HOST-PLASMID INTERACTIONS AND REGULATION OF CLONED GENE EXPRESSION IN RECOMBINANT CELLS

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Abstract

Host-plasmid interactions and the regulation of cloned gene expression have been studied in recombinant cells, primarily *Saccharomyces cerevisiae*. The plasmids employed contain the inducible yeast *GAL1*, *GAL10*, or hybrid *GAL10-CYC1* promoter cloned upstream of the *Escherichia coli lacZ* gene (for β-galactosidase). The promoters are controlled by the galactose regulatory circuit, and the yeast strains contain a *reg1* mutation which interferes with catabolite repression. Therefore, cloned *lacZ* gene expression could be induced by galactose addition to glucose-containing medium. Batch and continuous fermentations were performed to study the effects of cloned gene product synthesis on the host cell, and to determine the important factors influencing the kinetics of growth, induction, and cloned gene product formation.

The inducer (galactose) level had a strong influence on cloned *lacZ* gene expression. In batch culture, the initial specific rate of β-galactosidase synthesis increased with galactose concentration. A galactose concentration of 0.4% (in 0.4% glucose medium) was sufficient for high levels of expression that were approximately proportional to plasmid number (to an average plasmid number of at least 10). The relative galactose level, or galactose/glucose ratio, was also important, and cloned gene expression increased as this ratio increased.

Plasmid stability and promoter strength were also important factors. β-galactosidase production from an *ARS1* plasmid was an order of magnitude lower than for the same plasmid with the 2μ origin. The effects of promoter strength were studied in both batch and continuous fermentations. Although at the expense of a growth rate reduction of 16%, the rate of expression from the stronger *GAL1* promoter in batch culture was 3–5 times higher than for the *GAL10* or *GAL10-CYC1* promoters. In continuous culture, steady-state β-galactosidase specific activity

with the *GAL1* promoter was 2–4 times greater than that for the *GAL10-CYC1* promoter. Despite lower plasmid stability (72% versus 86%) and lower biomass concentration (0.98 versus 1.2 g/L), productivity was approximately 3 times higher with the stronger *GAL1* promoter system.

Carbon-limited continuous fermentations were utilized to study the effects of dilution rate and induction of cloned gene expression on the recombinant system. Selection for faster growing cells, and the concentrations of glucose and galactose, were important in determining chemostat dynamics. For the *GAL10-CYC1* promoter system, biomass concentration and β-galactosidase specific activity increased with decreasing dilution rate. The biomass trend is apparently due to the growth efficiency obtained at the various dilution rates, and the utilization of the inducer as an additional carbon source. Plasmid stability dropped after induction of *lacZ* gene expression and decreased with decreasing dilution rate. Despite lower plasmid stability and flow rate, overall productivity (activity/L/hr) was substantially higher at low dilution rate.

A temperature-sensitive (ts) strain of *S. cerevisiae* was constructed and characterized. In this strain, cloned *lacZ* gene expression can be induced by either a temperature-shift (T-shift) from 30°C to 35°C or by galactose addition. In batch culture at 35°C, cloned gene expression induced by T-shift was comparable or better than that observed for galactose induction under the same conditions. Higher levels were observed, however, for galactose induction at the preferred temperature of 30°C. At either temperature, the best levels of β-galactosidase synthesis were obtained for the ts strain in galactose-containing medium.

Theoretical yield factors have been estimated for recombinant cells to investigate the effect of plasmid-directed synthesis on metabolic stoichiometry. The analysis is based upon detailed accounting of ATP utilization for nutrient uptake

and biosynthesis. The microorganism considered was *E. coli*; however, the method can be extended to other organisms for which metabolic ATP requirements can be reasonably estimated.

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CHAPTER 1

INTRODUCTION

The current and potential applications of recombinant DNA technology are numerous. In industry, processes employing recombinant microorganisms have been successful in the production of pharmaceuticals and other compounds. The productivity of such processes depends on many factors ranging from the interactions between host cell and plasmid to downstream protein separation and purification. Further research at all levels will improve the competitive status of recombinant systems.

The thesis research presented here focuses primarily on processes at the cellular level: host-plasmid interactions and regulation of cloned gene expression. Numerous genetic and environmental factors influence cloned gene expression and can be manipulated to improve product synthesis. Previous research in this area has centered primarily on the bacterium *Escherichia coli*, a procaryote. The use of eucaryotic cells for cloned gene product synthesis may have several advantages over procaryotic systems, particularly when post-translational modifications are required. The eucaryotic microorganism *Saccharomyces cerevisiae* (Bakers' Yeast) possesses some of the benefits of procaryotic cells and of higher-level eucaryotes. Although not as well characterized as *E. coli*, *S. cerevisiae* is one of the best understood eucaryotic cells. Furthermore, its extensive use over the years has shown *S. cerevisiae* to be a safe and successful industrial microorganism.

High levels of cloned gene expression are desired; however, the synthesis of large quantities of cloned gene product can be detrimental to the host cell and reduce productivity. To minimize the deleterious effects, inducible plasmid promoters may be employed, and the time and extent of plasmid gene expression regulated. In this research, *GAL* promoters were used to regulate cloned gene ex-

pression in *S. cerevisiae*. The *GAL* promoters are controlled by the yeast galactose regulatory circuit; therefore, the induction of cloned gene product synthesis, and its effects, could be studied.

The primary goals of this research were to determine the effects of plasmid-directed synthesis on the host cell, and to examine the influence of several factors on *GAL* regulated cloned gene expression. The thesis is organized in the following manner. Chapter 2 reviews yeast plasmid stability, the galactose regulatory circuit, and other topics pertinent to this work. In Chapter 3 the materials and methods common to many of the chapters are summarized. Included are the yeast strains and plasmids employed, the experimental procedures, and the analytical methods. Results are presented in Chapters 4–7.

Chapter 4 includes the results of batch and continuous fermentations designed to study the effects of inducer level, medium composition, plasmid stability, and plasmid promoter strength on the induction and extent of *GAL* regulated cloned gene expression. The importance of galactose (inducer) utilization as an additional carbon source is also discussed. The effects of growth rate (dilution rate), plasmid promoter strength, and induction of cloned gene expression during carbon-limited continuous culture are presented in Chapter 5. This chapter describes the interdependence of growth, cloned gene product synthesis, inducer metabolism, plasmid stability, dilution rate, and promoter strength. Steady-state and transient results are presented, and the importance of selection and sugar concentrations in the chemostat are discussed. Chapter 6 deals with the construction and characterization of a temperature-sensitive yeast strain in which *GAL* regulated cloned gene expression is controlled by temperature. Induction of plasmid gene expression by temperature-shift is compared with induction by galactose addition in batch and continuous culture. In Chapter 7, theoretical yield factors are estimated to inves-

tigate the effect of plasmid-directed synthesis on the metabolic stoichiometry of the cell. The calculations are performed for *E. coli*.

