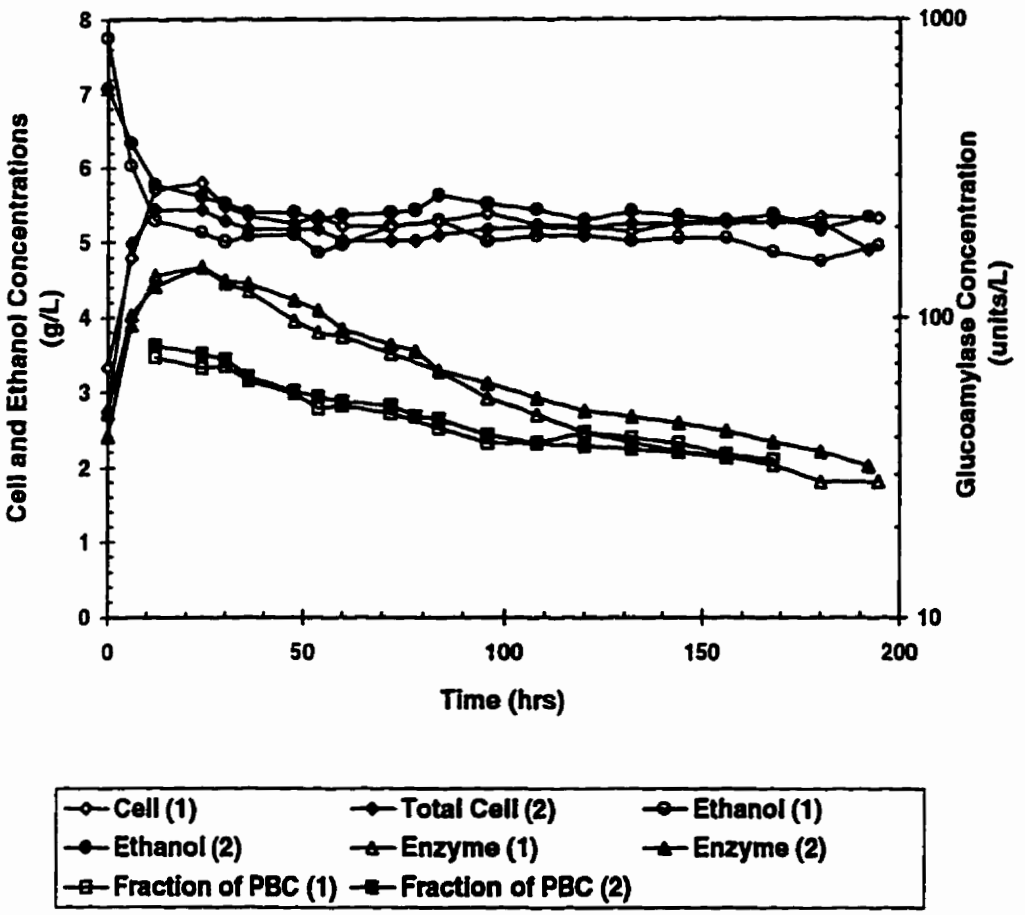


Figure 3.3. Reproducibility Check in Free Suspension Continuous Fermentation (Temperature = 30 °C, Aeration Rate = 1.0 VVM, Dilution Rate = 0.1 h^{-1}).

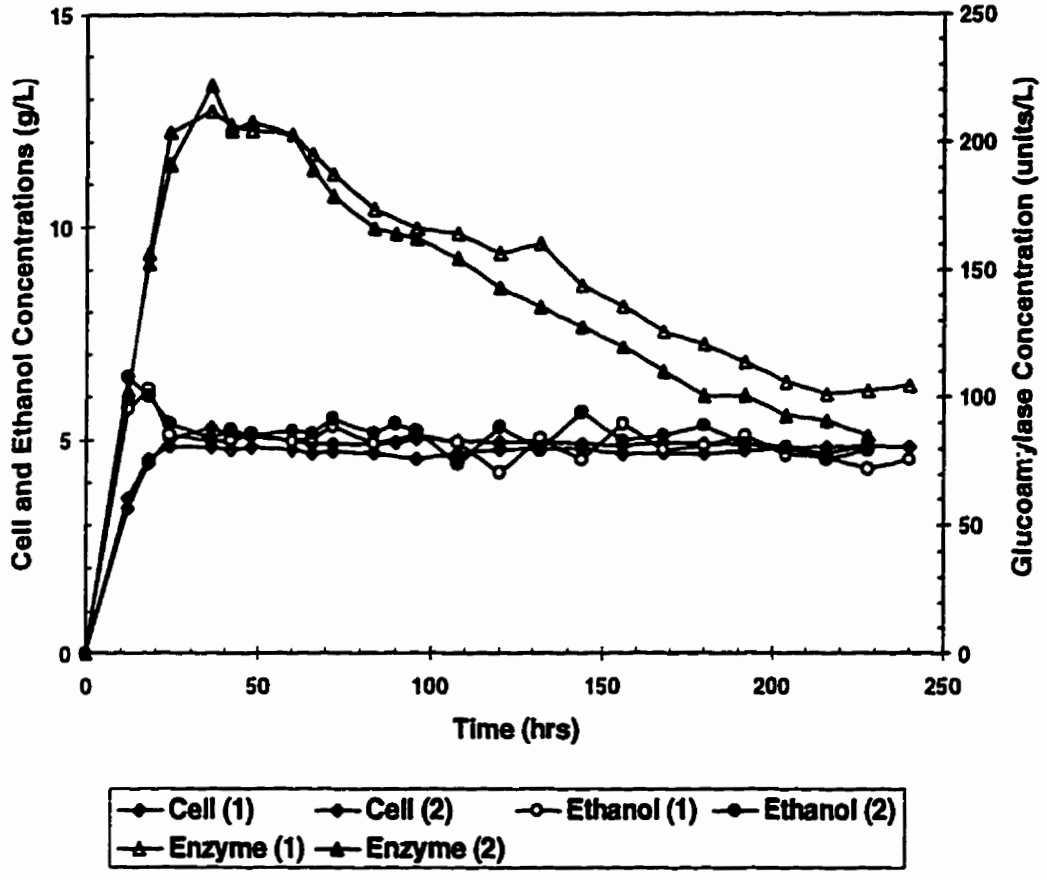


Figure 3.4. Reproducibility Check in Continuous Fermentation with Immobilized Cell System (Temperature= 30 °C, Aeration Rate = 1.0 VVM, Dilution Rate = 0.1 h^{-1}).

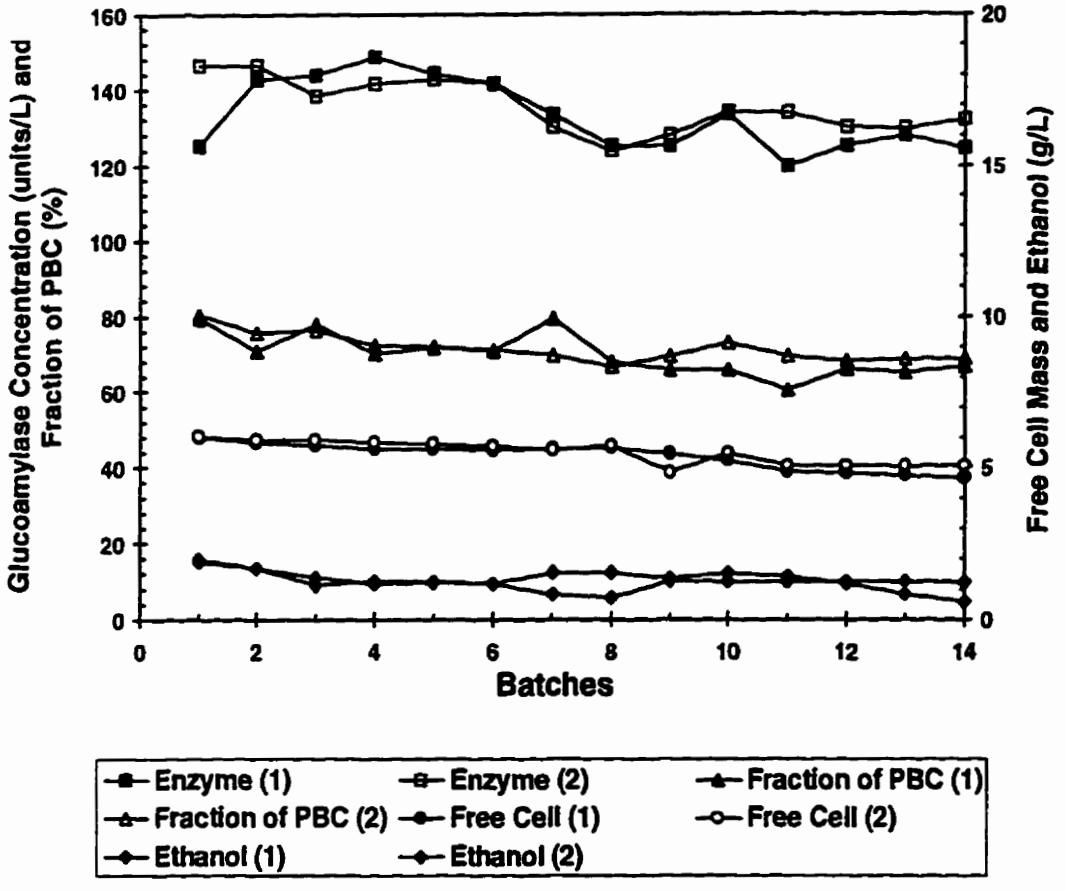


Figure 3.5. Reproducibility Check in Repeated Batch Fermentation with Immobilized Cell System (Temperature = 30 °C, Aeration Rate = 1.0 VVM).

CHAPTER 4

SUSPENSION RECOMBINANT YEAST CULTURE

4.1. Introduction

This part of the research was focused on the following questions:

1) What is the growth characteristics of the recombinant yeast? Is the production of the recombinant protein growth-associated?
2) What is the nature of the plasmid instability in the recombinant yeast? Are the 2 μm based yeast shuttle plasmids stable? And if not, to what extent?
3) How do fermentation conditions, growth rates and growth media affect the plasmid stability and the recombinant protein production?
4) How are the metabolic pathways in the yeast related to the recombinant protein formation.
5) What is the advantages and disadvantages of using an airlift bioreactor for the recombinant yeast fermentation.

4.2. Batch Suspension Fermentation

4.2.1. Batch Culture in Airlift Bioreactor (ALR)

Batch suspension cultures were carried out in a 1.5 liter airlift bioreactor (1.2 liter working volume) at 30 °C and an aeration rate of 1.0 VVM. Figure 4.1 shows a typical time course of batch culture for glucoamylase secretion using YPG nonselective medium. Cell growth was typically diauxic. Initially glucose concentration was high, after a short lag

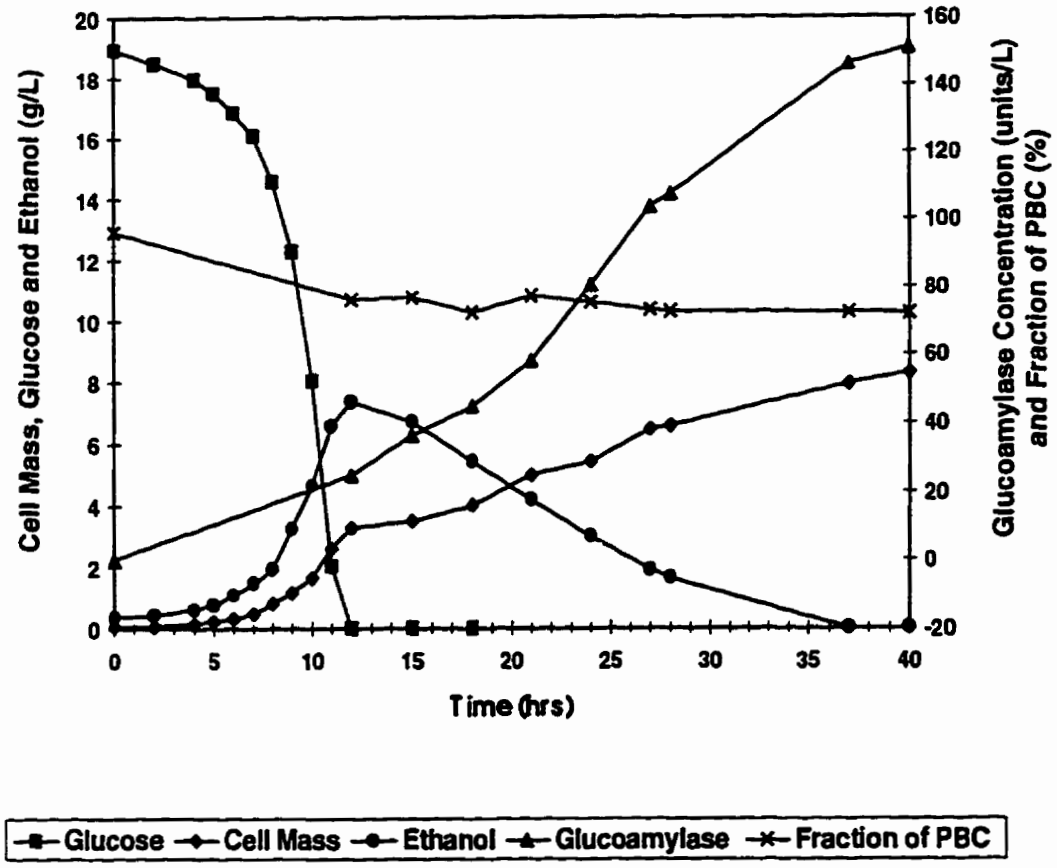


Figure 4.1. Typical Time Course of Batch Culture in Airlift Bioreactor (PBC – Plasmid-Bearing Cells).

period the cells, using glucose as carbon source, grew rapidly in the first exponential phase. After about 12 hours, the glucose was completely consumed and ethanol reached a maximum concentration. Then the cells began to utilize ethanol as carbon source and another exponential growth followed. Total cell concentration achieved a maximum value after about 36 hours and then remained constant in a stationary phase due to the exhaustion of ethanol. The total cell concentration included both plasmid-bearing and plasmid-free cells. Glucoamylase concentration increased with increasing cell concentration during the two exponential growth phases, reaching its maximum when cell growth approached stationary phase. This suggested that glucoamylase secretion was growth-associated.

Plasmid stability was also examined during batch culture by following the fraction of plasmid-bearing cells. Figure 4.1 shows that the fraction of plasmid-bearing cells decreased with culture time, indicating that plasmid loss occurred during the batch culture. Figure 4.1 also shows that the fraction of plasmid-bearing cells fell more rapidly during the first growth phase on glucose than during the second growth phase on ethanol. This is because the specific growth rate in the first exponential growth phase was higher than that in the second exponential phase. The more the cell divided, the more likely they lost plasmids. It appears that the segregational instability mainly contributed to plasmid loss.

4.2.2. Batch Culture in Stirred-Tank Bioreactor (STR)

Batch suspension cultures were also performed in a 2.0 L stirred-tank bioreactor (1.5 L working volume) at 30 °C, 500 rpm and 1.0 VVM using the YPG nonselective medium. Typical batch fermentation profiles are shown in Figure 4.2. The patterns of the fermentation profiles in the stirred-tank bioreactor are quite similar to those in the airlift bioreactor. The recombinant yeast also underwent a diauxic growth, and the glucoamylase production was growth-associated as well. These findings agree well with results reported for protein production with other recombinant yeast strains (Gu et al, 1989; Coppella and Dhurjati, 1990; Turner et al., 1991).

The fermentation speed in the STR was faster than in the ALR. In about 24 hours the batch fermentation in the STR had achieved stationary phase; in contrast, it took about 36 hours to reach stationary phase in the ALR. This is because of the slower ethanol growth phase in the ALR caused by the low availability of dissolved oxygen. A semilogarithmic plot (Figure 4.3) clearly shows that the specific growth rate during the second growth phase (using ethanol as carbon source) was smaller in ALR (0.060 h^{-1}) than in STR (0.097 h^{-1}). However, the specific growth rates during the first growth phase (using glucose as carbon source) were nearly the same (0.38 h^{-1}) in both the STR and ALR bioreactors (Figure 4.3). The results can be explained by the dissolved oxygen profiles during the batch cultures, which are presented in Figure 4.4. During the first growth phase the dissolved oxygen (DO) was above 50% air saturation for both the STR and ALR, but in second growth phase the DO in the ALR dropped to about 10% air

saturation. Although the critical DO for yeast is about 3% air saturation (Bailey and Ollis, 1986), the lower DO in the ALR might have caused cells to grow slowly.

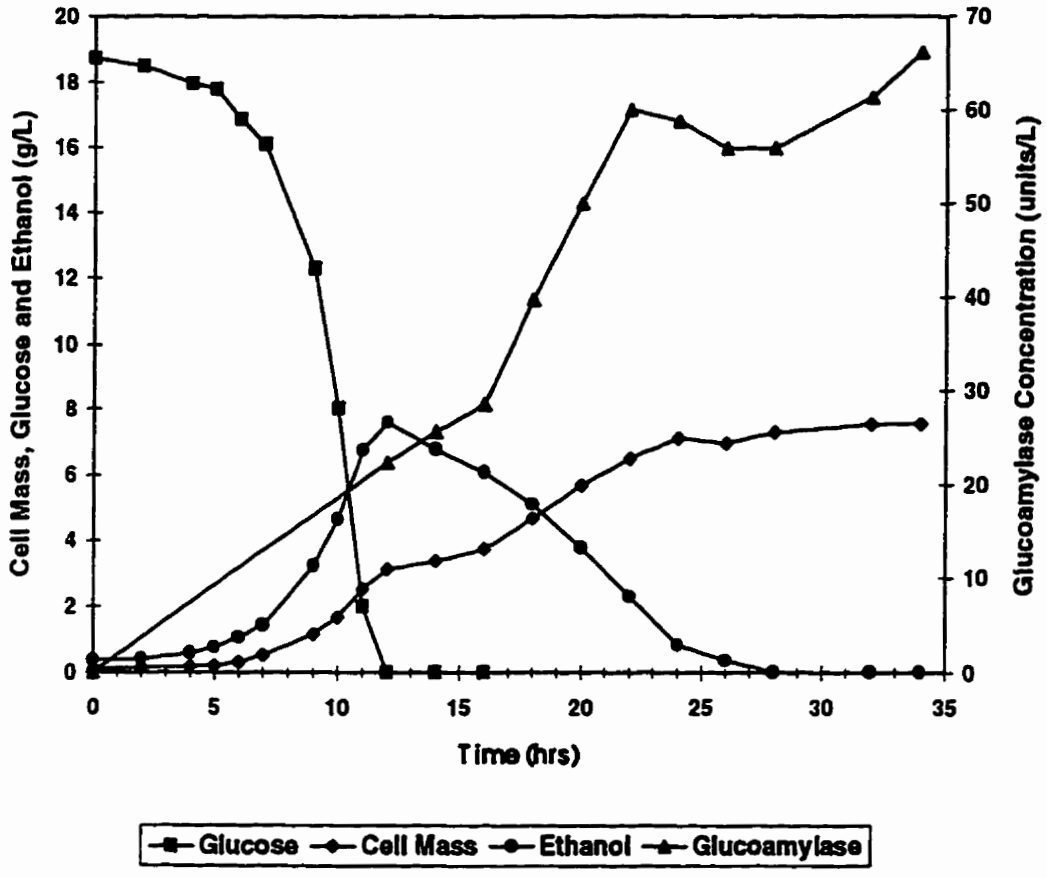


Figure 4.2. Typical Time Course of Batch Culture in Stirred-Tank Bioreactor.

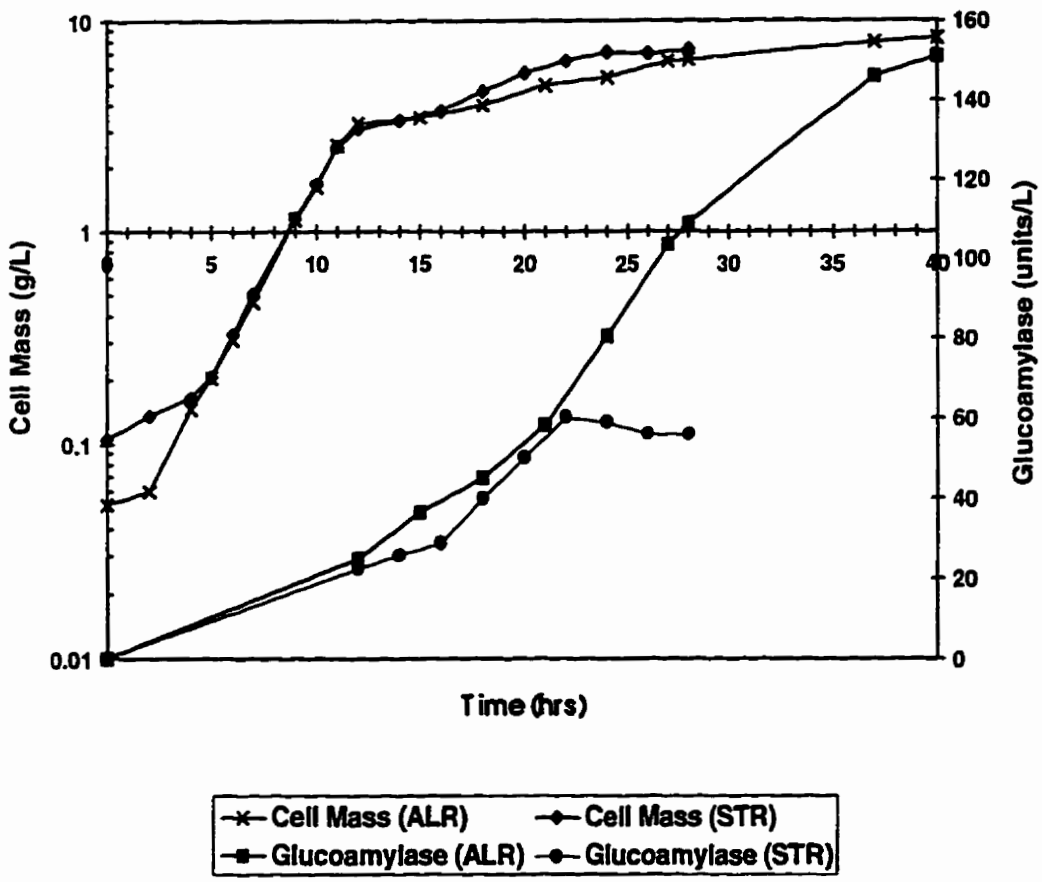


Figure 4.3. Comparison of Cell and Glucoamylase Concentrations between Stirred-Tank and Airlift Bioreactors.

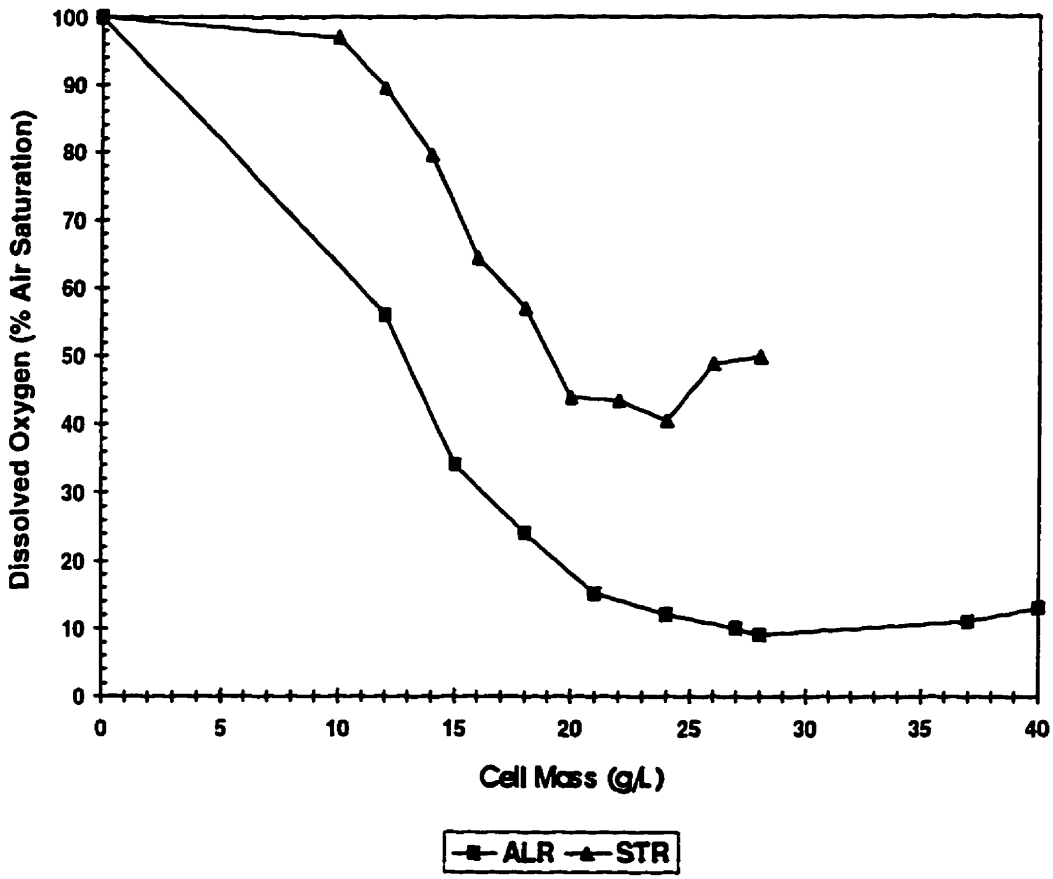


Figure 4.4. Comparison of Dissolved Oxygen Profiles between Stirred-Tank and Airlift Bioreactors.

The final glucoamylase concentration in the ALR (153 units/L) was much higher than that in the STR (66 units/L) although the final cell concentrations in both bioreactors were nearly the same (Figure 4.3). This result may be related to the difference in the specific growth rate in the second growth phase. It appears that the recombinant protein yield decreased with increasing specific growth rate of the recombinant yeast. The relationship between the recombinant protein yield and specific growth rate will be further discussed in the next section.

4.2.3. Effect of Specific Growth Rate on Recombinant Protein Yield

Figures 4.3 and 4.4 demonstrated that dissolved oxygen tension in the bioreactor affects the specific growth rate, and the recombinant protein production was directly related to the specific growth rate. In order to further test this phenomenon, a batch culture fermentation was performed in the STR bioreactor using lower aeration rate (0.4 VVM). Due to decreased dissolved oxygen in the bioreactor, the batch fermentation was prolonged but the final glucoamylase concentration increased from 61 to 91 units/L. Thus, the enhanced recombinant protein yield due to the slower growth rate was shown again.

The specific growth rates for both exponential phases were determined from the semilogarithmic plots of cell concentration versus time as shown in Figure 4.5. The glucoamylase yields were evaluated from linear regression of glucoamylase concentrations to cell concentrations as shown in Figure 4.6. The parameter estimates are listed in Table 4.1. Table 4.1 shows that the specific growth rates in the first growth phase (on glucose)

were nearly identical for the three different situations, so were the glucoamylase yields. But during the second growth phase (on ethanol), the specific growth rates were different and the glucoamylase yield decreased with increasing specific growth rate. The recombinant protein yield was significantly correlated with the specific growth rate.

Table 4.1. Effect of Specific Growth Rate on Recombinant Protein Yield during Batch Culture in STR and ALR.

	STR (1 VVM)		STR (0.4 VVM)		ALR (1 VVM)	
	First Growth Phase	Second Growth Phase	First Growth Phase	Second Growth Phase	First Growth Phase	Second Growth Phase
Specific Growth Rate (h^{-1})	0.386	0.097	0.376	0.072	0.390	0.060
Glucoamylase Yield (Enzyme Units/g cell)	6.59	10.86	6.66	18.42	6.73	25.01

This finding is consistent with the literature on foreign protein expression in the recombinant yeast *S. cerevisiae* (Gu et al., 1989; Da Silva and Bailey, 1991; Hardjito et al., 1992). It is also in agreement with the results reported for protein production with recombinant bacteria (Bentley and Kompala, 1989; Sayadi et al., 1989; Satyagal and Agrawal, 1989; Bentley et al., 1990; Flichinger and Rouse, 1993). There may be two reasons for explaining this phenomenon. Several researchers had reported that the copy number of plasmid increased with decreasing specific growth rate (Sayadi et al., 1987;

Bentley and Kompala, 1989; Satyagal and Agrawal, 1989). Increasing protein yield accompanied by decreasing specific growth rate is possibly due to increasing the copy number, even though the copy number was not measured in this work. If the copy number was the same regardless of the specific growth rate, increasing protein yield would be due to an increase in DNA transcription efficiency as a consequence of the increase in the intracellular promoter concentration (Hardjito et al., 1992). The experimental findings of Park and Ryu (1990) supported this assumption. In a two-stage continuous system with a recombinant *E. coli* K12ΔH1Δ*trp*/pPLc23*trp*A, they investigated the effect of dilution rate on Trpα production. The plasmid content (mg DNA per mg cells) was found to be practically the same independent of the dilution rate; however the protein yield (units of protein per mg cells) decreased as dilution rate increased. In other words, the transcription efficiency decreased with increasing dilution rate.

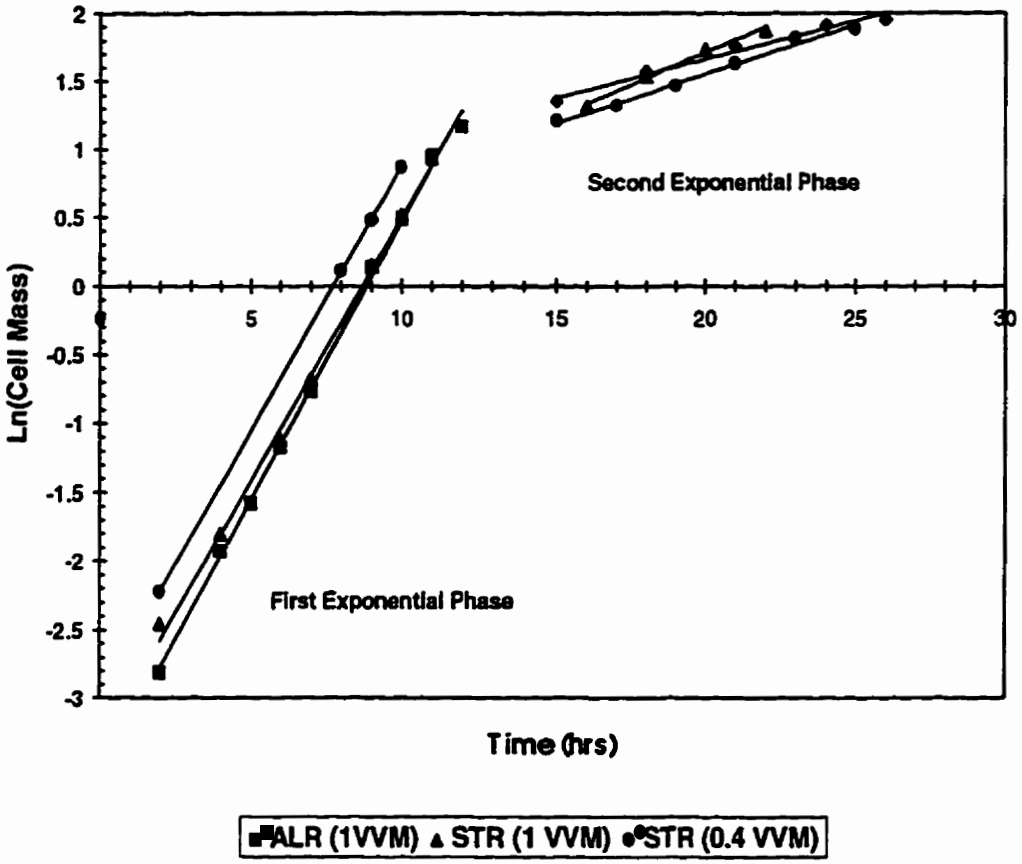


Figure 4.5. Determination of Specific Growth Rates by Semilogarithmic Plot of Cell Concentration versus Time in Two Different Exponential Growth Phases.

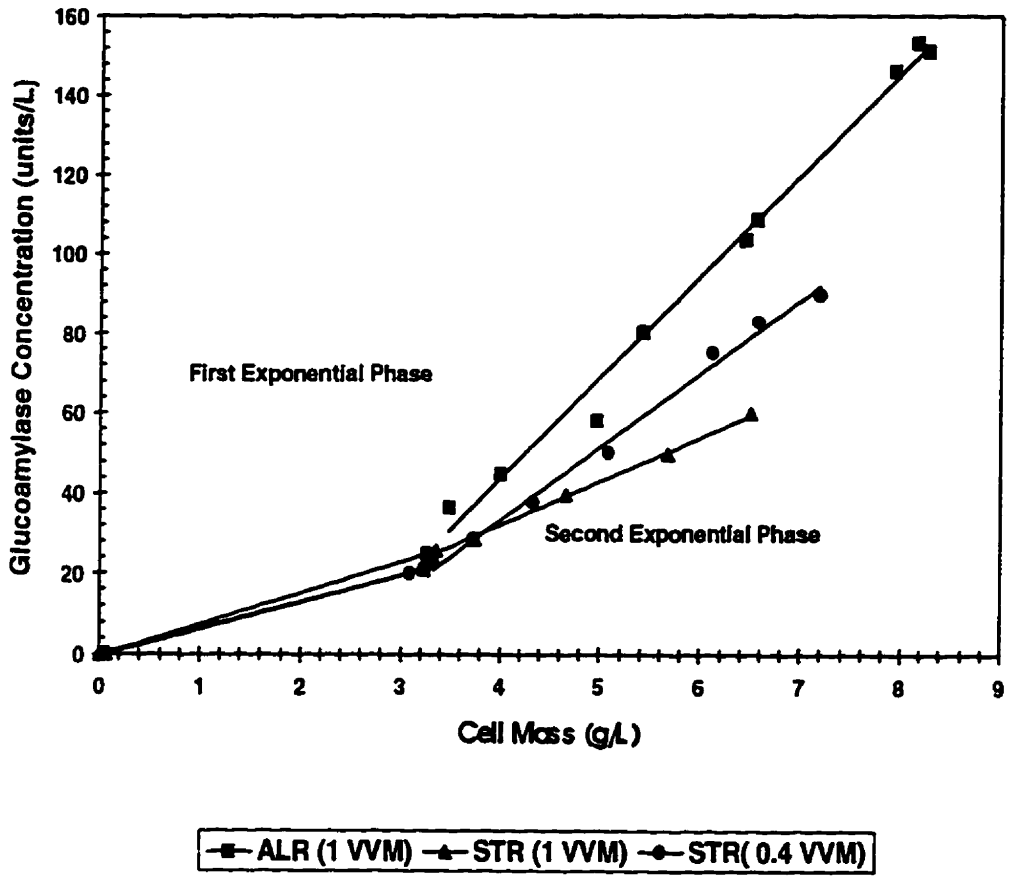


Figure 4.6. Determination of Recombinant Protein Yield by Linear Regression of Glucoamylase Concentration with regard to Cell Concentration in Two Different Exponential Growth Phases.

4.2.4. Metabolic Pathways in Recombinant Protein Production

Some of the important metabolic pathways identified in yeast is presented in Figure 4.7. At low carbonhydrate concentration and good aeration the glucose is metabolized by the Emden Meyerhof Parnas (EMP) pathway to pyruvate, which is then oxidized by the tricarboxylic acid (TCA) cycle to carbon dioxide and water (glucose oxidation pathway). Most of the ATP generation occurs in the TCA cycle accompanied by the formation of intermediates for the biosynthesis of cellular materials and other metabolites. However, in anaerobic conditions or in conditions of high glucose flux the repression of mitochondrial function can occur and the TCA cycle will be repressed. Another problem which arises in conditions which repress mitochondrial function is that NADH cannot be oxidized by the cytochrome system. Thus, NADH generated in the glyceraldehyde 3-phosphate to 1,3-diphosphoglycerate step can only be oxidized by passing on the hydrogen atoms to acetaldehyde, resulting in the formation of ethanol (Berry and Brown, 1987). Under these conditions, glucose was mainly metabolized to ethanol (glucose fermentation pathway). Figure 4.7 also shows that under aerobic conditions *Saccharomyces cerevisiae* can be grown on ethanol or acetate as the sole carbon source (ethanol oxidation pathway). In these cases, the TCA cycle functions normally, but the role of the EMP pathway is reversed and it functions to convert acetate into the hexose and pentose molecules required for cellular biosynthesis. This is called gluconeogenesis.

Because of the metabolic features discussed above, when yeast *S. cerevisiae* is inoculated into an environment of high glucose concentration, a diphasic growth curve is

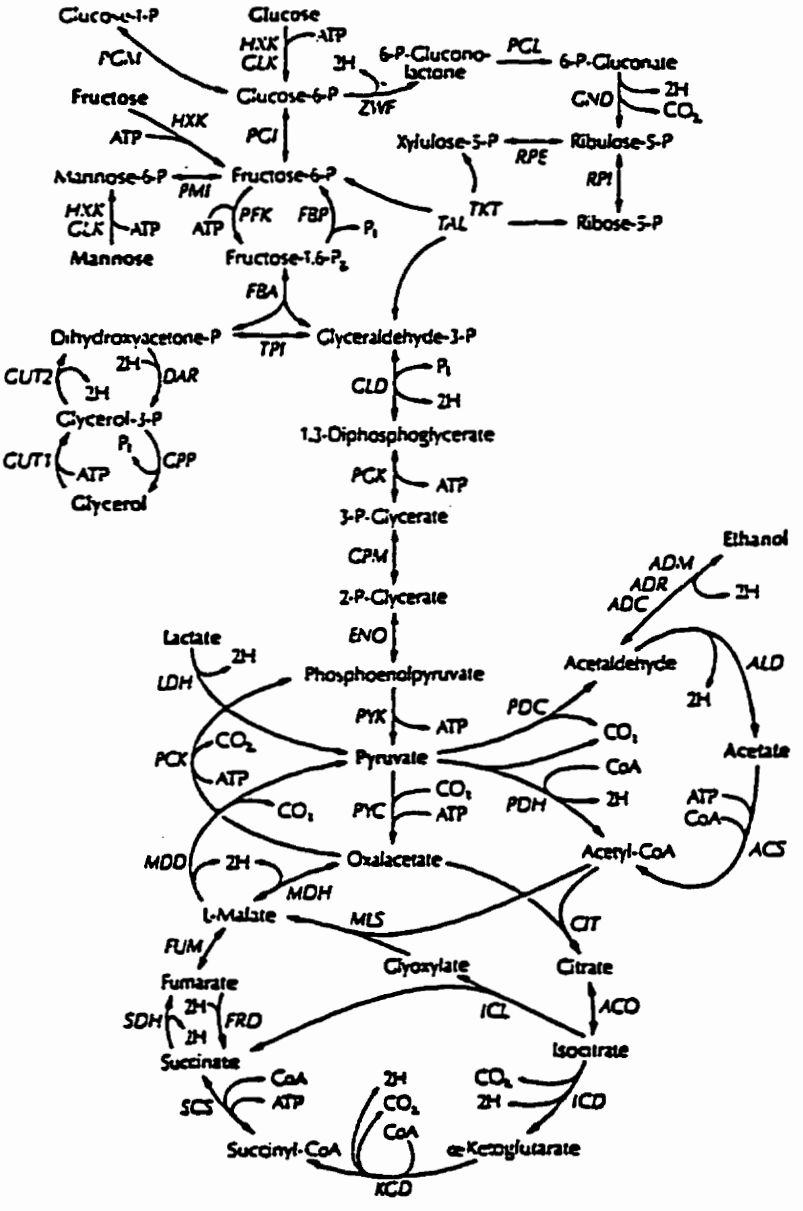


Figure 4.7. Pathways of Yeast Intermediary Metabolism (Berry and Brown, 1987).

observed. After an initial lag, the first exponential growth occurs. In this phase, the glucose is mainly fermented to ethanol and aerobic respiration is repressed due to the Crabtree effect. When the glucose has been depleted another lag phase occurs, and then relatively slower aerobic growth proceeds as the ethanol is metabolized to carbon dioxide and water. The typical diauxic growth was shown for the present recombinant yeast (Figures 4.1 and 4.2). Since the respiratory capacity of recombinant yeast is much lower than that of nonrecombinant yeast strains (Gopa et al., 1989), the Crabtree effect is more significant for recombinant yeast strains. Glucose fermentation and ethanol production will occur at glucose concentrations greater than 0.1 g/L even under aerobic condition (Coppella and Dhurjati, 1990).

Knowing the metabolic pathways in yeast is useful for optimizing the recombinant protein production. It is very likely that the formation of the recombinant protein is only associated with oxidative pathways. From Table 4.1 it can be seen that the glucoamylase yields in the first growth phase were much smaller than those in the second growth phase. The reason is that in the initial growth phase glucose fermentation dominated and most of the glucose was used for ethanol production due to the Crabtree effect, but a low level of glucose oxidation still occurred since repression of respiration by glucose is never total (Piper and Kirk, 1991). The glucoamylase yield in this phase was low since the cell growth in the fermentation pathway did not contribute to the production of glucoamylase and only the glucose oxidation pathway was associated with glucoamylase formation. This finding was further confirmed by the experimental results of complete anaerobic

fermentation (using nitrogen sparging) with the current recombinant yeast strain. No appreciable glucoamylase was detected under the strict anaerobic condition.

In the second growth phase, ethanol made in earlier fermentative phase was metabolized aerobically. Figures 4.1 and 4.2 shows that most of the glucoamylase was produced in the ethanol growth phase. Glucoamylase formation was significantly growth-associated in this pathway (See Figure 4.6). It is noteworthy that in both glucose and ethanol oxidation pathways, the TCA cycle functions actively. Therefore, it may be concluded that in order to produce recombinant protein efficiently, the TCA must be active and yeast should go through aerobic metabolism. This conclusion is reasonable because it is the TCA cycle that provides the precursors required for protein synthesis.

The Crabtree effect is the shift in metabolism from glucose oxidation to glucose fermentation at glucose levels greater than the threshold concentration, which ranges from 50 to 130 mg/L (Piper and Kirk, 1991; Pirt and Kurowski, 1970). Apparently the Crabtree effect is not beneficial for recombinant protein production since yeast undergoes anaerobic fermentation by conversion of glucose to undesired ethanol, which is not associated with protein formation. The Crabtree effect is controlled by glucose level in the media because the threshold concentration is so low. Enhancing oxygen transfer alone cannot overcome the Crabtree effect in batch culture since the glucose level is usually high. However, fed-batch or continuous operation modes may be used to reduce the Crabtree effect because it can keep low glucose concentration in the bioreactor for a longer period.

4.2.5. Application of Airlift Bioreactor to Recombinant Protein Production

The final glucoamylase concentration, glucoamylase productivity and the glucoamylase yield based the consumed glucose in both ALR and STR bioreactors are presented in Table 4.2. Although the fermentation speed was faster in the STR than in the ALR, the final glucoamylase concentration was more than doubled in the ALR, resulting in higher glucoamylase productivity. Furthermore, the enzyme yield based on the glucose consumed was much higher in the ALR than in the STR. These results indicate that the ALR for recombinant yeast fermentation is attractive. Because the recombinant protein yield decreases with increasing growth rate, it is not worthwhile to enhance fermentation speed by using high aeration rate or agitation speed. Airlift bioreactors provide good hydrodynamics and mass transfer characteristics at low capital and operating costs. Therefore they may be suitable for production of recombinant protein by employing recombinant yeast strains.

Table 4.2. Comparison of Glucoamylase Productivity between STR and ALR Bioreactors.