Specific conclusions are found at the end of each chapter. General conclusions for Chapters 4–7 are presented in Chapter 8. Finally, Appendices A and B include the preparatory medium optimization work required for the experimental investigations.

CHAPTER 2

BACKGROUND

2.1 Recombinant Yeast

2.1.1 General Properties

Yeast have long been considered successful industrial microorganisms. With the advent of molecular techniques for cloning and manipulating yeast genes and transforming yeast cells [1–2], recombinant yeast, in particular *Saccharomyces cerevisiae*, have shown promise for the production of heterologous proteins. Recombinant systems employing *S. cerevisiae* occupy a niche between those employing procaryotic and higher-level eucaryotic cells, and possess some advantages of both [3–4]. *S. cerevisiae* is not as well-characterized as the bacterium *Escherichia coli*, and its expression vectors are less versatile and produce lower levels of cloned gene product. Even so, *S. cerevisiae* has attributes that may make it the organism of choice in certain applications. It is able to secrete recombinant proteins and, as a eucaryote, can glycosylate proteins (although not in the manner done by higher cells). A great deal is known about yeast cell physiology and molecular biology, and *S. cerevisiae* is one of the best characterized eucaroytic cells. Furthermore, *S. cerevisiae* is generally considered safe, has been successfully cultivated on an industrial scale for years, and, relative to mammalian cells, can be grown in relatively inexpensive nutrient medium.

2.1.2 Yeast Plasmids

Yeast plasmid vectors fall into five major categories: integrating vectors, autonomously replicating vectors, centromere vectors, episomal vectors, and linear vectors containing telomeric DNA [1,5–6]. These plasmids have a number of common features. Most are shuttle vectors capable of propagating in *S. cerevisiae* or *E. coli*. They harbor an *E. coli* origin of replication and antibiotic resistance gene which allow replication and amplification of the plasmid in *E. coli*. The vectors also contain a yeast selectable marker allowing selection for plasmid-containing

yeast cells. This marker is often an essential gene complementing a mutation in the host strain, but drug resistance genes (e.g., conferring resistance to the aminoglycoside antibiotic G418 [5,7]) have also been utilized. The yeast sequence allowing replication varies and depends on the vector category.

Integrating plasmids [1,5–6] carry a sequence allowing integration into homologous chromosomal DNA, rather than an origin of replication. Replication occurs with the chromosomes, and, depending on the construction of the sequence, the integrated plasmids are 99–100% stable. Disadvantages include low transformation efficiency and low copy number (unless many copies of the plasmid are integrated).

Autonomously replicating plasmids [1,5–6,8] typically contain yeast chromosomal *ARS* sequences that function as origins of replication. Replication is controlled in the same manner as chromosomal DNA; however, segregational instability is high and plasmids tend to segregate to the mother cell. Due to the high degree of instability and the additional growth of plasmid-free cells, the percent of the population containing plasmids may be a low as 5% even in selective medium. Average copy number has been reported between 1 and 50 copies per cell. *ARS* plasmids efficiently transform yeast cells, and a low frequency of integration into the host chromosomes has been observed.

Centromere plasmids [1,5–6] contain a centromere (*CEN*) sequence in addition to the *ARS* fragment. This greatly increases plasmid stability (the loss per division is only 1–2%) but decreases copy number to one. Linear plasmids containing telomeric DNA [5–6] are maintained at higher copy number, but have reduced stability.

Episomal plasmids [1,5–6] utilize the replication system of the endogenous yeast 2μ plasmid. These vectors have been the most successful for the expression of recombinant proteins. Copy numbers have been reported between 5 and 100,

and are generally higher than for the *ARS* plasmids. Partitioning of the plasmid between mother and daughter cells is nearly unbiased [8], and plasmid stability is reasonably high. The vectors also transform yeast cells efficiently.

The 2μ origin of replication alone is insufficient for effective plasmid-partitioning. A *REP3 cis*-acting locus and the *REP1* and *REP2 trans*-acting proteins of the endogenous 2μ plasmid are also required [9–11]. In [cir$^+$] strains (which harbor the endogenous 2μ plasmid) only the origin and *REP3* sites are required on the episomal vector. The *REP1* and *REP2* proteins are supplied by the 2μ circle. In [cir^o] strains, however, there are no endogenous 2μ circles, and these sites must also be included in the plasmid construct. Commonly, [cir$^+$] strains are utilized, and the 2.2 kilobase EcoRI fragment (containing the 2μ origin, *REP3* site, and one of the two inverted repeats) of the B form of the 2μ circle is cloned into the hybrid vector [5].

In general, 2μ replication is controlled in the same manner as yeast chromosomal DNA — replication is initiated only once during the cell cycle [12]. However, the endogenous 2μ plasmid has an additional mechanism allowing plasmid amplification when copy number is reduced [11]. The *FLP* gene product of the 2μ circle catalyzes site specific recombination at a site in the inverted repeats, causing interconversion between the A and B 2μ forms [13]. This *FLP* inversion protein also appears to mediate 2μ copy number amplification by enabling multiple replications from one initiation event [11, 14–15]. Such amplification may compensate for the drop in copy number expected from imperfect plasmid segregation, and could play a role in the maintenance of 2μ hybrid vectors as well.

2.2 Yeast Promoters

Yeast promoters [6, 16–17] contain four basic *cis*-acting elements: an mRNA initiation site (I), a TATA box, an upstream activation site (UAS), and, in some cases, an operator site (O). The promoters are large compared to those in procaryotes; sequences more than 80 base pairs (bp), and in some cases more than 450 bp, upstream from the initiation site are critical. This size varies for promoters of different genes. *E. coli* promoters, on the other hand, are all approximately the same size, and promoter elements lie within a 45 bp region.

In yeast promoters, there may be a single or multiple initiation sites. Between 60 and 120 nucleotides upstream of the mRNA initiation site(s) is a TATA element. In some cases, as for the *CYC1* promoter, multiple TATA boxes are observed. The variable distance between the I site and TATA element contrasts with the fixed distance of 25–30 nucleotides in higher eucaryotes. In yeast, the TATA element is directional, necessary for transcription, and does not appear to specify the initiation region (as it does in the promoters of higher-level eucaryotic cells). Precise spacing between the TATA and I sites is not critical.