	ALR	STR
Final Glucoamylase Concentration (units/L)	153.19	66.22
Fermentation Time (h)	36	24
Glucoamylase Productivity (units/L.h)	4.26	2.76
Glucoamylase Yield Based on Glucose (units/g glucose)	8.06	3.48

4.3. Continuous Suspension Culture

Continuous culture experiments were performed for the following reasons: 1) to further test the relationship between recombinant protein production and metabolic pathways in yeast; 2) to examine the effects of dilution rate (growth rate) on plasmid stability and recombinant protein productivity; and 3) to make a comparison between nonselective and selective media.

4.3.1. Time Course for Continuous Culture in Nonselective Medium

Typical fermentation profiles for continuous cultures in YPG nonselective medium at dilution rate 0.10 h^{-1} are shown in Figure 4.8 (STR, original strain), Figure 4.9 (ALR, original strain), Figure 4.10 (STR, selected strain), and Figure 4.11 (ALR, selected strain). All these Figures show similar patterns. Continuous operation started about 12 hours after batch operation to provide cell build-up. Following the initiation of continuous feeding, cell concentration began to increase with culture time. After about 36 hours or 3 to 4 time's reactor volume of feeding, total cell concentration achieved a constant value. Ethanol concentration, pH and glucose concentration (not shown) also reached nearly constant values, indicating that the continuous culture approached its steady state. Recombinant protein concentration (glucoamylase) in the bioreactor, however, never achieved steady-state. Glucoamylase concentration increased with increasing cell mass and reached its maximum value after about 30 hours, and then decreased continuously throughout the culture process. After about 120 hours (16 generations), the enzyme con-

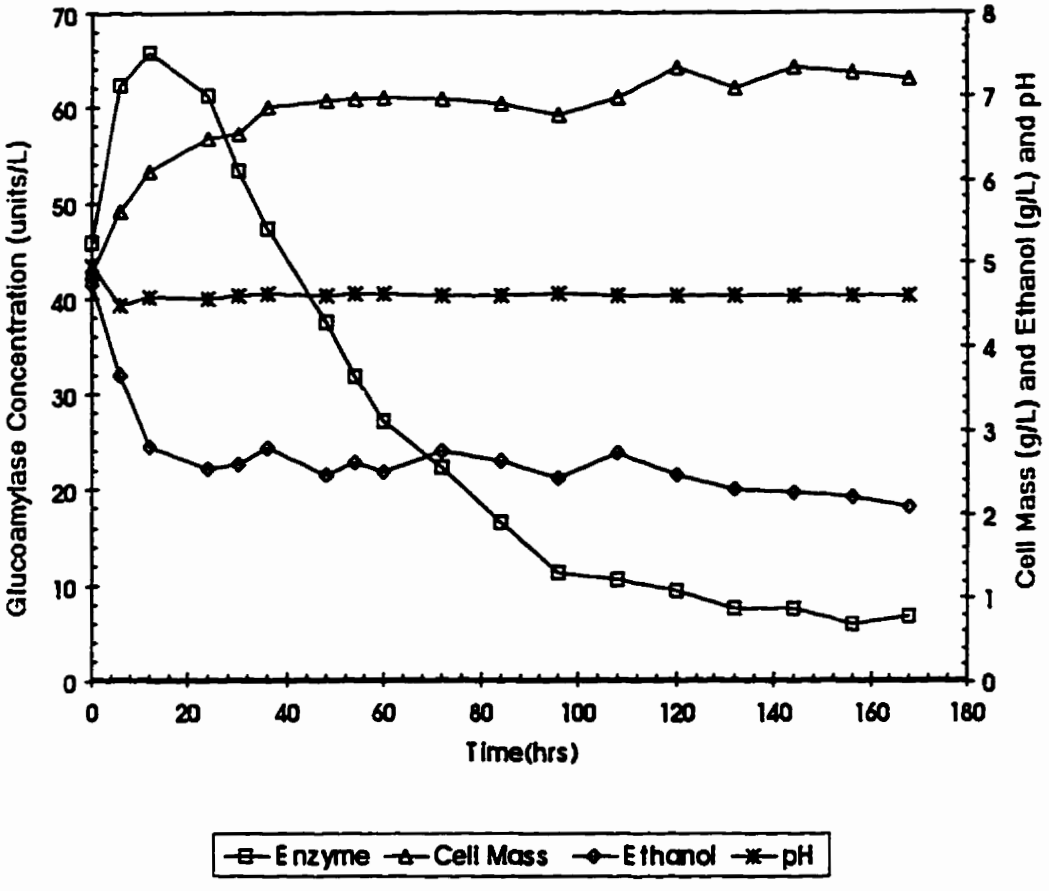


Figure 4.8. Continuous Suspension Culture in Stirred-Tank Bioreactor Using Original Strain at T = 30 °C, D = 0.1 h^{-1}, VVM = 1.0, RPM = 500 and G_0 = 20.0 g/L.

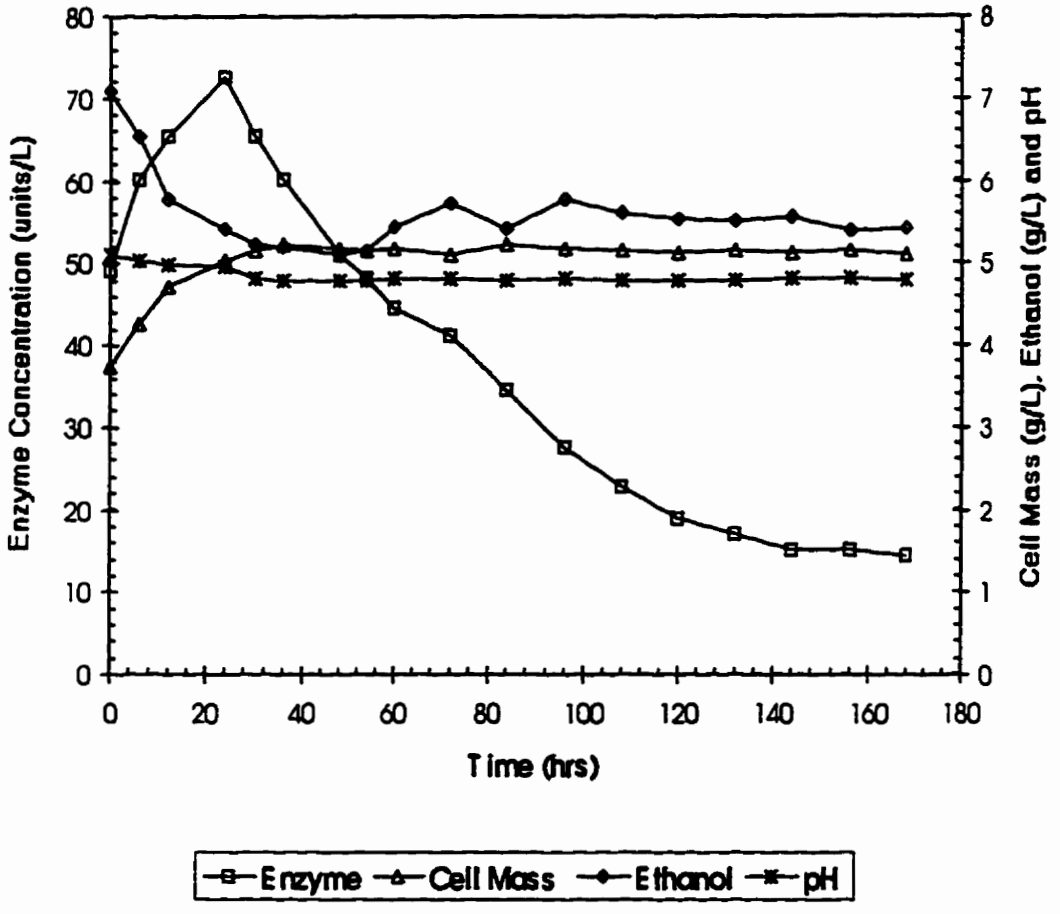


Figure 4.9. Continuous Suspension Culture in Airlift Bioreactor Using Original Strain at T = 30 °C, D = 0.1 h^{-1}, VVM = 1.0 and G_0 = 20.0 g/L.

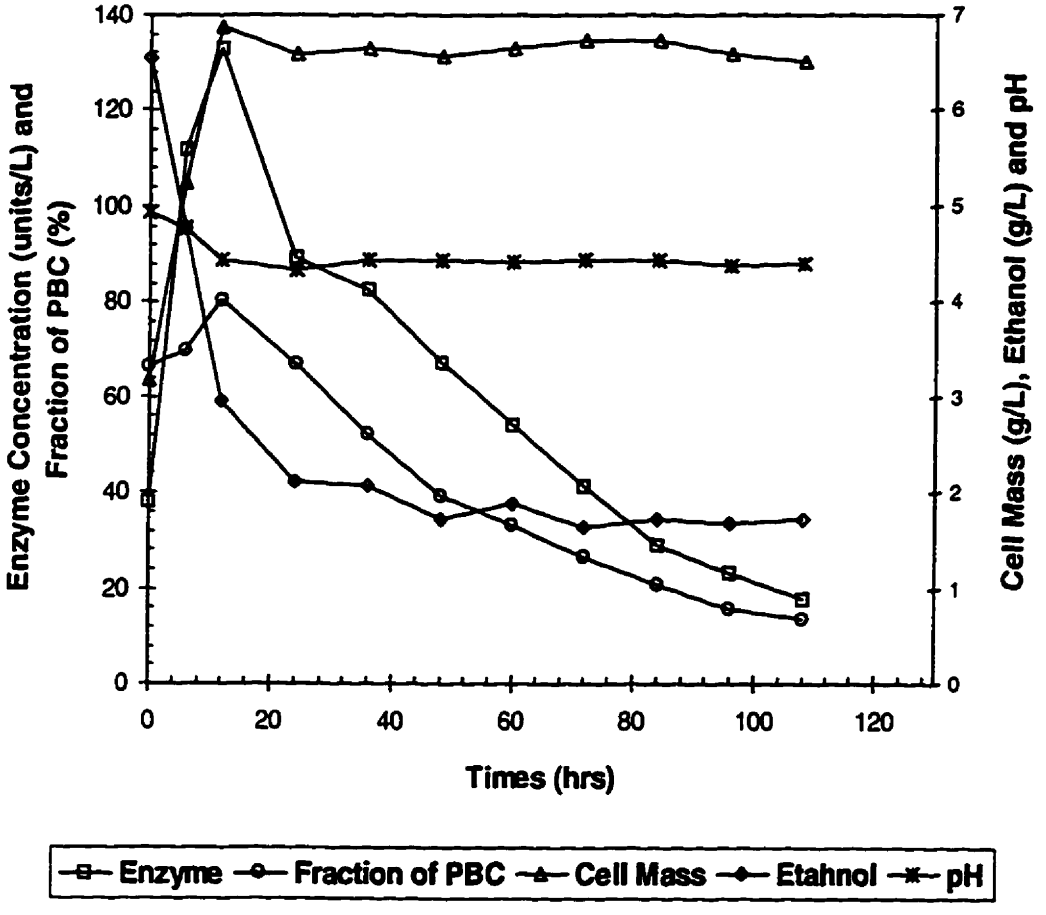


Figure 4.10. Continuous Suspension Culture in Stirred-Tank Bioreactor Using Selected Strain at T = 30 °C, D = 0.1 h^{-1}, VVM = 1.0, RPM = 500 and G_0 = 20.0 g/L.

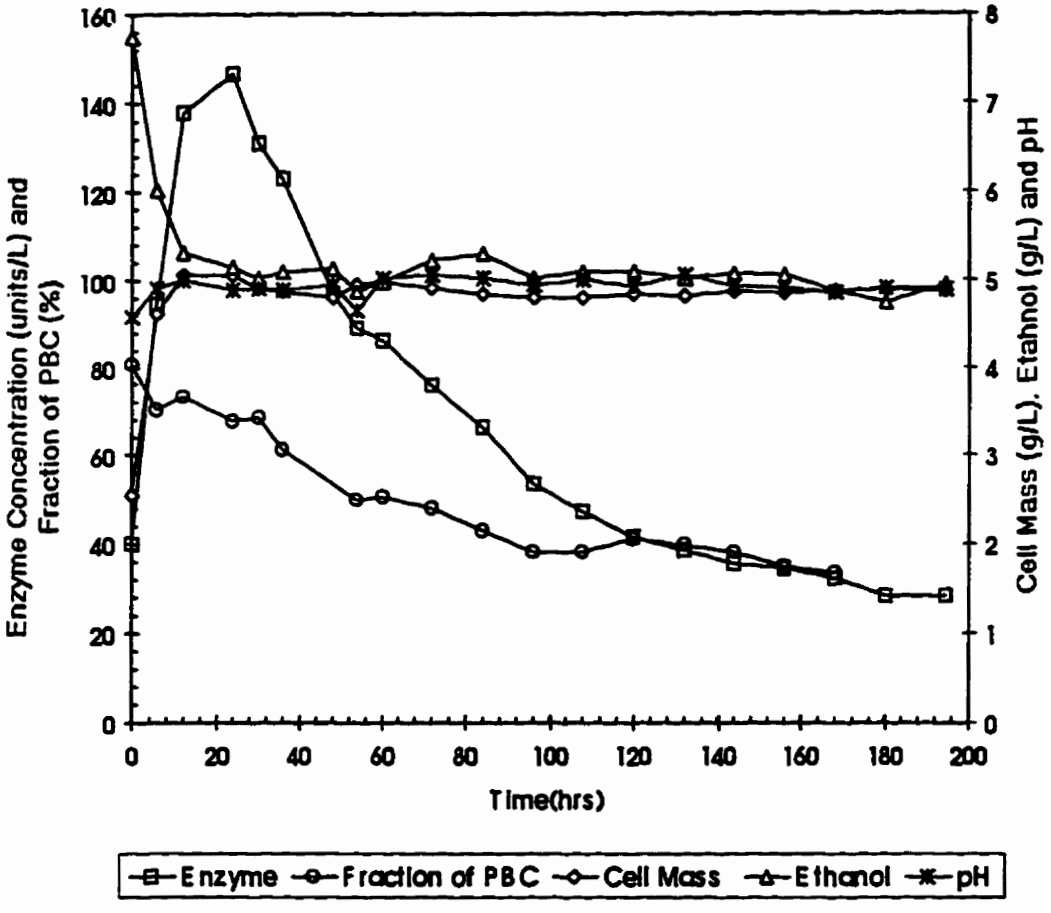


Figure 4.11. Continuous Suspension Culture in Airlift Bioreactor Using Selected Strain at T = 30 °C, D = 0.1 h^{-1}, VVM = 1.0 and G_0 = 20.0 g/L.

centration dropped to about one-sixth of its maximum value. Evidently, the decay of enzyme concentration in the bioreactor is due to the plasmid instability. Even though the total cell mass in the bioreactor remained nearly constant, the fraction of the plasmid-bearing cell continuously decreased (Figures 4.10 and 4.11). Growth advantage of plasmid-free cells could be another reason that was responsible for the drop of the proportion of plasmid- bearing cells. During the continuous cell culture process, cells lacking the plasmid compete successfully with plasmid-bearing cells in mixed culture. Eventually, the plasmid-bearing cells will be overtaken by plasmid-free cells.

In continuous culture experiments using the selected strain, both the fraction of plasmid-bearing cells and enzyme concentration were followed. Figures 4.10 and 4.11 show that both the enzyme concentration and the fraction of plasmid-bearing cells decreased with culture time. Thus, the proportion of plasmid-bearing cells was directly related to glucoamylase concentration in the bioreactor. This relationship was also found at different dilution rates in continuous suspension culture (See Section 4.3.4).

4.3.2. Comparison between Original and Selected Strains

The original strain represented the recombinant *S. cerevisiae* C468/pGAC9 originally obtained from ATCC. The selected strain was obtained according to a screening protocol described in the Chapter 3 (Section 3.2). The comparisons of enzyme concentration between the original and selected strains in continuous suspension culture in the YPG nonselective medium are demonstrated in Figure 4.12 (in STR) and Figure 4.13 (in ALR).

Figures 4.12 and 4.13 show that by using the selected strain the enzyme concentration doubled in both STR and ALR bioreactors even though the total cell concentrations in the bioreactor were a little lower than that using the original strain. This is because after the double pressure selection, the selected strain contained a high proportion of plasmid-bearing cells. These cells contained both the glucoamylase and LEU2 genes and could encode glucoamylase effectively. It is interesting to note that the decay of the enzyme concentration in the reactor followed an exponential pattern and the first-order decay rate constant for selected strain was slightly smaller (Figures 4.12 and 4.13). This suggests that the plasmid stability was enhanced after the recombinant cells experienced double selection pressure. Since the growth rate is very slow in the double selection medium, it is not practical to use the double selection medium in industrial fermentation. But double selection could be an effective way to screen and maintain recombinant cells during the inoculum preparation stage. In this research, screening under double selection pressure was performed every three months in order to keep the engineered genetic properties of the present recombinant yeast. Then the selected strain was maintained in the double selection medium.

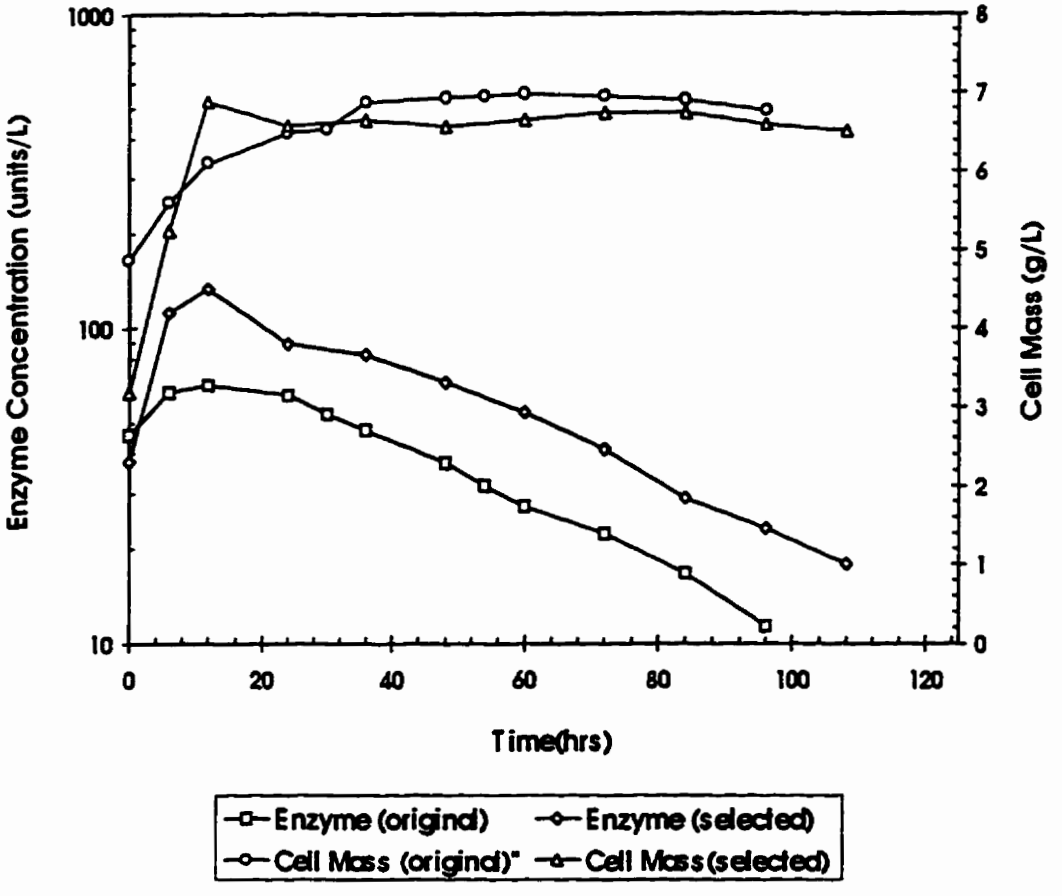


Figure 4.12. Comparison of Glucoamylase Concentration between Original and Selected Strains in Continuous Suspension Culture in Stirred-Tank Bioreactor (T = 30 °C, D = 0.1 h^{-1}, VVM = 1.0, RPM = 500 and G_0 = 20.0 g/L).

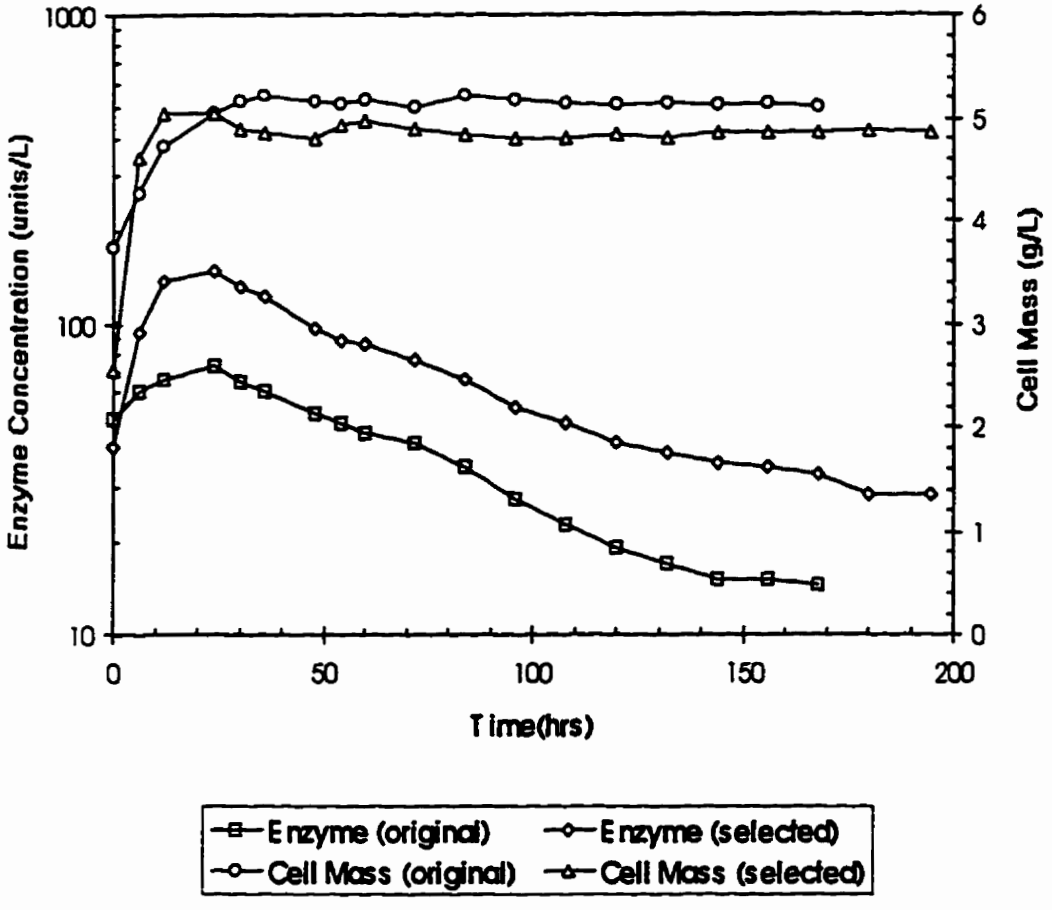


Figure 4.13. Comparison of Glucoamylase Concentrations between Original and Selected Strains in Continuous Suspension Culture in Airlift Bioreactor (T = 30 °C, D = 0.1 h^{-1}, VVM = 1.0 and G_0 = 20.0 g/L).

4.3.3. Plasmid Instability Kinetics in Continuous Culture

4.3.3.1. Mathematical Analysis

Assuming that the cells are in balanced growth, according to Imanaka and Aiba (1981), the change in plasmid-bearing cells in the continuous culture system can be described by:

$$\frac{dX^+}{dt} = \mu^+ X^+ - p\mu^+ X^+ - DX^+ = (\mu^+ - p\mu^+ - D)X^+ \qquad (4.1)$$

The analytical solution of Eq. (4.1) is as follows:

$$X^+ = X_0^+ \exp(\mu^+ - p\mu^+ - D)t = X_0^+ \exp(-k_f t) \qquad (4.2)$$

where $k_f = -(\mu^+ - p\mu^+ - D)$, a first-order decay rate constant, which is always positive because $\mu^+ \le D$. Eq. (4.2) shows that the decay of plasmid-bearing cells follows first-order kinetics and the concentration of plasmid-bearing cells decreases with culture time during continuous culture. Since the total cell concentration X_T is observed constant during the culture process (See Figure 4.17), the fraction of plasmid-bearing cells ($F = X^+ / X_T$) also follows an exponential decay pattern:

$$F = F_0 \exp(-k_f t) \qquad (4.3)$$

Similarly, the change in plasmid-free cells in the culture system can be described as follows:

$$\frac{dX^-}{dt} = \mu^- X^- + p\mu^+ X^+ - DX^- \qquad (4.4)$$

Eq. (4.2) and (4.4) can be solved analytically to give:

$$X^- = X_0^- \exp[(\mu^- - D)t] + [\, p\mu^+ X_0^+ / \delta \,]\{\exp[(\mu^- - D)]t - \exp(-k_f t)\} \qquad (4.5)$$

where $\delta = \mu^- - \mu^+ + p\mu^+$, which is always positive because $\mu^- \geq \mu^+$. Eq. (4.5) shows that the concentration of plasmid-free cells increases with culture time. Letting $B = X^-/X^+$ and dividing Eq. (4.5) by Eq. (4.2), one obtains:

$$B = \frac{X^-}{X^+} = \frac{X_o^-}{X_o^+} exp(\delta\ t) + \frac{p\mu^+}{\delta}\left[exp(\delta\ t) - 1\right] \tag{4.6}$$

Differentiating Eq. (4.6) and rearranging the result yields:

$$\frac{dB}{dt} = \delta\left[\frac{X_o^-}{X_o^+} exp(\delta\, t)B + \frac{p\mu^+}{\delta}[exp(\delta\, t) - 1]\right] + p\mu^+ \tag{4.7}$$

Substituting Eq. (4.6) into Eq. (4.7) gives:

$$\frac{dB}{dt} = (\mu^- - \mu^+ + p\mu^+)B + p\mu^+ \tag{4.8}$$

According to Eq. (4.8), the slope of a plot of $\frac{dB}{dt}$ versus B is:

$$C_1 = \mu^- - \mu^+ + p\mu^+ \tag{4.9}$$

and the intercept of the plot is:

$$b = p\mu^+ \tag{4.10}$$

According to Eq. (4.2), a semilogarithmic plot of X^+ versus time t yields a slope of:

$$C_2 = \mu^+ - p\mu^+ - D = -k_f \tag{4.11}$$

Solving Eq. (4.9), (4.10) and (4.11) for the specific growth rate of plasmid-bearing cells, the specific growth rate of plasmid-free cells and probability of plasmid loss gives:

$$\mu^+ = C_2 + b + D \tag{4.12}$$

$$\mu^- = C_1 + C_2 + D \tag{4.13}$$

$$p = \frac{b}{C_2 + b + D} \tag{4.14}$$

Eq. (4.12), (4.13) and (4.14) can be used to estimate the specific growth rate of plasmid-bearing cells (μ^+), the specific growth rate of plasmid-free cells (μ^-), and probability of plasmid loss (p) at different dilution rates from experimental data. These equations derived above could be a useful tool for analysis of plasmid instability in recombinant cells.

4.3.3.2. Effects of Dilution Rates on Plasmid Stability

Chemostat cultures in the YPG nonselective medium were performed with *recombinant S. cerevisiae* at various dilution rates (0.05, 0.10, 0.20, 0.25 h^{-1}) in the airlift bioreactor. The percentage of plasmid-bearing cells were determined during continuous culture. The results are plotted as a function of the culture time in Figure 4.14, which shows that the fall in fraction of plasmid-bearing cells followed an exponential decay pattern, in agreement with Eq. (4.3). Other workers (Futcher and Cox, 1984; Impoolsup et al., 1989a) had also reported an exponential decay pattern. From Figure 4.14 it can be seen that the apparent plasmid loss rate increases with increasing dilution rates. Since the dilution rate corresponds to the cell specific growth rate in chemostat culture, it appears that the more the cells divide, the more likely they lose plasmids. However, parallel lines were obtained when the fractions of plasmid-bearing cells were plotted against the number of generations (Figure 4.15). No difference in plasmid loss per generation at different dilution rates was observed. As the doubling time decreases with increasing dilution rate, there are more generations at higher dilution rates for a fixed culture time. Based on generation, the plasmid loss per generation is independent of the dilution rate. In contrast,

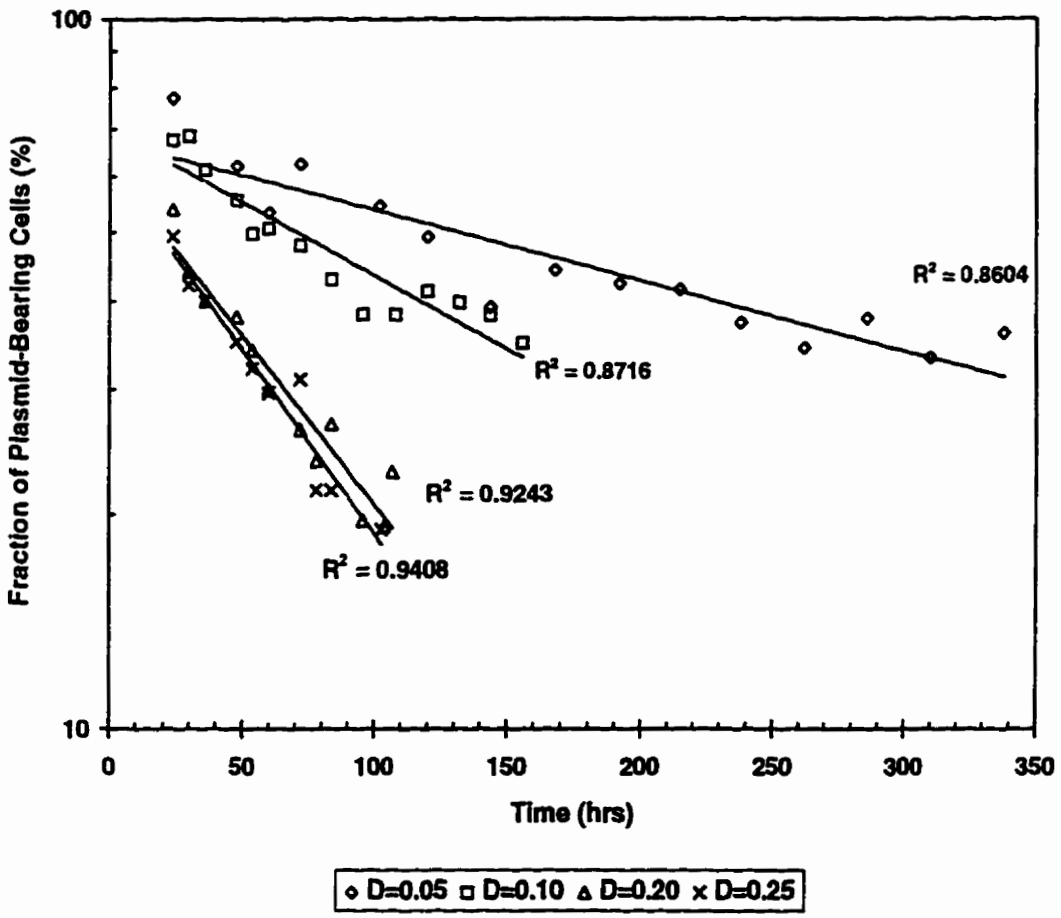


Figure 4.14. Effects of Dilution Rate and Time on Plasmid Stability during Continuous Culture in Airlift Bioreactor Using Nonselective Medium (T = 30 °C, VVM = 1.0 and G_0 = 20.0 g/L).

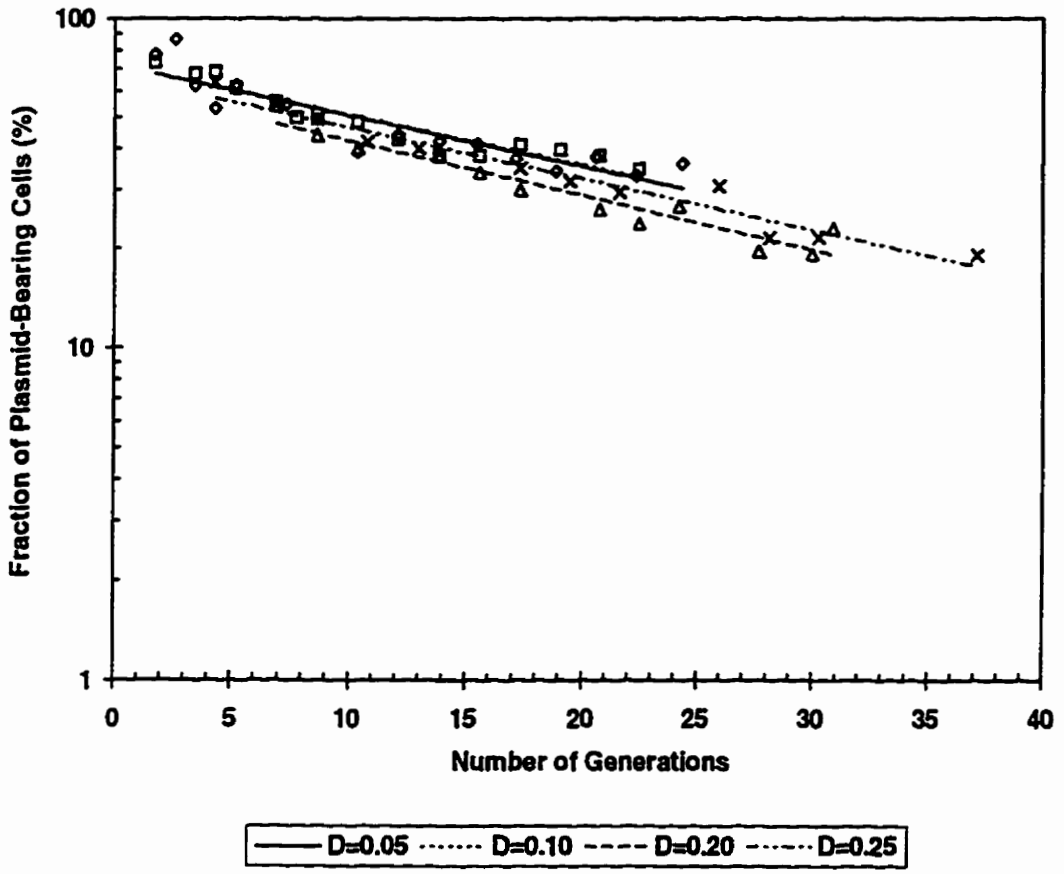


Figure 4.15. Effects of Dilution Rate and Generation Number on Plasmid Stability during Continuous Culture in Airlift Bioreactor Using Nonselective Medium (T = 30 °C, VVM = 1.0 and G_0 = 20.0 g/L).

Impoolsup et al. (1989a) and Hardjito et al. (1993) reported that segregational instability (plasmid loss/per generation) increased with increasing dilution rates in a culture of a recombinant yeast in non-selective medium. The effect of dilution rate seems to relate to the medium used. When recombinant *S. cerevisiae* yeasts were continuously grown in selective medium, plasmid instability was reported to decrease with increasing dilution rates (Parker and DiBiasio, 1987; Lee and Hassan, 1988; Caunt et al., 1989; Da Silva and Bailey, 1991).

4.3.3.3. Estimation of Instability Parameters

The following discussion refers to growth conditions when the specific growth rate of the plasmid-bearing cells is not significantly different from plasmid-free cells. It can be shown that if the fraction of plasmid-bearing cells (F) follows a exponential decay pattern, then plotting dB/dt versus B will give a straight line with a slope of $C_1 = k_f$ and an intercept of $b = k_f$. If F declines according to an exponential pattern (Eq.(4.3)), then we have:

$$B = \frac{X^-}{X^+} = \frac{1-F}{F} = \frac{1-F_0\,exp(-k_f t)}{F_0\,exp(-k_f t)} \tag{4.15}$$

Differentiating Eq. (15) and rearranging the result yields:

$$\frac{dB}{dt} = k_f \cdot \left[\frac{1-F_0\,exp(-k_f t)}{F_0\,exp(-k_f t)}\right] + k_f \tag{4.16}$$

Substituting Eq. (4.15) into Eq. (4.16) gives:

$$\frac{dB}{dt} = k_f B + k_f \tag{4.17}$$

From a semilogarithmic plot (Figure 4.14), values of k_f at different dilution rates can be obtained by linear regression. From Eq. (11) and (17), we have $C_2 = -k_f$, $C_1 = k_f$ and $b = k_f$ respectively. Using Eq. (12), (13) and (14), the specific growth rate of plasmid-bearing cells ($\mu^+ = C_2 + b + D = D$), the specific growth rate of plasmid-free cells ($\mu^- = C_1 + C_2 + D = D$), probability of plasmid loss ($p = b/(C_2 + b + D) = k_f/D$) at different dilution rates were estimated. The values of μ^+, μ^-, p, and k_f at various dilution rates are compiled in Table 4.3. The dB/dt versus B plot for each dilution rate is illustrated in Figure 4.16.

Table 4.3. Estimated Values of Instability Parameters During Continuous Culture of Recombinant *S. cerevisiae* (ATCC 20690) in Airlift Bioreactor.

D (h^{-1})	0.05	0.10	0.20	0.25
C_1 (h^{-1})	0.0025	0.0048	0.0106	0.0121
C_2 (h^{-1})	-0.0025	-0.0048	-0.0106	-0.0121
b (h^{-1})	0.0025	0.0048	0.0106	0.0121
μ^+ (h^{-1})	0.05	0.10	0.20	0.25
μ^- (h^{-1})	0.05	0.10	0.20	0.25
p	0.0500	0.0480	0.0530	0.0484
k_f (h^{-1})	0.0025	0.0048	0.0106	0.0121
k_f (generation^{-1})	0.0347	0.0333	0.0367	0.0336

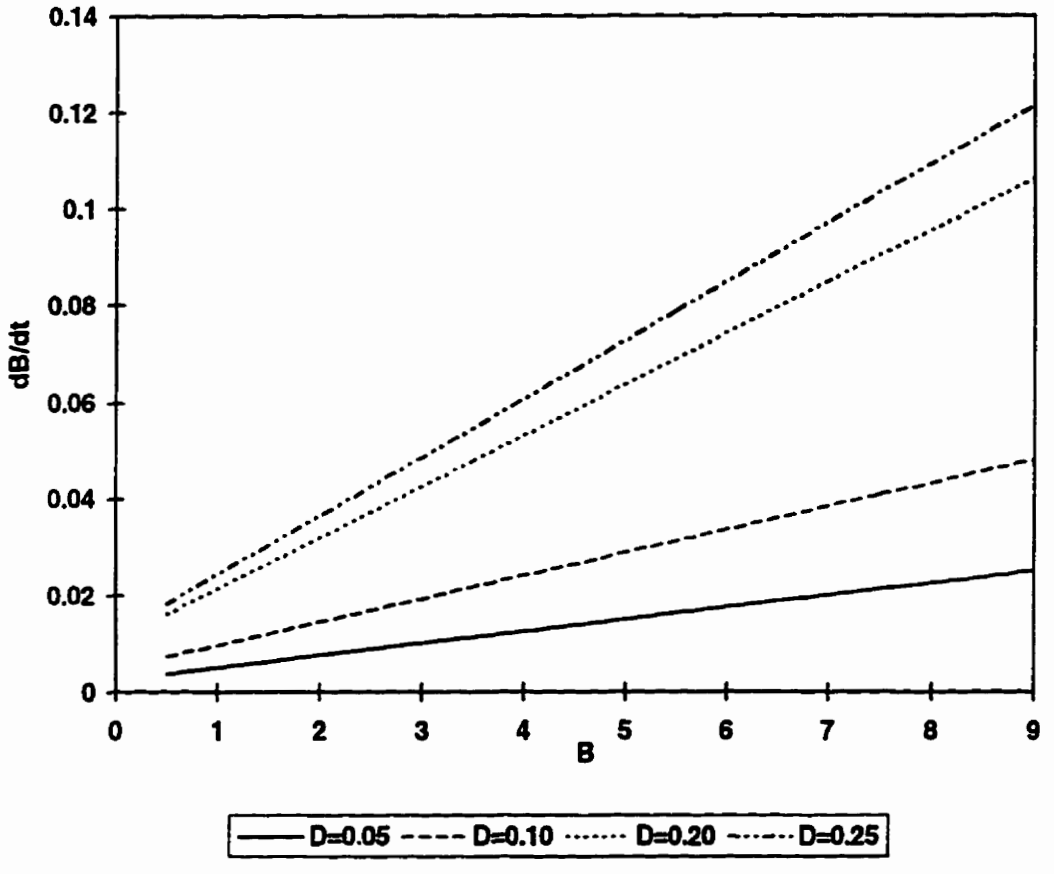


Figure 4.16. dB/dt versus B Plots at Different Dilution Rates.

4.3.3.4.Growth Rate Differences

For recombinant cells, both structural instability, segregational instability, and growth rate difference can contribute to plasmid loss. Structural instability is often caused by the DNA's deletion, insertion, recombination, and other events, while segregational instability is caused by a bias in partitioning of plasmids between mother and daughter cells during cell division (Primrose et al., 1981; Ryu and Lee, 1988). In addition, the plasmid-free cells usually have a growth advantage over plasmid-bearing cells in non-selective medium because the presence of the foreign plasmid places additional "stress and burden" on host cells. The excess metabolic load is due to the requirement to replicate and express the foreign DNA (Seo and Bailey, 1985; Birnaum and Bailey, 1991). During cell culture, the plasmid-free cells tend to overwhelm the original population of plasmid-bearing cells due to their higher growth rate.