Upstream activating and operator sites mediate positive and negative transcriptional control, respectively. In both cases, control is effected at a distance from the genes, precise upstream spacing does not appear critical, and the sites function in either orientation. UASs can lie up to 450 bp upstream of I, and are required for initiation of transcription. These sequences cannot lie downstream of the TATA box and I site. The primary function of the UAS is to regulate gene expression. Unlike the −35 sequence in *E. coli*, UAS sequences are generally not conserved, indicating that different proteins interact at different UASs.

The *trans*-acting proteins involved in transcription initiation are not well understood. RNA polymerase II is essential for transcription; however, its precise

interaction with the *cis*-acting elements is not known. Specific regulatory proteins interacting at the UASs have been identified for some genes. One example is the *GAL4* protein (of the galactose regulatory circuit) which interacts with the upstream activating site and initiates transcription of the *GAL1* and *GAL10* genes.

2.3 Galactose Regulatory Circuit and Catabolite Repression

The uptake and catabolism of galactose by yeast is regulated by the galactose regulatory circuit [18–20]. Galactose utilization requires the induction of four structural genes: *GAL2*, *GAL1*, *GAL7*, and *GAL10*. The *GAL2* gene product is a permease which transports exogeneous galactose into the cell. The *GAL1*, *GAL7*, and *GAL10* genes encode the Leloir pathway enzymes responsible for converting the internal galactose into glucose-1-phosphate. Galactokinase (*GAL1* gene product) catalyzes the reaction from galactose to galactose-1-phosphate. Galactose-1-phosphate uridyltransferase (*GAL7* protein) and uridine diphosphoglucose 4-epimerase (*GAL10* protein) convert galactose-1-phosphate to glucose-1-phosphate. A constitutive enzyme, phosphoglucomutase (*GAL5* gene product), converts glucose-1-phosphate to glucose-6-phosphate which enters the glycolytic pathway.

Induction of transcription of the genes for the permease and Leloir pathway enzymes requires galactose, and is controlled by at least three regulatory genes: *GAL4*, *GAL80*, and *GAL3* [18–20]. The *GAL3* gene is required for normal rapid induction by galactose. The *GAL4* gene codes for an activator protein which is made constitutively [21–22], and is required for transcription of the *GAL2*, *GAL1*, *GAL7*, and *GAL10* genes. The *GAL80* gene encodes a repressor protein which, in the absence of galactose, prevents activation of transcription at the promoter sites. In one model for the interaction [18], the *GAL80* protein binds to the *GAL4* protein and prevents transcription from the *GAL* promoters. Galactose interacts with the repressor, releasing the *GAL4* protein, which is then free to activate transcription. *GAL80* transcription may also be positively controlled by the *GAL4* protein, and negatively by the *GAL80* repressor protein [23].

The *GAL7*, *GAL1*, and *GAL10* genes for the Leloir pathway enzymes are clustered on chromosome II [18–19]. *GAL1* and *GAL10* share the same upstream

activating site (UAS_G) and are transcribed divergently on separate DNA strands [24–27]. The *GAL4* activator has been shown to bind to four related 17 bp sequences within the UAS_G [28]. The DNA binding domain of the *GAL4* protein is distinct from the region required for transcriptional activation [20, 29]. Repression by the *GAL80* protein also does not appear to prevent DNA binding [28], and the *GAL4* protein might be bound to the DNA in both the uninduced and induced states. The interaction site for the *GAL80* repressor lies near the carboxyl terminus of the *GAL4* protein [30]. Since the carboxyl terminus is also thought to interact with a transcription factor [29], one possible mechanism of repression would be binding of the *GAL80* repressor to the transcriptional activation site of the *GAL4* protein [30]. This mechanism would be consistent with the model for the galactose regulatory circuit described above.

Induction of the *GAL* promoters is repressed by glucose [18–20]. The *GAL80* protein does not mediate this repression [31–32]; however, direct evidence does implicate the *GAL4* protein in carbon catabolite repression of the galactose system [28, 33]. One possible mechanism is the interference by glucose or a metabolite with the binding of the *GAL4* activator protein to the DNA. Another mechanism, independent of the *GAL4* protein, has been observed: the *GAL* regulatory site, placed upstream of the intact *HIS3* promoter and gene, renders *HIS3* gene expression subject to glucose repression [34].

Yeast carbon catabolite repression is not well understood. The mechanisms appear to be different from those of *E. coli*, and cyclic AMP is not directly involved [20]. Many mutants have been isolated, and glucose repression may include both global and specific mechanisms. Results also indicate that protein phosphorylation may play a role in catabolite derepression [17,20].

The *reg1* mutation interferes with glucose repression of galactokinase (*GAL1*

gene product), epimerase (*GAL10* gene product), and invertase, and may be allelic to the *hex2* repression mutation [20, 35–37]. Evidence suggests that the *reg1* mutation may function early in the regulation process [20].