As shown in Table 4.3, there is negligible difference between the growth rates of plasmid-bearing and plasmid-free cells at the dilution rates tested, which is in agreement with the experimental measurements of the maximum specific growth rates of the plasmid-bearing and plasmid-free cells for the current recombinant yeast strains (Innis et al., 1987). No growth rate differences contribute to the apparent instability of the plasmids. This finding agrees with the observation that the total cell concentration remained nearly constant during the experiments (See Figure 4.17), even though the population of the plasmid-bearing cells continuously decreased (Figure 4.14), otherwise the total cell mass would increase during the culture process. This result is also consistent with the finding

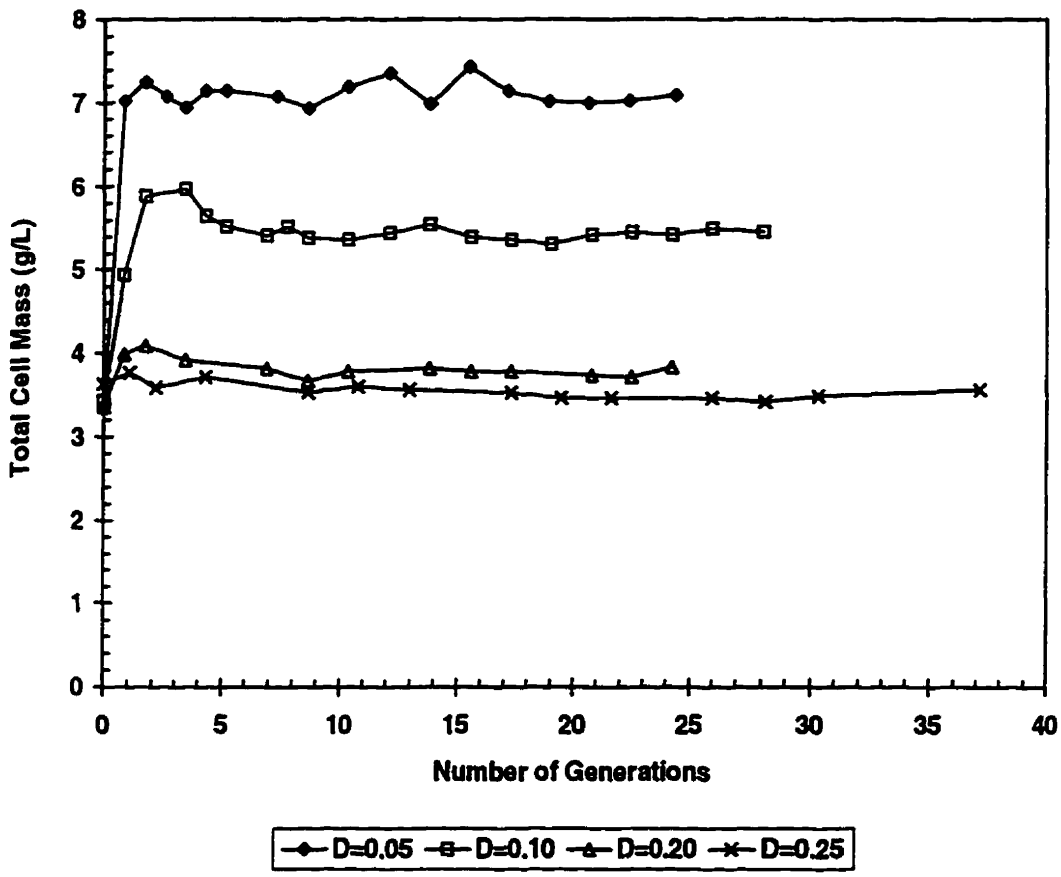


Figure 4.17. Total Cell Mass at Different Dilution Rates during Continuous Culture Process (T = 30 °C, VVM = 1.0 and G_0 = 20.0 g/L).

by Impoolsup et al. (1989b). Our findings with the recombinant yeast are different from those found with recombinant *E. coli*. For recombinant *E. coli*, it is well-documented that there is a difference in growth rates between plasmid-free and plasmid-bearing cells (Seo and Bailey, 1985; Kim and Shuler, 1990b; Birnbaum and Bailey, 1991; Flickinger and Rouse, 1993). The disparity between recombinant yeast and recombinant *E. coli* may be explained by the difference in plasmid copy number. For 2 μm based yeast episomal plasmid (YEp), the copy number per cell ranges from 5 to 40 (Caunt et al., 1988), while recombinant *E. coli* plasmids usually have much higher copy numbers (Seo and Bailey, 1985; Ryu and Lee, 1988; Kim and Shuler, 1990b; Togna et al., 1993). The higher the plasmid copy number, the greater the additional amount of energy required to maintain that DNA within the host cell, and consequently the lower the relative specific growth rate (Kim and Shuler, 1990b; Birnbaum and Bailey, 1991). In addition, recombinant *E. coli* generally has stronger promoters and a higher level of cloned gene expression than *S. cerevisiae*.

4.3.3.5. Probability of Plasmid Loss (p)

In modelling plasmid instability, the probability of plasmid loss (p) due to structural instability and/or segregational instability is an important kinetic parameter. We have shown a new methodology to evaluate this parameter (p). It can be seen in Table I that the probability of plasmid loss (p) is nearly constant at all dilution rates tested. So it can concluded that the dilution rate has no significant effect on the plasmid stability. This finding is contrary to those found in a recombinant *E. coli* (Mosrati et al., 1993) and in

another recombinant yeast (Impoolsup et al., 1989a). If the genetic factors (plasmid composition , structure, copy number etc.) have a dominant effect on the plasmid stability, then for a certain plasmid the probability of the plasmid loss is most likely the same under different environmental conditions.

This conclusion can be further confirmed by examining the first-order decay rate constant (k_f). When expressed as hour^{-1} , the values of k_f show a tendency to increase with increasing dilution rates. However, when it is expressed as generation^{-1}, the values of k_f are nearly constant for all the dilution rates tested. The result can be easily explained by the constant probability of plasmid loss. It was already shown that the first-order decay rate constant (k_f) can be described as:

$$k_f = D - \mu^+ + p\mu^+ \tag{4.18}$$

The first term $(D - \mu^+)$, reflects the effect of growth difference and the second term $p\mu^+$ reflects the effect of structural and segregational instability on plasmid loss. If there is no growth rate difference between plasmid-bearing and plasmid-free cells, we have:

$$\mu^+ = \mu^- = D \tag{4.19}$$

Thus Eq. (4.18) can be reduced to:

$$k_f = pD \qquad (h^{-1}) \tag{4.20}$$

It can be seen from Eq. (4.20) that k_f, expressed in h^{-1} , increases with increasing dilution rates. For a balanced growth in continuous culture, the doubling time or generation time (t_d) can be calculated as:

$$t_d = \frac{ln2}{D} \qquad (h^{-1}) \tag{4.21}$$

Then k_f, expressed in generation^{-1}, will become:

$$k_f = pDt_d = ln2 \times p \qquad (\text{generation}^{-1}) \tag{4.22}$$

Eq. (4.22) shows that if the probability of plasmid loss (p) is constant, k_f (generation^{-1}) is constant over different dilution rates.

If the difference in specific growth rates of plasmid-bearing and plasmid free cells are negligible, the three-parameter model of plasmid instability kinetics can be simplified to a one-parameter model as follows:

$$X^+ = X_0^+ exp(-pDt) \tag{4.23}$$

$$X^- = X_0^- + X_0^+ [1 - exp(-pDt)] \tag{4.24}$$

For the current recombinant yeast, the probability p can be considered independent of the dilution rate D. In the present case an average value of p could be calculated as 0.0499. Based on p = 0.0499, the first-order decay rate constants for different dilution rates were calculated and are listed in Table 4.4. Table 4.4 shows good agreement between predicted values of k_f and measured values of k_f. The model can be also applied to situations when probability of plasmid loss (p) is dependent on the dilution rate. For example, Impoolsup et al. (1989b) found that when expressed in units of generation^{-1}, the values of first-order decay rate constants (k_f) had a tendency to increase with increasing growth rates. It may be deduced from Eq. (4.22) that the probability of plasmid loss (p) increased with increasing dilution rates. Higher instability at higher dilution rate was also reported for

other recombinant yeast under nonselective condition (Hardjito et al., 1993). Increased probability of plasmid loss at higher dilution rates in a nonselective medium is possibly due to genetic errors during faster growth (Impoolsup et al., 1989b). Variable plasmid loss probability can be easily incorporated into the present model.

The present model may be also useful for analysing plasmid instability kinetics in a selective medium. It was noted that contrary to nonselective conditions, less plasmid loss per generation was observed at higher dilution rates under selective conditions (Parker and DiBiasio, 1987; Lee and Hassan, 1988). Assuming growth of plasmid-free cells is negligible under selective pressure, then specific growth rate of plasmid-bearing cells would be equal to dilution rate during chemostat culture ($\mu^* = D$). Thus, Eq. (4.22) may be also suitable for selective systems. Based on the Eq. (4.22), it may be concluded that probability of plasmid loss (p) decreases with increasing dilution rate in a selective system. Attempts have been made to explain this effect (DiBiasio and Sardonini, 1986; Parker and DiBiasio, 1987), but actual mechanisms remain unclear. In a selective medium, plasmid-free cells could still propagate to some degree since a metabolite being excreted into the medium by plasmid-bearing cells may support the growth of plasmid-free cells (Sardonini and DiBiasio, 1987). Increase in dilution rate decreases the concentration of the metabolite, hence, causing the washout of plasmid-free cells.

Table 4.4. Comparison between Values of Measured and Predicted k_f.

D (h^{-1})	0.05	0.10	0.20	0.25
Measured k_f (h^{-1})	0.0025	0.0048	0.0106	0.0121
Predicted k_f (h^{-1})	0.0025	0.0050	0.0100	0.0125

4.3.3.6. Conclusions

A simple kinetic model was derived for expressing plasmid stability in a recombinant yeast in continuous culture. The plasmid loss followed first-order kinetics, which agreed well with the experimental results. A novel method was developed for estimation of the specific growth rates of plasmid-bearing and plasmid-free cells, and the probability of plasmid loss. In the present case, the intrinsic probability of plasmid loss remained constant and independent of the dilution rate, and the specific growth rates of the plasmid-bearing and plasmid-free populations were identical. However, the model can be applied to situations when differences exist in the above parameter values.

4.3.4. Effect of Dilution Rate On Recombinant Protein Expression

Besides the fraction of plasmid-bearing cells, concentrations of the recombinant protein (glucoamylase) were also followed during continuous cultures in the airlift bioreactor using the YPG nonselective medium. The effects of the dilution rate and time on glucoamylase production are shown in Figure 4.18. The data taken at the dilution rate 0.25 h^{-1} was not included as glucose was not limiting at this dilution rate and the residual glucose level interfered with glucoamylase measurement. Like the fraction of the plasmid-bearing cells, the decline of recombinant protein concentrations also followed first-order kinetics. Figure 4.18 also shows increased decay rate of recombinant protein expression (as function of time) at higher dilution rates. However, the decay rates were nearly constant at different dilution rates when the glucoamylase activity was plotted against the number of generations (Figure 4.19). Thus, the recombinant protein expression was directly related to the fraction of plasmid-bearing cells. This further confirms the assumption of first-order kinetics for plasmid loss.

It is evident from Figure 4.18 that the enzyme concentrations decreased with increasing dilution rates. This is because of a metabolic shift. At lower dilution rates the metabolism of recombinant yeast was mainly respiratory. Such conditions gave efficient conversion of carbon source to cell and the biomass remained at a higher level, resulting in higher enzyme production. But at higher dilution rates glucose fermentation dominated as most of the glucose was used for ethanol production due to the Crabtree effect. Under these conditions biomass yield dropped to a lower level and cell concentrations were

lower but ethanol concentrations were higher, corresponding to a lower enzyme concentration. The relationship between dilution rate, cell mass and ethanol concentration is shown in Figure 4.20. Cell concentrations decreased with increasing dilution rates but ethanol concentrations increased with increasing dilution rates. It has been reported that the Crabtree effect could be induced by a high glucose concentration or by a high influx of glucose even when the concentration of glucose was low (10-100 mg/L) (van Dijken and Scheffers, 1984). Experimental data showed that the glucose concentrations in the bioreactor were very low (consistently below 50 mg/l) at all dilution rates (0.05, 0.10 and 0.20 h^{-1}) used in the present continuous cultures. The Crabtree effect was caused by a high influx of glucose.

The relationship between the specific enzyme activity (or enzyme yield) and the dilution rate during continuous culture is shown in Figure 4.21. It can be seen that the specific enzyme activity decreased with increasing dilution rate. This is also due to the shift in metabolism from glucose oxidation to glucose fermentation at higher dilution rates; more glucose was fermented to ethanol via glucose fermentation pathway. The estimated fraction of the cells produced by anaerobic pathway increased with increasing dilution rate (See Table 4.5). But the fermentative pathway is not associated with enzyme production. Thus, the specific enzyme activity or enzyme yield declined to a lower level at higher dilution rates. Furthermore, enzyme yield also decreased with increasing specific growth rate (dilution rate) as discussed in Section 4.2.3.

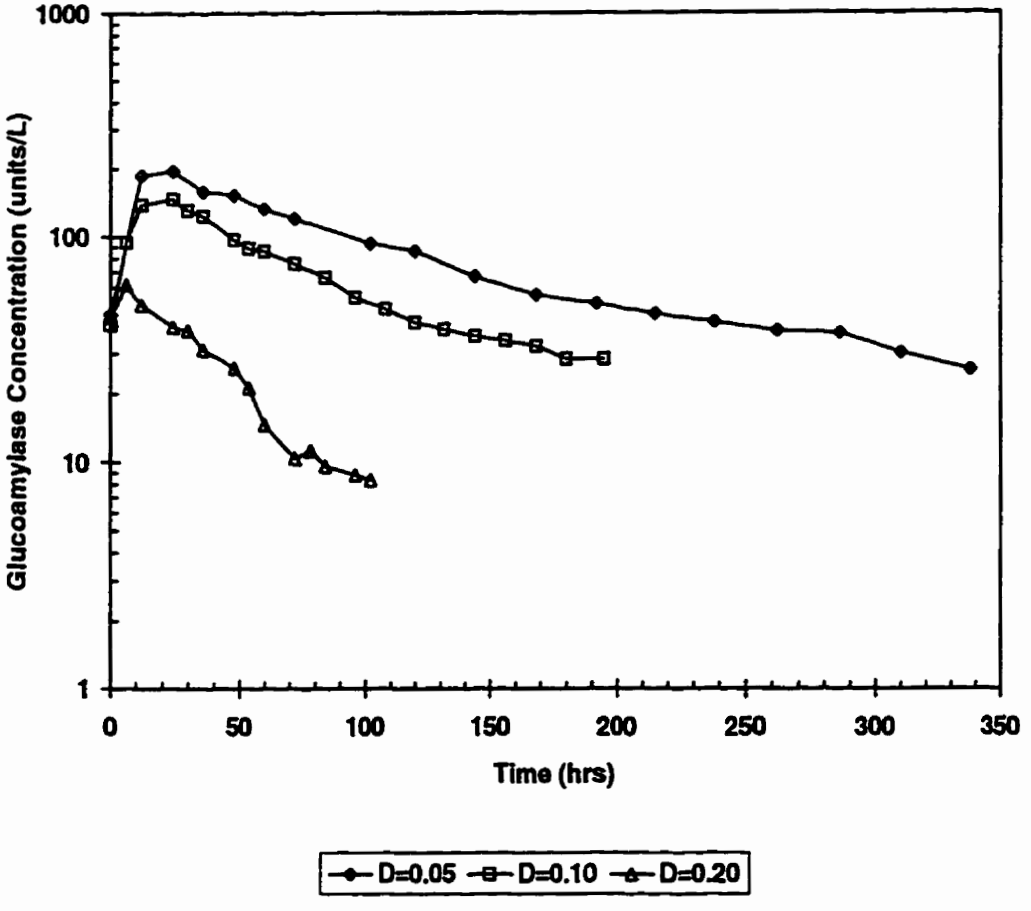


Figure 4.18. Effects of Dilution Rate and Time on Glucoamylase Expression during Continuous Culture in Airlift Bioreactor (T = 30 °C, VVM = 1.0 and G_0 = 20.0 g/L).

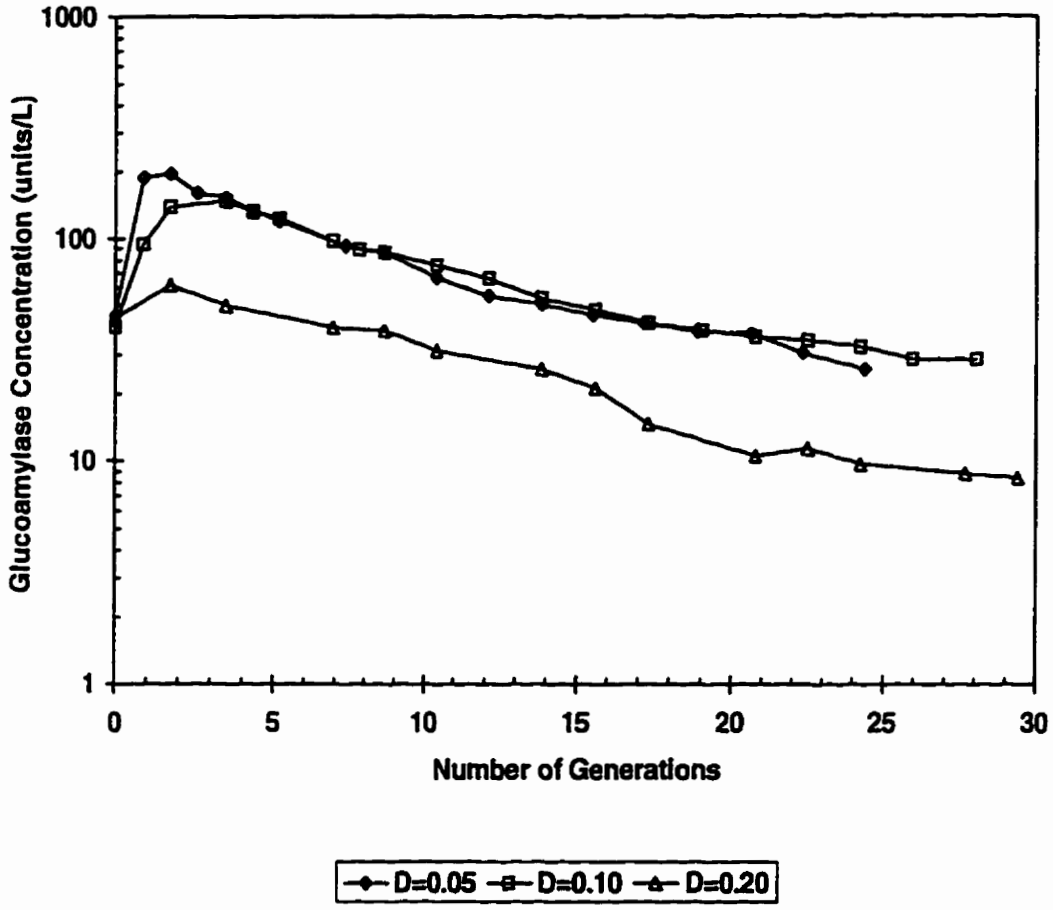


Figure 4.19. Effects of Dilution Rate and Generation Number on Glucoamylase Expression during Continuous Culture in Airlift Bioreactor (T = 30 °C, VVM = 1.0 and G_0 = 20.0 g/L).

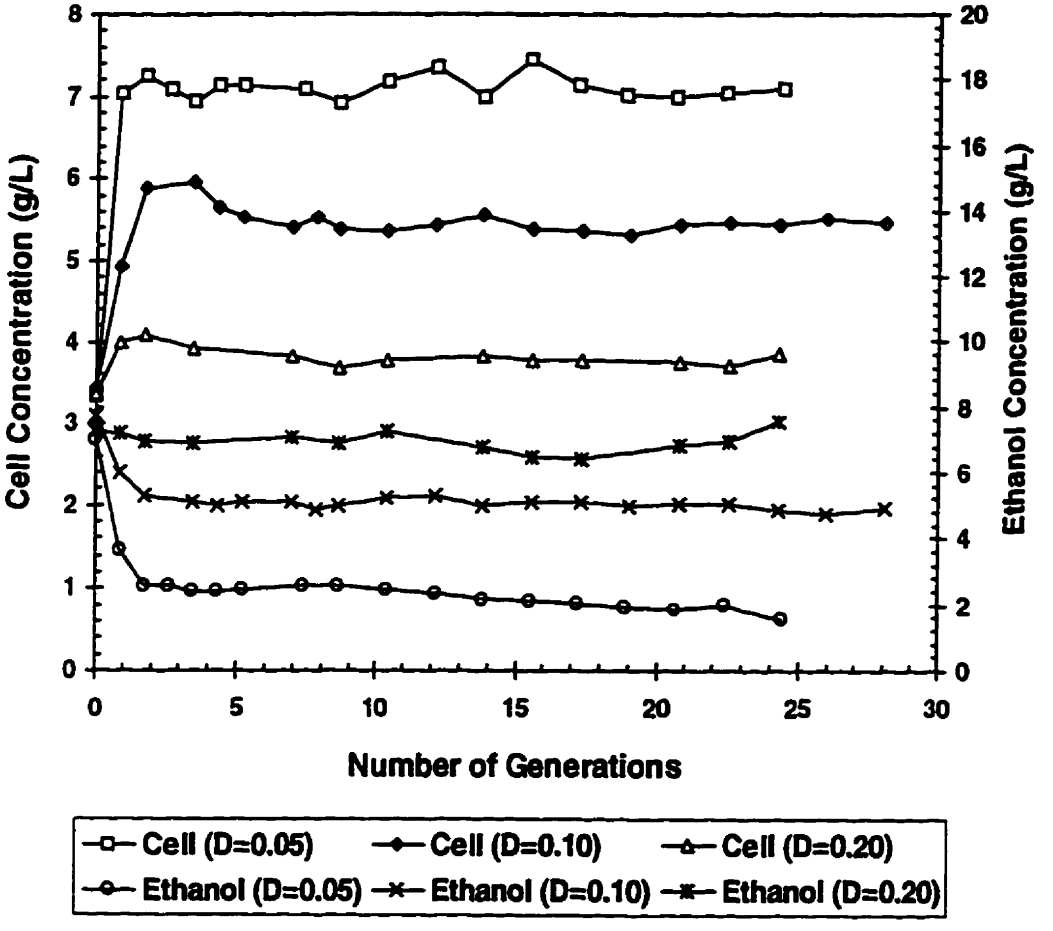


Figure 4.20. Effects of Dilution Rate on Cell and Ethanol Concentrations during Continuous Culture in Airlift Bioreactor (T = 30 °C, VVM = 1.0 and G_0 = 20.0 g/L).

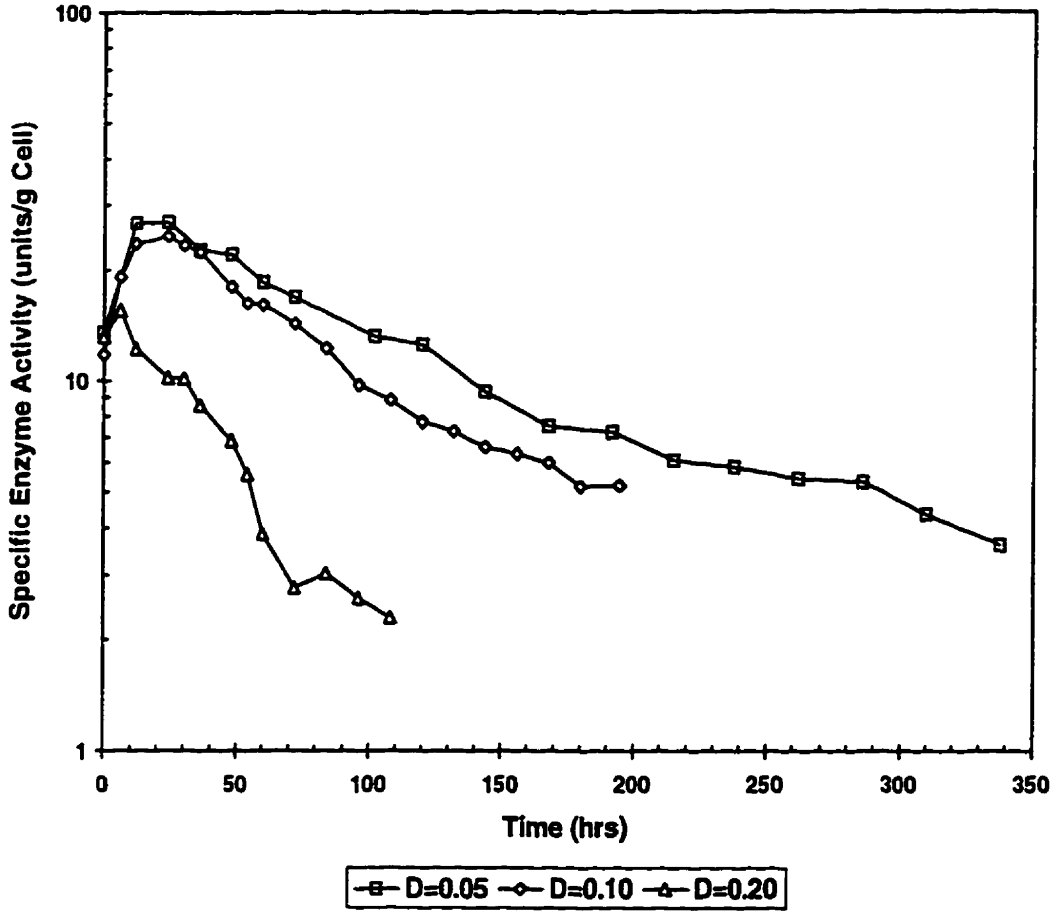


Figure 4.21. Effects of Dilution Rate on Specific Enzyme Activity during Continuous Culture in Airlift Bioreactor (T = 30 °C, VVM = 1.0 and G_0 = 20.0 g/L).

Table 4.5. Metabolic Pathway Analysis during Continuous Culture in ALR.

Dilution Rate D (h^{-1})	0.05	0.10	0.20
Total Cell Mass X (g/L)	7.0680	5.4463	3.7849
Ethanol Concentration E (g/L)	2.2967	4.8626	7.0343
Cell Mass Contributed from Glucose Fermentation X_1 (g/L) = $Y_{X/G}.E/Y_{G/X}$	0.5659	1.2405	1.7945
Cell Mass Contributed from Glucose Oxidation X_2 (g/L) = X - X_1	6.5021	4.2058	1.9904
Fraction of Cells Produced in Glucose Fermentation (X_1/X)	0.0800	0.2278	0.4741
Fraction of Cells Produced in Glucose Oxidation (X_2/X)	0.9200	0.7728	0.5259

4.3.5. Comparison between ALR and STR Bioreactors

Continuous culture experiments using nonselective medium were also performed in the STR bioreactor. Comparisons of the enzyme, cell and ethanol concentrations between the ALR and STR bioreactors in continuous culture (dilution rate = 0.10 h^{-1}) are presented in Figure 4.22 (using original strain) and Figure 4.23 (using selected strain). Figures 4.22 and 4.23 show that the enzyme concentrations were higher in the ALR bioreactor even though the cell concentrations were lower. Therefore, the enzyme yield or specific enzyme activity was lower in the STR bioreactor, which is consistent with the results obtained in batch fermentation as discussed in Section 4.2.3. It is also interesting to note

that the decay rate of enzyme production was higher in the STR bioreactor than in the ALR bioreactor (Figures 4.22 and 4.23). The enzyme concentration fell more rapidly in the STR bioreactor because the fraction of plasmid-bearing cells in the STR declined faster than in the ALR (Figure 4.24). The agitation was possibly responsible for the increased apparent plasmid instability in the STR bioreactor. When a recombinant *E. coli* B/pTG201 was grown in a nonselective medium, Huang et al. (1990) observed that the plasmid stability and enzyme productivity decreased with the increase in agitation speed from 50 to 200 rpm. They concluded that a mild agitation rate was favourable for the maintenance of plasmid stability. In the present case, the agitation rate used was high (500 rpm), which might result in a decreased plasmid stability in the STR bioreactor. Although the cell mass was higher in the STR bioreactor, the enzyme production was poorer due to the increased plasmid instability (Figure 4.22 and 4.23).

In the STR bioreactors, the energy required for the movement of the fluid is introduced focally at one point in the reactor via a stirrer. The shear stress will be greatest near the stirrer and dissipate with distance from it. The vigorous fluid movement and the wide variation of shear force in the reactor may have a detrimental effect on plasmid stability (Huang et al., 1990). In contrast, in the absence of agitation a regular and predictable pattern of bulk circulation generated by injected air exists in the ALR bioreactors. Shear distribution is homogeneous throughout the ALR bioreactors. The gentle and approximately uniform environment is very suitable for living cells. It is true that STR bioreactors can provide higher dissolved oxygen level and generate higher cell

concentration. But this may be more than offset by the increased plasmid instability caused by the agitation. Due to a relatively low critical dissolved oxygen level, ALR bioreactors are able to provide adequate oxygen for the recombinant yeast. The potential use of ALR bioreactors in commercial cultivation of recombinant yeast cells is very promising.

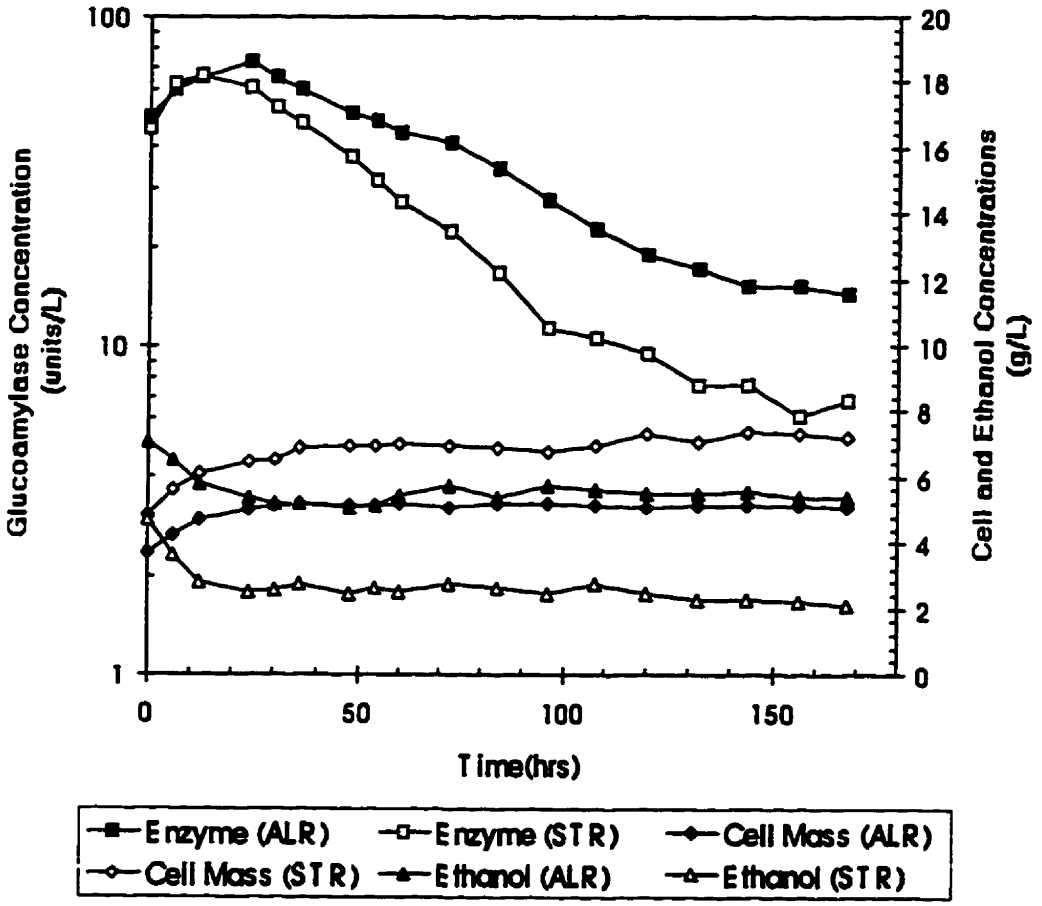


Figure 4.22. Comparisons of Enzyme, Cell and Ethanol Concentrations between ALR and STR Bioreactors in Continuous Culture Using Original Strain (D = 0.10 h^{-1}, T = 30 °C , VVM = 1.0, RPM = 500 and G_0 = 20.0 g/L).

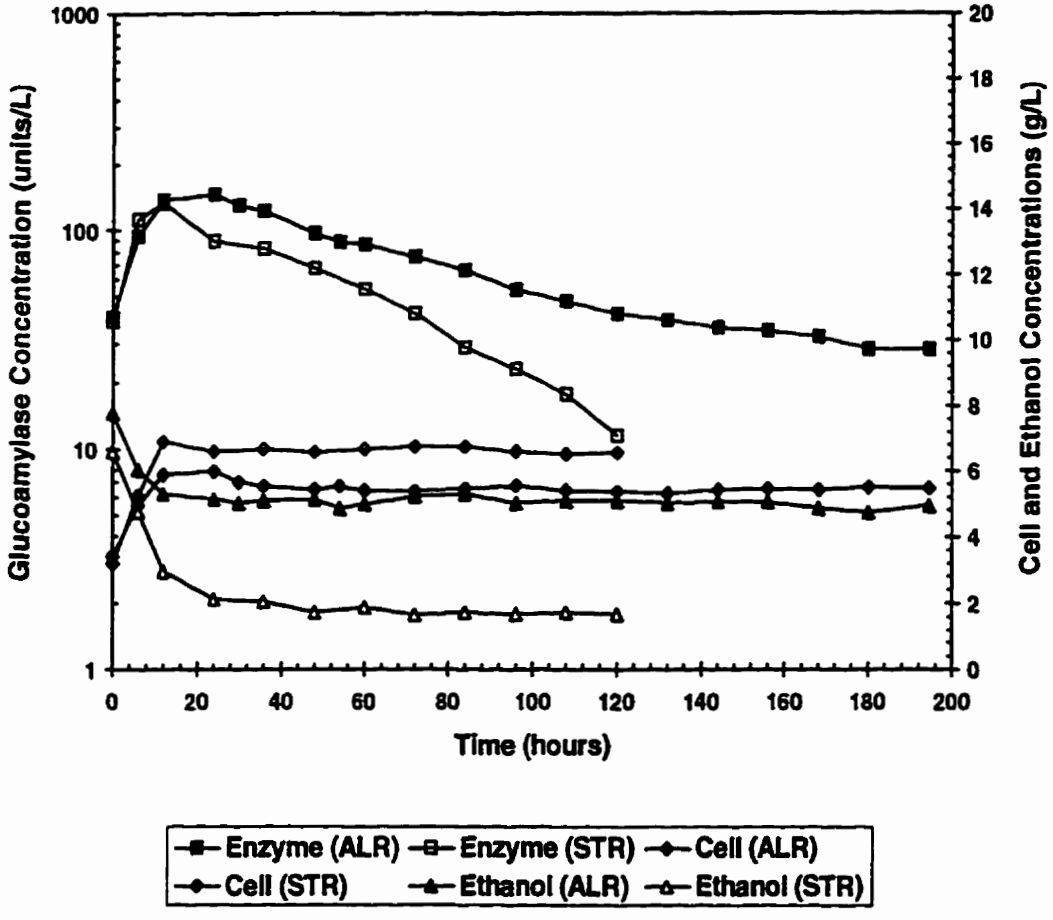


Figure 4.23. Comparisons of Enzyme, Cell and Ethanol Concentrations between ALR and STR Bioreactors in Continuous Culture Using Selected Strain (D = 0.10 h^{-1}, T = 30 °C , VVM = 1.0, RPM = 500 and G_0 = 20.0 g/L).

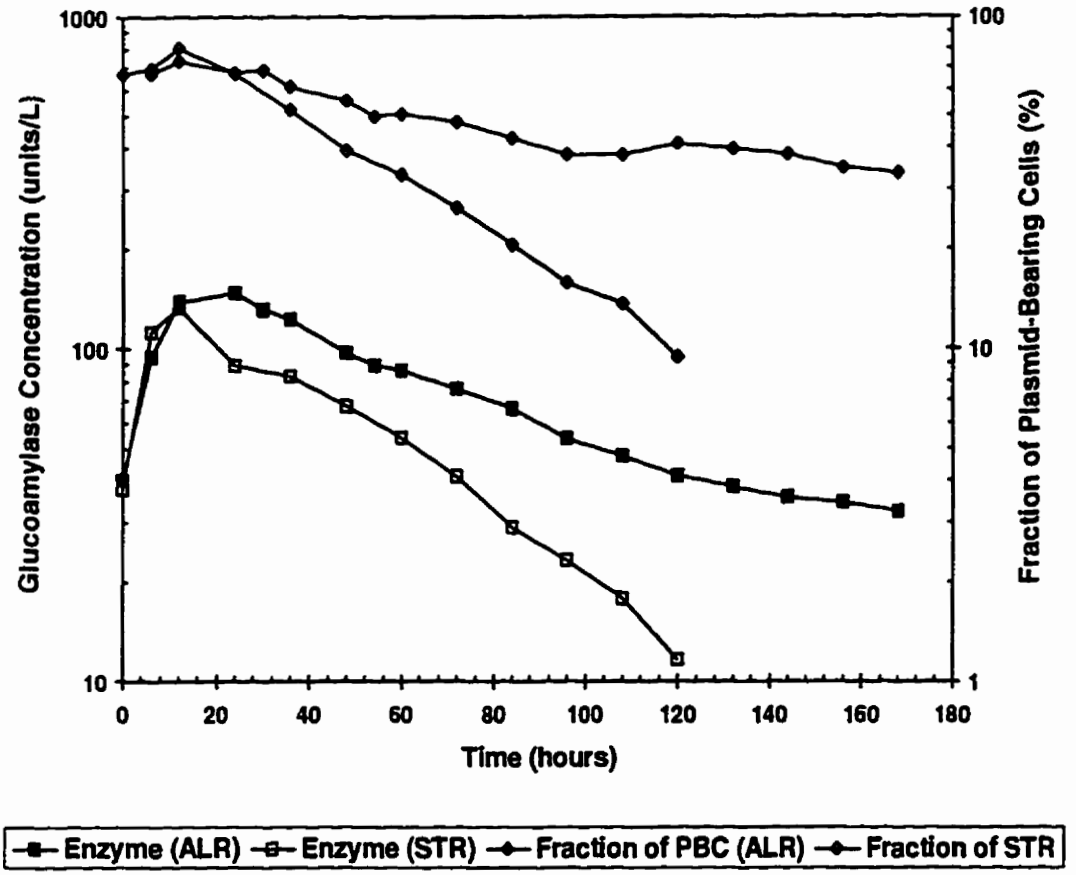


Figure 4.24. Correlation between Enzyme Production and Plasmid Loss During Continuous Culture in ALR and STR Bioreactors Using Selected Strain (D = 0.10 h^{-1}, T = 30 °C , VVM = 1.0, RPM = 500 and G_0 = 20.0 g/L).

4.3.6. Continuous Culture in a Selective Medium

Using a YNB selective medium with a glucose concentration of 10 g/L, continuous culture was performed in the airlift bioreactor to examine the effect of selection pressure. After the bioreactor was operated in batch mode for 24 hours, the continuous culture was initiated at a dilution rate = 0.10 h^{-1}. As shown in Figure 4.25, a decline in enzyme activity was observed prior to reaching steady state condition. This is probably due to the adaptation of cells from solely ethanol consumption to a glucose utilization during the start-up period. The ethanol concentration increased from 1 g/L at the beginning of continuous culture to 1.50 g/L at steady state (after 24 hours) and remained nearly constant thereafter. The total cell concentration at steady state was around 2.90 g/L. The enzyme concentration decreased during the culture process even though the fraction of plasmid-bearing cells remained constant (Figure 4.25). Decoupling between plasmid loss and enzyme production was observed in the selective medium. In contrast, in the nonselective medium, both the fraction of plasmid-bearing cells and enzyme activity decayed exponentially; thus, plasmid loss correlated directly with the decline of enzyme production (Figure 4.26).

It appears that a population of cells which were capable of leucine synthesis, but were less efficient in glucoamylase production had gradually supplanted the original population during the continuous culture in selective medium. The YNB selective medium imposes only one selection pressure which is based on the leucine gene (*LEU2*) inserted in the plasmid. Carrying plasmids and expressing *LEU2* gene for leucine synthesis were

necessary for host cells to grow in the selective medium. However, the expression of glucoamylse gene was not needed for survival. In order to reduce the metabolic load, the expression of glucoamylase might have been turned off. In this case, the plasmids would be maintained for the synthesis of leucine without producing glucoamylase. It was also possible that the plasmids lost a part or the whole glucoamylase gene by deletion. The reduced plasmid size was an advantage for recombinant cells since increased plasmid size required increased amount of energy (Cheah et al., 1987). Furthermore, since the host cell was a *LEU2* auxotrophic strain, it was likely that an integration of the *LEU2* gene encoded on the plasmid into the host chromosome occurred as a result of a homologous recombination event (Kuriyama et al., 1992). The host cells which reacquired the leucine gene did not need plasmids to survive. They could grow in the selective medium and appeared to contain the plasmids; but they were not able to synthesize glucoamylase. The decoupling between plasmid loss and enzyme production in the selective medium may be explained by the above mechanisms. The decay in enzyme production in selective medium was mainly caused by the structural instability of the plasmids. In contrast, the plasmid instability in the nonselective medium was mainly segregational, plasmid loss occurred because of the bias partitioning of plasmids between mother and daughter cells.

The fraction of the plasmid-bearing cells never reached 100%. It remained at 80-86% during culture. In a selective medium the plasmid-free cells are expected to be washed out, nevertheless, many researchers reported the existence of plasmid-negative cells in a selective medium. It was shown that plasmid-free cells could survive in the

selective medium because an essential amino acid that was excreted into the medium by the plasmid-bearing cells could support the growth of plasmid-free cells (Dibiasio and Sardonini, 1986; Sardonini and Dibiasio, 1987). Kleinman et al. (1986) also reported that the selective medium only reduced the instability but could not remove it completely. In the present case, leucine could be synthesized by plasmid-positive cells and secreted into the medium. The small amount of amino acid is probably enough to keep a certain portion of plasmid-free cells viable in the bioreactor.

Comparison of the specific enzyme activity between selective and nonselective media is shown in Figure 4.27. As expected, higher specific enzyme activity and higher stability of glucoamylase production were observed in the selective medium. But higher cell mass and enzyme concentration were obtained in the nonselective medium (Figure 4.28). Therefore, the selective medium is not an economical medium especially if the higher cost of this medium is considered.

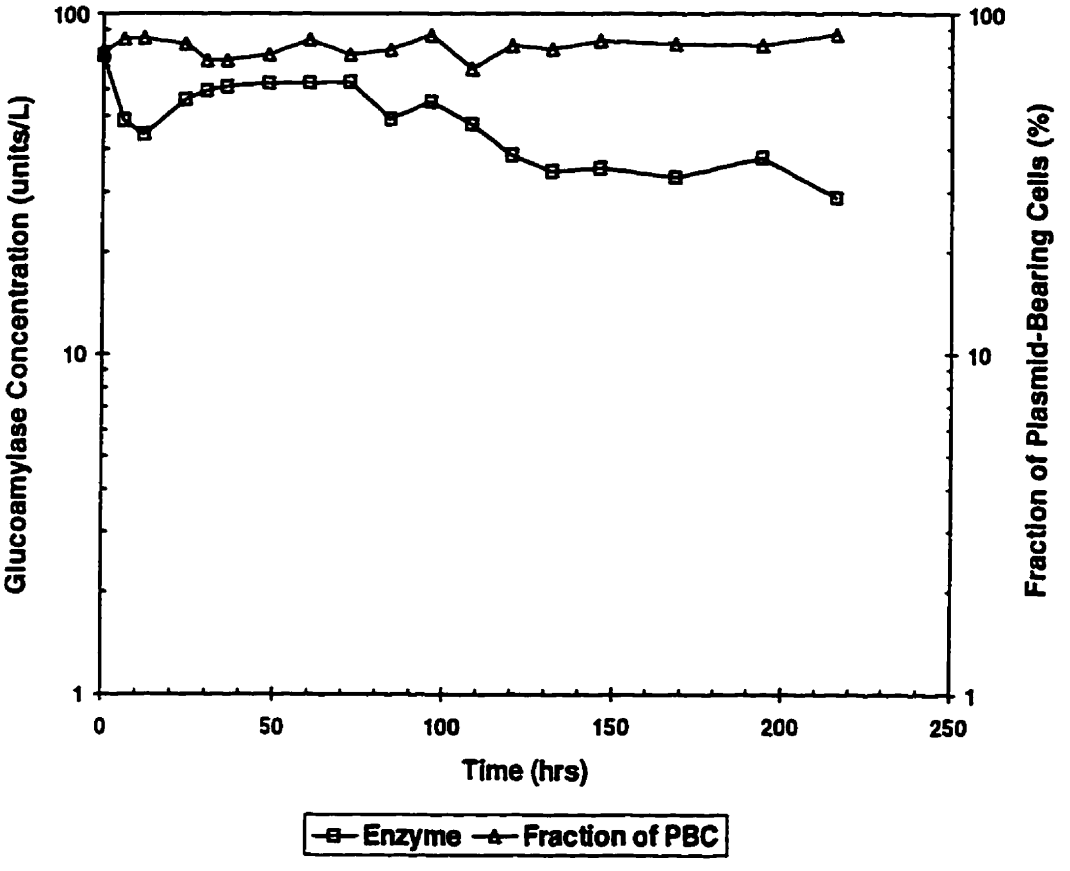


Figure 4.25. Correlation between Fraction of Plasmid-Bearing Cells and Enzyme Concentration during Continuous Culture in ALR Bioreactor Using Selective Medium ($D = 0.10\ h^{-1}$, VVM = 1.0, T = 30 °C and $G_0 = 10$ g/L).