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CHAPTER 3

MATERIALS AND METHODS

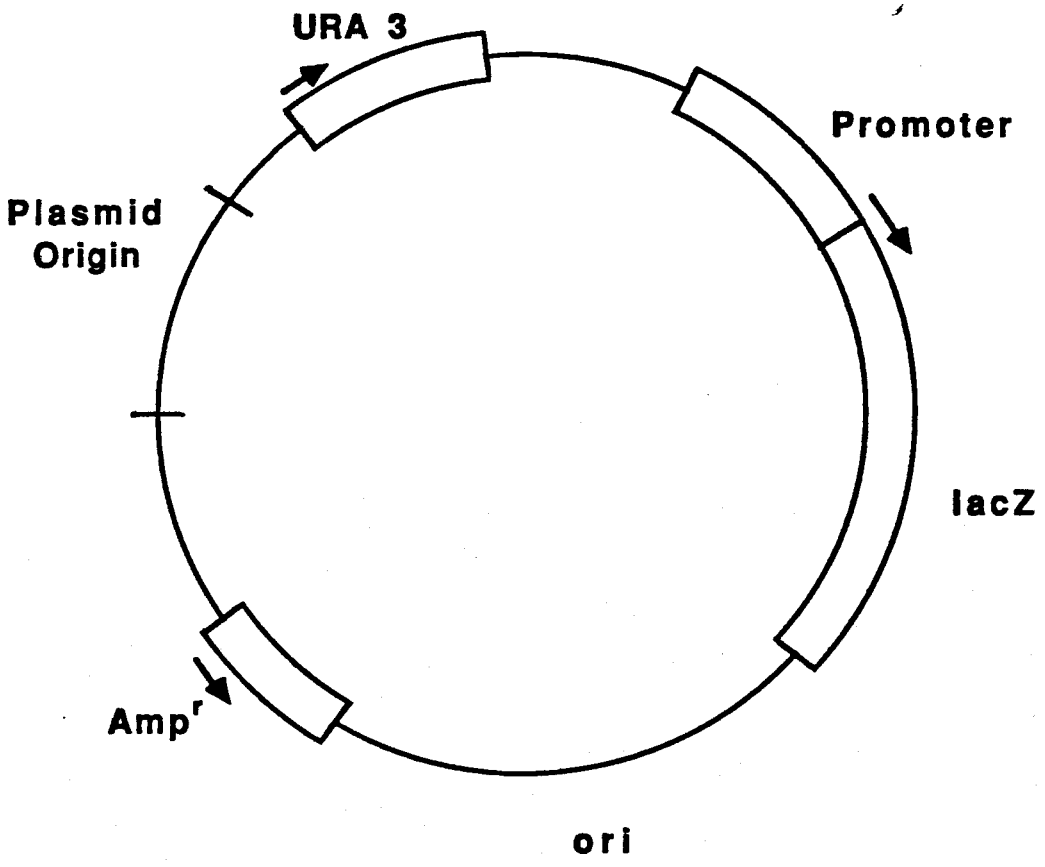
3.1 Yeast Strains and Plasmids

Saccharomyces cerevisiae strain D603 was the primary strain utilized in this research. D603 (constructed by F. Srienc [1–2]) is the a/α homozygous diploid of strain YM603 (*MAT*a *ade2-101 ura3-53 his3 lys2-801 met reg1-501*). YM603 was provided by M. Johnston. An important feature of YM603 and D603 is the *reg1* mutation which inhibits catabolite repression by glucose. These strains are [cir$^+$]: the cells harbor the endogenous yeast 2μ plasmid.

S. cerevisiae strains D603-i and D603-2i were used as control (constant "copy number") strains. D603-i and D603-2i (constructed by F. Srienc [2]) were derived from D603, and have one and two copies, respectively, of plasmid pLG2ARS1 integrated into the chromosomes. pLG2ARS1 is identical to pLGSD5 except at the origin of replication where two *ARS1* fragments replace the 2μ region of pLGSD5.

All of the plasmids utilized in this work are shuttle vectors which contain origins of replication and selectable markers allowing transformation into both *E. coli* and *S. cerevisiae*. Plasmids pLGSD5 [3], pLGARS1 [4], pRY121, and pRY123 [5–6] were derived from the same plasmid and have similar construction. The general plasmid maps are shown in Figure 3.1. The four plasmids contain the *E. coli* ColE1 origin and β-lactamase gene (conferring ampicillin resistance) of pBR322 [4], and the *S. cerevisiae URA3* gene. The *URA3* gene complements the *ura3* mutation in each host strain described above, providing a selective advantage to plasmid-containing cells.

Plasmid pLGSD5 (provided by L. Guarente) was the primary plasmid used in this research. pLGSD5 contains the 2μ origin and a *GAL10-CYC1* hybrid promoter (from *S. cerevisiae*) controlling transcription of the *E. coli lacZ* gene (for β-galactosidase). The 2μ fragment includes the origin of replication and the *REP3* (or *STB*) site from the 2μ circle [4, 7–8]. The hybrid promoter contains the 365



	Promoter	Plasmid Origin
pLGSD5 :	GAL10-CYC1	2μ
pLGARS1 :	GAL10-CYC1	ARS1
pRY121 :	GAL1	2μ
pRY123 :	GAL10	2μ

Figure 3.1 General Structure of Plasmids pLGSD5, pLGARS1, pRY121, and pRY123

base-pair *GAL10-1* intergenic region containing the *GAL* upstream activating site (UAS_G). The *GAL10* end of the region is fused to a *CYC1* fragment which includes the TATA box, transcription start site, and the first 4 base-pairs of the *CYC1* gene [3]. The hybrid promoter is fused to the *E. coli lacZ* gene (containing all but the extreme amino-terminal end of the gene) [5]. The UAS_G confers galactose inducibility on the *lacZ* gene, and β-galactosidase production is, therefore, controlled by the galactose regulatory circuit.

Plasmid pLGARS1 (provided by F. Srienc) is identical to pLGSD5 except at the origin of replication where the *ARS1* sequence replaces the 2μ fragment [4]. Plasmids pRY121 and pRY123 (provided by R. Yocum) retain the 2μ origin, but have promoter regions different from pLGSD5. pRY121 contains the *GAL1* promoter region, including the upstream sequences. The *lacZ* gene is fused to the *GAL1* amino-terminus at amino acid 33. Plasmid pRY123 is similar to pRY121 except that the *GAL1-GAL10* promoter is inverted and the *lacZ* gene is fused to the *GAL10* amino-terminus at amino acid 47 [5–6].

An additional plasmid, pNN219, was utilized in the temperature-sensitive strain construction. This vector is described in Chapter 6.

3.2 Culture Media

The complete, non-selective medium used was YPD medium [9], containing yeast extract (10 g/L, Difco), peptone (20 g/L, Difco), and glucose (20 g/L).

Minimal media included SD minimal [9] and carbon-limited (C-limited) medium, supplemented as necessary. SD minimal medium contains glucose (20 g/L) and nitrogen base without amino acids (6.7 g/L, Difco); adenine sulfate (20 mg/L), L-histidine-HCl (20 mg/L), L-lysine-HCl (30 mg/L), L-methionine (20 mg/L), and uracil (20 mg/L) were added when required. In order to obtain reasonable biomass yields during C-limited growth in the chemostat, an optimized C-limited medium was developed (see Appendix A). The composition is: glucose (4.0 g/L), nitrogen base without amino acids (6.7 g/L, Difco), adenine-sulfate (100 mg/L), L-histidine-HCl (80 mg/L), L-lysine-HCl (150 mg/L), and L-methionine (100 mg/L). For cultivation of plasmid-free cells, uracil (20 mg/L) was added. Under inducing conditions, galactose concentration was 4.0 g/L. This medium is not stable at room temperature (see Appendix B), and was refrigerated at 4°C until used.