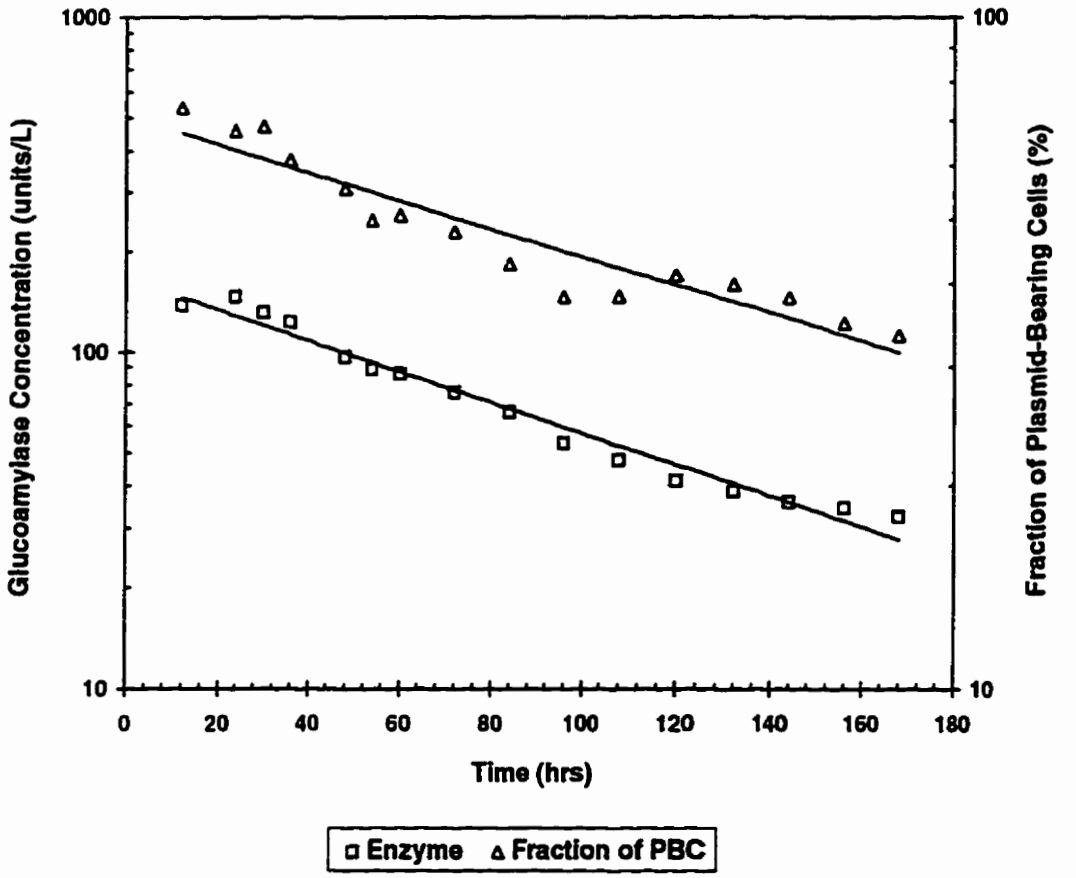


Figure 4.26. Correlation between Fraction of Plasmid-Bearing Cells and Enzyme Concentration during Continuous Culture in ALR Bioreactor Using Nonselective Medium ($D = 0.10\ h^{-1}$, VVM = 1.0, T = 30 °C and $G_0 = 20$ g /L).

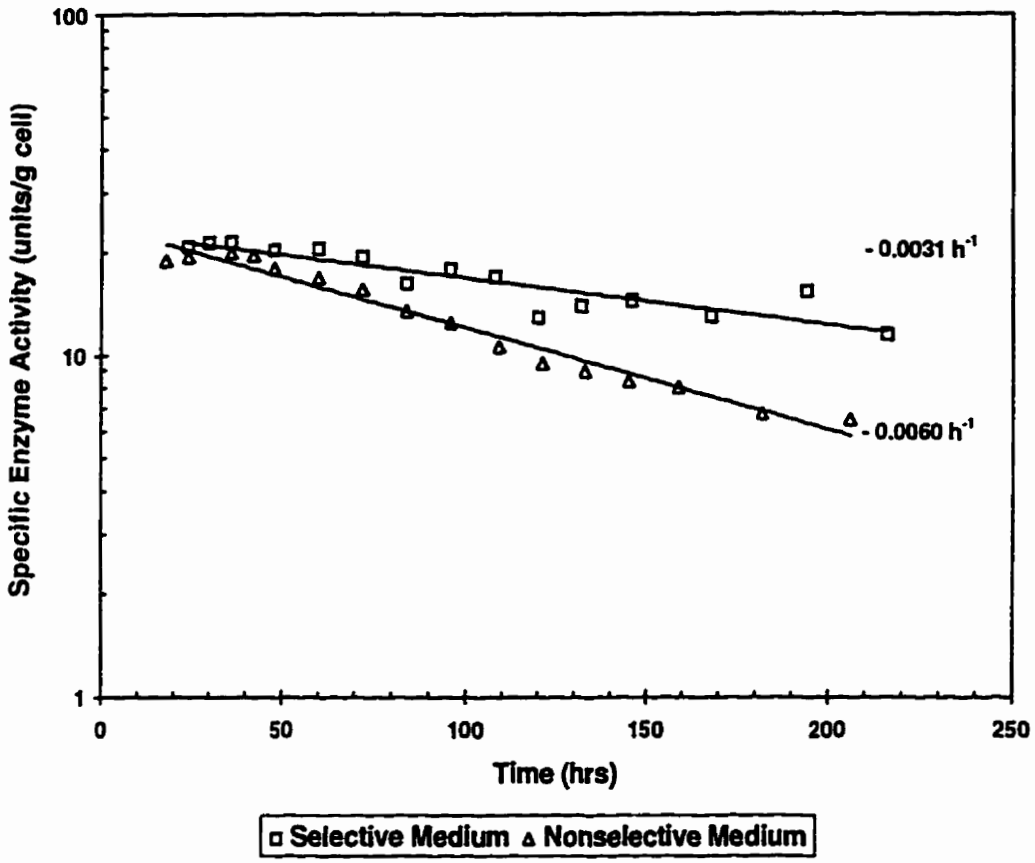


Figure 4.27. Comparison of Specific Enzyme Activity between Selective and Nonselective Media during Continuous Culture in ALR ($D = 0.10\ h^{-1}$, VVM = 1.0, T = 30 °C, $G_0 = 10$ /L).

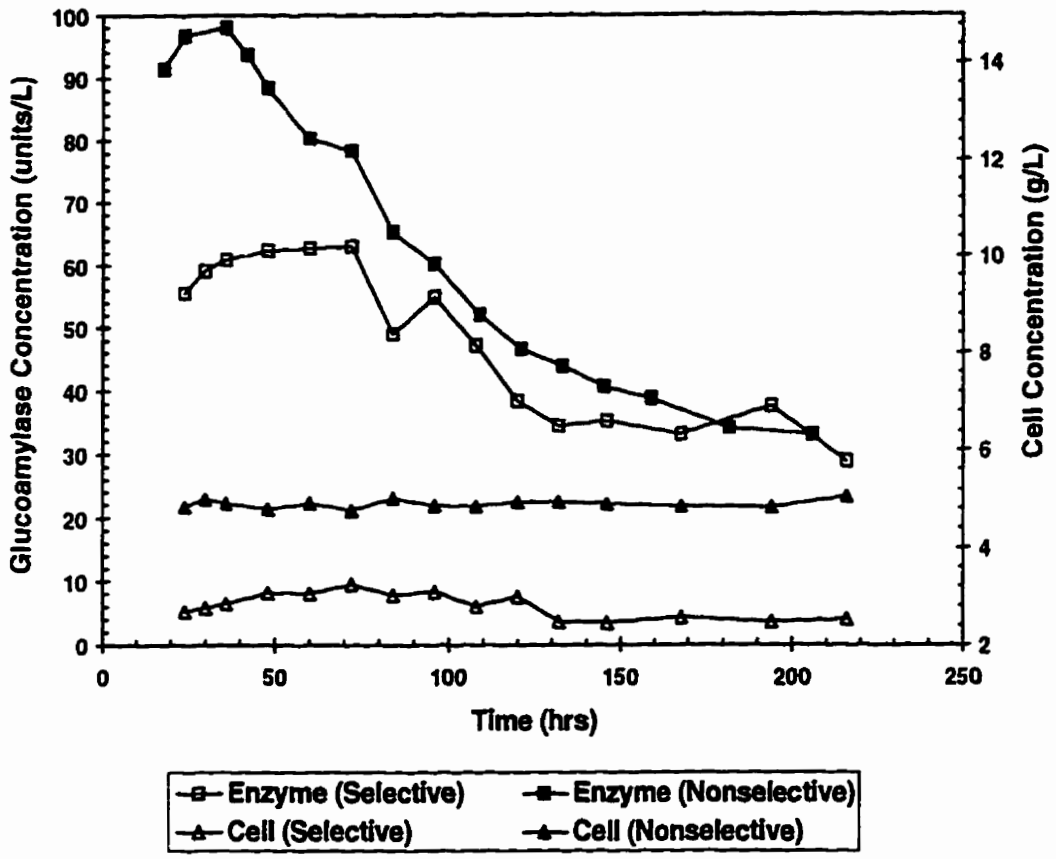


Figure 4.28. Comparison of Cell Mass and Enzyme Concentration between Selective and Nonselective Media during Continuous Culture in ALR (D = 0.10 h^{-1}, VVM = 1.0, T = 30 °C and G_0 = 10 /L).

CHAPTER 5

MODELING OF RECOMBINANT *S. CEREVISIAE*

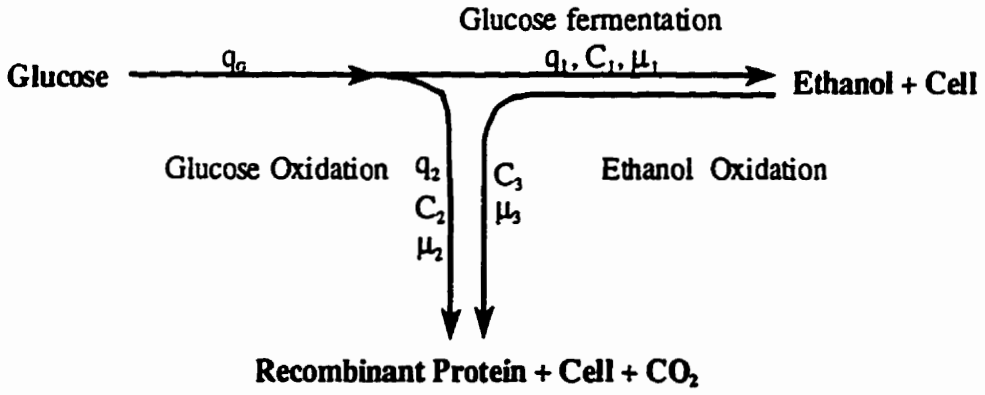
5.1 Introduction

Many genetic and environmental factors must be considered in developing a recombinant cell bioprocess. Quantitative modeling of recombinant cells is very useful for organizing the literature and experimental information, understanding the nature of recombinants, and design, scale-up, operation, control and optimization of recombinant bioprocesses.

In this chapter, a metabolic model describing cell growth, substrate consumption, recombinant protein production and plasmid segregation of recombinant yeast is proposed by utilizing the information obtained in this work and the available knowledge in the literature. Although the model's simplicity and feasibility for engineering applications are emphasized, the key metabolic features of yeast are still incorporated into the model. Experimental results in both batch and continuous fermentations of the recombinant *S. cerevisiae* C468/pGAC9, are used to evaluate the accuracy and feasibility of the model in describing the observed phenomena. To test the generality of the model, verification is also performed with sets of experimental data from independent studies for other recombinant yeast strains.

5.2. Model Development

The model is based on the following assumptions and concepts, which can be derived from both the experimental results obtained in this work and the known metabolic and enzyme expression and regulation features of yeast. Glucose fermentation, glucose oxidation and ethanol oxidation represent the three metabolic pathways in the recombinant yeast *S. cerevisiae*, which can be simplified as shown below (See "Nomenclature" for explanation of notations):



All the essential enzymes in the glucose fermentation pathway are lumped as a pacemaker enzyme pool C_1 and all the key enzymes in glucose oxidation pathway are grouped together as a pacing enzyme pool C_2. Glucose fermentation and glucose oxidation can occur simultaneously. Their specific growth rates (μ_1 or μ_2) are regulated by the activity of the pacing enzyme pools (C_1 or C_2) responsible for individual pathway. C_1 is induced by the high glucose level while C_2 is repressed. The latter corresponds to the Crabtree effect: when the glucose flux becomes too large for the cell's oxidative capacity to

handle, excess glucose is fermented to ethanol and the oxidative enzymes are attenuated to restrict glucose oxidation (Coppella and Dhurjati, 1990; Sonnleitner and Kappeli, 1986). The effects of glucose flux (q_G) on the activities of the pacing enzyme pools can be described according to the following expressions:

$$C_1 = L(t)^* \frac{1 + k_a q_G}{k_b + k_a q_G} \tag{5.1}$$

$$C_2 = L(t)^* \frac{1 + k_c q_G}{1 + k_c k_d q_G} \tag{5.2}$$

$L(t)$ is incorporated to consider the adaptive characteristics in the modes of control and regulation of the enzyme activities. The values of C_1 and C_2 are normalized and vary between 0 and 1. The forms of the above two equations were adapted from van Dedem and Moo-Young (1973). Function $L(t)$ represents the time lag of the transient state between inoculation and the onset of exponential growth. Based on the experimental observation of the sigmoidal increase in specific growth rate during the lag phase, $L(t)$ is approximated by the following exponential function:

$$L(t) = \begin{cases} exp\left[-(t_{lag} - t)^2\right] & t < t_{lag} \\ 1 & t \geq t_{lag} \end{cases} \tag{5.3}$$

where t_{lag} is lag time, which depends on the recombinant strain, medium used and inoculum conditions.

As glucose is depleted in batch culture, ethanol and acetaldehyde dehydrogenases are induced gradually and ethanol is metabolized by progressive oxidation to acetaldehyde

and acetate and is then converted to acetyl-CoA (Coppella and Dhurjati, 1990; Berry and Brown, 1987). The outcome of this metabolism is the onset of respiratory growth on ethanol. The pacemaker enzyme pool in ethanol oxidation pathway is denoted as C_3. The magnitude of C_3 depends on the glucose level and the availability of dissolved oxygen. It was reported that ethanol is assimilated after glucose decreases below a critical concentration, which ranges from 0.13 g/L (Piper and Kirk, 1991) to 0.5 g/L (Pirt and Kurowski, 1970). In this model, in order to take into account the time required for induction and synthesis of the key enzymes for the ethanol oxidation pathway (lag phase for ethanol assimilation), it is assumed that ethanol is assimilated only after glucose is completely exhausted (no glucose flux). The above assumption can be formulated into the following equation:

$$C_3 = \begin{cases} 0 & q_G > 0 \\ 1 & q_G = 0 \end{cases} \tag{5.4}$$

The limiting substance is the carbon source. G denotes glucose and E denotes ethanol. Cell growth in each metabolic pathway follows a modified Monod equation described by:

$$\mu_1 = \frac{\mu_{1,max} C_1 G}{K_1 + G} \tag{5.5}$$

$$\mu_2 = \frac{\mu_{2,max} C_2 G}{K_2 + G} \tag{5.6}$$

$$\mu_3 = \frac{\mu_{3,max} C_3 E}{K_3 + E} \tag{5.7}$$

The carbon fluxes from the three metabolic pathways in yeast are quantitatively additive (Sonnleitner and Kappeli, 1986); therefore, the specific growth rates associated with the three metabolic events are additive as well:

$$q_G = q_1 + q_2 = \frac{\mu_1}{Y^F_{X/G}} + \frac{\mu_2}{Y^O_{X/G}} \tag{5.8}$$

$$\mu = \mu_1 + \mu_2 + \mu_3 \tag{5.9}$$

It is apparent that ethanol production is only associated with the glucose fermentation pathway and is growth-associated. Ethanol is produced in the glucose fermentation pathway and then consumed in the ethanol oxidation pathway as described below:

$$\frac{dE}{dt} = Y_{E/X}\mu_1 X - \mu_3 X / Y_{X/E} \tag{5.10}$$

In addition, it may be assumed that the formation of the recombinant protein is only associated with the oxidative pathways. As discussed in Section 4.2.4, this assumption was justified by our experimental results. Glucoamylase formation has been shown to be growth-associated with plasmid-bearing cells in the present case (Figure 5.1). This observation is consistent with results reported for protein production with other recombinant yeasts (Gu et al., 1989; Coppella and Dhurjati, 1990; Sode et al., 1988; Turner et al., 1991). Consequently, the production rate of glucoamylase may be given as:

$$\frac{dP}{dt} = \alpha_2\mu_2 X^+ + \alpha_3\mu_3 X^+ \tag{5.11}$$

Finally, a negligible growth difference between plasmid-bearing and plasmid-free cells and a constant probability of plasmid loss (p) were found in the present recombinant strain

(See Section 4.3.3). Based on these observations, the segregational plasmid loss kinetics may be expressed as follows:

$$\frac{dX^+}{dt} = (1-p)\mu X^+ \tag{5.12}$$

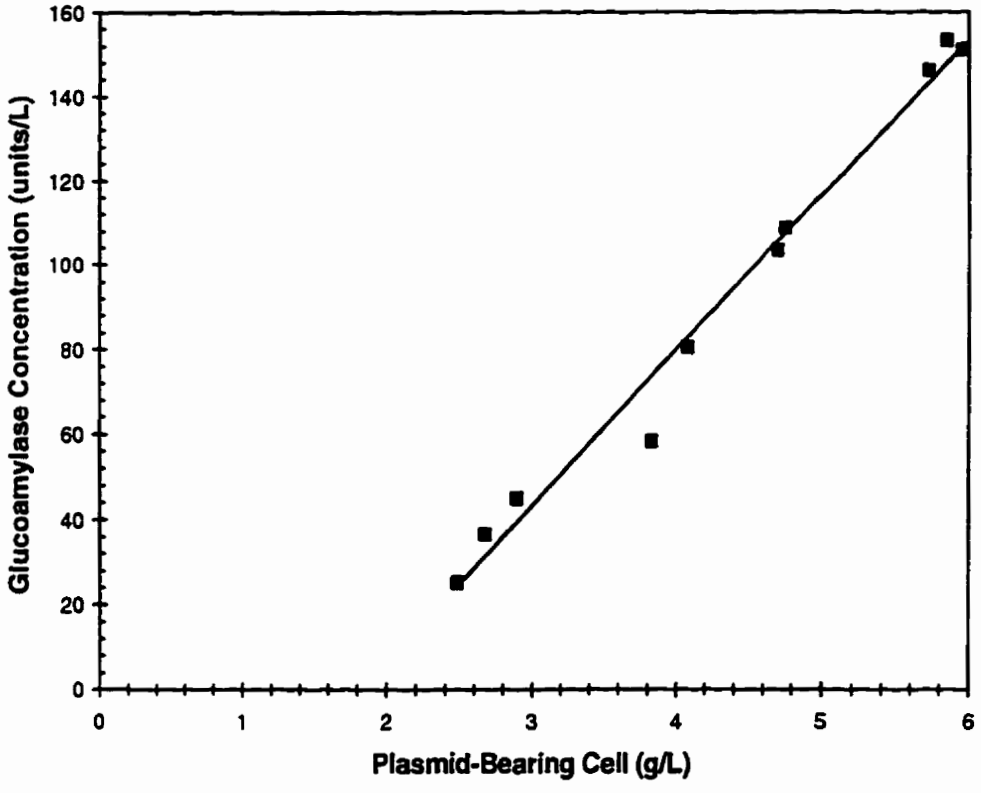


Figure 5.1. Linear Regression of Glucoamylase Concentration to Plasmid-Bearing Cell Concentration in Batch Culture in Airlift Bioreactor.

5.3. Model Simulation in Batch Culture

5.3.1 System Equations for Batch Culture

When the above model is applied to a batch fermentation process, the following dynamic equations are generated:

Glucose (*G*): $$\frac{dG}{dt} = -q_G X \tag{5.13}$$

Total Cell Mass (*X*): $$\frac{dX}{dt} = \mu X \tag{5.14}$$

Plasmid-Bearing Cell (X^+): $$\frac{dX^+}{dt} = (1-p)\mu X^+ \tag{5.12}$$

Ethanol (*E*): $$\frac{dE}{dt} = Y_{E/X}\mu_1 X - \mu_3 X / Y_{X/E} \tag{5.10}$$

rProtein (*P*): $$\frac{dP}{dt} = \alpha_2\mu_2 X^+ + \alpha_3\mu_3 X^+ \tag{5.11}$$

Fraction of PBC (*F*): $$F = \frac{X^+}{X} \times 100 \tag{5.15}$$

The batch culture model requires 15 equations and 18 parameters. Using the parameter estimates from experimental data, the model can be solved simultaneously for six variables (*G*, *X*, X^+, *E*, *P* and *F*) by C computer programming.

5.3.2. Parameter Estimation

The parameter values used for model simulation are summarized in Table I. The values of all these parameters are within the range of literature values. Of the 18 parameters, 14 were estimated from the experimental results, 4 were derived from literature. All

parameter values were considered constant and experimentally easily accessible. In order to obtain the parameters associated with the glucose fermentation pathway ($Y^{F}_{X/G}$, $Y_{E/X}$ and $\mu_{1,max}$), a complete anaerobic batch fermentation was carried out using nitrogen sparging. Then the yield coefficients ($Y^{F}_{X/G}$ and $Y_{E/X}$) were determined from a linear regression of glucose or ethanol to cell dry weight and the maximum specific growth rate for glucose fermentation ($\mu_{1,max}$) was evaluated from a semilogarithmic plot of cell concentration (X) versus time (t). After $Y^{F}_{X/G}$ was determined, $Y^{O}_{X/G}$ and α_2 were estimated from aerobic batch fermentation. Similarly, the parameters for the ethanol oxidation pathway ($Y_{X/E}$, $\mu_{3,max}$ and α_3) were obtained from the experimental data of oxidative growth on ethanol. The maximum specific growth rate for glucose oxidation $\mu_{2,max}$ was taken from the literature (Coppella and Dhurjati, 1990; Piper and Kirk, 1991). The saturation constants K_1, K_2 and K_3 were obtained from published data (Coppella and Dhurjati, 1990; Sonnleitner and Kappeli, 1986). Lag time t_{lag} was evaluated from experimental data, which is the time required to achieve exponential growth after inoculation. The probability of plasmid loss (p) was determined from the continuous fermentation data according to the method described in Section 4.3.3. The regulation coefficients of the pacemaker enzyme pools (k_a, k_b, k_c and k_d) were estimated from the continuous fermentation data and were then adjusted according to published information on yeast metabolic pathways (van Dedem and Moo-Young, 1973); Coppella and Dhurjati, 1990; Berry and Brown, 1987).

Table 5.1. Summary of Parameters for Recombinant *Saccharomyces cerevisiae*.

Parameters	Dimension	This study	Literature
p	dimensionless	0.05	N/A
$Y_{X/G}^{F}$	g cell/g glucose	0.12	0.05-0.10, 0.15
$Y_{X/G}^{O}$	g cell/g glucose	0.48	0.49 , 0.50
$Y_{X/E}$	g cell/g ethanol	0.65	0.53 , 0.72
$Y_{E/X}$	g ethanol/g dcw	3.35	3.0
α_2	units/g dcw	32.97	N/A
α_3	units/g dcw	33.80	N/A
$\mu_{1,max}$	h^{-1}	0.38	0.30, 0.40-0.45
$\mu_{2,max}$	h^{-1}	0.25	0.25 , 0.25-0.3
$\mu_{3,max}$	h^{-1}	0.06	0.10
K_1	g/L	0.10	0.1-0.5
K_2	g/L	0.01	0.005
K_3	g/L	0.5	0.48
k_a	(g glucose/g dcw.h)$^{-1}$	25	N/A
k_b	(dimensionless)	10	N/A
k_c	(g glucose/g dcw.h)$^{-1}$	1	N/A
k_d	(dimensionless)	4	N/A
t_{lag}	h	2.5	N/A

Note: N/A = not available

5.3.3. Simulated Results for Present Recombinant Yeast

The batch culture model consists of 5 differential equations and 10 analytical equations. These 15 equations must be solved simultaneously in order to evaluate the fermentation variables, which are glucose concentration (G), total cell concentration (X), plasmid-bearing cell concentration (X^+), ethanol concentration (E), recombinant protein concentration (P) and the fraction of plasmid-bearing cells (F). The differential equations were solved numerically using a fourth order Runge-Kutta algorithm while Newton's method was used for solving the analytical equations. The step size taken was 0.2 hour. Solution of the model equations was programmed in C code and was run in a Turbo C++ environment (Borland Inc.). A printout of the source code is presented in Appendix F. Graphical representation of the model output and experimental data were carried out with Excel software. A comparison between the model-simulated and experimental results for the present recombinant yeast (C468/pGAC9) culture in airlift bioreactor is presented in Figure 5.2.

Figure 5.2 shows excellent agreement between model predictions and experimental results for glucose, cell mass, ethanol, glucoamylase (recombinant protein) and fraction of plasmid-bearing cells during batch fermentation of recombinant *S. cerevisiae* strain C468/pGAC9. Using the modified Monod equation, the model successfully predicted diauxic growth, glucose consumption, ethanol production and ethanol utilization after glucose exhaustion. With the assumption that the formation of recombinant protein is only

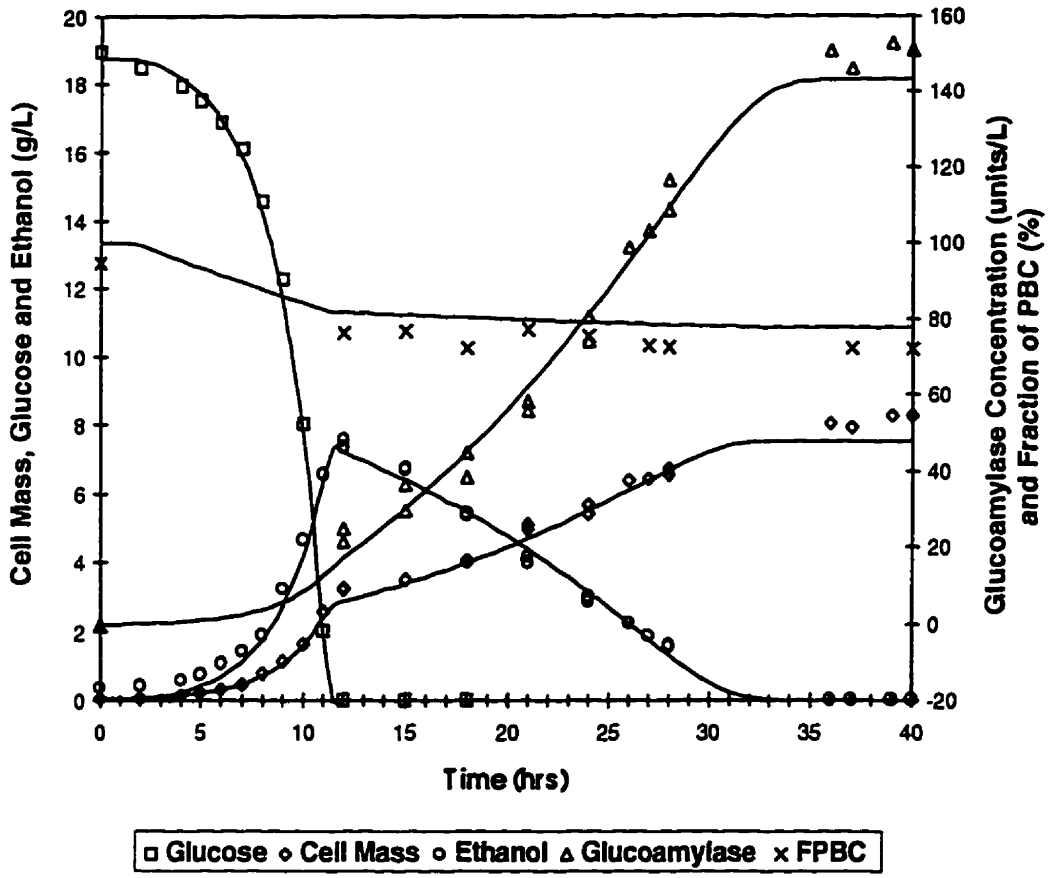


Figure 5.2. Comparison between Model Simulation (solid line) and Experimental Data (markers) for Batch Fermentation with Recombinant Yeast Strain C468/pGAC9 Producing Glucoamylase (data from current studies).

associated with the oxidative growth, the model also simulated well the production of glucoamylase. The proposed plasmid loss kinetics also agrees well with the experimental observations. Both measured and simulated fractions of plasmid-bearing cells are nearly constant during the slower ethanol growth phase. This indicates that the segregation instability decreases with decreasing growth rate. Finally, it is very encouraging that the proposed model simulated well the first lag phase on glucose, the diauxic lag on ethanol and the stationary phase, which are usually difficult to describe.

4.3.4. Independent Verification of Proposed Model

Data on the production of extracellular Epidermal Growth Factor (hEGF) using recombinant *S. cerevisiae* strain AB103.1 pYαEGF-25 was obtained from Coppella and Dhurjati (1990). Because it is a different recombinant yeast strain, growth associated coefficients for hEGF production (α_2 and α_3) were adjusted to 3.11 mg hEGF/ g dcw according to the authors' reported value. Since the lag time t_{lag} depends on recombinant strains, medium used and inoculum conditions, it was changed to 5.0 hr according to the experimental data. Only two other parameters ($Y^P_{X/G}$ and K_I) were adjusted based on the authors' reported values ($Y^P_{X/G}$ from 0.12 to 0.154 g cell/g glucose and K_I from 0.1 to 2.1 g/L). With four adjusted parameters, the model successfully predicted the dynamics of glucose consumption, cell growth, ethanol production and utilization, hEGF production and plasmid loss during batch fermentation of recombinant yeast strain AB103.1 pYαEGF-25. Figure 5.3 shows that the simulated results by the proposed model agree well with the experimental observations. With the same probability of plasmid loss (p)

determined in the current study, the model was able to simulate the fraction of plasmid-bearing cells of the cited recombinant strain. Coppella and Dhurjati also developed an excellent structured model to describe the recombinant yeast (Coppella and Dhurjati, 1990). But their model required 43 equations and 48 parameters and some parameters were difficult to estimate. Its complexity poses a number of difficulties for its practical use (e.g. parameter estimations). In contrast, the present model requires 15 equations and 18 experimentally accessible parameters. It is more realistic and useful for industrial applications.

In order to further test the proposed model, another set of experimental data on the growth of recombinant *S. cerevisiae* SEY2102/pRB58 were obtained from Parker and Seo (1992). The host strain SEY2102 contained the plasmid pBR58, which encodes for intracellular invertase. The dynamics of cell growth, glucose consumption and ethanol metabolism were simulated by the model. Due to inadequate data, the simulation for invertase production could not be presented. Figure 5.4 shows a remarkable agreement between the model simulations and the reported data. In executing the model, the same parameter values listed in Table I were used except the value of $\mu_{3,max}$ and t_{lag}, which were set to 0.10 h^{-1} and 1.5 h respectively to match the observed values. Thus, the general applicability of the present model was further confirmed.

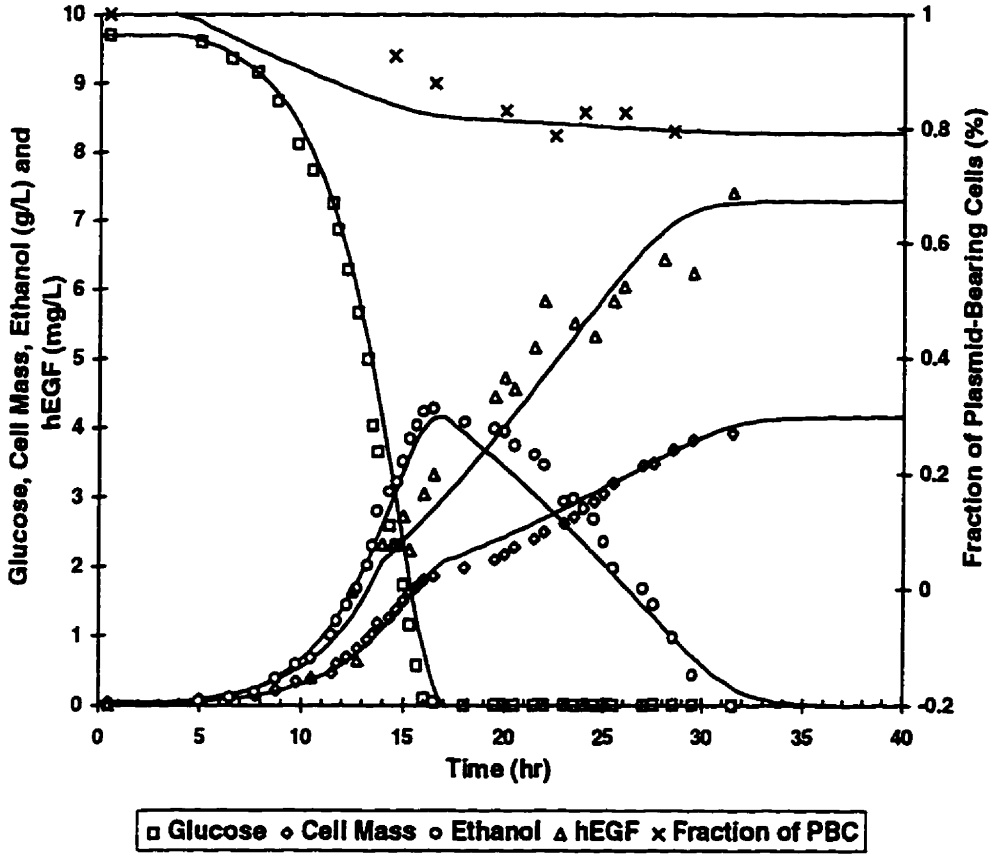


Figure 5.3. Comparison between Model Simulation (solid line) and Experimental Data (markers) for Batch Fermentation with Recombinant Yeast Strain AB103.1/pYαEGF-25 Producing Human Epidermal Growth Factor (data from Coppella and Dhurjati (1990)).

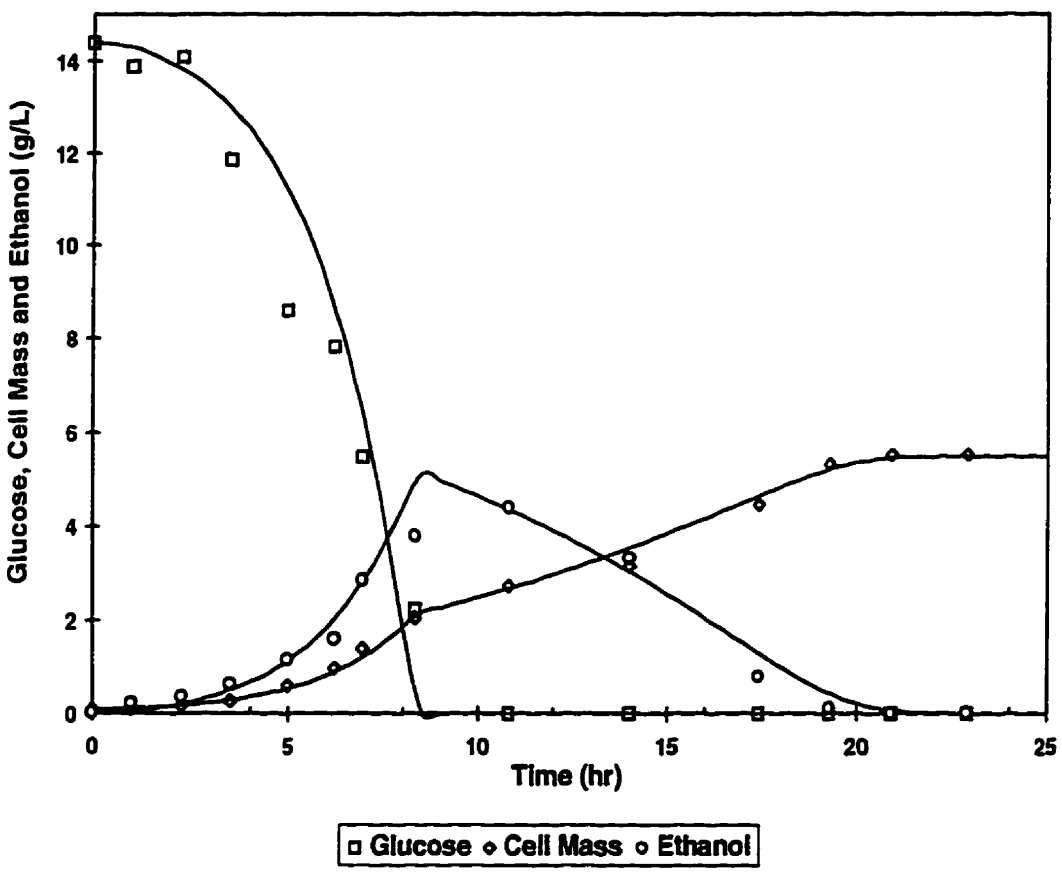


Figure 5.4. Comparison between Model Simulation (solid line) and Experimental Data (markers) for Batch Fermentation with Recombinant Yeast Strain SEY2102/pRB58 Producing Invertase (data from Parker and Seo (1992).

5.4. Model Simulation in Continuous Culture

5.4.1. System Equations in Continuous Culture

In continuous culture, glucose was fed into the bioreactor and ethanol was produced continuously. Since glucose fermentation and ethanol oxidation pathways are unlikely to function simultaneously, the ethanol oxidation pathway may be switched off. When the proposed model is applied to the steady state continuous culture, the following system equations may be formed:

$$\mu_3 = 0 \qquad \text{(no ethanol oxidation)} \tag{5.16}$$

$$\mu = \mu_1 + \mu_2 = D \tag{5.17}$$

$$q_G = q_1 + q_2 = \frac{\mu_1}{Y^P_{X/G}} + \frac{\mu_2}{Y^O_{X/G}} \tag{5.18}$$

$$\mu_1 = \frac{\mu_{1,max} C_1 G}{K_1 + G} \tag{5.19}$$

$$\mu_2 = \frac{\mu_{2,max} C_2 G}{K_2 + G} \tag{5.20}$$

$$C_1 = \frac{1 + k_a q_G}{k_b + k_a q_G} \tag{5.21}$$

$$C_2 = \frac{1 + k_c q_G}{1 + k_c k_d q_G} \tag{5.22}$$

$$q_G = D*(G_0 - G) / X \tag{5.23}$$

Equations (17) to (23) can be solved simultaneously by using Maple V to get μ_1, μ_2, $\mu = \mu_1 + \mu_2$, C_1, C_2, q_G, X, G (Examples of Maple V code for solving the system

equations are listed in Appendix G. Then the following dynamic equations may be obtained based on the proposed model and mass balance:

Plasmid-Bearing Cell (X^+): $$\frac{dX^+}{dt} = \mu X^+ - p\mu X^+ - DX^+ \quad \textbf{(5.24a)}$$

$$X^+ = X_0^+ exp(-pDt) \quad \textbf{(5.24b)}$$

Plasmid-Free Cell (X^-): $$\frac{dX^-}{dt} = \mu X^- + p\mu X^+ - DX^- \quad \textbf{(5.25a)}$$

$$X^- = X_0^- + X_0^+ [1 - exp(-pDt)] \quad \textbf{(5.25b)}$$

rProtein (P): $$\frac{dP}{dt} = \alpha_2 \mu_2 X^+ - DP \quad \textbf{(5.26a)}$$

$$P = P_0 exp(-Dt) + \frac{P_0}{(1-p)} [exp(-pDt) - exp(-Dt)]$$

Where **(5.26b)**

$$P_0 = \alpha_2 \mu_2 X_0^+ / D$$

Ethanol (E): $$\frac{dE}{dt} = Y_{E/X} \mu_1 X - DE = 0 \quad \textbf{(5.27a)}$$

$$E = Y_{E/X} \mu_1 X / D \quad \textbf{(5.27b)}$$

Equations 5.24a and 5.25a did not include a term for decline of cell population due to cell death since the experimental results indicated that the attrition of cell population due to death was negligible under the conditions the experiments were carried out. The

continuous culture model consists of 12 equations and 13 parameters. Using the parameters listed in Table 5.1, the model can be solved analytically to give glucose concentration (G), total cell concentration (X), plasmid-bearing cell concentration (X^*), ethanol concentration (E), and recombinant protein concentration (P).

5.4.2. Comparison between Model Predictions and Experimental Results

Comparison between the simulated and experimental results for the continuous culture of the present recombinant yeast (C468/pGAC9) in the airlift bioreactor is presented in Table. 5.2. Excellent agreement between the experimental data and model predictions was achieved for the glucose, cell, ethanol, glucoamylase concentrations and specific glucose consumption rate at different dilution rates. Therefore, the proposed model can simulate not only the batch culture but also the continuous culture. Its applicability was further tested.

5.4.3. Effect of Dilution Rate

Effects of dilution rate on G, X, E, q_G and P_0 are shown in Figure 5.5. In Figure 5.5, lines represent model simulations and points (markers) represent experimental results. X and P_0 are predicted to decrease with increasing dilution rate. In contrast, G and q_G increase with increasing dilution rate. Initially, E increases with increasing dilution rate and reaches a maximum value at dilution rate = 0.35 h^{-1}, then declines rapidly when dilution rate approaches the wash-out point. The wash-out dilution rate predicted by the model is 0.415 h^{-1}. When dilution rate is close to the wash-out point, G rises rapidly to the feed glucose concentration (G_0) while X and P_0 drop to zero. As shown in Figure 5.6, there is

a dilution rate at which the glucoamylase productivity is maximized. The dilution rate to achieve the maximum productivity is estimated to be 0.1 h^{-1}.

Table 5.2. Comparison between Measured and Predicted Results in Continuous Suspension Culture in Airlift Bioreactor (T = 30 °C, VVM =1).