An additional minimal medium was developed to obtain amino acid + purine-limited growth (see Appendix A). This medium has the same composition as the supplemented SD minimal medium except that the histidine concentration is 16 mg/L. Therefore, the ratios of adenine, histidine, lysine, and methionine are the same as in the C-limited medium.

Deionized or distilled water was used in all media. Media for the continuous fermentations were prepared using only distilled water. For batch cultivation, all media were buffered to pH 4.4 with citrate buffer (0.05 M). (Although the measured buffer pH at 0.05 M was 4.5, the pH of the medium was 4.4.)

YPD plates and supplemented SD minimal plates contain Bacto-agar (20 g/L, Difco) in addition to the constituents above. In YPG and glycerol minimal plates,

3% (v/v) glycerol replaces the glucose. The composition of the presporulation plates was yeast extract (8 g/L), peptone (3 g/L), glucose (100 g/L), and Bacto-agar (20 g/L). The sporulation plates contained potassium acetate (10 g/L), yeast extract (1 g/L), glucose (0.5 g/L), Bacto-agar (20 g/L), adenine-sulfate (20 mg/L). L-histidine-HCl (20 mg/L), L-lysine HCl (30 mg/L), L-methionine (20 mg/L), and uracil (20 mg/L). The recipes for all plates were from *Methods in Yeast Genetics* [9].

3.3 Batch Cultivation

3.3.1 Experimental Procedure

Batch experiments were performed in shake flasks. Culture tubes containing 5 mL medium were inoculated from single colonies and incubated in a shaker for ca. 24 hr. Erlenmeyer flasks (250 mL or 300 mL) containing 50 mL medium buffered to pH 4.4 were inoculated from the "overnight" cultures. Inoculum size was 1.0% (v/v). Temperature (usually 30°C) and oscillation speed were controlled in an air shaker (New Brunswick Scientific) at 250 RPM or a water bath shaker (Precision Scientific) at 100 oscillations/min.

During growth, samples were withdrawn for optical density (biomass) measurements, ONPG tests, and other assays. Growth rates were determined from optical density measurements made every 45 min to 1 hr during exponential growth. Linear regression of optical density versus time was used to calculate μ.

3.3.2 Induction Methods

The induction of cloned *lacZ* gene expression was studied during batch cultivation. Induction experiments were performed in two ways; the cells were either induced in exponential phase or throughout the experiment.

In the first method, "overnight" cultures were grown in glucose medium at the appropriate temperature, and used to inoculate flasks under the same conditions. During exponential growth (at approximately 0.09 g cells/L), β-galactosidase production was induced by galactose addition, temperature-shift (T-shift), or both. Biomass and β-galactosidase specific activity were then determined over the initial induction transient (the 2-2.5 generations until the end of the exponential growth phase — at a final cell density of approximately 0.43 g cells/L).

In the second method, the cells were under inducing conditions for the entire experiment. "Overnight" cultures in galactose-containing medium (and/or at the

appropriate temperature) were used to inoculate flasks under the same conditions. Samples for biomass and β-galactosidase specific activity measurements were taken during both exponential and stationary phases.

For both methods, growth-rates were calculated from the optical density (biomass) measurements, and β-galactosidase specific activity from the results of ONPG tests. All of the analytical methods are described in section 3.5 below.

3.4 Continuous Fermentations

3.4.1 Chemostat Operation

Continuous fermentations were performed in a 2L LH 500 Series Research Fermentor. The fermentor configuration is illustrated in Figure 3.2. During operation, the vessel was covered to prevent any possible light-mediated reactions in the medium (see Appendix B). The impellor speed (driven by an LH Model 502D Direct Drive Agitator) was 600 RPM, and mixing was enhanced by baffles in the vessel. All pumps utilized were Masterflex peristaltic pumps (Cole-Parmer). Medium was pumped from a reservoir at 4°C through a double-break tube into the fermentor. Flowrate was measured with a flowtube in the feed line. The working volume of 1L was maintained by an overflow exit tube. Both feed and exit flowrates were controlled with the same pump and pump drive. The pumphead and tubing were larger for the exit stream to ensure constant volume; therefore, a small amount of air also exited with this stream.

Temperature was maintained at 30°C or 35°C with an LH Model 503 Temperature Control Module. Fermentor temperature was sensed via a thermocouple input, and a heater probe was activated accordingly. When required, a cold finger was used to maintain the correct temperature. Fermentor pH was measured with an Ingold pH probe. A pH controller (Horizon, Model 5997) regulated pH at 4.5 ± 0.05 through the addition of 1N NaOH. Air flowrate was maintained at 1L/min $\pm$ 10% (LH Model 504 Air Control Module). The air was sparged into the vessel through four small sparge holes below the impellors on the agitator shaft, and passed through a condenser before exiting the fermentor. (A small fraction was also pumped out through the waste line.) Filters were used on all entering and exiting air streams. Samples were withdrawn from the fermentor through a sample tube.