D (h^{-1})	0.05	0.10	0.20
G_0 (g/L)	19.00	19.00	19.00
G (g/L)	0.0041	0.0108	0.0497
Predicted G (g/L)	0.0033	0.0124	0.0665
X (g/L)	7.0680	5.4463	3.7849
Predicted X (g/L)	7.4056	5.5253	3.3088
E (g/L)	2.2967	4.8626	7.0343
Predicted E (g/L)	2.2643	4.6784	7.5488
q_G ($g.g^{-1}.h^{-1}$)	0.1326	0.3441	0.9902
Predicted q_G ($g.g^{-1}.h^{-1}$)	0.1283	0.3432	1.1495
P_0 (units/L)	194.81	146.65	51.97
Predicted P_0 (units/L)	202.59	128.36	41.04
Predicted μ_1 (h^{-1})	0.0039	0.0217	0.1164
Predicted μ_2 (h^{-1})	0.0461	0.0783	0.0836

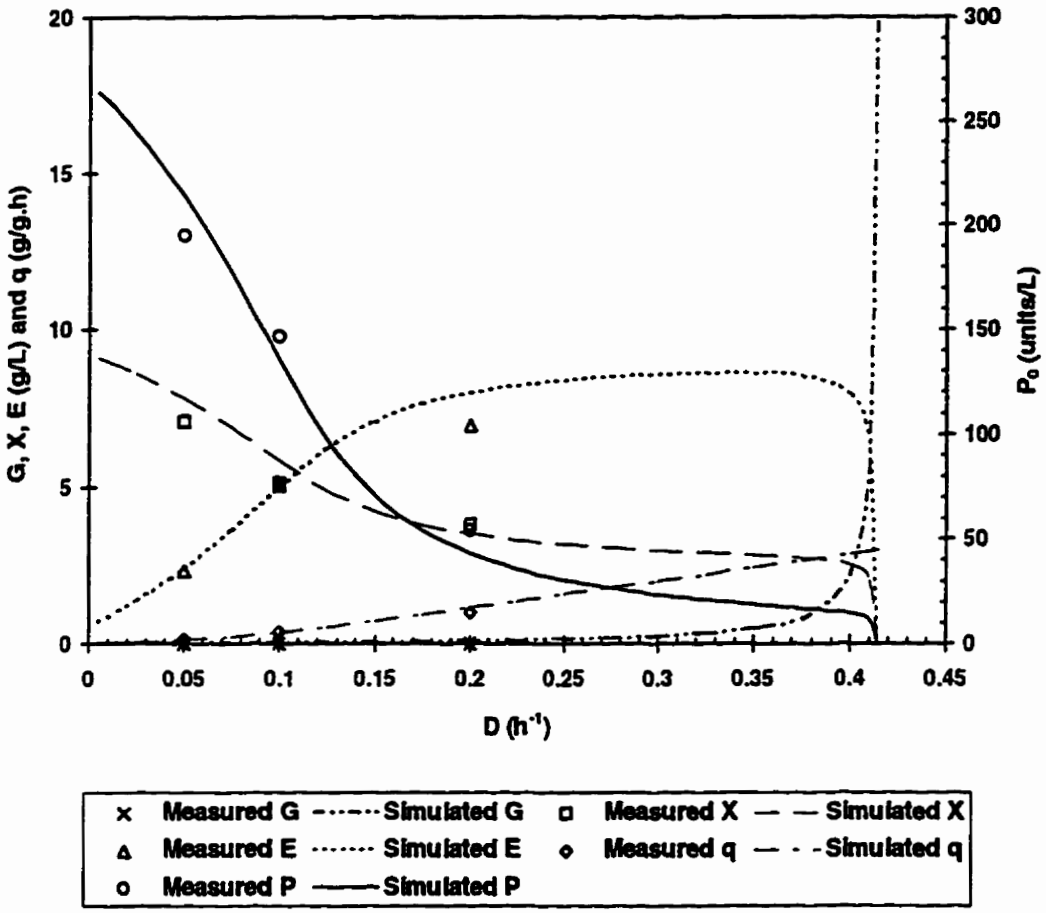


Figure 5.5. Effect of Dilution Rate on *G*, *X*, *E*, *q* and P_0 during Continuous Fermentation in Airlift Bioreactor with Recombinant Yeast Strain C468/pGAC9 Producing Glucoamylase.

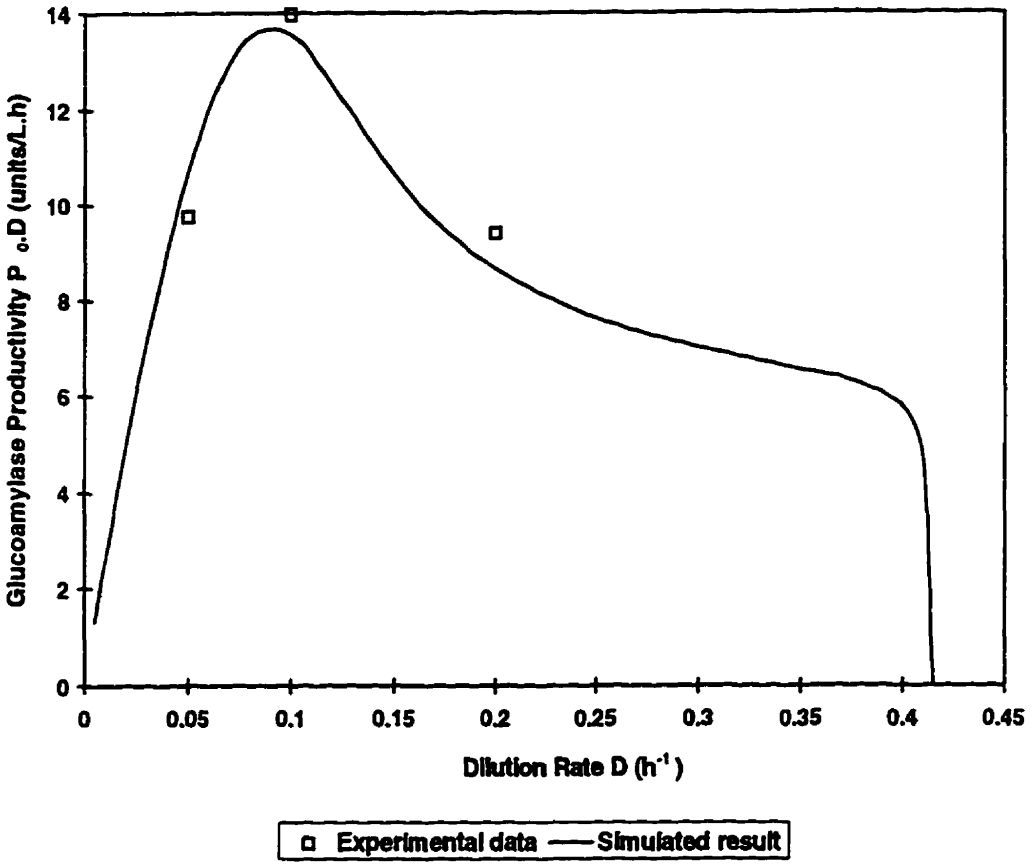


Figure 5.6. Effect of Dilution Rate on Glucoamylase Productivity during Continuous Fermentation in Airlift Bioreactor with Recombinant Yeast Strain C468/pGAC9 Producing Glucoamylase.

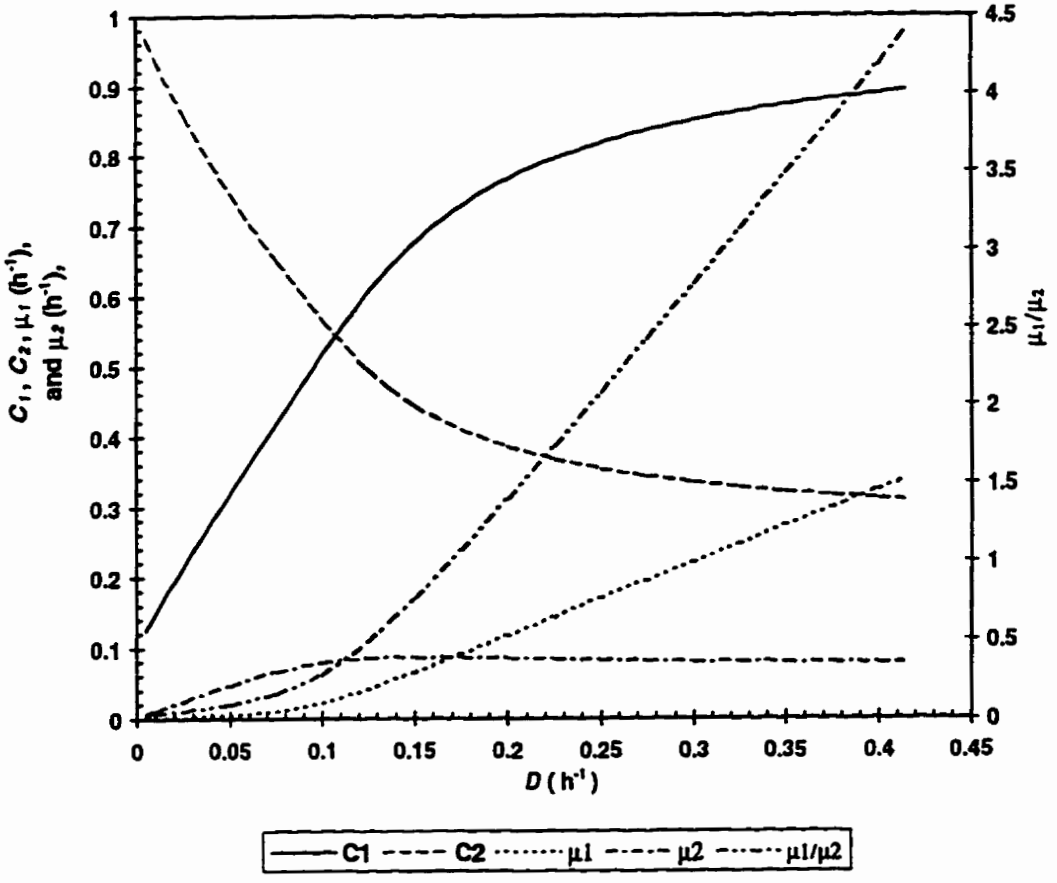


Figure 5.7. Effect of Dilution Rate on C_1, C_2, μ_1, μ_2 and μ_1/μ_2 during Continuous Fermentation in Airlift Bioreactor with Recombinant Yeast Strain C468/pGAC9.

The effects of dilution rate on the activities of the pacemaker enzyme pools for glucose fermentation and glucose oxidation pathways (C_1 and C_2) are presented in Figure 5.7. C_1 increases with increasing dilution rate since the glucose flux increases with increasing dilution rate and C_1 is induced by the glucose flux. In contrast, C_2 decreases with increasing dilution rate because of the repression by high glucose flux. Both C_1 and C_2 tend to be constant at high dilution rate, corresponding to full induction of C_1 and full repression of C_2. The specific growth rate for glucose fermentation (μ_1) is directly proportional to the dilution rate due to increasing C_1 and glucose concentration (G). The specific growth rate for glucose oxidation (μ_2) first increases with increasing dilution rate, then tends to be nearly constant at higher dilution rate, indicating that a saturated respiratory capacity is reached (Sonnleitner and Kappeli, 1986). The ratio of μ_1 to μ_2 increase steadily with increasing dilution rate, suggesting that glucose oxidation is dominant at low dilution rate and glucose fermentation predominates at high dilution rate.

5.4.4. Plasmid Loss and Enzyme Production Decay Kinetics

As derived in Section 5.4.1, the plasmid loss kinetics can be described by Equation (5.24b) as shown below:

$$X^+ = X_0^+ exp(-pDt) = X_0^+ exp(-k_f t) \qquad \textbf{(5.24b)}$$

where $k_f = pD$, a first-order decay rate constant for plasmid loss. Equation (5.24b) shows that the decay of plasmid-bearing cells follows first-order kinetics and the concentration of plasmid-bearing cells decreases with culture time during continuous culture.

On the other hand, the decay kinetics for enzyme production can be described by Equation (5.26b). Since the probability of plasmid loss (p) is usually small, Equation (5.26b) may be simplified as the following:

$$P = P_0 exp(-pDt) = P_0 exp(-\gamma_f t) \tag{5.28}$$

where γ_f is a first-order decay rate constant for enzyme production. Equation (5.28) shows that like the concentration of plasmid-bearing cells, the decay of enzyme concentration also follows first-order kinetics. From Equations (5.24b) and (5.28), it is interesting to note that, theoretically, the enzyme concentration decay rate constant (γ_f) is the same as the plasmid loss constant (k_f) at a fixed dilution rate, that is:

$$\gamma_f = k_f = pD \tag{5.29}$$

In order to test the proposed models for the plasmid loss and enzyme production decay kinetics, continuous suspension cultures were performed in the airlift bioreactor at different dilution rates. As shown in Figure 5.8, both plasmid-bearing cell and enzyme concentration followed an exponential decay pattern, which agrees well with Equations (5.24b) and (5.28). Apparent first-order decay constants for different dilution rates are listed in Table 5.3. As shown in Table 5.3, the apparent kinetic constants for plasmid loss and enzyme production decay decreased with increasing dilution rate. The results are consistent with Equation (5.29); the first-order decay constants are expected to be directly proportional to the dilution rate. The probability of plasmid loss (p) due to structural instability and/or segregational instability is an important parameter in studying recombi-

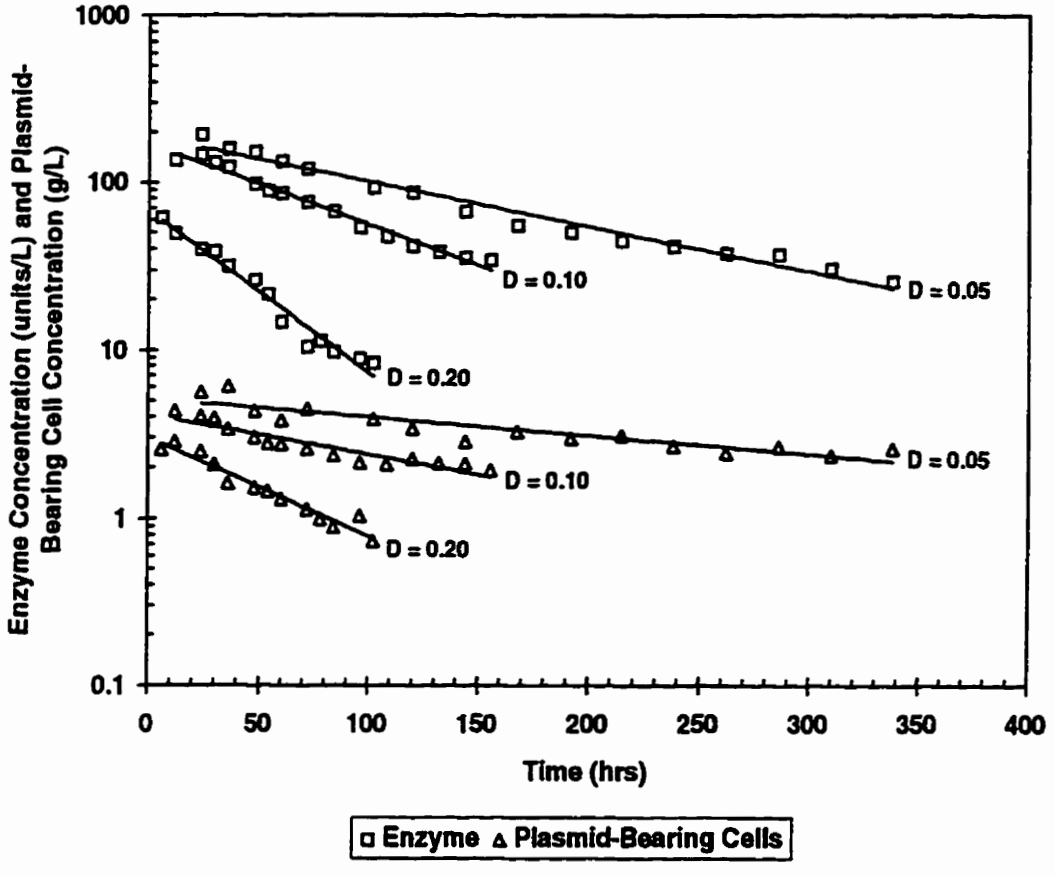


Figure 5.8. Relationship between Plasmid-Bearing Cell Concentration and Glucoamylase Concentration in Continuous Suspension Culture of Recombinant Yeast Strain C468/pGAC9.

nant cell kinetics. The values of the plasmid loss probability for different dilution rates can be calculated using Equation (5.29) based on the first-order decay rate constants determined by the experiments. The estimated values of p at different situations are listed in Table 5.3. It can be seen that the probability of plasmid loss is nearly constant for all dilution rates tested. The assumption of a constant probability of plasmid loss is proved.

According to Equation (5.29), the first-order plasmid loss constants (k_f) should be the same as the first-order enzyme production decay constants (γ_f). But the results in Table 5.3 shows that the observed values of γ_f are higher than those of k_f. This may be explained by the following reasons. First, during culture the plasmids might have been losing a part or the whole glucoamylase gene by deletion while retaining the *LEU2* gene. It was also possible that the expression of glucoamylase turned off in order to reduce the metabolic load. Furthermore, an integration of the *LEU2* gene encoded on the plasmid into the host chromosome might occur as a result of homologous recombination event (Kuriyama et al., 1992) since the host cell was a *LEU2* auxotrophic strain. In the above cases, the resulted cells were capable of leucine synthesis without producing glucoamylase. Such cells showed positive results in plasmid detection since the YNB selective medium used for measuring the fraction of the plasmid-bearing cell imposes only one selection pressure which is based on the leucine gene (*LEU2*) inserted in the plasmid. Finally, the plasmid copy number might have been decreasing with increasing culture time due to uneven partition between mother and daughter cells. For the 2 μm based yeast plasmid (Yep), the copy number per cell usually ranges from 5 to 40 (Caunt et al., 1988).

It may take several generations for plasmid-bearing cells to lose all plasmids. Overall, the plasmid copy number may decrease during culture. The cells with lower copy number still show positive result in plasmid detection but produce less glucoamylase. Because of the above reasons, the enzyme production may decay faster than the plasmid-bearing cell concentration.

According to Equation (5.29), the probability of plasmid loss (p) can also be estimated based on the first-order decay constants of the enzyme production (γ_f). Table 5.3 shows that such calculated values of p are higher than the corresponding estimated values of p based on first-order plasmid loss constants (k_f). This is because the former include both the segregational and structural instability, as discussed in the last section.

Table 5.3. First-Order Plasmid Loss and Enzyme Decay Constants in Continuous Suspension Culture of Recombinant Yeast Strain C468/pGAC9.

D (h^{-1})	0.05	0.10	0.20
Measured k_f (h^{-1})	0.0026	0.0052	0.0122
p (dimensionless) based on k_f	0.0520	0.0520	0.0610
Measured γ_f (h^{-1})	0.0058	0.0099	0.0223
p (dimensionless) based on γ_f	0.1160	0.0990	0.1115

CHAPTER 6

IMMOBILIZED RECOMBINANT YEAST CULTURE

6.1. Introduction

Through the experimental results and modelling discussed in Chapters 4 and 5, a better understanding of the nature of the recombinant yeast was obtained. In the following sections, effects of immobilization on the behaviour of the recombinant yeast will be addressed.

Use of immobilization as an alternative method for maintaining genetic stability under nonselective conditions has been examined (Kumar and Schugerl, 1990). Enhanced genetic stability and productivity of recombinant products were observed in both immobilized recombinant *E. coli* and recombinant *S. cerevisiae*. However, a general explanation for the enhancement is still lacking. In this work, a novel immobilized-cell-film airlift bioreactor (See Figure 3.1.) is developed by employing cotton cloth sheets to immobilize yeast cells by attachment. The performance of the immobilized-cel-film bioreactor is compared with the corresponding free cell system in both continuous and repeated batch culture modes to evaluate the efficiency and feasibility of the proposed bioreactor. A mathematical model is developed to describe the plasmid loss and enzyme production decay kinetics in the immobilized cell system. A general explanation for the enhancement by immobilization is sought. It is the intent of this work to gain a better

understanding of the nature of the immobilized recombinant yeast through experiments and modelling.

6.2. Continuous Culture in Immobilized-Cell Bioreactor

6.2.1. Model Development

An idealised model is used to characterize the immobilized cell system as illustrated in Figure 6.1. As shown in Figure 6.1, the idealized biofilm has a uniform density of cells attached to the support and a locally uniform thickness with negligible internal and external mass transfer resistance. These assumptions may be justified for the present immobilized-cell-film airlift bioreactor because of the thin biofilm (less than 2.0 mm) and strong hydrodynamic circulation and mixing in the bioreactor. It is also assumed that no growth difference between the plasmid-bearing and plasmid-free cells exists, which was confirmed for the present recombinant yeast in previous studies. Under these assumptions, an identical specific growth rate for both the biofilm and suspended plasmid-bearing and plasmid-free cells in the immobilized-cell-film bioreactor may be justified. The specific growth rate of the immobilized cell system at a fixed dilution rate is denoted as μ_i.

In the immobilized-cell-film airlift bioreactor, cells grow on the cloth film and are continuously detached into the medium due to hydrodynamic forces. It is unlikely that additional free space will be available for re-entry of the released cells onto the fully-covered cloth surface again (Ghose, 1988). Assuming a constant value of plasmid loss

probability (p), the change of plasmid-bearing cells in the biofilm can be expressed as the net result of cell growth in the biofilm, plasmid loss and cell detachment from the biofilm:

$$\frac{dX_i^+}{dt} = \mu_i X_i^+ - p\mu_i X_i^+ - k_{det} X_i^+ = (\mu_i - p\mu_i - k_{det}) X_i^+ \tag{6.1}$$

Integrating Equation (6.1) gives the following:

$$X_i^+ = X_{i,o}^+ exp(\mu_i - p\mu_i - k_{det})t = X_{i,o}^+ exp(-k_i t) \tag{6.2}$$

where

$$k_i = -(\mu_i - p\mu_i - k_{det}) \tag{6.3}$$

Similarly, the change in plasmid-free cells in the biofilm can be described as:

$$\frac{dX_i^-}{dt} = \mu_i X_i^- + p\mu_i X_i^+ - k_{det} X_i^- \tag{6.4}$$

A sum of Equations (6.1) and (6.4) yields:

$$\frac{dX_i}{dt} = \mu_i X_i - k_{det} X_i \tag{6.5}$$

where $X_i = X_i^+ + X_i^-$, denoting the total biofilm cell mass. Assuming that at steady state the total biofilm cell mass remains constant during continuous culture, Equation (6.5) becomes:

$$\frac{dX_i}{dt} = \mu_i X_i - k_{det} X_i = 0 \quad or \quad k_{det} = \mu_i \tag{6.6}$$

Equation (6.6) shows that at steady state, the cell detachment rate constant (k_{det}) equals to the specific growth rate of the biofilm (μ_i). Substituting Equation (6.6) into (6.3) gives the following:

$$k_i = p\mu_i \tag{6.7}$$

On the other hand, the net accumulation of freely suspended plasmid-bearing cells in the immobilized-cell bioreactor can be described by four processes: cell growth in the medium, growth-related plasmid loss, cell detachment from the biofilm and cell wash-out. These processes can be expressed by the following differential equation:

$$\frac{dX^+}{dt} = \mu_i X^+ - p\mu_i X^+ + k_{det} X_i^+ - DX^+ \tag{6.8}$$

Substituting Equation (6.6) into Equation (6.8) and rearranging the result give the following:

$$\frac{dX^+}{dt} = \mu_i (X^+ + X_i^+) - p\mu_i X^+ - DX^+ \tag{6.9}$$

Similarly, the net change of freely suspended plasmid-free cells in the immobilized-cell bioreactor can be described as:

$$\frac{dX^-}{dt} = \mu_i (X^- + X_i^-) + p\mu_i X^+ - DX^- \tag{6.10}$$

Adding Equation (6.9) to (6.10) yields:

$$\frac{dX}{dt} = \mu_i (X^+ + X_i^+) + \mu_i (X^- + X_i^-) - D(X^+ + X^-) \tag{6.11}$$

where $X = X^+ + X^-$, denoting the total suspended cell concentration in the immobilized-cell bioreactor. It was observed that at steady state the total suspended cell concentration remained constant during continuous culture ($dX/dt = 0$), thus Equation (6.11) becomes:

$$\mu_i (X^+ + X_i^+) + \mu_i (X^- + X_i^-) = DX^+ + DX^- \tag{6.12}$$

From Equation (6.12) the following equations may be deduced:

$$\mu_i (X^+ + X_i^+) = DX^+ \tag{6.13}$$

$$\mu_i(X^- + X_i^-) = DX^- \tag{6.14}$$

Substituting Equations (6.13) and (6.7) into Equation (6.9) gives:

$$\frac{dX^+}{dt} = -k_i X^+ \tag{6.15}$$

Integrating Equation (6.15) yields:

$$X^+ = X_0^+ exp(-k_i t) \tag{6.16}$$

From Equation (6.2) and (6.16), it is interesting to note that based on the idealized biofilm model, plasmid loss kinetics for both biofilm and suspended cells in the immobilized cell system follow first-order decay pattern with same decay rate constant. In addition, rearranging Equation (6.12) and substituting $X = X^+ + X^-$ and $X_i = X_i^+ + X_i^-$ into it gives:

$$\mu_i X + \mu_i X_i = DX \quad or \quad \mu_i = \frac{DX}{X + X_i} \tag{6.17}$$

Equation (6.17) can be used to estimate the specific growth rate in the immobilized-cell system (μ_i).

Since glucoamylase formation is growth-associated, a quasi-steady-state enzyme mass balance in the immobilized-cell bioreactor can be described as following:

$$DP = Y_{P/X}\mu_i X^+ + Y_{P/X}\mu_i X_i^+ \tag{6.18}$$

or

$$P = \frac{Y_{P/X}\mu_i}{D}(X^+ + X_i^+) \tag{6.19}$$

Substituting Equations (6.2) and (6.16) into Equation (6.19) gives:

$$P = \frac{Y_{P/X} X_0^+}{D} exp(-k_i t) + \frac{Y_{P/X} X_{i,0}^+}{D} exp(-k_i t) \quad (6.20)$$

Differentiating Equation (6.20) results in:

$$\frac{dP}{dt} = -\frac{k_i Y_{P/X} X_0^+}{D} exp(-k_i t) - \frac{k_i Y_{P/X} X_{i,0}^+}{D} exp(-k_i t) \quad (6.21)$$

Rearranging Equation (6.21) and substituting Equation (6.20) into Equation (6.21) yields:

$$\frac{dP}{dt} = -k_i P = -\gamma_i P \quad (6.22)$$

Where γ_i is an enzyme concentration decay rate constant in the immobilized cell system. Equation (6.22) shows that the enzyme concentration decay rate constant (γ_i) is the same as the plasmid loss constant (k_i), that is:

$$\gamma_i = k_i = p\mu_i \quad (6.23)$$

Substituting Equation (6.17) into (6.23) gives:

$$\gamma_i = k_i = pD\frac{X}{X + X_i} \quad (6.24)$$

Comparing Equations (5.29) with (6.24), it is obvious that first-order decay rate constants of immobilized-cell systems are always smaller than those of corresponding free cell systems ($k_i < k_f$ and $\gamma_i < \gamma_f$). In other words, the apparent plasmid loss and enzyme production decay rates are reduced in the immobilized-cell systems.

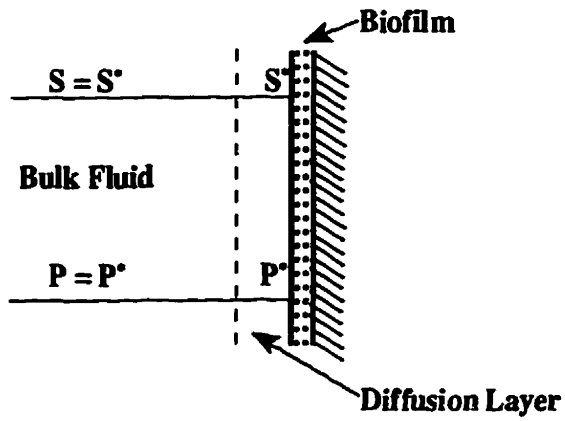


Figure 6.1. Schematic Diagram of Idealized Biofilm.

6.2.2. Continuous Fermentation with Immobilized Cells

After the immobilization procedure described in Section 3.5.2, the immobilized-cell-film airlift bioreactor was filled with fresh YPG nonselective medium and continuous feeding was initiated immediately. Figure 6.2 shows typical results for a continuous run in the immobilized-cell-film airlift bioreactor for the original strain at a dilution rate of 0.1 h^{-1}. Glucose was completely consumed (below 10 mg/L) and ethanol achieved its maximum value after 12 hours. After 36 hours, the ethanol concentration became constant around 4.6 g/L and the pH remained constant at pH = 5.0. Free cells in the medium initially increased and then reached a constant value of 5.40 g/L after 36 hours, indicating steady-state. At steady-state, the cloth-bound cell concentration was about 7.72 g/L (relative to the medium) or 5.43 mg/cm^2 (relative to the cloth area), which was similar to the result (5 mg/cm^2) obtained by Joshi and Yamazaki (1984).

Enzyme concentration in the bioreactor achieved its maximum value after 36 hours and remained constant for about 30 hours before decreasing. The maximum enzyme concentration in the immobilized cell system was 60% higher than that of the corresponding free cell system. The decline in the enzyme concentration was due to plasmid loss rather than the instability of the enzyme. The enzyme concentration would tend to reach a constant steady state value even if the enzyme were unstable. In addition, batch cultures in stationary phase gave nearly constant enzyme activity for 12 hours or more under growth conditions. No extracellular proteases that would adversely affect glucoamylase were found in strains of *S. cerevisiae* (Bilinski et al., 1990).

Using the selected strain, continuous fermentation was also carried out in immobilized-cell-film airlift bioreactor. The typical results were shown in Figure 6.3. The fermentation time course with the selected strain was similar to that with the original strain. However, with the selected strain the enzyme concentration doubled even though the cell concentrations were nearly the same for both strains. As in free suspension culture, the selected strain also showed better production performance than the original strain in the immobilized cell system.

As controls, continuous free suspension cultures were performed in the airlift bioreactor (without the cotton cages) in order to make a comparison between free cell and immobilization systems. The culture medium and conditions were exactly the same as in the immobilized-cell-film bioreactor. Comparison of the enzyme concentrations between the free and immobilized cell systems for both the original and selected strains are presented in Figure

6.4. As shown in Figure 6.4, the enzyme concentration in the immobilized-cell system was higher and declined much slower than the corresponding free suspension culture. Loss of the recombinant protein expression (glucoamylase) was significantly reduced in the immobilized cell system. Thus, enhanced productivity and stability in production of the recombinant protein was achieved in the immobilized-cell-film airlift bioreactor.

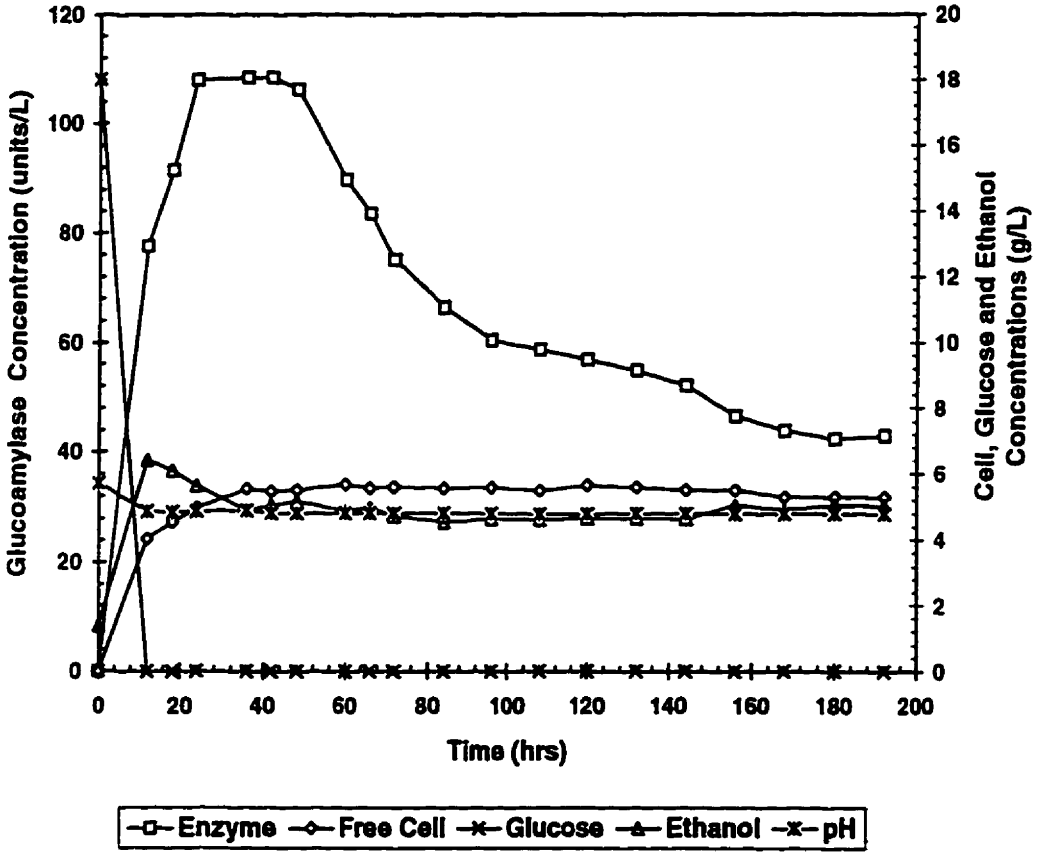


Figure 6.2. Continuous Recombinant Yeast Fermentation in Immobilized-Cell-Film Bioreactor Using Original Strain. Temperature = 30 °C, Aeration rate = 1.0 VVM, Dilution rate = 0.1 h^{-1}, G_0 = 20.0 g/L.

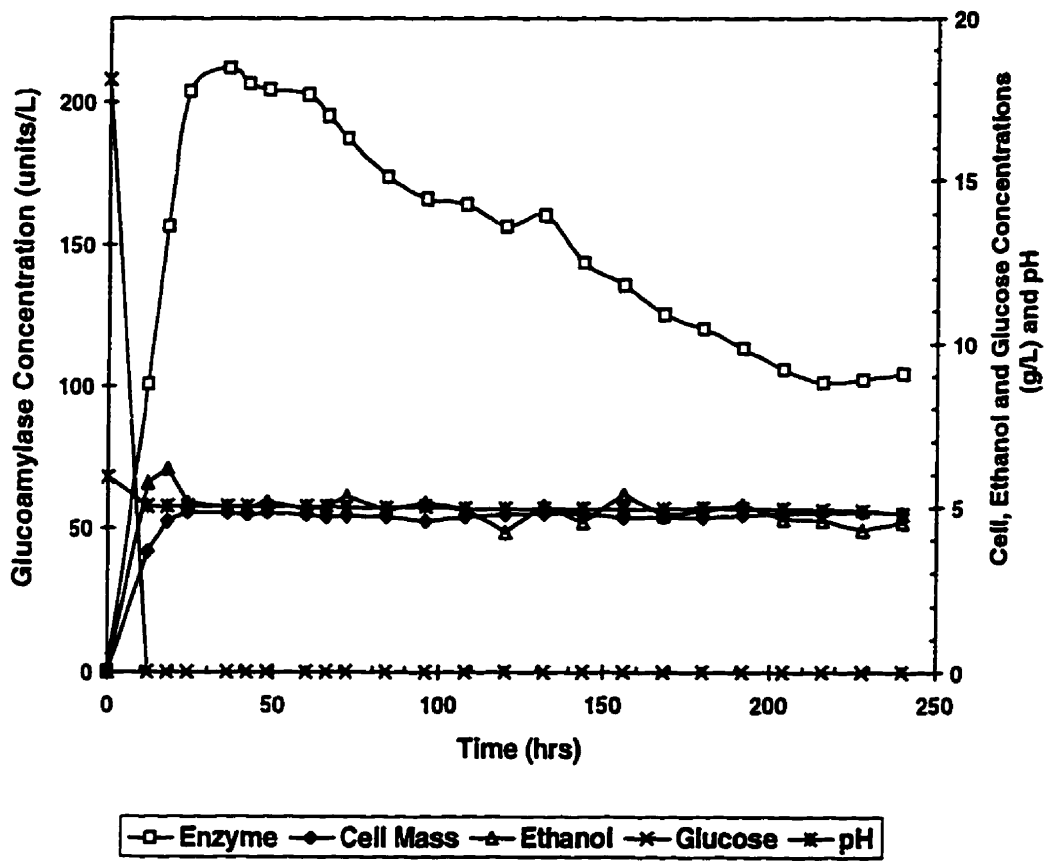


Figure 6.3. Continuous Recombinant Yeast Fermentation in Immobilized-Cell-Film Bioreactor Using Selected Strain. Temperature = 30 °C, Aeration Rate = 1.0 VVM, Dilution Rate = 0.1 h^{-1}, G_0 = 20.0 g/L

The more stable glucoamylase production in the immobilized cell systems is possibly the result of retention of a higher proportion of plasmid-bearing cells in the immobilization matrix. The hypothesis is that the attached yeast film behaves as a plasmid-bearing cell reservoir and continuously generate plasmid-bearing cells. This observation is consistent with the occurrence of higher fractions of plasmid-bearing cells in the medium from the immobilized cell system. Figure 6.5 shows that the fraction of the plasmid-bearing cells in the effluent of the immobilized cell system was higher and decreased relatively more slowly than that of the free cell suspension. Although we were unable to follow the fraction of plasmid-bearing attached cells due to sampling difficulty, the data presented in Figure 6.5 provides a strong indication that the immobilized cells maintained a higher proportion of plasmid-bearing cells for a longer time. The lower decay rate in enzyme concentration in the immobilized cell system further supports this view. The effect of immobilization on plasmid stability will be discussed further in Section 6.2.3.

The free cell concentrations in both the free and immobilized cell systems were nearly the same at steady state (Figures 6.6). Both biofilm and free cell growths contribute to the free cell mass in the immobilized cell system. Besides the free cell mass, the cloth-bound cell mass was also present in the immobilized-cell-film bioreactor. Therefore, the total cell concentration in the immobilized cell system was much higher than that of free cell system. Overall, the higher cell concentration plus higher fraction of plasmid bearing cells were responsible for the substantially higher enzyme concentration obtained in the immobilized cell system (Figure 6.5).

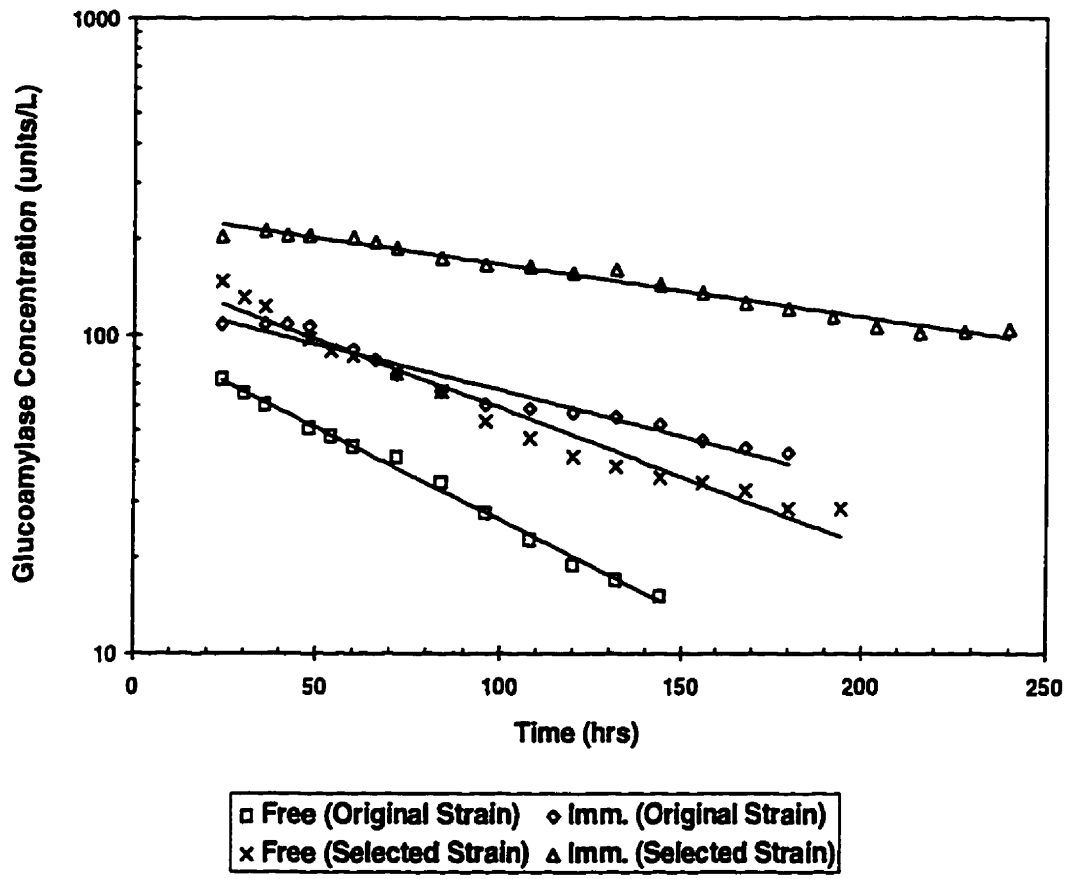


Figure 6.4. Comparison of Enzyme Concentrations between Free and Immobilized Cell Systems for Original and Selected Strains (Temperature = 30 °C, Aeration Rate = 1.0 VVM, Dilution Rate = 0.1 h^{-1}, G_0 = 20.0 g/L).

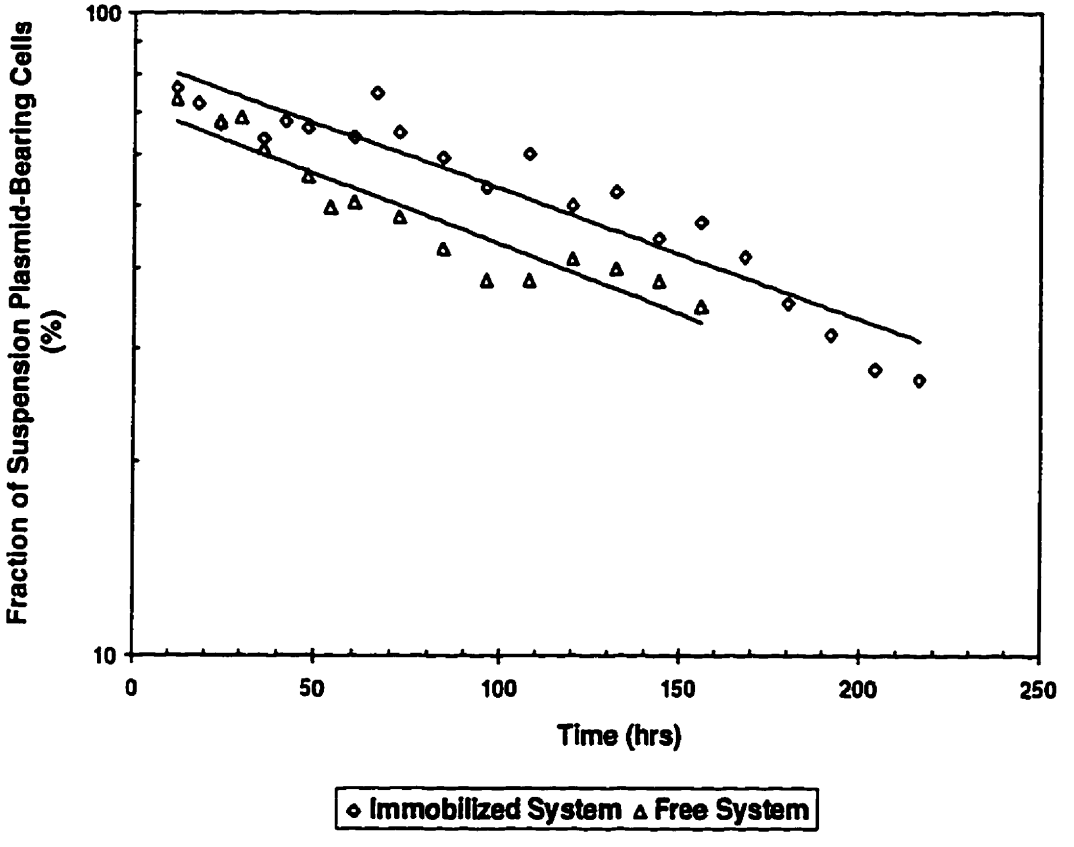


Figure 6.5. Comparison of Plasmid-Bearing Cell Fractions in Effluent of Free and Immobilized Cell Systems (Selected Strain; Temperature = 30 °C, Aeration Rate = 1.0 VVM, Dilution Rate = 0.1 h^{-1}, G_0 = 20.0 g/L).

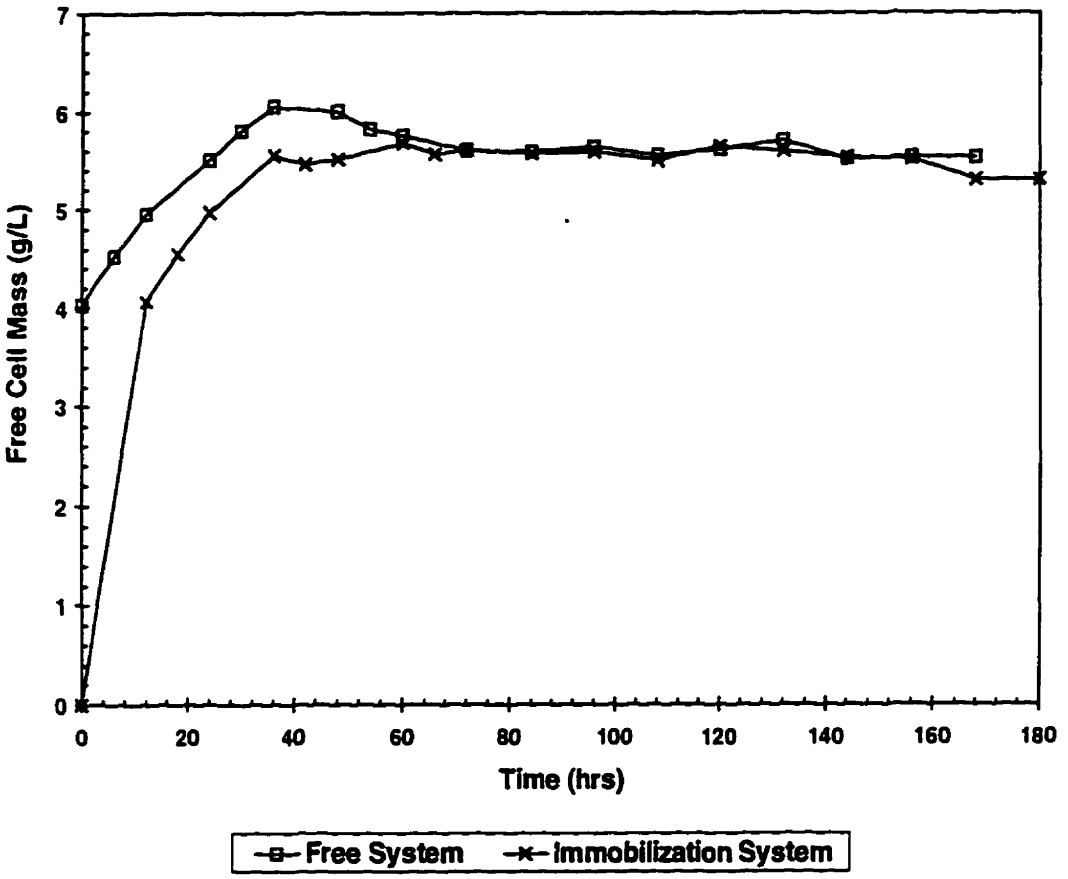


Figure 6.6. Comparison of Free Cell Mass between Free and Immobilized Cell Systems (Original Strain; Temperature = 30 °C, Aeration Rate = 1.0 VVM, Dilution Rate = 0.1 h^{-1}, G_0 = 20.0 g/L).

6.2.3. Effects of Immobilization on Plasmid Loss and Enzyme Productivity

The effects of immobilization on plasmid loss and enzyme production at different dilution rates are shown in Figure 6.7 ($D = 0.10\ h^{-1}$) and Figure 6.8 ($D = 0.20\ h^{-1}$). From the nearly linear correlation it is evident that both the plasmid-bearing cell concentration (in medium) and enzyme concentration in the immobilized-cell system can be approximated by first-order decay kinetics, which is in good agreement with the mathematical description given in Section 6.2.1. Based on the idealized biofilm model, Equations (6.2), (6.15) and (6.22) have been derived for describing the plasmid loss and enzyme production decay kinetics in the immobilized cell system. Equations (6.2), (6.15) and (6.22) show that plasmid loss (for both biofilm and suspended cells) and enzyme production decay follow first-order kinetics with same decay rate. The established relationships may provide a useful way to examine plasmid loss kinetics in biofilm since it is usually difficult to follow the fraction of the plasmid-bearing cells in the biofilm directly.

The measured first-order decay constants in the immobilized cell system are listed in Table 6.1. Comparing Table 6.1 with Table 5.3, we see that the first-order plasmid loss and enzyme production decay constants of the immobilized cell system were less than half of those of the corresponding free system at different dilution rates. Thus, the apparent genetic instability of the recombinant yeast was significantly reduced in the immobilized cell system. The finding of improved genetic stability by immobilization is consistent with the literature on recombinant protein expression in both cultures of recombinant *E. coli* (De Taxis du Poet et al., 1986; De Taxis du Poet et al., 1987; Nasri et al., 1987; and

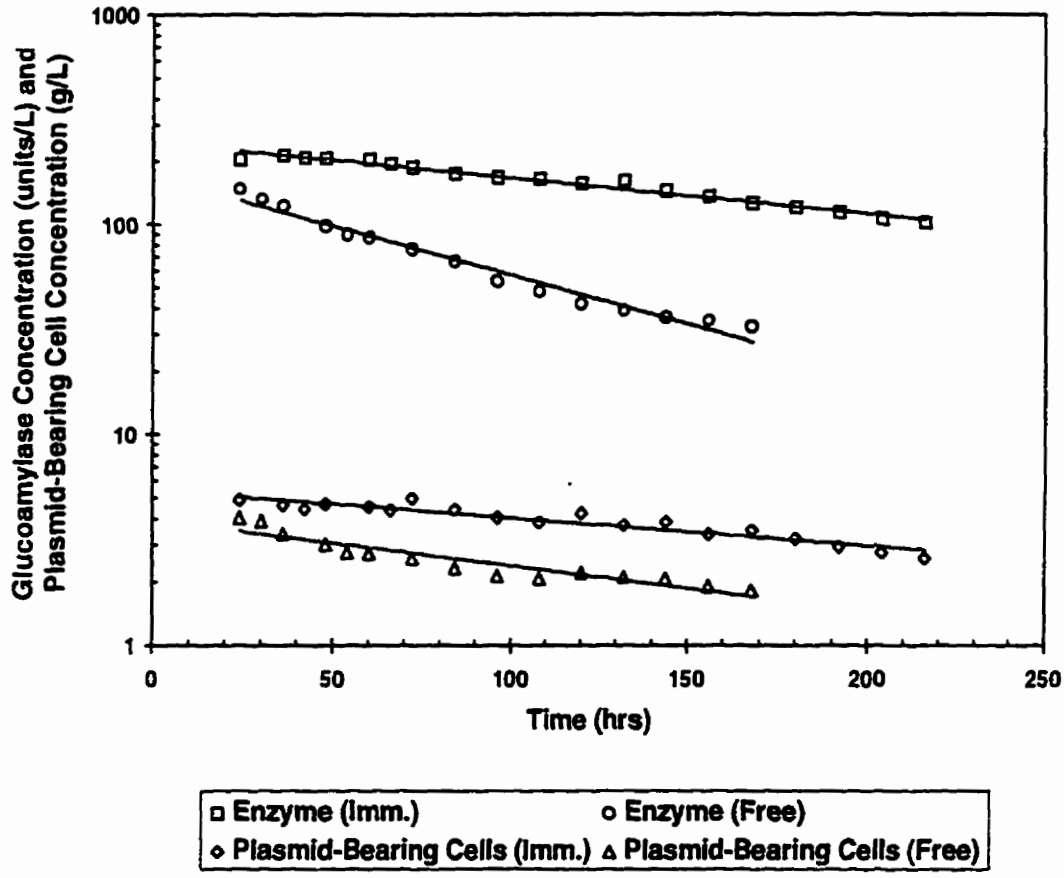


Figure 6.7. Comparison of Plasmid Loss and Glucoamylase Production between Free and Immobilized Cell Systems at Dilution rate = 0.1 h^{-1}. (Selected Strain; Temperature = 30 °C, Aeration Rate = 1.0 VVM, G_0 = 20.0 g/L).

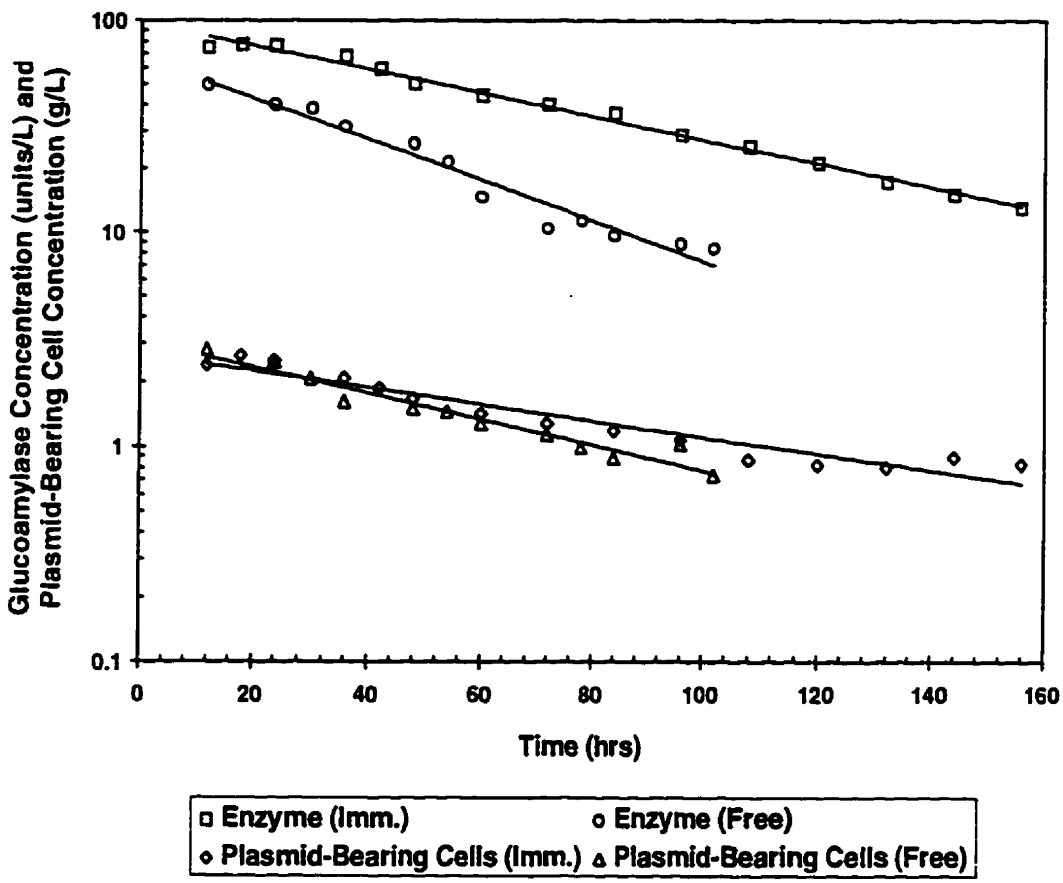


Figure 6.8. Comparison of Plasmid Loss and Glucoamylase Production between Free and Immobilized Cell Systems at Dilution rate = 0.2 h^{-1}. (Selected Strain; Temperature = 30 °C, Aeration Rate = 1.0 VVM, G_0 = 20.0 g/L).

Nasri et al., 1988) and recombinant *S. cerevisiae* (Sode et al., 1988; Walls and Gainer, 1989; Yang and Shu, 1996). However, a general explanation for the above improved plasmid stability is still lacking. The proposed model may help us to understand the stabilizing mechanisms. As derived in the model development section, the first-order decay constants of the immobilized cell system can be described by Equation (6.24) as shown below:

$$\gamma_i = k_i = p\mu_i = pD\frac{X}{X + X_i} \tag{6.24}$$

while the first-order decay constants of the corresponding free cell system can be expressed as Equation (5.29) as shown below:

$$\gamma_f = k_f = pD \tag{5.29}$$

Comparing Equations (5.29) with (6.24), it is seen that the decay constants of the immobilized cell system are always less than those of the corresponding free cell system as long as the biofilm is existing in the bioreactor ($X_i > 0$). Further analysis shows that the reduced apparent decay constant is due to a reduced specific growth rate in the immobilized cell system. According to Equations (5.17) ($\mu = D$) and (6.17) ($\mu_i = \frac{DX}{X + X_i}$), the specific growth rate of the immobilized cells is always lower than that of the corresponding free cells at same dilution rate. Equation (6.24) also shows that the apparent decay constants decrease with increasing biofilm concentration but increase with increasing dilution rate because of the changes in the specific growth rate. These observations are confirmed by the experimental results listed in Table 6.1.

Table 6.1. Measured and Predicted First-Order Decay Constants in Immobilized Cell Systems at Different Dilution Rates.

D (h^{-1})	0.10	0.20
X (g/L)	5.40	3.91
X_i (g/L)	7.72	5.83
Predicted μ_i (h^{-1})	0.0412	0.0803
*Measured k_i (h^{-1})	0.0031	0.0089
Predicted k_i (h^{-1})	0.0022	0.0049
Measured γ_i (h^{-1})	0.0038	0.0128
Predicted γ_i (h^{-1})	0.0041	0.0090

* first-order plasmid loss constant of the suspended cell in the immobilized cell system

It was reported that segregational plasmid instability increases with increasing specific growth rate in culture of recombinant yeast in nonselective medium (Hardjito et al., 1993; Impoolsup et al., 1989b). Similar observation can be made for the present recombinant strain (Figure 5.8): the more the cells divide, the more likely they lose plasmids. Therefore, an reduced specific growth may be responsible for the improved apparent plasmid stability in an immobilized cell system. Assuming an identical probability of plasmid loss for both free and immobilized cell systems, the apparent first-order decay constants for plasmid loss and enzyme production in the immobilized cell system can be

estimated by using Equation (6.24) and the values of p obtained in the corresponding free cell system. Table 6.1 shows that a reasonable agreement between the predicted and measured first-order decay constants was achieved. It is also noted from Table 6.1 that the predicted decay constant is less than the corresponding measured one. This is because the actual immobilized cell system may deviate from the assumed idealized biofilm model. Thus, in the present case the reduced specific growth rate alone is adequate to explain the enhanced apparent stability in the immobilized cell system.

Equation (6.24) also shows that the apparent decay constants are directly proportional to the probability of plasmid loss. In general, enhanced plasmid stability by immobilization may be also due to a reduced probability of plasmid loss. The probability of plasmid loss in immobilized cell system may be reduced due to increased plasmid copy number, close contact, the effects of compartmentalization and mass transfer. It was found that the copy number of the plasmid increased with decreasing specific growth rate (Sayadi et al., 1987; Bentley and Kompala, 1989). Since the immobilized cells grow slower, an increased plasmid copy number could be another reason for the increased plasmid stability in immobilized cell systems. There is also some evidence that budding of immobilized cells may be delayed while decoupled DNA replication and polysaccharide synthesis continue (Ghose, 1988). Additional DNA replication will change the gene level of the offspring of the immobilized cells. In the case of the immobilized recombinant cells, the delay of the budding combined with continuing plasmid DNA replication would result in increased plasmid copy number and ensure that the offspring cells would be more likely to bear plasmids. Finally, the

close contact of immobilized cells may facilitate the unbiased partition of plasmids between mother and daughter cells during cell division. Therefore, segregational plasmid loss is less likely to happen in the immobilized cell system.

The proposed model successfully describes the experimental results and provides useful information for better understanding the stabilizing mechanism of the immobilized recombinant cells. It may be concluded that a reduced specific growth rate coupled with an increased plasmid copy number is a general explanation for the enhanced apparent plasmid stability in an immobilized cell system.

6.2.4. Effect of Feeding Glucose Concentration

Using various inlet glucose feed concentrations (G_0), continuous fermentation experiments were also performed in the immobilized-cell-film bioreactor at dilution rate 0.1 h^{-1}. Figure 6.9 is a comparison of glucoamylase concentration between the free and immobilized cell system at a feed glucose concentration G_0 = 10 g/L. Again higher enzyme concentration and slower decay rate of enzyme production were observed with the immobilized cell system. The enhanced effect of immobilization on enzyme production was further confirmed.

The effect of the glucose feed concentration on the enzyme production in the immobilized cell system is shown in Figure 6.10. As expected, the enzyme concentrations increased with increasing glucose concentrations since higher cell concentrations was obtained at higher glucose concentration (see Figure 6.11 and Table 6.2). Nearly parallel lines are

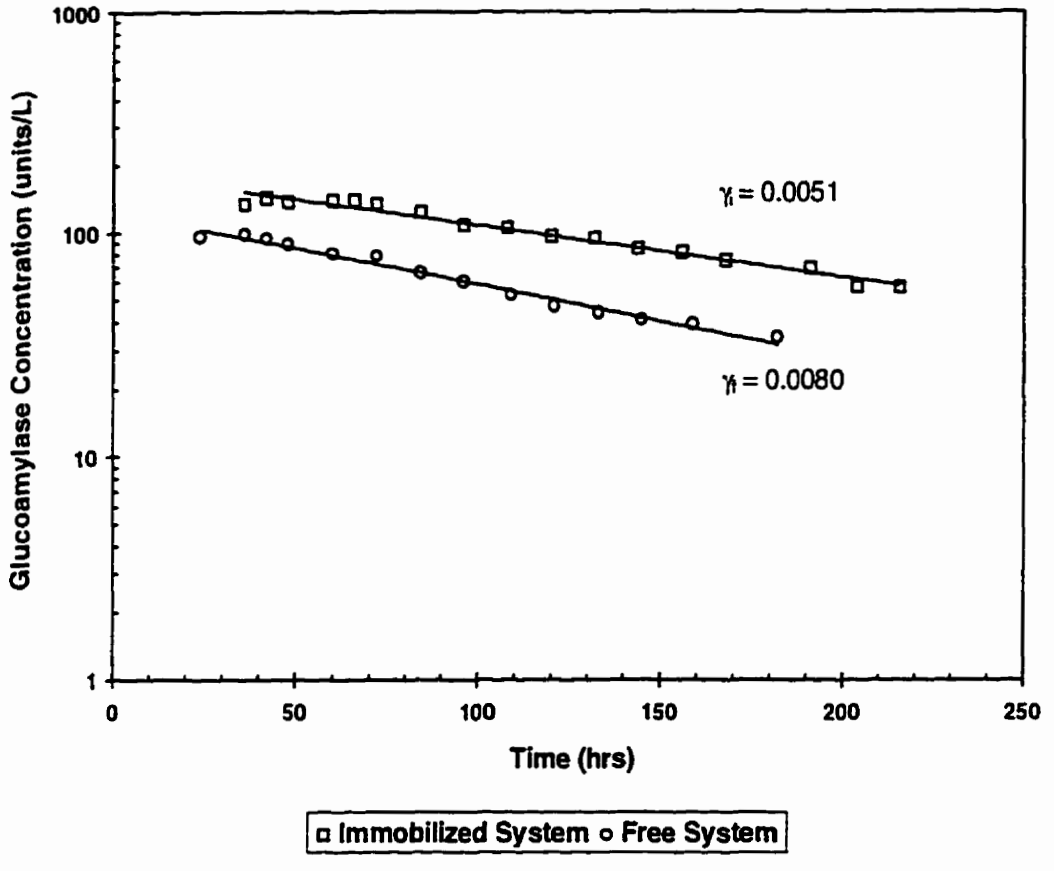


Figure 6.9. Comparison of Glucoamylase Concentration between Free and Immobilized Cell System at Glucose Feed Concentration (G_0) = 10 g/L and Dilution Rate = 0.1 h^{-1}. (Selected Strain; Temperature = 30 °C, Aeration Rate = 1.0 VVM).

observed in Figure 6.10, indicating that the decay rates of the enzyme production at different feeding glucose concentrations were nearly identical. As listed in Table 6.2, the first-order enzyme production decay constants predicted by the proposed model (Equation 6.24) are fairly close to the measured ones. The effect of the feeding glucose concentration could also be described by the proposed model.

Table 6.2. Measured and Predicted First-Order Decay Constants in Immobilized Cell Systems at Different Feed Glucose Concentrations.

G_o (g/L)	5.0	10.0	20.0
X (g/L)	3.15	4.48	5.40
X_i (g/L)	3.68	5.79	7.72
Predicted μ_i (h^{-1})	0.0461	0.0436	0.0412
Measured γ_i (h^{-1})	0.0045	0.0051	0.0038
Predicted γ_i (h^{-1})	0.0046	0.0043	0.0041

Figure 6.11 shows that ethanol concentration decreased with decreasing feed glucose concentration. When the feed glucose concentration was equal to 5 g/L the ethanol concentration was nearly zero; glucose was completely oxidized by yeast via the glucose

oxidation pathway. With increasing feeding glucose concentration, glucose flux became too large for the cell's oxidative capacity to handle and excess glucose was fermented to ethanol. At the feed glucose concentration G_0 = 20 g/L, a large fraction of glucose was converted to ethanol due to the Crabtree effect; although the enzyme concentration in the bioreactor was the highest, the enzyme yield based on total glucose consumed was the lowest. From this point of view, 10 g/L was the optimum feed glucose concentration in continuous fermentation with the immobilized cell bioreactor.

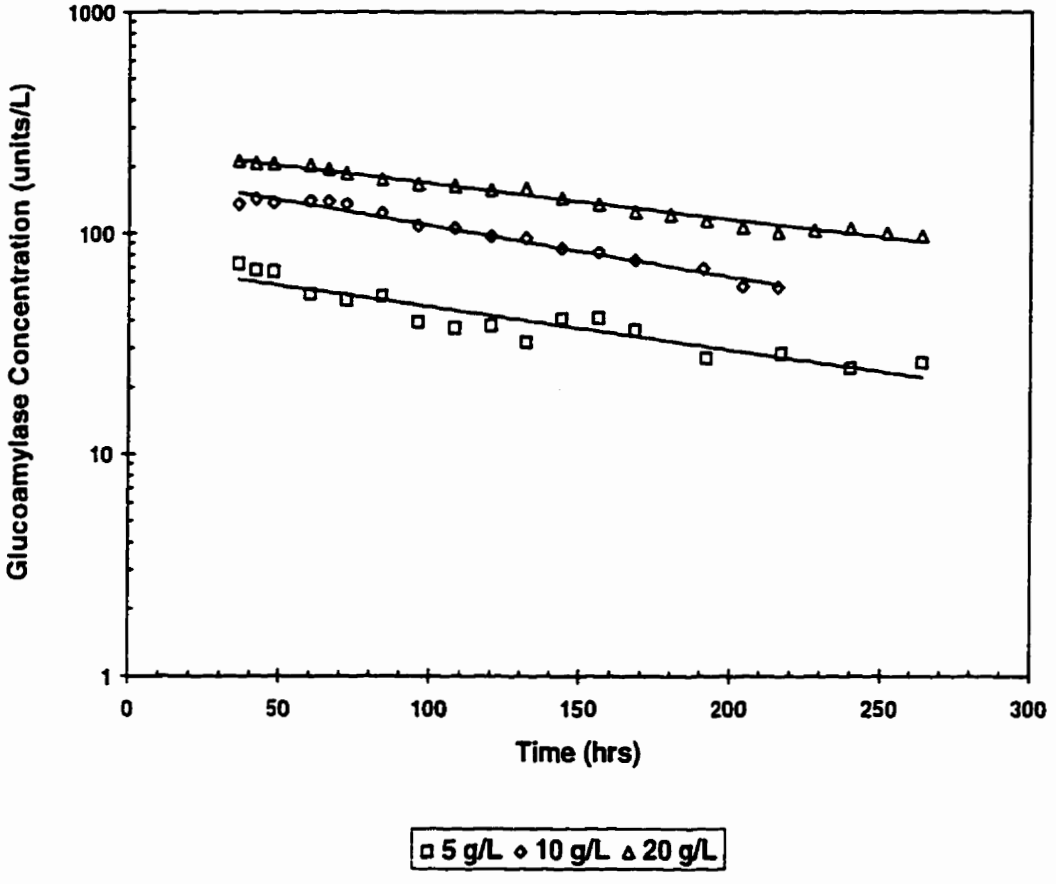


Figure 6.10. Effect of Glucose Feed Concentration (G_0) on Enzyme Production in Immobilized Cell System at Dilution Rate = 0.1 h^{-1}. (Selected Strain; Temperature = 30 °C, Aeration Rate = 1.0 VVM)

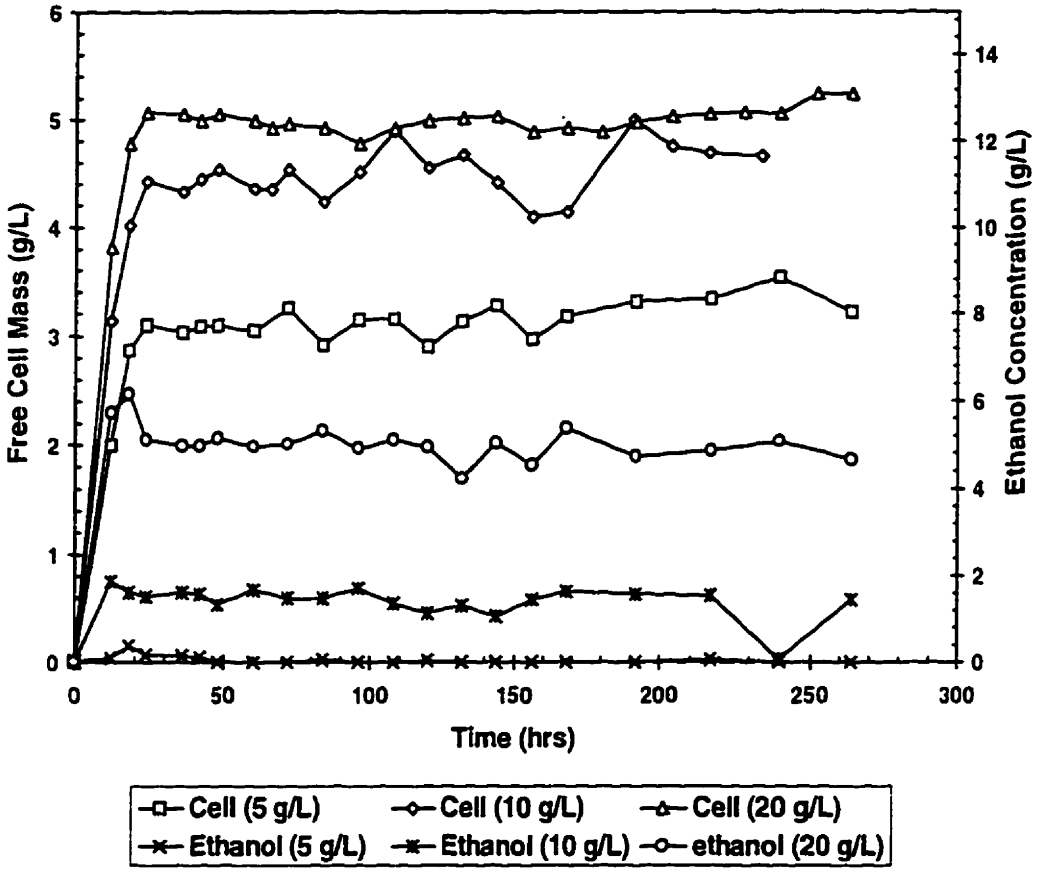


Figure 6.11. Effect of Glucose Feed Concentration (G_0) on Free Cell Mass and Ethanol Concentration in Immobilized Cell System at Dilution Rate = 0.1 h^{-1}. (Selected Strain; Temperature = 30 °C, Aeration Rate = 1.0 VVM)

6.3. Repeated Batch Fermentation in Immobilized-Cell Bioreactor

By using yeast film as resident inoculum, repeated batch fermentations were performed in the immobilized-cell-film airlift bioreactor to further investigate the effect of immobilization. The detailed procedures for the repeated batch fermentation was described in Section 3.5.4. Results for two repeated batch fermentation experiments are shown in Figure 6.12. Good reproducibility was obtained between the two experiment runs. Each batch lasted 24 hours and the immobilized-cell bioreactor was run for two weeks (14 batches). Over the two week period of fermentation, both the fraction of plasmid-bearing cells and enzyme concentrations were nearly constant. Stable enzyme production performance was achieved. In contrast, in free suspension repeated batch fermentations significant plasmid loss and enzyme production decay were observed. As shown in Figure 6.13, both the fraction of plasmid-bearing cells and enzyme concentration decreased over the culture period.

Although the continuous studies showed that the plasmid loss and the decay of the enzyme production were reduced in the immobilized cell system, the instability still occurred (see Figures 6.7 and 6.8). By operating the immobilised-cell-film bioreactor in repeated batch mode, the stability of the recombinant yeast was further improved. This is the result of maintaining a higher proportion of plasmid-bearing cells in the immobilization matrix. As discussed in the Section 6.2.3, the yeast film could be a dynamic reserve of highly concentrated plasmid-bearing cells having a high plasmid copy number and less segregarional instability, therefore providing for an enhanced genetic stability. In the

repeated batch fermentations, the fermented culture was completely separated from the yeast film which was retained in the immobilized cell bioreactor at the end of each batch fermentation. Evidently, the film retained nearly constant proportion of plasmid-bearing cells. This proportion of plasmid-bearing cells was equal to or higher than the proportion in the liquid suspension. The free plasmid-bearing cells generated from the yeast film were unlikely to lose plasmids due to the short culture period. In continuous culture, however, because of the longer culture period, the free cells continuously divided for many generations and tended to lose the plasmids. Gradually, the population of plasmid-free cells increased with the culture time, causing lower recombinant protein production.

Use of the microbial films as resident inocula make it easy to operate repeated batch fermentations in the proposed immobilized cell bioreactor. In commercial fermentations, batch operation is usually preferred. Generally, a seed culture must be prepared in 2 to 3 stages of increasing volume before starting a main batch fermentation. A part of the old fermented culture may be used as an inoculum to repeat a batch fermentation at the expense of a reduced product yield. But the fermented culture often contains toxic substances which inhibit the growth of the microorganisms and the production of the desired products. The harvested cells may be also used to inoculate the batch fermentation. However, harvest and readdition of cells raise the cost of fermentation and increase the risk of contamination. Genetic instability could be another serious problem associating with free suspension repeated fermentations. Using the proposed immobilized cell bioreactor can overcome all the above problems.

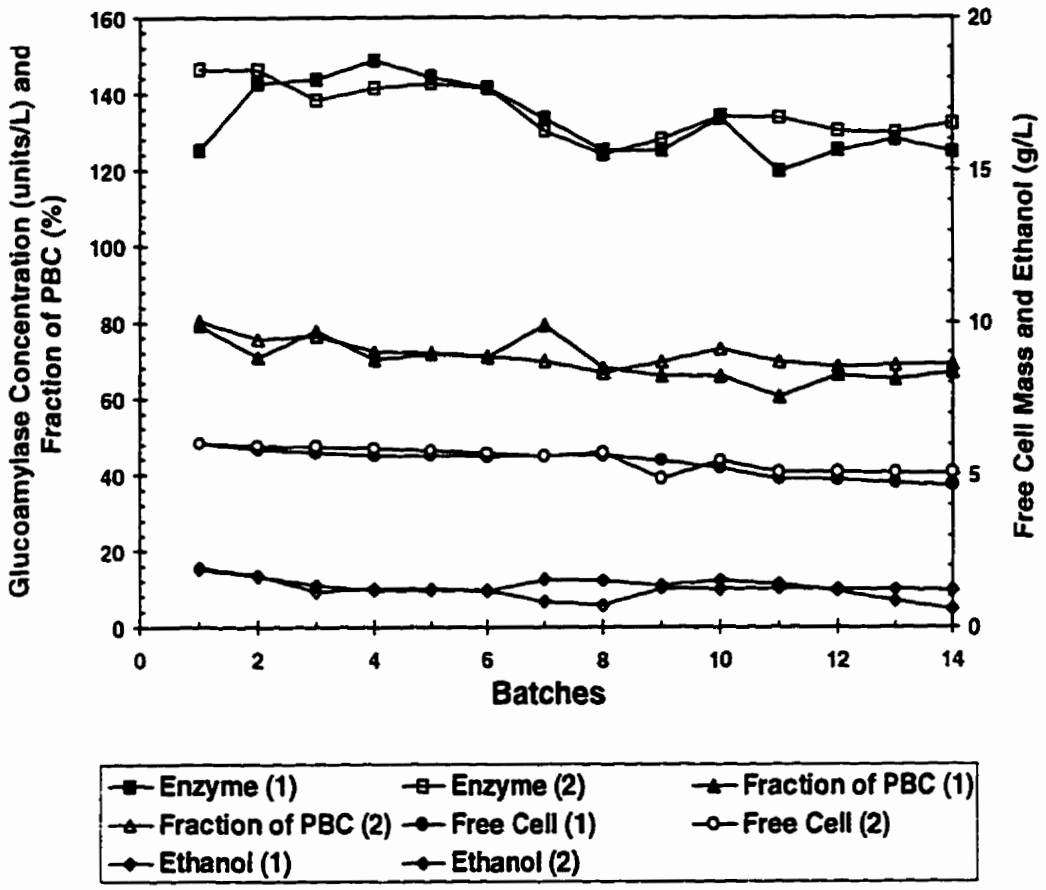


Figure 6.12. Results for Two Repeated Batch Fermentation Experiments in Immobilized-Cell-Film Airlift Bioreactor. (Selected Strain; Temperature = 30 °C, Aeration Rate = 1.0 VVM, Each Batch Lasted 24 hours).

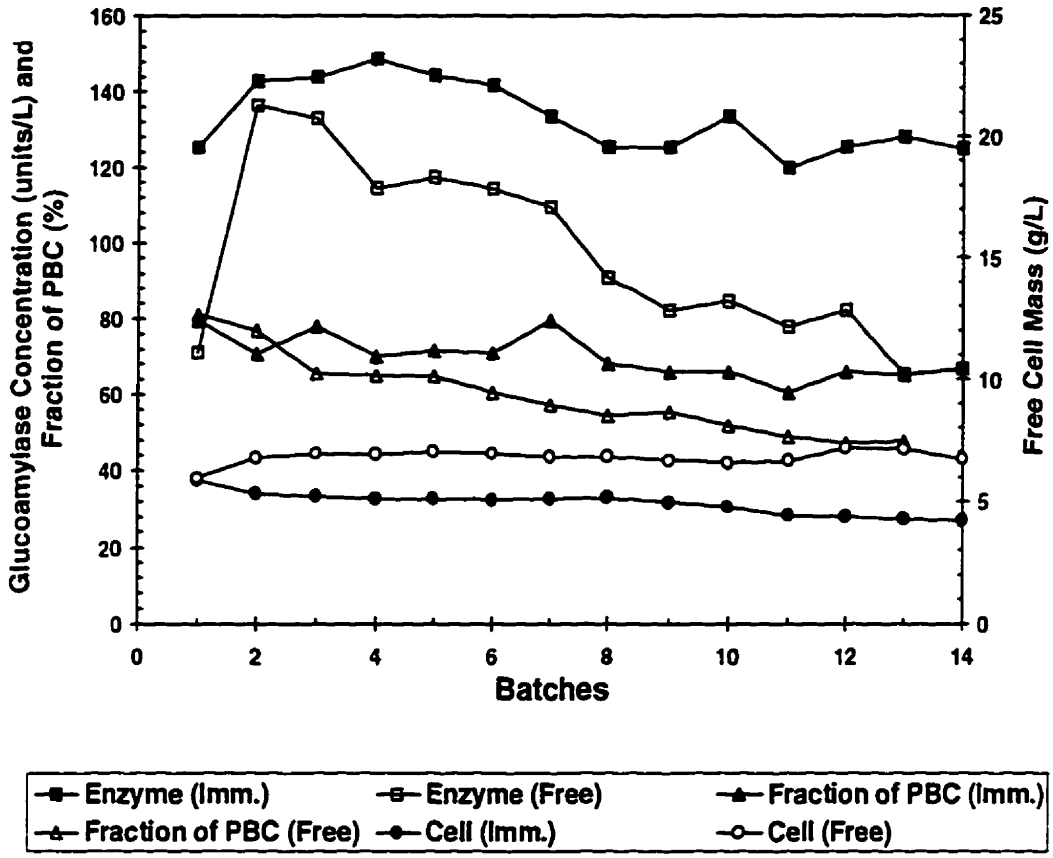


Figure 6.13. Comparison between Free and Immobilized Cell Systems in Repeated Batch Fermentation. (Selected Strain; Temperature = 30 °C, Aeration Rate = 1.0 VVM, Each Batch Lasted 24 hours).

6.4. Advantages of Proposed Immobilized Cell Bioreactor

Using the model recombinant yeast, good performance in production of the recombinant protein was achieved with the novel design of an immobilized-cell-film airlift bioreactor. Due to its unique features, the proposed immobilized-cell bioreactor could offer the following advantages:

1) Suitable for uses of the soft, highly porous supporting materials such as cotton cloth, polyurethane foam sheet and cellulosic sponge cloth for cell immobilization. The immobilization procedure is simple, convenient and efficient.
2) Substrate channelling, dead zone, bed blockage, CO_2 build-up and mass transfer limitation, which are likely to happen in a packed-bed immobilized cell bioreactor, can be avoided.
3) Good operating performance and enhanced liquid-solid mass transfer are achieved because of a relatively high liquid-solid slip velocity. Three-phase slurry airlift bioreactors are not suitable for the soft and porous supporting materials due to their low density. Even after loading with biomass, they still have a density close to that of the continuous liquid phase. While the immobilized-cell particles circulating through the slurry airlift bioreactor, they tend to block the downcomer if the particle loading increases to certain level. In addition, liquid-solid mass transfer limitation may occur in the slurry bioreactor due to a low liquid-solid velocity.
4) Reduction of genetic instability of recombinant cells due to immobilization can be achieved.
5) High cell concentration and gene product productivity can be realized.

6) Suitable for repeated-batch and continuous operations for longer periods of time.

7) The system offers enhanced operating efficiency and better economics.

With all these advantages, its potential use in commercial cultivation of recombinant or non-recombinant cells is promising.

CHAPTER 7

CONCLUSIONS AND RECOMMENDATIONS

7.1. Conclusions

Fundamental engineering studies were carried out on protein production by free suspension and immobilized cells of a recombinant yeast, *S. cerevisiae* C468/pGAC9. Based on the results obtained, the following conclusions may be drawn.

1. Batch culture studies indicated that in the YPG nonselective medium, the recombinant yeast underwent diauxic growth and the recombinant protein production was growth-associated. The recombinant protein yield (units of glucoamylase per gram cells) was correlated with specific growth rate. The recombinant protein yield decreased with increasing specific growth rate. This is possibly due to an increased plasmid copy number or /and an increase in DNA transcription efficiency as a result of the reduced specific growth rate.

2. The importance of metabolic pathways in yeast with regard to recombinant protein formation was analyzed. Production of glucoamylase was shown to be associated with oxidative growths of the recombinant yeast (both glucose and ethanol oxidation pathways). In order to produce recombinant protein efficiently the TCA cycle must function actively since the TCA cycle provides the intermediates required for protein synthesis. At high glucose level, secretion of the recombinant protein was repressed

because glucose fermentation dominated and most of the glucose was used for ethanol production due to the Crabtree effect. The Crabtree effect was induced by high glucose concentration in batch culture and high glucose flux in continuous culture.

3. Results of the continuous cultures confirmed the high instability of the model recombinant yeast in free suspension when growing in the YPG nonselective medium. The changes in the fraction of plasmid-bearing cells and glucoamylase activity followed an exponential decay pattern. Expressed as a function of time, the decay rates of both the plasmid-bearing cell fraction and glucoamylase expression increased with increasing dilution rates. If expressed as a function of cell generations, the decay rates were nearly constant over the dilution rates tested. A novel method is proposed to evaluate the instability parameters μ^+, μ^+ and p. The results indicated that the growth rate difference between plasmid-bearing and plasmid-free cells was negligible. This was probably due to the low copy number of the 2 μm based yeast shuttle vector. Thus, the contribution of preferential growth to apparent plasmid instability was negligible. The estimated values of probability of plasmid loss ($p = 0.05$) were nearly constant at different dilution rates. No significant effect of growth rates on plasmid instability was observed. When the model recombinant yeast was cultured continuously in the YNB selective medium, no apparent plasmid loss was observed. However, the glucoamylase production still declined during the culture period. The decoupling between the plasmid loss and enzyme production in the selective medium was attributed to the structural instability.

4. Increased enzyme production was achieved in the airlift bioreactor compared to the stirred-tank bioreactor. Higher glucoamylase productivity (70 %) was obtained in batch cultures. In continuous suspension culture, it was also shown that the enzyme production decreased more rapidly in the stirred-tank bioreactor because plasmid loss was faster than in the airlift bioreactor. This was possibly due to the vigorous fluid movement and wide variation of shear forces caused by agitation. Airlift bioreactors provide more uniform hydrodynamic and mass transfer characteristics. Double selection pressure was used to select and maintain the recombinant strain. The selected strain showed better production performance than the original strain in both airlift and stirred-tank bioreactors.

5. Based on this work and the literature, a semi-structured metabolic model was developed for describing cell growth, substrate consumption, ethanol formation and utilization, plasmid loss and recombinant protein production of recombinant yeast. By combining the Monod equation and segregational instability kinetics with the key characteristics in yeast metabolism, the proposed model has features of simplicity and accuracy. The proposed model simulated the Crabtree effect, diauxic growth, plasmid loss and recombinant protein production. The model contains 18 parameters which can be estimated from either experimental data or literature information. Most of the parameters are general and suitable for different yeast recombinant strains. When the model was applied to the batch and continuous fermentations of the present recombinant yeast (C468/pGAC9), excellent agreement between model predictions and experimental results was achieved for glucose, cell mass, ethanol, glucoamylase and fraction of plasmid-bearing

cells. Tests of the proposed model were also carried out with published experimental data from independent researchers. The model successfully predicted the experimental data for different recombinant yeast strains. The simplicity, feasibility, accuracy and generality of the model make it very useful for designing, scaling-up, controlling and optimizing recombinant yeast fermentation processes.

6. An immobilized-cell-film airlift bioreactor was developed for the recombinant yeast. The performance of the immobilized cell system was compared with the corresponding free suspension cell system at different dilution rates using the YPG nonselective medium. Enhanced enzyme productivity and production stability in the immobilized cell system were observed. Experimental data indicated that the immobilized cells maintained a higher proportion of plasmid-bearing cells for longer periods under continuous operation. By operating the immobilized-cell-film bioreactor in repeated batch mode, the stability of the recombinant yeast was further improved. This is possibly the result of retention of a higher proportion of plasmid-bearing cells in the immobilization matrix. The attached yeast film behaved as a plasmid-bearing cell reservoir and continuously generated plasmid-bearing cells, providing for enhanced apparent plasmid stability.

7. A mathematical model was developed to describe plasmid loss and enzyme production decay kinetics in the immobilized recombinant yeast system based on an idealized biofilm model. Correlation between plasmid loss and enzyme production decay was established. The proposed model successfully described the enhanced effects observed in the

immobilized cell systems. A general explanation for the reduced genetic instability by immobilization was sought through the modelling. The improved apparent stability in an immobilized cell system may be explained by a reduced specific growth rate accompanying by an increased plasmid copy number. The modelling may help us to understand the nature of the immobilized recombinant cells and design better immobilized cell bioreactors.