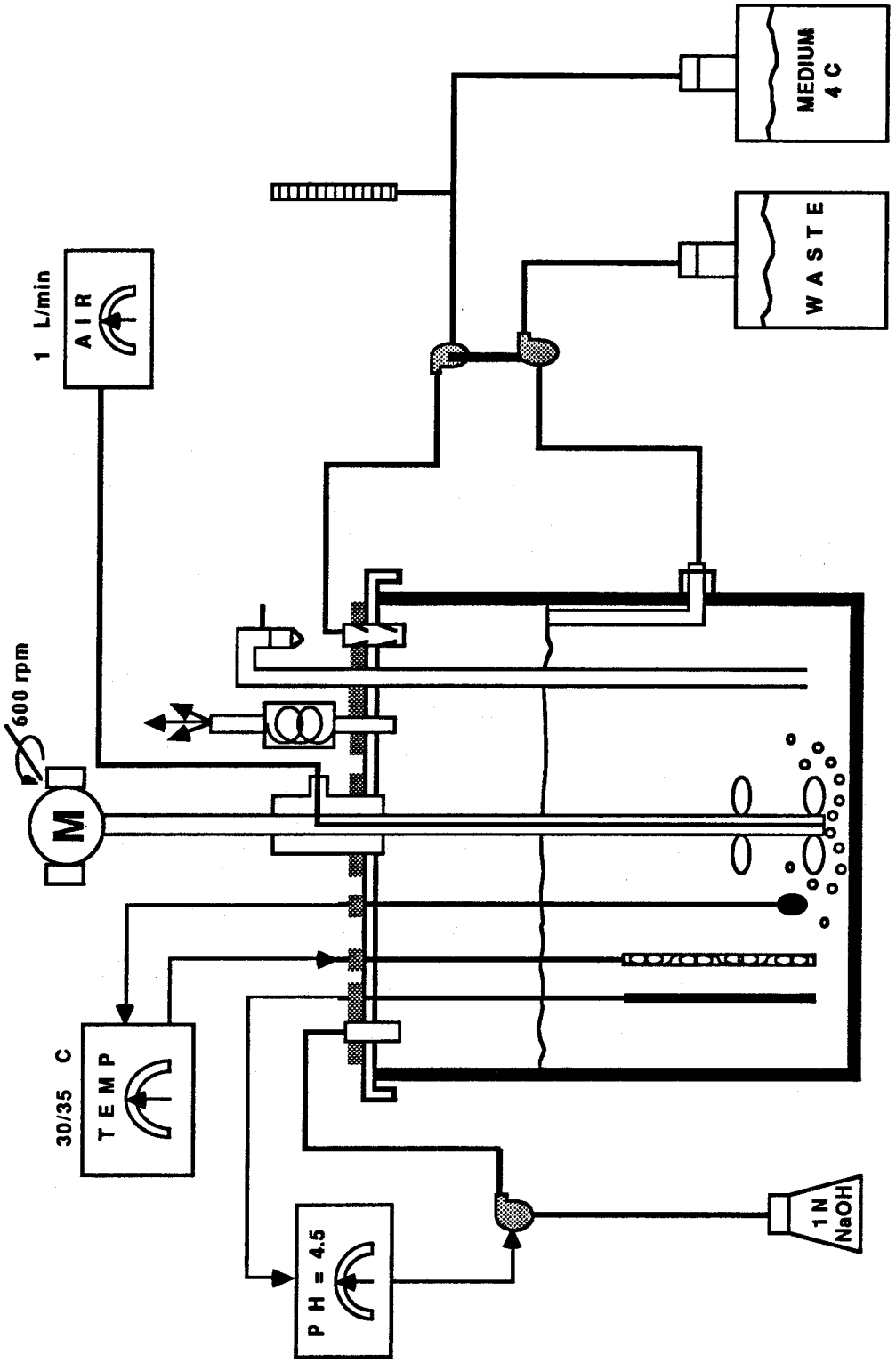


Figure 3.2 Fermentor Configuration

3.4.2 Experimental Procedure

The general procedure for the continuous fermentations was to attain an uninduced steady-state in glucose medium, induce the cells by galactose addition or T-shift, and then follow the transient to an induced steady-state.

A single yeast colony was used to inoculate two test-tube cultures, each containing 6 mL of the appropriate glucose medium. The tubes were placed in a shaker at 30°C for ca. 24 hr, and the cultures were used for inoculating the fermentor.

After preparing the medium, 5% H_2SO_4 was added to bring medium pH below 4.5. The medium was filter-sterilized through a 0.2 μm capsule filter, and refrigerated at 4°C. One liter of medium was pumped into the fermentor, and aerated, heated, and brought to the proper pH before inoculation. Cells were inoculated into the vessel with a syringe through an inoculation port and septum. Inoculum size was 1.0%. Batch cultivation proceeded until late exponential phase. At this time, the feed pump was turned on and continuous cultivation initiated.

OD_{590} measurements (biomass) indicated when the uninduced steady-state was attained. Samples were then withdrawn and the steady-state re-established before induction of cloned *lacZ* gene expression by T-shift or galactose addition. In the latter case, the feed was switched to glucose + galactose medium at the same time a pulse of galactose was injected into the fermentor. The amount of galactose added in the pulse was calculated to raise the galactose concentration in the vessel to that in the glucose + galactose feed. Glucose and galactose concentrations, biomass concentration, and β-galactosidase specific activity were followed during the transient period before the attainment of an induced steady-state.

At each steady-state, biomass concentration, β-galactosidase specific activity, sugar concentrations, and plasmid stability were determined.

3.5 Experimental Procedures

3.5.1 Yeast Transformation

S. cerevisiae was transformed by the lithium acetate method of Ito et al. [10] with the modifications of Kuo and Campbell [11]. Yeast cells were cultivated in YPD medium until mid-exponential phase. 10 mL of culture was harvested by centrifugation, washed with deionized water, washed with 0.1M lithium acetate, and resuspended in 2 mL lithium acetate (0.1M). After incubation at 30°C for 30 min, the cells were centrifuged and resuspended in 0.125 mL lithium acetate (0.1M). Plasmid DNA (1–1.5 μg) was mixed with 0.05 mL cell suspension in sterile Eppendorf tubes and incubated at 30°C for 30 min. 0.6 mL 40% polyethylene glycol 4000 in 0.01 M Tris Buffer (pH 7.6) was added, and the mixture was incubated at 30°C for 1 hr. The cells were heat shocked at 42°C for 5 min and the tubes then placed on ice. 200 μL of the cell suspension was spread on each selective plate, and the plates were incubated at 30°C until transformants appeared.

3.5.2 Mating and Sporulation of Yeast

Crosses to obtain diploid strains were performed in the following manner. Cells of the two haploid strains were mixed in a small drop of sterile water on a YPD plate. The plate was left upright at room temperature for 7–8 hr. The crosses were then streaked onto selective plates (on which only the diploid cells can grow) and placed in a 30°C incubator. To verify that the colonies which appeared on the selective plates were indeed diploid strains, the cells were sporulated.

To sporulate the cells, single colonies were transferred from selective plates to presporulation plates and incubated at 30°C for 3 days. Single colonies were then transferred from the presporulation to sporulation plates. After incubating at 30°C for 4 days, cells from the sporulation plates were checked under a microscope. The presence of tetrads confirmed that sporulation had taken place, and that the

cells were diploids.

3.5.3 Biomass Concentration Determination

Biomass concentrations (g cell dry weight/L) were determined from optical density measurements at 590 nm (Bausch & Lomb Spectronic 21) and a correlation between biomass concentration and OD_{590}: 1.49 g cells L^{-1}/OD_{590}. Appropriate dilutions were made to ensure that measurements were in the linear region of the correlation ($OD_{590} < .300$).

In order to establish the correlation, OD_{590} and biomass concentration were measured for shake flask and steady-state fermentor samples. Cell dry weight was determined by filtration or centrifugation. Samples were filtered through preweighed 0.22 μm Millipore filters and rinsed with deionized water. The filters were then transferred to an 80°C drying oven for ca. 24 hr, and placed in a dessicator to cool. Alternatively, samples were centrifuged, washed twice with distilled water, and resuspended in distilled water. The cell suspensions were transferred to preweighed aluminum pans, placed in the 80°C drying oven for ca. 24 hr, and transferred to a dessicator to cool. Cell dry weight was determined by the difference in weight of the filter (or pan) and the filter (or pan) + cells.