8. The immobilized-cell-film airlift bioreactor appears to be practical and efficient. Its advantages include the maintenance of genetic stability, high cell concentration and cloned gene product productivity, suitability for repeated batch or continuous operations for long periods of time, stable reactor operation, enhanced operation efficiency and better economics. Its potential use in commercial cultivation of recombinant or non-recombinant cells is promising.

7.2. Recommendations

The following further studies may be recommended regarding the potential applications of the proposed mathematical models and the developed immobilized cell bioreactor.

1. In this work, batch and continuous cultures of the model recombinant yeast have been studied. Examining fed-batch fermentation could be an interesting topic since fed-batch operation mode offers several advantages (Parker and Seo, 1992). Effect of different feeding strategies on cell growth, plasmid instability and recombinant protein production may be investigated. Test of the proposed model against the data obtained in fed-batch fermentations of recombinant yeast may be performed.

2. Specific growth rate plays an important role in plasmid loss and recombinant protein production. Plasmid copy number, which is a function of genetic and environmental factors, is one of the most important considerations in recombinant cell systems. It may be useful to examine the relationship between plasmid copy number, promoter strength, specific growth rate, and recombinant protein yield.

3. The proposed models may be further generalized by studying more recombinant yeast strains. Based on the proposed model, an interface program could be developed for controlling and optimizing fermentation processes employing the recombinant yeasts.

4. Both immobilization and cyclic fermentation systems were shown to have an enhanced effects on stability and productivity. More significant effects may be expected by combining both immobilization and cyclic operation. Effects of cyclic shifts in temperature, dissolved oxygen, dilution rate, feed rate and substrate concentration in an immobilized cell system may be investigated.

5. Application of the immobilized cell bioreactor to other recombinant or non-recombinant cells may be recommended. Fermentations may be conducted on a pilot-plant scale to assess the efficiency and feasibility of the bioreactor. Problems relating to scale-up should be examined.

6. In the immobilized-cell-film airlift bioreactor, the cotton cloth may be replaced by other soft and porous materials such as polyurethane foam sheets, cellulosic sponge cloth etc. The comparative studies of cell immobilization techniques would provide useful information for designing immobilized cell bioreactors.

APPENDIX A: PLASMID MAP FOR pGAC9

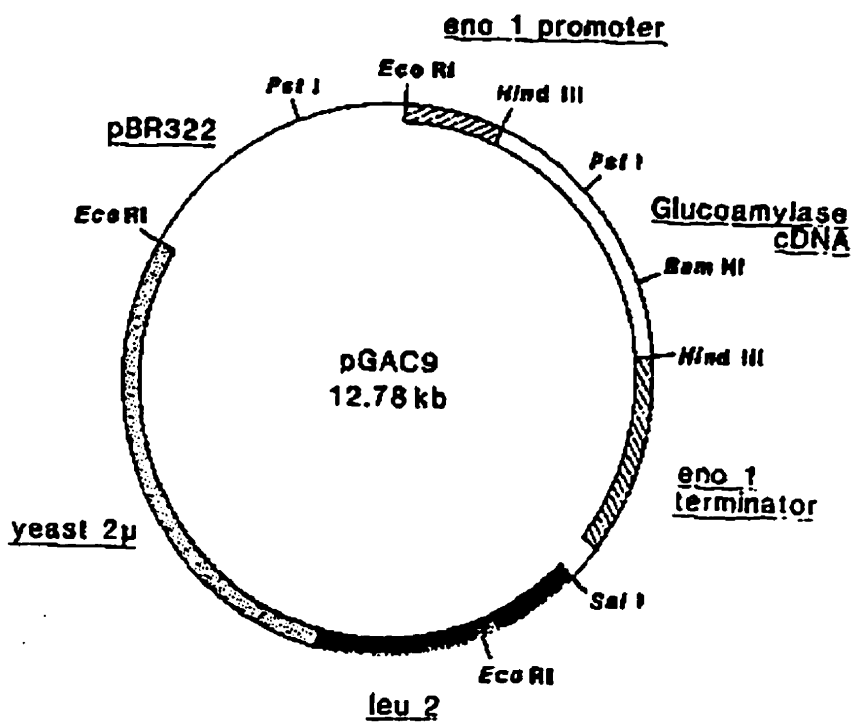
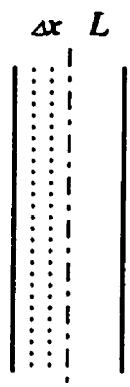


Figure A.1. An Illustration of Genetic Map for Plasmid pGAC9 (Nunberg et al., 1988)

APPENDIX B. MODELING OF IMMOBILIZATION SYSTEM UNDER MASS TRANSFER LIMITATION



Internal Mass Transfer:

$$D_e \frac{d^2 s}{dx^2} - \frac{v_{max} s}{K_s + s} = 0 \qquad \text{(B.1)}$$

with boundary condition:

$$x = 0, \quad \frac{ds}{dx} = 0 \qquad \text{(B.2)}$$

where

s is the limiting substrate concentration

x is the distance from the centreline

D_e is the effective diffusivity of the limiting substrate

External Mass Transfer:

$$x = L, \; D_e \frac{ds}{dx} = k_s (s - s_0) \qquad \text{(B.3)}$$

where

L is the film thickness

k_s is the solid-liquid mass transfer coefficient

Solving Eqs. (1), (2) and (3) gives the effectiveness factor (η) as following Equation:

$$\eta = f(\phi, B_i) \qquad \text{(B.4)}$$

where

where

$$B_i\ (Biot\ number) = \frac{k_s L}{D_e} \qquad \textbf{(B.5)}$$

$$\phi\ (Thiele\ number) = L\sqrt{\frac{v_{max}}{K_s D_e}} \qquad \textbf{(B.6)}$$

Assuming that the specific growth rate of the suspended cells in the immobilized-cell system (in bulk liquid) is μ , then the specific growth rate of the immobilized cells (cell film) can be described by $\mu_i = \eta\mu$. At steady state during continuous culture, it can shown that:

$$\mu X + \eta\mu X_i = DX \qquad \textbf{(B.7)}$$

or

$$\mu = \frac{DX}{X + \eta X_i} \qquad \textbf{(B.8)}$$

and

$$\mu_i = \frac{\eta DX}{X + \eta X_i} \qquad \textbf{(B.9)}$$

Plasmid loss rate constant for the suspended cells:

$$k = p\mu = p\frac{DX}{X + \eta X_i} \qquad \textbf{(B.10)}$$

Plasmid loss rate constant for the immobilized cells:

$$k_i = p\mu_i = p\frac{\eta DX}{X + \eta X_i} \qquad \textbf{(B.11)}$$

When $\eta \rightarrow I$ (an idealized biofilm), Eqs. (B.10) and (B.11) are reduced to Eq. (6.24) as shown below:

$$k_i = k = p\frac{DX}{X + X_i} \qquad \textbf{(6.24)}$$

Under any mass transfer limitation conditions, average plasmid loss rate constant in the immobilization system can be calculated as:

$$k_{i,\,average} = \frac{kX + k_i X_i}{X + X_i} = p\frac{DX}{X + X_i} \qquad \textbf{(B.12)}$$

Comparing Eq. (B.12) with Eq. (6.24), it can be seen that the average plasmid loss rate constant is the same as the plasmid loss rate constant by assuming an idealized biofilm.

APPENDIX C. EFFECTS OF INITIAL GLUCOSE CONCENTRATION IN BATCH CULTURE

The proposed model was used to simulate the effects of initial glucose concentrations on residual glucose, cell mass, ethanol, glucoamylase and fraction of plasmid-bearing cells in batch culture. The simulated results are shown in Figures C1, C2, C3, C4, and C5. Glucoamylase concentrations are predicted to increase with increasing initial glucose concentrations; but fermentation time and plasmid loss are not significantly different at different initial glucose concentrations. Using a high initial glucose concentration should facilitate the productivity of recombinant protein. However, ethanol concentration increases with increasing initial glucose concentration (Figure C3). Increased ethanol concentrations can cause a reduction in yeast growth rate and viability (Berry and Brown, 1987), especially when ethanol concentrations exceed 20 g/L (Roostaazad, 1993). Therefore, too high initial glucose concentration will have an adverse effect on the fermentation of recombinant yeast. A suitable initial glucose concentration may range from 20 to 40 g/L.

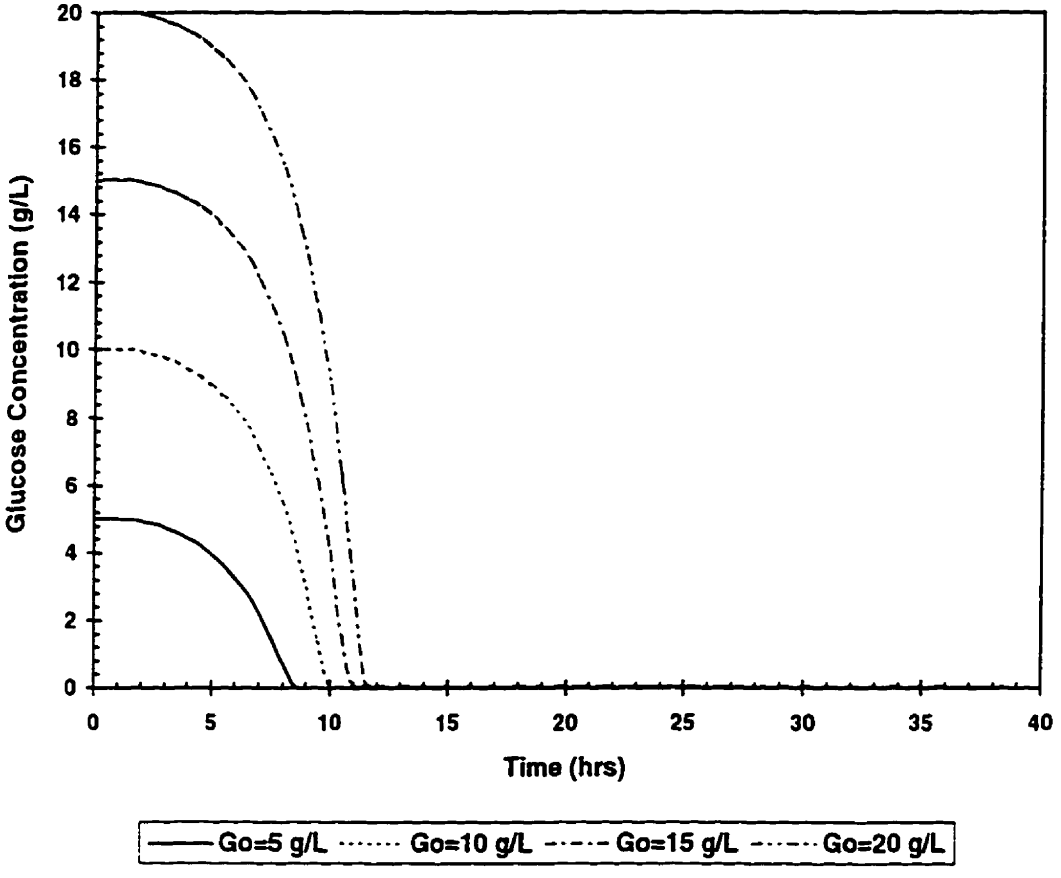


Figure C.1. Effect of Initial Glucose Concentration on Residual Glucose in Batch Culture.

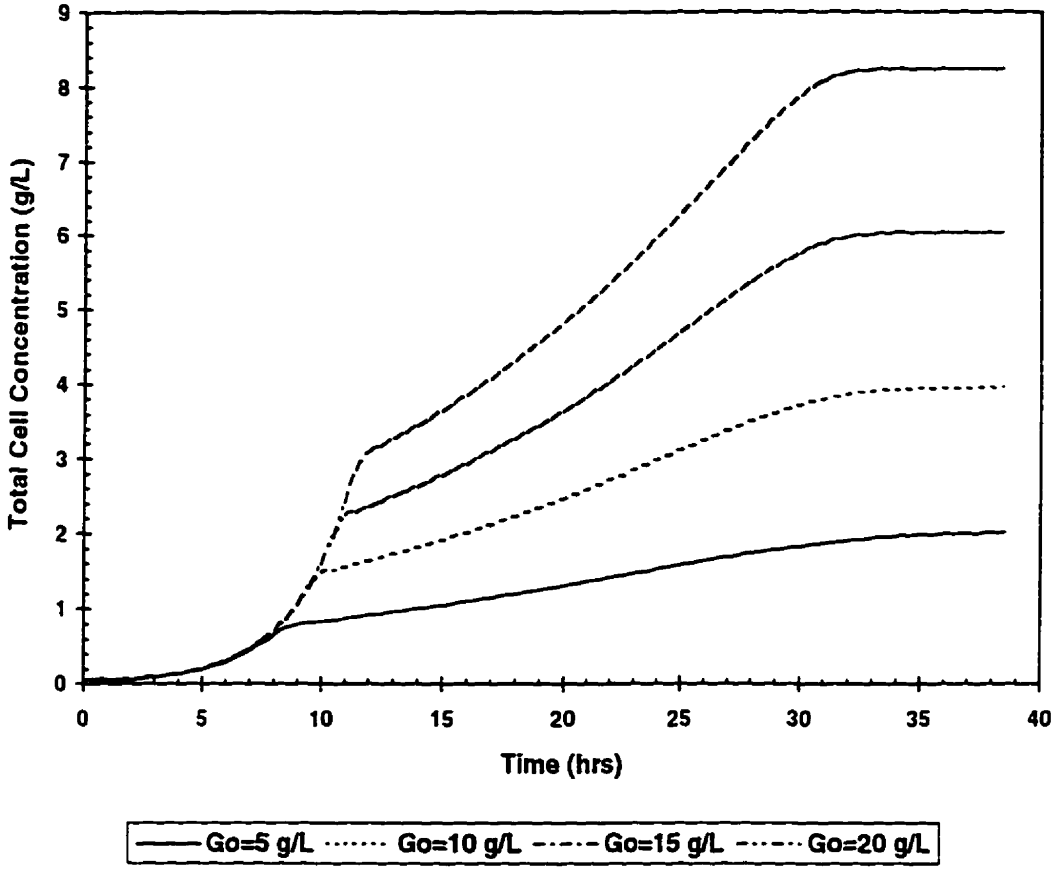


Figure C.2. Effect of Initial Glucose Concentration on Total Cell Concentration in Batch Culture.

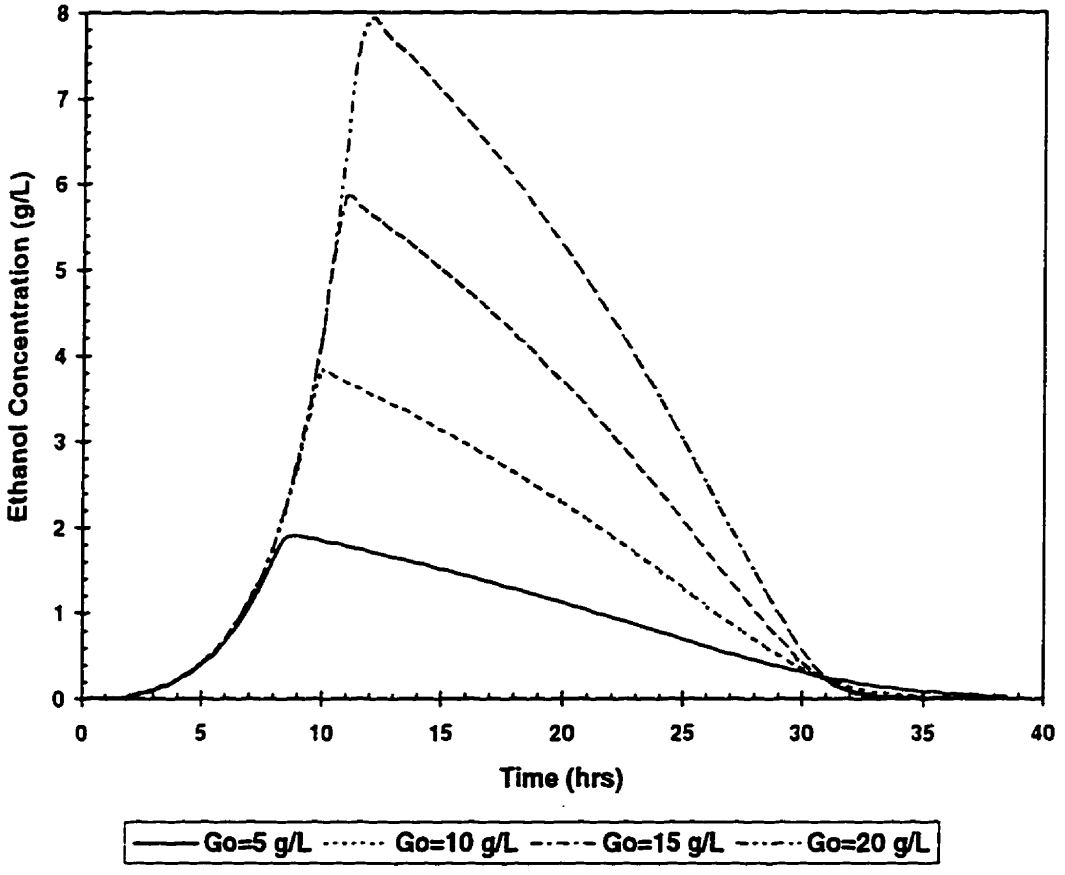


Figure C.3. Effect of Initial Glucose Concentration on Ethanol Concentration in Batch Culture.

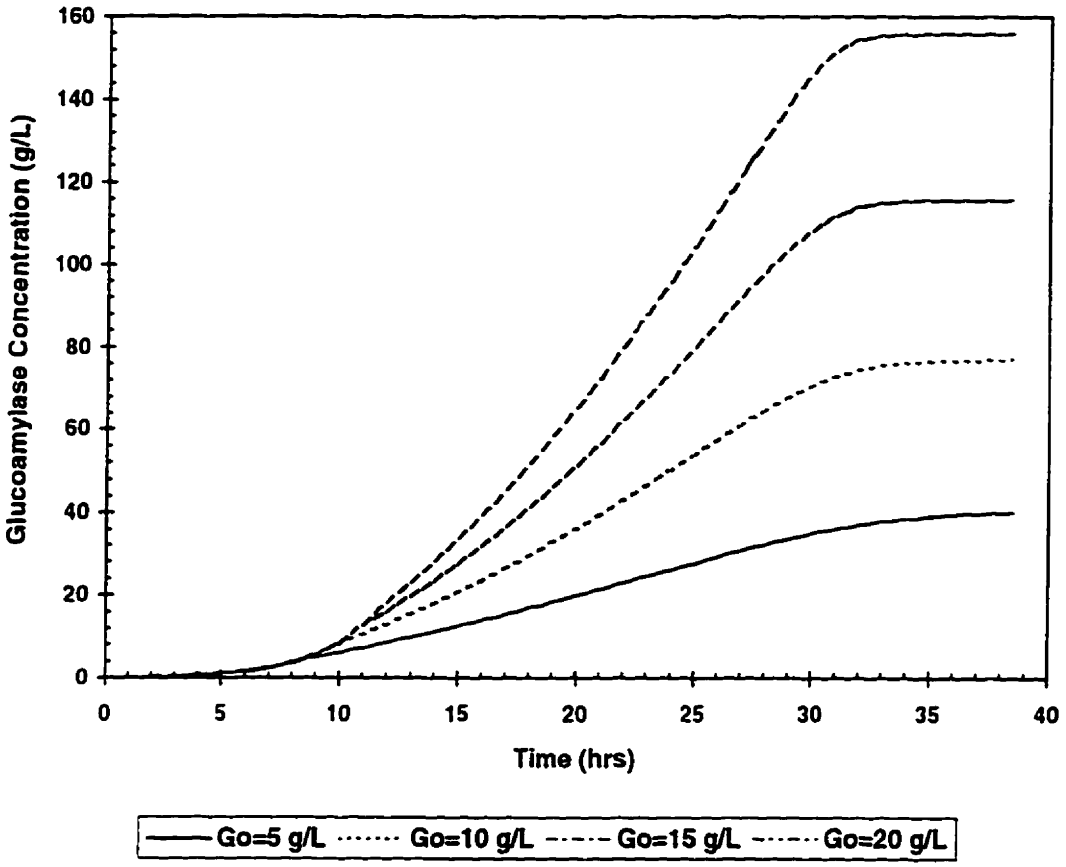


Figure C.4. Effect of Initial Glucose Concentration on Glucoamylase Concentration in Batch Culture.

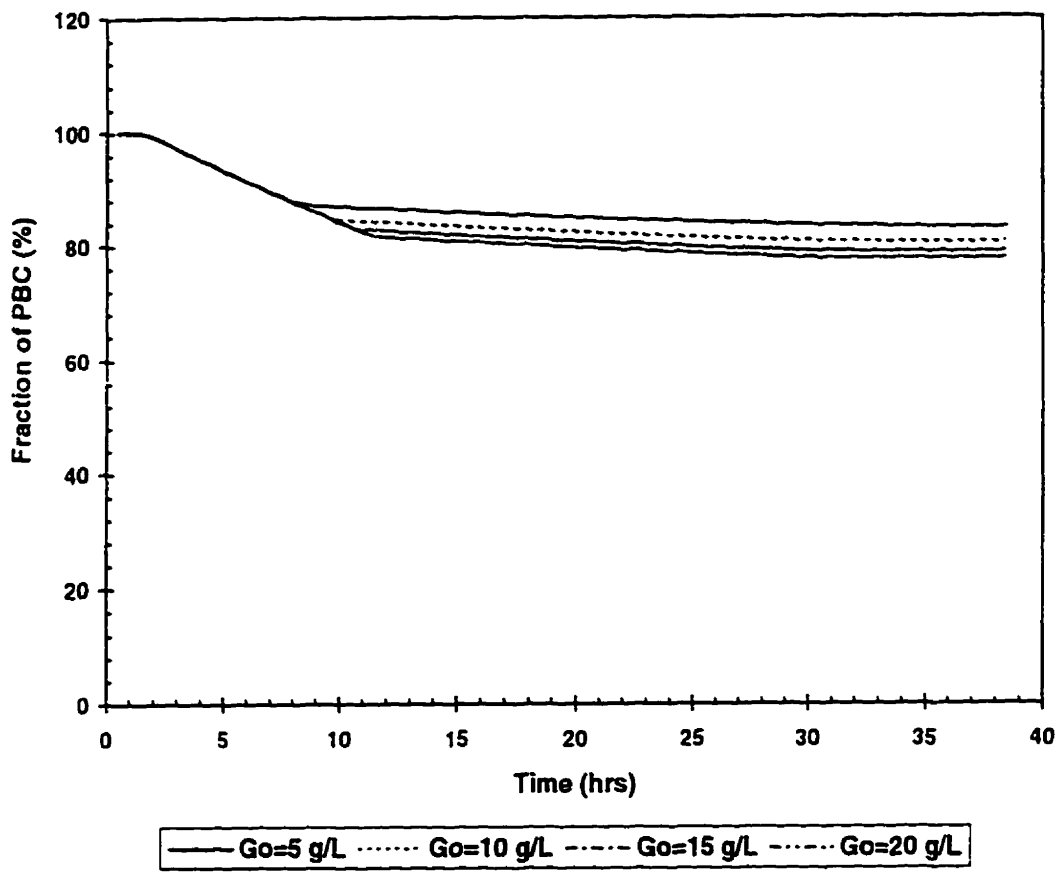


Figure C.5. Effect of Initial Glucose Concentration on Fraction of Plasmid-Bearing in Batch Culture.

APPENDIX D. COMPARISON OF ORIGINAL AND SELECTED STRAINS IN SHAKE FLASK EXPERIMENTS

Table D.1. Comparison of Cell and Enzyme Concentrations between Original and Selected Strains in Shake Flask Experiments (YPG medium, T=30 °C, RPM=250 and G_0= 20.0 g/L).

		Original Strain			Selected Strain		
Triplicates		24 hour	36 hour	48 hour	24 hour	36 hour	48 hour
# 1	Enz. Con. (units/L)	24.71	90.61	133.03	39.13	135.91	226.52
# 2	Enz. Con. (units/L)	23.89	82.37	135.91	38.30	140.03	220.34
# 3	Enz. Con. (units/L)	26.77	96.86	142.09	41.19	133.03	224.46
# 1	Cell Mass (g/L)	3.07	5.84	8.14	3.01	5.93	7.69
# 2	Cell Mass (g/L)	3.02	6.04	8.19	3.08	5.66	7.91
# 3	Cell Mass (g/L)	3.05	6.02	8.24	3.06	5.88	7.53

Table D.2. Comparison of Specific Enzyme Activity (units/g cell) between Original and Selected Strains in Shake Flask Experiments (YPG medium, T=30 °C, RPM=250 and G_0= 20.0 g/L).

	Original Strain			Selected Strain		
Triplicates	24 hr	36 hr	48 hr	24 hr	36 hr	48 hr
# 1	8.05	15.50	16.35	12.98	22.91	29.44
# 2	7.93	13.63	16.60	12.44	24.75	27.84
# 3	8.79	16.09	17.23	13.46	22.63	29.81

APPENDIX E. EXPERIMENTAL AND SIMULATION DATA

E. 1. Batch Fermentation with Recombinant Yeast C468/pGAC9

E.1.1. Simulation Data

t (h)	G (g/L)	X (g/L)	X* (g/L)	E (g/L)	P (units/L)	F (%)
0	18.75	0.05	0.05	0	0	100
0.2	18.75	0.05	0.05	0	0.0003	100
0.4	18.7499	0.05	0.05	0	0.0009	100
0.6	18.7497	0.0501	0.0501	0	0.0024	100
0.8	18.7493	0.0502	0.0502	0.0001	0.0053	100
1	18.7483	0.0504	0.0504	0.0004	0.0104	100
1.2	18.7458	0.0509	0.0509	0.0013	0.0178	100
1.4	18.7404	0.0518	0.0517	0.0031	0.0277	99.80695
1.6	18.7307	0.0532	0.0531	0.0067	0.0408	99.81203
1.8	18.7151	0.0555	0.0552	0.0126	0.0578	99.45946
2	18.6924	0.0587	0.0583	0.0211	0.0795	99.31857
2.2	18.6622	0.063	0.0622	0.0326	0.1064	98.73016
2.4	18.625	0.0682	0.0671	0.0467	0.1381	98.3871
2.6	18.5829	0.074	0.0726	0.0627	0.1734	98.10811
2.8	18.5392	0.0801	0.0783	0.0793	0.2104	97.75281
3	18.4959	0.0862	0.0839	0.0958	0.2478	97.33179
3.2	18.4424	0.0937	0.0908	0.1161	0.2921	96.90502
3.4	18.3843	0.1018	0.0982	0.1382	0.34	96.46365
3.6	18.3212	0.1106	0.1063	0.1622	0.3919	96.11212
3.8	18.2527	0.1201	0.115	0.1883	0.4481	95.75354
4	18.1782	0.1305	0.1244	0.2166	0.5088	95.32567
4.2	18.0973	0.1418	0.1346	0.2474	0.5745	94.92243
4.4	18.0094	0.154	0.1456	0.2808	0.6456	94.54545

(continue)

4.6	17.9139	0.1674	0.1575	0.3171	0.7225	94.08602
4.8	17.8101	0.1818	0.1705	0.3566	0.8057	93.78438
5	17.6974	0.1975	0.1844	0.3995	0.8958	93.36709
5.2	17.5749	0.2146	0.1995	0.446	0.9932	92.96365
5.4	17.4419	0.2332	0.2159	0.4966	1.0986	92.58148
5.6	17.2973	0.2533	0.2336	0.5516	1.2126	92.22266
5.8	17.1403	0.2752	0.2527	0.6113	1.336	91.82413
6	16.9697	0.299	0.2734	0.6762	1.4695	91.43813
6.2	16.7844	0.3248	0.2958	0.7467	1.614	91.07143
6.4	16.5831	0.3529	0.32	0.8232	1.7702	90.67725
6.6	16.3644	0.3834	0.3463	0.9064	1.9393	90.32342
6.8	16.1268	0.4165	0.3746	0.9968	2.1222	89.93998
7	15.8687	0.4525	0.4053	1.0949	2.3202	89.56906
7.2	15.5884	0.4916	0.4385	1.2015	2.5343	89.19854
7.4	15.2839	0.5341	0.4744	1.3173	2.7659	88.82232
7.6	14.9531	0.5802	0.5133	1.4431	3.0166	88.46949
7.8	14.5938	0.6303	0.5553	1.5797	3.2877	88.1009
8	14.2036	0.6847	0.6007	1.7281	3.581	87.73185
8.2	13.7798	0.7438	0.6499	1.8893	3.8984	87.37564
8.4	13.3195	0.808	0.703	2.0643	4.2417	87.00495
8.6	12.8196	0.8777	0.7606	2.2544	4.6132	86.65831
8.8	12.2768	0.9534	0.8227	2.4608	5.015	86.29117
9	11.6874	1.0356	0.89	2.6849	5.4497	85.94052
9.2	11.0475	1.1249	0.9627	2.9282	5.9199	85.58094
9.4	10.3529	1.2218	1.0414	3.1924	6.4286	85.2349
9.6	9.5989	1.327	1.1263	3.479	6.9788	84.87566
9.8	8.7808	1.4411	1.2182	3.7901	7.5739	84.53265
10	7.8934	1.565	1.3175	4.1274	8.2177	84.1853
10.2	6.9312	1.6993	1.4246	4.4931	8.9139	83.83452
10.4	5.8889	1.8449	1.5403	4.8893	9.6668	83.48962
10.6	4.7611	2.0024	1.665	5.3179	10.481	83.15022
10.8	3.5439	2.1726	1.7992	5.7802	11.3614	82.81322
11	2.2384	2.3555	1.9428	6.2758	12.3133	82.4793
11.2	0.8717	2.5479	2.0933	6.7934	13.3431	82.15786
11.4	0	2.6954	2.2083	7.1473	14.4156	81.92847

(continue)

18.6	0	4.019	3.2275	5.111	50.6782	80.30605
18.8	0	4.0631	3.2611	5.0431	51.8761	80.26138
19	0	4.1077	3.2951	4.9745	53.085	80.21764
19.2	0	4.1527	3.3294	4.9053	54.305	80.17434
19.4	0	4.1982	3.364	4.8354	55.536	80.12958
19.6	0	4.244	3.3989	4.7648	56.7782	80.08718
19.8	0	4.2904	3.4341	4.6936	58.0314	80.04149
20	0	4.3371	3.4697	4.6217	59.2958	80.00046
20.2	0	4.3843	3.5055	4.5491	60.5712	79.95575
20.4	0	4.4319	3.5417	4.4758	61.8578	79.91381
20.6	0	4.48	3.5782	4.4019	63.1556	79.87054
20.8	0	4.5285	3.615	4.3273	64.4644	79.82776
21	0	4.5774	3.6521	4.252	65.7843	79.78547
21.2	0	4.6268	3.6895	4.176	67.1152	79.74194
21.4	0	4.6766	3.7272	4.0994	68.4571	79.69893
21.6	0	4.7268	3.7652	4.0221	69.81	79.65643
21.8	0	4.7775	3.8035	3.9442	71.1737	79.61277
22	0	4.8285	3.8422	3.8656	72.5482	79.57337
22.2	0	4.8801	3.8811	3.7863	73.9335	79.52911
22.4	0	4.932	3.9203	3.7064	75.3293	79.48702
22.6	0	4.9844	3.9599	3.6259	76.7356	79.44587
22.8	0	5.0371	3.9997	3.5447	78.1522	79.40482
23	0	5.0903	4.0398	3.4629	79.5789	79.36271
23.2	0	5.1439	4.0802	3.3805	81.0157	79.32114
23.4	0	5.1978	4.1208	3.2974	82.4622	79.2797
23.6	0	5.2522	4.1617	3.2138	83.9183	79.23727
23.8	0	5.3069	4.2029	3.1296	85.3836	79.19689
24	0	5.362	4.2444	3.0449	86.8579	79.15703
24.2	0	5.4175	4.286	2.9596	88.3409	79.11398
24.4	0	5.4732	4.328	2.8737	89.8321	79.07623
24.6	0	5.5293	4.3701	2.7874	91.3313	79.03532
24.8	0	5.5858	4.4124	2.7006	92.8378	78.99316
25	0	5.6425	4.455	2.6134	94.3513	78.95436
25.2	0	5.6994	4.4977	2.5258	95.8711	78.91532
25.4	0	5.7566	4.5406	2.4378	97.3967	78.87642

(continue)

25.6	0	5.8141	4.5836	2.3494	98.9273	78.83593
25.8	0	5.8717	4.6267	2.2608	100.4622	78.7966
26	0	5.9294	4.67	2.1719	102.0005	78.76008
26.2	0	5.9873	4.7133	2.0828	103.5412	78.72163
26.4	0	6.0453	4.7566	1.9936	105.0833	78.68261
26.6	0	6.1033	4.8	1.9044	106.6257	78.64598
26.8	0	6.1613	4.8433	1.8152	108.1669	78.60841
27	0	6.2192	4.8865	1.7261	109.7055	78.5712
27.2	0	6.277	4.9297	1.6372	111.2399	78.53592
27.4	0	6.3346	4.9726	1.5486	112.7682	78.49904
27.6	0	6.3919	5.0153	1.4604	114.2884	78.46337
27.8	0	6.4489	5.0578	1.3728	115.7982	78.42888
28	0	6.5053	5.0998	1.2859	117.295	78.39454
28.2	0	6.5612	5.1415	1.1999	118.776	78.36219
28.4	0	6.6164	5.1826	1.115	120.2379	78.32961
28.6	0	6.6708	5.223	1.0313	121.6772	78.29646
28.8	0	6.7242	5.2627	0.9492	123.09	78.26507
29	0	6.7765	5.3016	0.8688	124.4718	78.23508
29.2	0	6.8274	5.3394	0.7905	125.818	78.20547
29.4	0	6.8768	5.3761	0.7145	127.1231	78.17735
29.6	0	6.9244	5.4114	0.6412	128.3816	78.14973
29.8	0	6.9701	5.4453	0.571	129.5875	78.1237
30	0	7.0135	5.4776	0.5041	130.7345	78.10081
30.2	0	7.0545	5.508	0.4411	131.8162	78.07782
30.4	0	7.0928	5.5364	0.3822	132.8266	78.05662
30.6	0	7.1282	5.5626	0.3277	133.76	78.03653
30.8	0	7.1605	5.5866	0.278	134.6118	78.01969
31	0	7.1895	5.6081	0.2333	135.3786	78.00403
31.2	0	7.2153	5.6272	0.1936	136.0589	77.98983
31.4	0	7.2379	5.6439	0.1589	136.6532	77.97704
31.6	0	7.2573	5.6583	0.1291	137.1643	77.96701

(continue)

31.8	0	7.2737	5.6705	0.1038	137.5969	77.95895
32	0	7.2874	5.6806	0.0828	137.9575	77.95098
32.2001	0	7.2986	5.6889	0.0654	138.2539	77.94509
32.4001	0	7.3078	5.6957	0.0514	138.4944	77.94001
32.6001	0	7.3151	5.7011	0.0401	138.6873	77.93605
32.8001	0	7.3209	5.7054	0.0312	138.8406	77.93304
33.0001	0	7.3255	5.7088	0.0241	138.9613	77.93052
33.2001	0	7.3291	5.7115	0.0186	139.0557	77.92908
33.4001	0	7.3319	5.7135	0.0143	139.1292	77.92659
33.6001	0	7.334	5.7151	0.011	139.1861	77.9261
33.8001	0	7.3357	5.7164	0.0084	139.2301	77.92576
34.0001	0	7.337	5.7173	0.0065	139.2639	77.92422
34.2001	0	7.338	5.718	0.0049	139.2899	77.92314
34.4001	0	7.3387	5.7186	0.0038	139.3098	77.92388
34.6001	0	7.3393	5.719	0.0029	139.3251	77.92296
34.8001	0	7.3397	5.7194	0.0022	139.3367	77.92417
35.0001	0	7.3401	5.7196	0.0017	139.3457	77.92264
35.2001	0	7.3403	5.7198	0.0013	139.3525	77.92325
35.4001	0	7.3405	5.7199	0.001	139.3577	77.92248
35.6001	0	7.3407	5.7201	0.0007	139.3617	77.92309
35.8001	0	7.3408	5.7201	0.0006	139.3647	77.92202
36.0001	0	7.3409	5.7202	0.0004	139.367	77.92233
36.2001	0	7.341	5.7203	0.0003	139.3688	77.92263
36.4001	0	7.341	5.7203	0.0003	139.3701	77.92263
36.6001	0	7.3411	5.7203	0.0002	139.3712	77.92156
36.8001	0	7.3411	5.7203	0.0001	139.3719	77.92156
37.0001	0	7.3411	5.7204	0.0001	139.3725	77.92293
37.2001	0	7.3411	5.7204	0.0001	139.373	77.92293
37.4001	0	7.3411	5.7204	0.0001	139.3734	77.92293

(continue)

37.6001	0	7.3411	5.7204	0	139.3736	77.92293
37.8001	0	7.3412	5.7204	0	139.3738	77.92187
38.0001	0	7.3412	5.7204	0	139.374	77.92187
38.2001	0	7.3412	5.7204	0	139.3741	77.92187
38.4001	0	7.3412	5.7204	0	139.3742	77.92187
38.6001	0	7.3412	5.7204	0	139.3742	77.92187
38.8001	0	7.3412	5.7204	0	139.3743	77.92187
39.0001	0	7.3412	5.7204	0	139.3743	77.92187
39.2001	0	7.3412	5.7204	0	139.3744	77.92187
39.4001	0	7.3412	5.7204	0	139.3744	77.92187
39.6001	0	7.3412	5.7204	0	139.3744	77.92187
39.8001	0	7.3412	5.7204	0	139.3744	77.92187
40.0001	0	7.3412	5.7204	0	139.3744	77.92187

E.1.2. Experimental Data (present studies)

t (h)	G (g/L)	X (g/L)	E (g/L)	P (units/L)	F (%)
0	18.95	0.052	0.36		94.5
2	18.48	0.06	0.419		
4	17.946	0.145	0.592		
5	17.508	0.206	0.759		
6	16.853	0.31	1.074		
7	16.094	0.468	1.432		
8	14.56	0.785	1.905		
9	12.277	1.15	3.238		
10	8.024	1.631	4.645		
11	2.009	2.58	6.561	0	
12	0.00982	3.20285	7.3387	21.4163	
12	0.00625	3.25979	7.56816	24.8656	76.34
15	0.00714	3.48757	6.76184	29.6533	75.72
15	0.00654	3.51035	6.69789	36.4103	72.39
18	0.00543	4.00008	5.41418	38.3022	74.1
18	0.00458	4.05702	5.36007	44.8468	77.18
21		4.96814	4.15721	55.6	76.53
21		5.10481	3.99352	58.1676	75.26
24		5.4237	2.97815	74.1333	72.67
24		5.6856	2.87359	80.369	72.99
26		6.3918	2.23495	98.8444	71.8
27		6.44871	1.87406	103.459	72.5
28		6.5626	1.59917	108.787	72.24
28		6.7334	1.56334	116.554	70.78
36		8.04317	0	150.738	71.12
37		7.92928	0	146.085	72.05
39		8.27095	0	152.797	70.76
40		8.27095	0	150.969	71.75

E.2. Batch Fermentation with Recombinant Yeast AB103.1/pYαEGF-25

E.2.1. Simulation Data

t (h)	G (g/L)	X (g/L)	X* (g/L)	E (g/L)	P (mg/L)	F (%)
0	9.696	0.04	0.04	0	0	100
0.5	9.696	0.04	0.04	0	0	100
1	9.696	0.04	0.04	0	0	100
1.5	9.696	0.04	0.04	0	0	100
2	9.696	0.04	0.04	0	0	100
2.5	9.696	0.04	0.04	0	0	100
3	9.6959	0.04	0.04	0	0.0001	100
3.5	9.695	0.0403	0.0403	0.0003	0.0008	100
4	9.6891	0.0417	0.0416	0.0025	0.0029	99.76019
4.5	9.6678	0.0458	0.0455	0.0122	0.0068	99.34498
5	9.6251	0.0537	0.053	0.0321	0.0128	98.69646
5.5	9.5699	0.0639	0.0624	0.0579	0.0204	97.65258
6	9.5043	0.076	0.0736	0.0886	0.0293	96.84211
6.5	9.4265	0.0904	0.0868	0.125	0.0398	96.0177
7	9.3341	0.1074	0.1023	0.1682	0.0522	95.2514
7.5	9.2245	0.1277	0.1205	0.2194	0.0668	94.36179
8	9.0946	0.1517	0.1419	0.2801	0.084	93.53988
8.5	8.9407	0.1802	0.1671	0.3519	0.1043	92.7303
9	8.7588	0.2138	0.1966	0.4368	0.1282	91.9551
9.5	8.5438	0.2536	0.2313	0.5371	0.1564	91.20662
10	8.2904	0.3006	0.2718	0.6552	0.1895	90.41916
10.5	7.9922	0.356	0.3192	0.7941	0.2285	89.66292
11	7.6423	0.4211	0.3744	0.9569	0.2743	88.91
11.5	7.2332	0.4974	0.4385	1.1469	0.3282	88.15842
12	6.7574	0.5864	0.5128	1.3675	0.3915	87.44884
12.5	6.2073	0.6898	0.5982	1.6218	0.4657	86.72079
13	5.5772	0.8088	0.6959	1.9121	0.5527	86.04105

(continue)

13.5	4.8644	0.9445	0.8064	2.2387	0.6545	85.37851
14	4.0735	1.0969	0.9295	2.5984	0.7736	84.73881
14.5	3.2209	1.2642	1.0637	2.9812	0.9128	84.14017
15	2.3441	1.4417	1.2051	3.3663	1.0759	83.58882
15.5	1.509	1.6208	1.3469	3.7173	1.2685	83.10094
16	0.8027	1.7906	1.4806	3.9851	1.5001	82.68737
16.5	0.2864	1.9456	1.6021	4.132	1.7853	82.34478
17	0	2.0731	1.7017	4.1476	2.0852	82.0848
17.5	0	2.1292	1.7455	4.0353	2.2285	81.97915
18	0	2.1867	1.7902	3.9203	2.375	81.86765
18.5	0	2.2456	1.836	3.8026	2.5247	81.75989
19	0	2.3058	1.8827	3.6821	2.6778	81.65062
19.5	0	2.3674	1.9305	3.559	2.834	81.54516
20	0	2.4304	1.9792	3.433	2.9936	81.43515
20.5	0	2.4947	2.029	3.3044	3.1564	81.33242
21	0	2.5604	2.0797	3.173	3.3224	81.22559
21.5	0	2.6274	2.1314	3.039	3.4916	81.12202
22	0	2.6957	2.184	2.9023	3.6639	81.01792
22.5	0	2.7654	2.2375	2.7631	3.8392	80.91054
23	0	2.8362	2.292	2.6214	4.0174	80.81235
23.5	0	2.9082	2.3472	2.4773	4.1984	80.70972
24	0	2.9814	2.4033	2.331	4.3818	80.60978
24.5	0	3.0555	2.46	2.1828	4.5675	80.51055
25	0	3.1305	2.5173	2.0328	4.7552	80.41207
25.5	0	3.2062	2.5751	1.8814	4.9444	80.31626
26	0	3.2824	2.6332	1.729	5.1346	80.22179
26.5	0	3.3589	2.6915	1.5761	5.3253	80.1304
27	0	3.4353	2.7496	1.4232	5.5156	80.03959
27.5	0	3.5113	2.8073	1.2713	5.7046	79.95045
28	0	3.5863	2.8643	1.1212	5.8911	79.86783

(continue)

28.5	0	3.6598	2.9201	0.9741	6.0737	79.78851
29	0	3.7311	2.9741	0.8315	6.2505	79.71108
29.5	0	3.7993	3.0257	0.6951	6.4195	79.63835
30	0	3.8634	3.0741	0.567	6.578	79.56981
30.5	0	3.9221	3.1185	0.4495	6.7233	79.51098
31	0	3.9744	3.158	0.345	6.8526	79.45853
31.5	0	4.0192	3.1918	0.2554	6.9632	79.41381
32	0	4.0558	3.2194	0.1822	7.0537	79.37768
32.5	0	4.0843	3.2409	0.1253	7.1239	79.35019
33	0	4.1052	3.2567	0.0833	7.1756	79.33109
33.5	0	4.1199	3.2678	0.054	7.2118	79.31746
34	0	4.1298	3.2752	0.0342	7.2362	79.3065
34.5	0	4.1362	3.28	0.0214	7.252	79.29984
35	0	4.1403	3.2831	0.0132	7.2621	79.29619
35.5	0	4.1428	3.285	0.0081	7.2684	79.2942
36	0	4.1444	3.2862	0.005	7.2722	79.29254
36.5	0	4.1454	3.2869	0.003	7.2746	79.2903
37	0	4.146	3.2874	0.0019	7.2761	79.29088
37.5	0	4.1463	3.2877	0.0011	7.277	79.29238
38	0	4.1465	3.2878	0.0007	7.2775	79.29097
38.5	0	4.1467	3.2879	0.0004	7.2779	79.28956
39	0	4.1468	3.288	0.0003	7.2781	79.29005
39.5	0	4.1468	3.288	0.0002	7.2782	79.29005
40	0	4.1468	3.288	0.0001	7.2783	79.29005

E.2.2. Experimental Data (Coppella and Dhurjati, 1990)

t (h)	G (mm)	E (mm)	X (mm)	G (g/L)	E (g/L)	X (g/L)
0.5	101	0	1	9.696	0	0.04
5	100	0.5	2	9.6	0.048	0.08
6.5	97.5	1	2.5	9.36	0.096	0.1
7.75	95.5	2	3.5	9.168	0.192	0.14
8.75	91	4	5.5	8.736	0.384	0.22
9.75	84.5	6	8.5	8.112	0.576	0.34
10.5	80.5	7	9.5	7.728	0.672	0.38
11.5	75.5	10.5	11.5	7.248	1.008	0.46
11.75	71.5	12.5	15	6.864	1.2	0.6
12.25	65.5	15	17.5	6.288	1.44	0.7
12.75	59	17.5	20.5	5.664	1.68	0.82
13.25	52	21	23.5	4.992	2.016	0.94
13.5	42	24	25.5	4.032	2.304	1.02
13.75	38	29	29.5	3.648	2.784	1.18
14.33	27	32	31.5	2.592	3.072	1.26
14.67	24	33.5	34.5	2.304	3.216	1.38
15	18	36.5	37.5	1.728	3.504	1.5
15.33	12	40	40.5	1.152	3.84	1.62
15.67	6	42	42.5	0.576	4.032	1.7
16	1	44	45	0.096	4.224	1.8
16.5	0.5	44.5	46.5	0.048	4.272	1.86
18		42.5	49.5	0	4.08	1.98
19.5		41.5	52.5	0	3.984	2.1
20		41	54.5	0	3.936	2.18
20.5		39	57	0	3.744	2.28
21.5		37.5	60	0	3.6	2.4
22		36	62.5	0	3.456	2.5
23		30.5	65.5	0	2.928	2.62
23.5		31	68	0	2.976	2.72
24		29.5	71	0	2.832	2.84
24.5		28	73.5	0	2.688	2.94
25		24.5	76	0	2.352	3.04

(continue)

25.5		20.5	80	0	1.968	3.2
27		17.5	86.5	0	1.68	3.46
27.5		15	87	0	1.44	3.48
28.5		10	92	0	0.96	3.68
29.5		4.5	95.5	0	0.432	3.82
31.5		0	98	0	0	3.92

t (h)	*P (mm)*	*P (mg/L)*	*F (mm)*	*F (%)*
0.5	0	0	125	100
10.5	5	0.4		
12.75	8	0.64		
12.5	20.5	1.64		
14	29	2.32	116	92.8
14.5	29	2.32		
15	34	2.72		
15.33	28	2.24		
16	38	3.04		
16.5	41.5	3.32	110	88
19.5	55.5	4.44		
20	59	4.72	104	83.2
20.5	57	4.56		
21.5	64.5	5.16		
22	73	5.84	98.5	78.8
23.5	69	5.52		
24.5	66.5	5.32	103.5	82.8
25.5	73	5.84		
26	75.5	6.04	103.5	82.8
28	80.5	6.44	99.5	79.6
29.5	78	6.24		
31.5	92.5	7.4		

E.3. Batch Fermentation with Recombinant Yeast SEY2102/pRB58

E.3.1. Simulation Data

t (h)	G (g/L)	X (g/L)	X* (g/L)	E (g/L)
0	9.696	0.04	0.04	0
0.5	9.696	0.04	0.04	0
1	9.696	0.04	0.04	0
1.5	9.696	0.04	0.04	0
2	9.696	0.04	0.04	0
2.5	9.696	0.04	0.04	0
3	9.6959	0.04	0.04	0
3.5	9.695	0.0403	0.0403	0.0003
4	9.6891	0.0417	0.0416	0.0025
4.5	9.6678	0.0458	0.0455	0.0122
5	9.6251	0.0537	0.053	0.0321
5.5	9.5699	0.0639	0.0624	0.0579
6	9.5043	0.076	0.0736	0.0886
6.5	9.4265	0.0904	0.0868	0.125
7	9.3341	0.1074	0.1023	0.1682
7.5	9.2245	0.1277	0.1205	0.2194
8	9.0946	0.1517	0.1419	0.2801
8.5	8.9407	0.1802	0.1671	0.3519
9	8.7588	0.2138	0.1966	0.4368
9.5	8.5438	0.2536	0.2313	0.5371
10	8.2904	0.3006	0.2718	0.6552
10.5	7.9922	0.356	0.3192	0.7941
11	7.6423	0.4211	0.3744	0.9569
11.5	7.2332	0.4974	0.4385	1.1469
12	6.7574	0.5864	0.5128	1.3675
12.5	6.2073	0.6898	0.5982	1.6218
13	5.5772	0.8088	0.6959	1.9121
13.5	4.8644	0.9445	0.8064	2.2387

(continue)

14	4.0735	1.0969	0.9295	2.5984
14.5	3.2209	1.2642	1.0637	2.9812
15	2.3441	1.4417	1.2051	3.3663
15.5	1.509	1.6208	1.3469	3.7173
16	0.8027	1.7906	1.4806	3.9851
16.5	0.2864	1.9456	1.6021	4.132
17	0	2.0731	1.7017	4.1476
17.5	0	2.1292	1.7455	4.0353
18	0	2.1867	1.7902	3.9203
18.5	0	2.2456	1.836	3.8026
19	0	2.3058	1.8827	3.6821
19.5	0	2.3674	1.9305	3.559
20	0	2.4304	1.9792	3.433
20.5	0	2.4947	2.029	3.3044
21	0	2.5604	2.0797	3.173
21.5	0	2.6274	2.1314	3.039
22	0	2.6957	2.184	2.9023
22.5	0	2.7654	2.2375	2.7631
23	0	2.8362	2.292	2.6214
23.5	0	2.9082	2.3472	2.4773
24	0	2.9814	2.4033	2.331
24.5	0	3.0555	2.46	2.1828
25	0	3.1305	2.5173	2.0328
25.5	0	3.2062	2.5751	1.8814
26	0	3.2824	2.6332	1.729
26.5	0	3.3589	2.6915	1.5761
27	0	3.4353	2.7496	1.4232
27.5	0	3.5113	2.8073	1.2713
28	0	3.5863	2.8643	1.1212
28.5	0	3.6598	2.9201	0.9741
29	0	3.7311	2.9741	0.8315
29.5	0	3.7993	3.0257	0.6951

(continue)

30	0	3.8634	3.0741	0.567
30.5	0	3.9221	3.1185	0.4495
31	0	3.9744	3.158	0.345
31.5	0	4.0192	3.1918	0.2554
32	0	4.0558	3.2194	0.1822
32.5	0	4.0843	3.2409	0.1253
33	0	4.1052	3.2567	0.0833
33.5	0	4.1199	3.2678	0.054
34	0	4.1298	3.2752	0.0342
34.5	0	4.1362	3.28	0.0214
35	0	4.1403	3.2831	0.0132
35.5	0	4.1428	3.285	0.0081
36	0	4.1444	3.2862	0.005
36.5	0	4.1454	3.2869	0.003
37	0	4.146	3.2874	0.0019
37.5	0	4.1463	3.2877	0.0011
38	0	4.1465	3.2878	0.0007
38.5	0	4.1467	3.2879	0.0004
39	0	4.1468	3.288	0.0003
39.5	0	4.1468	3.288	0.0002
40	0	4.1468	3.288	0.0001

E.3.2. Experimental Data (Parker and Seo, 1992)

t (h)	G (mm)	E (mm)	OD (mm)	G (g/L)	E (g/L)	OD	Cell (g/l)
0	64.35	0	9	14.4	0	0.1892	0.090816
1	62	3	12	13.87413	0.20979	0.234007	0.112323
2	63	5	17.5	14.0979	0.34965	0.345509	0.165844
3.5	53	9	24	11.86014	0.629371	0.547595	0.262846
5	38.5	16.5	36	8.615385	1.153846	1.281416	0.61508
6	35	23	42.5	7.832168	1.608392	2.030907	0.974836
7	24.5	40.5	47.5	5.482517	2.832168	2.894252	1.389241
9	10	54	53	2.237762	3.776224	4.273336	2.051201
11	0	63	57	0	4.405594	5.673407	2.723235
14	0	47.5	59	0	3.321678	6.537059	3.137788
17.5	0	11.5	64	0	0.804196	9.31598	4.471671
19	0	1.5	66.5	0	0.104895	11.1212	5.338176
21	0	0	67	0	0	11.52222	5.530667
23	0	0	67	0	0	11.52222	5.530667

E.4. Continuous Suspension Culture with Recombinant Yeast C468/pGAC9

E.4.1. Continuous Culture in Airlift Bioreactor (ALR)

Run No: CALR1 (Selected Strain, D = 0.05 h^{-1})

t (h)	X (g/L)	E (g/L)	P (units/L)	F (%)	pH
0	3.347726	7	44.89185		4.63
12	7.029549	3.645	187.3926	71.2327	5.6
24	7.24594	2.593	194.8059	77.5005	5.66
36	7.075105	2.591	158.9748	86.3535	4.67
48	6.938437	2.383	151.9733	62.1115	4.74
60	7.143439	2.419	132.2044	53.2795	4.73
72	7.143439	2.463	119.8489	62.606	4.77
102	7.075105	2.589	92.66667	54.625	4.64
120	6.927048	2.564	86.48889	49.22	4.7
144	7.188995	2.45	66.72	39.215	4.68
168	7.35983	2.367	55.22933	44.206	4.44
192	6.983993	2.143	50.41067	42.32	4.6
215	7.439553	2.12	45.1	41.515	4.32
238	7.13205	2.04	41.5	37.375	4.3
262	7.01816	1.903	38	34.27	4.32
286	6.995382	1.853	37	37.835	4.42
310	7.029549	1.973	30.39467	33.235	4.53
338	7.097883	1.568	25.576	36.11	4.36

Run No: CALR2 (Selected Strain, D = 0.10 h^{-1})

t (h)	X (g/L)	E (g/L)	P (units/L)	F (%)	pH
0	3.3251796	7.7482942	40.16717	92.6	4.58
6	4.7903107	6.0201451	94.222185	67.4	4.91
12	5.7059612	5.3018521	137.71005	73.3	4.99
24	5.7914913	5.1328929	146.65149	67.7	4.89
30	5.4814447	5.0037908	130.80077	68.6	4.91
36	5.3531495	5.0817719	122.67219	61.3	4.89
48	5.2569282	5.1138308	97.067186	55.6	4.93
54	5.3531495	4.8625582	88.938612	49.8	4.65
60	5.2248544	4.9760641	86.093611	50.7	5.02
72	5.2088175	5.2056753	75.931894	48	5.05
84	5.2783107	5.2931875	66.178606	42.9	5.02
96	5.3798777	5.0124553	53.579317	38.3	4.94
108	5.2302	5.0930358	47.482886	38.3	5
120	5.2088175	5.0904365	41.386456	41.3	4.92
132	5.1500155	5.009856	38.541455	39.9	5.05
144	5.2676194	5.0635763	35.696455	38.2	4.92
156	5.2943476	5.0453807	34.477169	34.9	4.9
168	5.2676194	4.8651576	32.445025	33.52	4.85
180	5.3424583	4.7425539	28.380738		4.9
194.5	5.3050388	4.9448717	28.380738		4.88

Run No: CALR3 (Selected Strain, D = 0.20 h^{-1})

t (h)	X (g/L)	E (g/L)	P (units/L)	F (%)	pH
0	3.354745	7.296	43.82883	84	4.66
6	3.986455	7.189	61.36036	64	4.83
12	4.09174	6.981	49.67267	69	4.93
24	3.916265	6.911	39.65465	63.3	4.77
30	3.81098	7.071	38.4024	54	4.84
36	3.6706	6.891	31.30631	44	4.82
48	3.775885	7.259	25.87988	40	4.84
54	3.81098	6.766	21.28829	38	4.75
60	3.775885	6.496	14.60961	34	4.8
72	3.775885	6.429	10.43544	30	4.76
78	3.74079	6.855	11.27027	26.3	4.75
84	3.705695	6.968	9.600601	23.8	4.79
96	3.832037	6.989	8.765766	26.8	4.77
102	3.74079	6.925	8.348348	19.6	4.71

Run No: CALR4 (Selected Strain, D = 0.25 h^{-1})

t (h)	X (g/L)	F (%)	pH
0	3.918765	73.6	4.6
3	4.123439	62.3	4.63
6	3.835379	63.8	4.68
12	3.797477	69	4.72
24	3.812638	63.8	4.76
30	3.774735	49.4	4.72
36	3.805057	42.1	4.74
48	3.736832	40	4.73
54	3.744413	35	4.73
60	3.683769	32	4.76
72	3.736832	29.6	4.86
78	3.691349	31	4.65
84	3.767154	21.6	4.7
103	3.812638	21.6	4.73

Run No: CALR 5 (Original Strain, D = 0.10 h^{-1})

t (h)	X (g/L)	P (units/L)	E (g/L)	pH
0	4.02	49.17	7.08	5.1
6	4.52	60.06	6.53	5.02
12	4.95	65.5	5.78	4.98
24	6	72.62	5.41	4.95
30	6.1	65.43	5.21	4.82
36	6.05	60.14	5.19	4.78
48	6	51.06	5.11	4.78
54	6.12	48.04	5.16	4.78
60	6.08	44.63	5.43	4.82
72	5.98	41.23	5.73	4.82
84	6.07	34.42	5.41	4.8
96	6.03	27.61	5.77	4.81
108	6.15	22.69	5.61	4.79
120	6.04	18.91	5.53	4.8
132	6.11	17.02	5.5	4.78
144	6.03	15.13	5.56	4.82
156	6.09	15.13	5.4	4.82
168	6.13	14.37	5.41	4.8

E.4.2. Continuous Culture in Stirred-Tank Bioreactor (STR)

Run No: CSTR1 (Selected Strain, D = 0.10 h^{-1})

t (h)	X (g/L)	E (g/L)	P (units/L)	F (%)	pH
0	3.183	6.551	37.83889	66.66667	4.94
6	5.25	4.769	111.9722	69.65517	4.77
12	6.883	2.963	132.8222	80.4878	4.43
24	6.583	2.116	89.19167	67.05539	4.33
36	6.655	2.057	82.62778	52.23881	4.44
48	6.568	1.718	67.18333	39.39394	4.43
60	6.65	1.886	54.05556	33.23442	4.42
72	6.735	1.639	41.31389	26.42643	4.43
84	6.74	1.715	28.95833	20.58824	4.43
96	6.588	1.68	23.16667	15.71429	4.38
108	6.505	1.712	17.76111	13.63636	4.4
120	6.55	1.672	11.58333	9.463722	4.4

Run No: CSTR2 (Selected Strain, D = 0.20 h^{-1})

t (h)	X (g/L)	E (g/L)	P (units/L)	F (%)	pH
0	3.37	6.495	51.35278	78.5058	5.02
6.5	5.163	5.206	82.24167	63.3123	4.78
12	5.038	5.066	80.31111	66.27198	4.76
24	5.35	4.753	81.08333	56.22642	4.76
30	5.31	4.701	77.60833	50.60953	4.76
36	4.935	4.882	67.56944	47.11647	4.8
47	4.96	4.566	60.23333	38.46154	4.73
54	5.305	4.825	57.91667	34.45774	4.73
60	4.905	4.767	48.65	31.71642	4.77
72	5.53	4.555	44.40278	28.66557	4.73
78	4.89	4.794	44.78889	26.60448	4.75
84	5.43	4.691	36.68056	24.4238	4.78

Run No: CSTR3 (Selected Strain, D = 0.30 h^{-1})

t (h)	X (g/L)	E (g/L)	P (units/L)	F (%)	pH
0	3.365	7.108	37.47852	80.29197	4.72
3	3.345	6.405	35.41926	66.14583	4.71
6	3.315	6.367	32.12444	63.16667	4.7
10	3.215	6.423	26.77037	62.42291	4.71
14	3.175	5.925	25.12296	57.51634	4.72
26	3.5	6.563	23.47556	48.96907	4.82
30	3.153	6.805	24.71111	47.90721	4.78
38	3.155	6.448	19.76889	42.09115	4.82
48	3.143	6.963	23.0637	39.42708	4.88

Run No: CSTR4 (Original Strain, D = 0.10 h^{-1})

t (h)	X (g/L)	P (units/L)	E (g/L)	pH
0	4.871	45.77	4.73	4.97
6	5.61	62.41	3.65	4.5
12	6.1	65.81	2.8	4.58
24	6.49	61.27	2.54	4.57
30	6.54	53.33	2.59	4.61
36	6.87	47.28	2.78	4.62
48	6.93	37.45	2.47	4.6
54	6.96	31.77	2.61	4.62
60	6.98	27.23	2.51	4.63

(continue)

72	6.95	22.32	2.75	4.61
84	6.9	16.64	2.63	4.61
96	6.76	11.35	2.43	4.63
108	6.97	10.59	2.73	4.6
120	7.33	9.46	2.46	4.6
132	7.09	7.57	2.3	4.6
144	7.34	7.57	2.25	4.61
156	7.28	6.05	2.2	4.61
168	7.2	6.81	2.08	4.61

E.4.3. Simulation Data (Effect of Dilution Rate)

D (h^{-1})	*G (g/L)*	*X (g/L)*	*E (g/L)*	*P (units/L)*	*q (g/g.h)*
0.005	0.000207	9.0842	0.690835	264.2994	0.011
0.01	0.000434	8.963	0.850266	259.5498	0.0223
0.025	0.001259	8.5707	1.357325	244.0192	0.05833
0.05	0.003289	7.3192	2.209227	200.87	0.12807
0.075	0.006669	6.8787	3.566505	177.0966	0.218
0.1	0.01241	5.4599	4.6294	126.9557	0.3432
0.125	0.02139	4.8623	6.187709	97.43043	0.5136
0.15	0.0345	4.1883	7.058704	70.8404	0.7151
0.175	0.04832	3.7623	7.605866	54.05957	0.928
0.2	0.0619	3.3815	7.4755	40.89	1.1437
0.225	0.08932	3.2959	8.188363	35.81263	1.3592
0.25	0.11873	3.1576	8.350058	30.48542	1.5741
0.275	0.15832	3.0515	8.463791	26.4766	1.7881

(continue)

0.3	0.21471	2.9657	8.540512	23.35023	2.0014
0.325	0.30167	2.8915	8.58262	20.83001	2.2141
0.35	0.4537	2.8197	8.580776	18.71366	2.4262
0.375	0.7882	2.7311	8.486505	16.79842	2.6378
0.4	2.1291	2.509	7.935596	14.37848	2.8491
0.41	5.5058	2.0258	6.448202	11.29983	2.9335
0.41476	19.949	0.007097	0.022655	0.039092	2.9737

$D\ (h^{-1})$	$\mu_1\ (h^{-1})$	$\mu_2\ (h^{-1})$	C_1	C_T	$\mu 1/\mu 2$
0.005	0.000097	0.004903	0.1241	0.9684	0.014971
0.01	0.000242	0.00976	0.1476	0.9385	0.009553
0.025	0.00101	0.02399	0.2146	0.8581	0.006901
0.05	0.00385	0.04615	0.3183	0.746	0.008306
0.075	0.00992	0.06508	0.4175	0.6507	0.011476
0.1	0.02163	0.07837	0.5156	0.5661	0.02174
0.125	0.04058	0.08442	0.606	0.4955	0.03947
0.15	0.06449	0.08551	0.6772	0.4443	0.070975
0.175	0.09025	0.08475	0.728	0.4092	0.112563
0.2	0.11642	0.08358	0.7668	0.3845	0.170325
0.225	0.1426	0.0824	0.7954	0.3665	0.21477
0.25	0.16865	0.08135	0.8176	0.3528	0.272017
0.275	0.19458	0.08042	0.8355	0.342	0.332307
0.3	0.22039	0.07961	0.8501	0.3333	0.395195
0.325	0.24609	0.07891	0.8623	0.3261	0.460669
0.35	0.27171	0.07829	0.8726	0.3201	0.529874
0.375	0.29726	0.07774	0.8815	0.3149	0.607005
0.4	0.32274	0.07726	0.8892	0.3105	0.726315
0.41	0.33292	0.07708	0.892	0.3089	0.932271
0.41476	0.33776	0.077	0.8933	0.30816	270.5418

E.5. Continuous Culture with Immobilized Recombinant Yeast C468/pGAC9

Run No: CICR1 (Original strain, D = 0.10 h^{-1})

t (h)	X (g/L)	P (units/L)	E (g/L)	pH
0	0	0	1.52	5.85
12	4.06	77.65	7.08	5.78
18	4.55	91.57	6.71	4.98
24	4.97	108.05	6.2	5.03
36	5.55	108.42	5.38	5.02
42	5.46	108.42	5.55	4.99
48	5.51	106.22	5.69	5.01
60	5.68	89.74	5.35	5
66	5.56	83.51	5.46	4.98
72	5.6	75.09	5.23	4.97
84	5.57	66.3	5	4.99
96	5.58	60.44	5.12	5.02
108	5.5	58.6	5.09	4.95
120	5.64	56.77	5.13	4.96
132	5.59	54.95	5.15	4.92
144	5.52	52.02	5.12	4.98
156	5.51	46.52	5.55	4.94
168	5.3	43.95	5.4	4.93
180	5.3	42.49	5.54	4.96
192	5.3	43	5.53	4.95
204	5.53	34.06	5.12	4.95
216	5.4	28.57	5.08	4.83
228	5.55	22.71	5.3	4.8
240	5.55	20.15	5.11	4.85
252	5.55	16.48	5.03	4.8
264	5.56	16.48	5.13	4.84
276	5.56	16.84	5.14	4.81

Run No: CICR2 (Selected Strain, D = 0.10 h^{-1})

t (h)	X (g/L)	E (g/L)	P (units/L)	F (%)	pH
0	0	0	0	81.62037	5.9
12	3.81728	5.72381	100.39	76.2963	5.03
18	4.775374	6.161905	156.38	71.94444	5.02
24	5.070172	5.114286	203.87	67.0463	5.03
36	5.055432	4.980952	212.08	63.61111	5.01
42	4.996472	4.971429	206.57	67.59259	5.01
48	5.055432	5.152381	204.64	66.11111	5
60	4.996472	4.952381	202.7	63.88889	5.03
66	4.922773	5.019048	194.99	74.90741	5.01
72	4.959623	5.314286	187.26	65	4.97
84	4.922773	4.895238	173.75	59.25926	4.96
96	4.775374	5.114286	166.03	53.33333	5.01
108	4.922773	4.933333	164.1	60.18519	4.93
120	4.996472	4.219048	156.38	50	4.93
132	5.018582	5.038095	160.24	52.5	4.95
144	5.033322	4.533333	143.63	44.44444	4.95
156	4.885923	5.371429	135.53	47.22222	4.95
168	4.922773	4.733333	125.49	41.66667	4.93
180	4.885923	4.857143	120.47	35.18519	4.96
192	4.981732	5.066667	113.52	31.48148	4.95
204	5.033322	4.647619	105.79	27.77778	4.95
216	5.055432	4.590476	101.16	26.85185	4.91
228	5.070172	4.304762	102.32	29.62963	4.9
240	5.055432	4.52381	104.25	23.7037	4.8
252	5.239681	4.809524	100.39	18.98148	4.83
264	5.239681	4.647619	96.53	24.62963	4.85
276	5.239681	4.447619	83.4	23.33333	4.85
288	5.622918	4.67619	78.77		4.83
300	5.645028	3.609524	71.43		4.8

Run No: CICR3 (Selected Strain, D = 0.20 h^{-1})

t (h)	X (g/L)	E (g/L)	P (units/L)	F (%)	pH
0	0	0	0		5.84
12	3.481087	6.75	74.20952	68.4285	4.91
18	3.902227	6.38	76.21905	67.2735	5.03
24	3.986455	6.41	75.80952	62.622	4.95
36	3.95136	6.32	67.39048	52.0695	4.97
42	3.972417	6.39	58.97143	46.662	4.99
48	3.916265	6.4	50.14286	42.4515	4.94
60	3.95136	6.37	44.12381	36.183	4.92
72	3.972417	6.51	40.11429	32.2035	4.93
84	3.937322	6.28	36.10476	30.0825	4.73
96	3.937322	6.41	28.78095	27.1215	4.73
108	3.88117	6.53	25.32381	22.239	4.75
120	3.775885	6.38	21.10476	21.588	4.73
132	3.81098	6.29	17.26667	20.8635	4.76
144	3.796942	6.16	14.9619	23.457	4.7
156	3.74079	6.37	13.04762	21.987	4.68

Run No: CICR4 (Selected Strain, D = 0.10 h^{-1}, G_0 = 10 g/L)

t (h)	X (g/L)	E (g/L)	P (units/L)	pH
0	0	0	0	6.28
12	3.1301	1.864	57.917	5.45
18	4.0216	1.608	86.875	5.55
24	4.4287	1.52	115.45	5.52
36	4.3234	1.612	135.14	5.52
42	4.4427	1.58	142.86	5.52
48	4.5339	1.326	136.68	5.4
60	4.3585	1.667	139.77	5.46
66	4.3514	1.48	139	5.32
72	4.5339	1.476	135.14	5.3
84	4.2321	1.712	123.56	5.31
96	4.5129	1.369	108.11	5.27
108	4.8989	1.137	105.02	5.15
120	4.548	1.313	96.528	5.22
132	4.6743	1.047	94.597	5.09
144	4.4076	1.432	84.944	5.21
156	4.0917	1.639	81.856	5.25
168	4.1409	1.567	75.292	5.2
191	5.0042	1.537	68.728	5.12
204	4.7445	0.076	57.144	5.03
216	4.6884	1.447	56.372	5.13
234	4.6533	0.082	44.017	5.41

Run No: CICR4 (Selected Strain, D = 0.10 h^{-1}, G_0 = 5.0 g/L)

t (h)	X (g/L)	E (g/L)	P (units/L)	pH
0	0	0.0393	0	6
12	1.986	0.088	36.916	5.25
18	2.8634	0.3828	55.374	5.1
24	3.095	0.1705	65.921	5.03
36	3.0249	0.1598	72.325	4.98
42	3.081	0.1147	67.805	4.98
48	3.088	0	66.675	4.94
60	3.0389	0	52.737	5.1
72	3.2495	0	49.724	5.3
84	2.9125	0.0548	51.23	4.9
96	3.1442	0	39.176	5.32
108	3.1512	0	36.916	5.32
120	2.8985	0.0313	38.046	4.91
132	3.1301	0	32.019	5.28
144	3.2705	0	40.683	5.38
156	2.9687	0	41.06	5.2
168	3.1723	0	36.163	5.45
192	3.3126	0	27.122	5.2
217	3.3337	0.051	28.252	5
240	3.5302	0	24.108	5.5
264	3.2144	0	25.615	5.1

E.6. Repeated Batch Culture in Immobilized Cell Bioreactor

Run No: RICR1 (First Run, 1 Batch = 24 hours)

Batches	*X (g/L)*	*E (g/L)*	*P (units/L)*	*F (%)*	*pH*
1	5.86	1.892	125.2	79.45	6.22
2	5.29	1.674	142.62	70.78	6.1
3	5.21	1.374	143.71	77.86	6.04
4	5.11	1.19	148.61	70.13	6.04
5	5.11	1.209	144.26	71.67	6.04
6	5.07	1.177	141.54	70.95	6.08
7	5.1	1.54	133.37	79.39	6.1
8	5.12	1.533	125.2	67.99	5.82
9	4.95	1.361	125.2	65.79	5.64
10	4.74	1.517	133.37	65.89	5.4
11	4.42	1.394	119.76	60.46	5.4
12	4.38	1.175	125.2	65.98	5.1
13	4.29	0.835	127.93	65.04	4.94
14	4.22	0.585	124.66	66.76	4.65

Run No: RICR1 (Second Run, 1 Batch = 24 hours)

Batches	X (g/L)	E (g/L)	P (units/L)	F (%)	pH
1	6.028	1.975	146.32	80.35	5.91
2	5.9275	1.681	146.32	75.67	5.81
3	5.9125	1.133	138.19	76.43	5.55
4	5.845	1.253	141.44	72.34	5.02
5	5.7875	1.225	142.66	71.97	4.92
6	5.69	1.196	141.44	71.09	5.11
7	5.5775	0.813	130.06	69.86	5.3
8	5.7225	0.704	123.96	66.78	5.25
9	4.8375	1.266	128.03	69.49	5.3
10	5.4625	1.232	134.12	72.98	5.45
11	5.0725	1.244	133.72	69.58	5.41
12	5.06	1.232	130.06	68.27	5.46
13	5.0425	1.227	129.65	68.63	5.4
14	5.0523	1.212	132.09	68.92	5.5

APPENDIX F. C CODE FOR THE PROPOSED MODEL

```
#include <stdio.h>
#include <math.h>
#include <stdlib.h>
#include <iostream.h>

/*function declarations*/

float Miu1(float g, float q, float t);
float Miu2(float g, float q, float t);
float Miu3(float q, float e);
float Miu(float g, float q, float e, float t);
float Q(float g, float q, float t);
float dQ(float g, float q, float t);
float C1(float q, float t);
float C2(float q, float t);
float Findq (float g, float q, float t);
float Fg(float q, float x);
float Fx(float g, float q, float e, float x, float t);
float Fxp(float g, float q, float e, float xp, float t);
float Fe(float g, float q, float e, float x, float t);
float Fp(float g, float q, float e, float xp, float t);
float L(float t);

/* model parameters */

static float Yxg1; /*cell yield for glucose fermentation*/
static float Yxg2; /*cell yield for glucose oxidation*/
static float Yex; /*ethanol yield for fermentation pathway*/
static float Yxe; /*cell yield for ethanol oxidation*/
static float Ypx2; /*glucoamylase yield for glucose oxidation pathway*/
static float Ypx3; /*glucoamylase yield for ethanol oxidation pathway*/
static float M1; /*specific growth rate for glucose fermentation*/
static float M2; /*specific growth rate for glucose oxidation*/
static float M3; /*specific growth rate for ethanol oxidation*/
static float K1; /*saturation constant for glucose fermentation*/
static float K2; /*saturation constant for glucose oxidation*/
```

```
static float K3; /*saturation constant for ethanol oxidation*/
static float ka; /*first regulation constant for C1*/
static float kb; /*second regulation constant for C1*/
static float kc; /*first regulation constant for C2*/
static float kd; /*second regulation constant for C2*/
static float ppl; /*probability of plasmid loss*/
static float tlag; /*lag time*/

main()
{
FILE *fout1;

/* fermentation variables */

float G, q, X, Xp, E, P;  /* Glucose, Glucose flux, Total biomass, Plasmid-bearing cell,
                             Ethanol, Glucoamylase */
float m1, m2, m3, c1, c2; /* Specific growth rates for glucose fermentation, glucose
                             oxidation and ethanol oxidation, pacing enzyme pools for
                             glucose fermentation and glucose oxidation */
float t, tfinal, delt; /* culture time, final culture time and time interval */

/* dummy variables in Runge-Kutta method */

float t1, t2, t3, t4;
float g1, g2, g3, g4;
float q1, q2, q3, q4;
float x1, x2, x3, x4;
float xp1, xp2, xp3, xp4;
float e1, e2, e3, e4;
float p1, p2, p3, p4;
float Fg1, Fg2, Fg3, Fg4;
float Fx1, Fx2, Fx3, Fx4;
float Fxp1, Fxp2, Fxp3, Fxp4;
float Fe1, Fe2, Fe3, Fe4;
float Fp1, Fp2, Fp3, Fp4;

fout1=fopen("model.dat","write");

/* getting the parameters data*/
```

```
Yxg1=0.12;
Yxg2=0.48;
Yex=3.35;
Yxe=0.65;
Ypx2=32.97;
Ypx3=33.80;
M1=0.38;
M2=0.25;
M3=0.06;
K1=0.1;
K2=0.01;
K3=0.50;
ka=25.0;
kb=10.0;
kc=1.0;
kd=4.0;
ppl=0.05;
tlag=2.5;

/* the initial data */

G=18.75;
q=5.0;
X=0.05;
Xp=0.05;
E=0.0;
P=0.0;
t=0.0;
tfinal=40.0;
delt=0.5;

fprintf(fout1,"\nt\t G\t X\t Xp\t E\t P\t q\t m1\t m2\t m3\t C1\t C2\n");


do
{

/* getting the first terms in R-K method */
t1=t;
g1=G;
```

```
if(g1<0.0)
  g1=0.0;
x1=X;
xp1=Xp;
e1=E;
if(e1<0.0)
  e1=0.0;
p1=P;
q1=Findq(g1, q, t1);

Fg1=Fg(q1, x1);
Fx1=Fx(g1, q1, e1, x1, t1);
Fxp1=Fxp(g1, q1, e1, xp1, t1);
Fe1=Fe(g1, q1, e1, x1, t1);
Fp1=Fp(g1, q1, e1, xp1, t1);

/* getting the second term in R-K method */

t2=t1+0.5*delt;
g2=g1+0.5*delt*Fg1;
if(g2<0.0)
  g2=0.0;
x2=x1+0.5*delt*Fx1;
xp2=xp1+0.5*delt*Fxp1;
e2=e1+0.5*delt*Fe1;
if(e2<0.0)
  e2=0.0;
p2=p1+0.5*delt*Fp1;
q2=Findq(g2, q, t2);

Fg2=Fg(q2, x2);
Fx2=Fx(g2, q2, e2, x2, t2);
Fxp2=Fxp(g2, q2, e2, xp2, t2);
Fe2=Fe(g2, q2, e2, x2, t2);
Fp2=Fp(g2, q2, e2, xp2, t2);

/* getting the third term in R-K method */

t3=t1+0.5*delt;
g3=g1+0.5*delt*Fg2;
```

```
if(g3<0.0)
  g3=0.0;
x3=x1+0.5*delt*Fx2;
xp3=xp1+0.5*delt*Fxp2;
e3=e1+0.5*delt*Fe2;
if(e3<0.0)
  e3=0.0;
p3=p1+0.5*delt*Fp2;
q3=Findq(g3, q, t3);

Fg3=Fg(q3, x3);
Fx3=Fx(g3, q3, e3, x3, t3);
Fxp3=Fxp(g3, q3, e3, xp3, t3);
Fe3=Fe(g3, q3, e3, x3, t3);
Fp3=Fp(g3, q3, a3, xp3, t3);

/* getting the fourth term in R-K method */

t4=t1+delt;
g4=g1+delt*Fg3;
if(g4<0.0)
  g4=0.0;
x4=x1+delt*Fx3;
xp4=xp1+delt*Fxp3;
e4=e1+delt*Fe3;
if(e4<0.0)
  e4=0.0;
p4=p1+delt*Fp3;
q4=Findq(g4, q, t4);

Fg4=Fg(q4, x4);
Fx4=Fx(g4, q4, e4, x4, t4);
Fxp4=Fxp(g4, q4, e4, xp4, t4);
Fe4=Fe(g4, q4, e4, x4, t4);
Fp4=Fp(g4, q4, e4, xp4, t4);

/* determing the new values of the fermentation variables */

t=t+delt;
G=G+delt*(Fg1/6.0+Fg2/3.0+Fg3/3.0+Fg4/6.0);
```

```
if(G<0.0)
  G=0.0;
X=X+delt*(Fx1/6.0+Fx2/3.0+Fx3/3.0+Fx4/6.0);
Xp=Xp+delt*(Fxp1/6.0+Fxp2/3.0+Fxp3/3.0+Fxp4/6.0);
E=E+delt*(Fe1/6.0+Fe2/3.0+Fe3/3.0+Fe4/6.0);
if(E<0.0)
  E=0.0;
P=P+delt*(Fp1/6.0+Fp2/3.0+Fp3/3.0+Fp4/6.0);
q=Findq(G, q, t);
m1=Miu1(G, q, t);
m2=Miu2(G, q, t);
m3=Miu3(q, E);
c1=C1(q, t);
c2=C2(q, t);

fprintf(fout1,"\n%-6.4f\t %-6.4f\t %-6.4f\t %-6.4f\t %-6.4f\t %-6.4f\t %-6.4f\t %-6.4f\t
%-6.4f\t %-6.4f\t %-6.4f\t %-6.4f",t, G, X, Xp, E, P, q, m1, m2, m3, c1, c2);

}
while(t<tfinal);

return 0;
}

/*fuctions definition*/

/* Glucose concentration dynamics */

float Fg(float q, float x)
{
float fg;
fg=-q*x;
return fg;
}

/* Cell concentration dynamics */

float Fx(float g, float q, float e, float x, float t)
{
float fx;
```

```
fx=Miu(g, q, e, t)*x;
return fx;
}

/*Plasmid-bearing cell concentration dynamics */

float Fxp(float g, float q, float e, float xp, float t)
{
float fxp;
fxp=(1.0-ppl)*Miu(g, q, e, t)*xp;
return fxp;
}

/* Ethanol concentration dynamics */

float Fe(float g, float q, float e, float x, float t)
{
float fe;
fe=Yex*Miu1(g, q, t)*x - Miu3(q, e)*x/Yxe;
return fe;
}

/* Glucoamylase concentration dynamics */

float Fp(float g, float q, float e, float xp, float t)
{
float fp;
fp=Ypx2*Miu2(g, q, t)*xp + Ypx3*Miu3(q, e)*xp;
return fp;
}

/* Specific growth rate for glucose fermentation */

float Miu1(float g, float q, float t)
{
float miu1;
miu1=L(t)*M1*g/(K1+g)*(1.0+ka*q)/(kb+ka*q);
return miu1;
}
```

```
/* Specific growth rate for glucose oxidation */

float Miu2(float g, float q, float t)
{
float miu2;
miu2=L(t)*M2*g/(K2+g)*(1.0+kc*q)/(1.0+kc*kd*q);
return miu2;
}

/* Specific respiratory growth on ethanol */

float Miu3(float q, float e)
{
float miu3;
if(q>0.0)
  miu3=0.0;
else
  miu3=M3*e/(K3+e);
return miu3;
}

/* Overal specific growth rate */

float Miu(float g, float q, float e, float t)
{
float miu;
miu=Miu1(g, q, t)+Miu2(g, q, t)+Miu3(q, e);
return miu;
}

/* Lag fuction L(t) */

float L(float t)
{
float Lt;
if (t <2.5)
  Lt=1.0/exp((tlag-t)*(tlag-t));
else
  Lt=1;
return Lt;
```

```
}

/* Function C1 */

float C1(float q, float t)
{
float c1;
c1=L(t)* (1.0+ka*q)/(kb+ka*q);
return c1;
}

/* Function C2 */

float C2(float q, float t)
{
float c2;
c2=L(t)*(1.0+kc*q)/(1.0+kc*kd*q);
return c2;
}

/* The following functions are used for determing q by Newton method */

/* Function Q=miu1/Yxg1+miu2/Yxg2-q */

float Q(float g, float q, float t)
{
float Q;
Q = Miu1 (g, q, t)/Yxg1 + Miu2(g, q, t)/Yxg2 - q;
return Q;
}

/* Function dQ/dq */

float dQ(float g, float q, float t)
{
float dQ;
dQ =L(t)* M1*g/(K1+g)*(ka*kb-
ka)*1.0/(kb+ka*q)*1.0/(kb+ka*q)/Yxg1+L(t)*M2*g/(K2+g)*(kc-
kc*kd)*1.0/(1.0+kc*kd*q)*1.0/(1.0+kc*kd*q)/Yxg2-1.0;
return dQ;
```

```
}

/* Function Findq */

float Findq (float g, float q, float t)
{
float dq;
do
{
 dq=-Q(g, q, t)/dQ(g, q, t);
 q=q+dq;
}
while (fabs(dq) >=1e-06);
return q;
}
```

APPENDIX G. EXAMPLES OF MAPLE V CODE FOR SOLVING CONTINUOUS CULTURE MODEL

```
>solve ({a+b=0.05, a/0.12+b/0.48=q, a=0.38*c*g/(0.10+g),b=0.25*d*g/(0.01+g),
>c=(1+25*q)/(10+25*q), d=(1+q)/(1+4*q), q=(0.95-0.05*g)/x}, {a, b, c, d, q, g,
>x});
```

$$\{b = .1475304194, x = -1.880248453, c = 4.415609859,$$
$$g = -.005493241210, d = -.4841469644,$$
$$a = -.09753041937, q = -.5053984544\}, \{$$
$$d = .7456922908, c = .3185159239, a = .003854750664,$$
$$b = .04614524934, g = .003289561261,$$
$$x = 7.405613417, q = .1282588583\}$$

```
>solve ({a+b=0.10, a/0.12+b/0.48=q, a=0.38*c*g/(0.10+g),b=0.25*d*g/(0.01+g),
>c=(1+25*q)/(10+25*q), d=(1+q)/(1+4*q), q=(1.90-0.10*g)/x}, {a, b, c, d, q, g,
>x});
```

$$\{q = -.5058707095, c = 4.400373925, d = -.4827919650,$$
$$x = -3.757164904, g = -.006396759073,$$
$$b = .2142726468, a = -.1142726468\}, \{$$
$$q = .3436457682, c = .5158985428, d = .5658449197,$$
$$x = 5.525336601, g = .01241459301, b = .07835001042,$$
$$a = .02164998958\}$$

```
>solve ({a+b=0.05, a/0.12+b/0.48=q, a=0.38*c*g/(0.10+g),b=0.25*d*g/(0.01+g),
>c=(1+25*q)/(10+25*q), d=(1+q)/(1+4*q), q=(3.80-0.20*g)/x}, {a, b, c, d, q, g,
>x});
```

$$\{q = -.4924279386,\ b = .3454551368,\ a = -.1454551368,$$
$$g = -.007252713626,\ x = -7.719810849,$$
$$d = -.5234257078,\ c = 4.894926204\},\ \{$$
$$q = 1.144421880,\ b = .08355916586,\ a = .1164408341,$$
$$x = 3.308824480,\ d = .3844643273,\ c = .7669030693,$$
$$g = .06654433776\}$$

where

a represents μ_1

b represents μ_2

c represents C_1

d represents C_1

g represents *G*

q represents *q*

x represents *X*

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