Cell dry weights were divided by the sample volume to determine biomass concentration, and correlated with the previously measured OD_{590}. The correlation showed no obvious dependence on cultivation method, chemostat dilution rate, or induction. The correlation was relatively constant (within experimental error) for all samples, so the average value of 1.49 g cells L^{-1}/OD_{590} was utilized.

3.5.4 Glucose and Galactose Assays

The samples for glucose and galactose assays were filtered through 0.2 μm filters and the cell-free samples frozen at −20°C until assayed.

Sigma Glucose Kit 510A (utilizing glucose oxidase and peroxidase) was used

to determine glucose concentration in the samples. Absorbance was read at 450 nm with a Shimadzu UV-160 spectrophotometer. Dilutions were made to keep the glucose concentrations within the proper range of 0.25–3.0 g/L. Optimized carbon-limited medium without glucose had no effect on the assay; the assay correctly predicted 0 g/L glucose. Therefore, very low concentrations of glucose (below 0.25 g/L) were estimated by omitting the initial 20-fold water dilution in the procedure, and adjusting the calculations accordingly.

Galactose concentrations were determined by an ultraviolet assay using galactose dehydrogenase described in *Methods of Enzymatic Analysis* [12]. The assays were performed at room temperature, and appropriate dilutions were made (the assay is accurate for galactose concentrations up to 0.3 g/L). 3.0 mL Tris Buffer (0.10 M, pH 8.6), 0.10 mL NAD solution (12.5 mg/mL, Sigma N-1636), and 0.20 mL sample were mixed in the cuvettes (1 cm light path). Extinction E_1 of the mixture was read (against air) at 340 nm in a Shimadzu UV-160 or UV-260 (slit = 2 nm) spectrophotometer. 0.02 mL galactose dehydrogenase (Boehringer Mannheim, ca. 25 U/mL) was then added to the cuvettes, and the absorbance at 340 nm followed until constant. Extinction E_2 was measured and $\Delta E = E_2 - E_1$ determined. Galactose concentration (g/L) was calculated as $\Delta E \times 0.480$ [12].

3.5.5 β-galactosidase Assay

β-galactosidase activity was assayed by ONPG tests on permeabilized yeast cells. The method is a modification of that described by Celniker et al.[4] Cell samples were centrifuged from the medium, resuspended in 0°C sodium phosphate buffer (0.02 M, pH 7), and placed on ice until permeabilization.

After spinning the cells out of the buffer and washing once with 0°C water, the cells were permeabilized with isopropanol as described by Srienc et al.[13] The cell pellet was resuspended in 2.5 mL 0°C sodium phosphate buffer (0.02 M, pH 7)

and 2.5 mL 0°C isopropanol, and incubated on ice for 10 min. The cells were then washed twice with 0°C water and resuspended in ca. 3 mL sodium phosphate buffer (0.02 M, pH 7). All spins were at 0°C–5°C, and the permeabilized cell suspensions were kept on ice until assayed.

The OD_{590} of each permeabilized sample was measured and used to determine biomass (g cells/L). ONPG tests were then conducted on the permeabilized cells. The Z buffer [14] for the tests contains $Na_2HPO_4 \cdot 7H_2O$ (16.1 g/L), $NaH_2PO_4 \cdot H_2O$ (5.5 g/L), KCl (0.75 g/L),$MgSO_4 \cdot 7H_2O$ (0.246 g/L), and β-mercaptoethanol (2.7 mL/L, added fresh daily). Z buffer pH was adjusted to 7.0 with 50% (w/v) NaOH. The ONPG solution (10 mg *o*-nitrophenyl-β-D-galactopyranoside/mL) was prepared fresh each day. The reaction mixture in the test cuvette was 2.4 mL Z buffer (prewarmed to 37°C), 0.30 mL ONPG solution, and 0.30 mL permeabilized cell suspension. In order to prevent any change in absorbance due to cell settling, the blank cuvette contained the same mixture except that 0.30 mL H_2O replaced the ONPG. Absorbance at 420 nm was followed with time at 37°C in a Shimadzu UV-260 spectrophotometer (slit = 5 nm). A rate assay program (UV-260 software) calculated the slope of the curve and a correlation coefficient. β-galactosidase specific activity (Abs_{420}/min/g cells) was determined by normalizing the activity (initial rate) by the mass of cells (g) in the cuvette.

Leaving samples on ice up to 24 hr before permeabilizing had no significant effect on measured β-galactosidase activities. Longer times on ice (before permeabilization) were not investigated. After permeabilization, cell samples could be left up to 1 week on ice with little change in β-galactosidase activities (5% change maximum).

3.5.6 Plasmid Stability Determination

The fraction of plasmid-containing cells was measured by comparing growth

on non-selective and selective plates. Cell suspensions were diluted and spread on YPD plates to obtain ca. 100 colonies/plate, and incubated at 30°C for approximately 3 days. Cells from individual colonies were then transferred to ura$^-$ minimal grid plates. The fraction of plasmid-containing cells was calculated as (number of colonies on ura$^-$ plates)/(total number transferred).

Between 400 and 600 colonies were transferred for each sample tested. Therefore, measured plasmid-containing fractions above 75% or below 25% have a statistical error of less than ± 3–4% (95% confidence interval). For plasmid-containing fractions above 90% or below 10%, error is less than ± 2–3% (95% confidence interval).

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CHAPTER 4

ENVIRONMENTAL AND GENETIC FACTORS INFLUENCING *GAL* REGULATED CLONED GENE EXPRESSION

4.1 Abstract

The effects of several genetic and environmental factors on cloned gene expression in recombinant *Saccharomyces cerevisiae* have been studied in batch and continuous fermentations. Plasmid gene expression was controlled by *GAL1*, *GAL10*, and *GAL10-CYC1* promoters regulated by the galactose regulatory circuit, and cloned gene product synthesis was induced by galactose addition. The factors studied include inducer level, medium composition, plasmid copy number, plasmid promoter strength, and plasmid stability.

In batch culture, a galactose level of 0.4% (in 0.4% glucose medium) was sufficient to induce cloned gene product synthesis from the *GAL10-CYC1* promoter at a rate approximately proportional to plasmid number (to an average plasmid number of at least 10). The relative inducer level, or galactose/glucose ratio, was also important in determining the extent of cloned gene expression.

The initial induction transients in batch culture were compared for strains containing plasmids with 2μ and *ARS1* origins. As expected, cloned gene product synthesis was much lower with the *ARS1* plasmid: average β-galactosidase specific activity was an order of magnitude lower. This was primarily due to the low plasmid stability of 7.5%. The influence of plasmid promoter strength was studied using the yeast *GAL1*, *GAL10*, and *GAL10-CYC1* promoters. The rate of increase in β-galactosidase specific activity after induction in batch culture was 3–5 times higher with the *GAL1* promoter. Growth rate under induced conditions, however, was 15% lower than in the absence of expression for this promoter system.

Continuous fermentations under carbon-limitation and amino acid + purine-limitation demonstrated the importance of medium optimization and characterization, and the selection which may occur after cloned gene induction under stringent conditions. In the latter medium, two populations of cells were quickly

established. In one no cloned gene product was formed, although the plasmid stability was measured at 100%. Very low expression levels were observed in this fermentation and can be attributed to the nutrient limitation, selection, and relative inducer level.

4.2 Introduction

Numerous genetic and environmental factors influence cloned gene product synthesis in recombinant microorganisms. Cell growth rate, medium composition, and genetic factors such as plasmid copy number and promoter strength are very important in determining productivity [1]. To improve the performance of recombinant systems, the effects and interdependence of the various factors must be understood.

High cloned gene expression levels are desired; however, this expression can have deleterious effects on the host cell and host-plasmid interactions must be considered. Plasmid replication and gene expression may compete for energy reserves, enzymes, precursors, and other necessary cell components, and thereby reduce cell growth rate and biomass yield [2]. To reduce the negative effects of cloned gene expression, plasmids with inducible promoters may be employed. The time and level of cloned gene product synthesis can then be controlled [3].

The promoters governing transcription of the Leloir pathway enzymes in *Saccharomyces cerevisiae* are one set of inducible promoters. Expression of genes cloned downstream of the *GAL7*, *GAL1*, or *GAL10* promoters is controlled by the yeast galactose regulatory circuit [4–5] and can be induced by the addition of galactose to the medium. In this system of regulation, the yeast *GAL4* gene encodes an activator protein required for transcription from the promoter sites, and the *GAL80* gene codes for a repressor. In a model for the interactions [5], the repressor binds the *GAL4* protein when no galactose is present, and prevents transcription. Upon addition, galactose binds to the repressor, releasing the activator protein which initiates transcription from the promoters. This system of regulation is subject to catabolite repression, and cells with mutations inhibiting this repression must be utilized for induction by galactose in glucose-containing

medium. The promoter for the *GAL1* gene (encoding galactokinase) is stronger than the *GAL10* promoter (regulating epimerase transcription), and, in the cell, galactokinase levels are 4 times higher than epimerase levels [6]. The galactose regulatory circuit is described in more detail in Chapter 2.

Cell growth and cloned gene product synthesis depend on the copy number and stability of the plasmid utilized. Ideally, higher copy numbers lead to higher expression levels. An optimum number of plasmids usually exists, however, beyond which the detrimental effects of high expression outweigh the positive effects of increasing the number of gene copies [7]. The level of transcription of each gene is as important as the absolute number of genes, and may depend on many factors. Plasmid stability also plays an important role in determining productivity. If plasmid stability is low, a large fraction of the population will produce no cloned gene product. In non-selective medium, the plasmid-free cells may take over the culture [3]. In selective medium, plasmid-free cells grow only until the plasmid-encoded selection protein is degraded or diluted by growth, and the plasmid-free cells reduce overall growth rate and yield [8–9].

The objective of this work was to study the effects of medium composition (including inducer level), plasmid stability, copy number, and plasmid promoter strength on cloned gene product synthesis in recombinant *S. cerevisiae*. Yeast promoters regulated by the galactose regulatory circuit were employed, and cloned gene expression was induced by galactose addition. The importance of absolute and relative galactose concentration was determined in batch culture, and expression was compared at three different gene dosages. In addition, the utilization of inducer (galactose) as a carbon source in the cells was studied. Cell growth and cloned gene expression were compared for plasmids with two different plasmid origins (conferring vastly different plasmid stabilities) and three different *GAL*

promoters. Finally, continuous fermentations were used to study the behavior of the system under very stringent growth conditions.

4.3 Materials and Methods

4.3.1 Yeast Strains and Plasmids

Saccharomyces cerevisiae diploid strain D603 was the host strain utilized in all experiments. D603 is described in Chapter 3; important features include *ura3* and *reg1* mutations. The *reg1* mutation inhibits catabolite repression by galactose.

S. cerevisiae D603-i and D603-2i were used as "constant copy number" strains. D603-i and D603-2i were constructed from D603 and contain one and two copies, respectively, of pLGARS2 (similar to pLGSD5) integrated into the chromosomes. Both strains are described in Chapter 3.

Plasmids pLGSD5, pRY121, pRY123, and PLGARS1 were utilized in this work. The general plasmid maps are shown in Chapter 3, Figure 3.1. pLGSD5, pRY121, and pRY123 contain the 2μ origin (and *REP3* site) and the *GAL10-CYC1*, *GAL1*, and *GAL10* promoters, respectively. The plasmid promoters control transcription of the *Escherichia coli lacZ* gene (for β-galactosidase). Plasmid pLGARS1 is identical to pLGSD5, except that the *ARS1* sequence replaces the 2μ fragment. All of the plasmids contain a *URA3* gene to complement the *ura3* mutation in the host. Further details on the plasmid constructions can be found in Chapter 3.

4.3.2 Culture Media

The primary growth medium was selective, carbon-limited medium supplemented with adenine, histidine, lysine, and methionine. Under inducing conditions, the initial galactose concentration was 4.0 g/L (equal to the initial glucose concentration). Selective amino acid + purine-limited medium (similar to supplemented SD minimal medium) was also utilized. For induction in this medium, the initial galactose concentration was 4.0 g/L (initial glucose concentration was 20 g/L). The media are described in Chapter 3 and Appendix A. Both were re-

frigerated at 4°C until used (see Appendix B). For batch culture, the media were buffered to pH 4.4 with 0.05 M citrate buffer.

YPD and ura$^-$ supplemented SD minimal plates were used in the plasmid stability determinations. The recipes are given in Chapter 3.

4.3.3 Experimental Methods

Batch experiments were performed at 30°C in shake flasks, as outlined in Chapter 3. For the batch induction experiments, β-galactosidase production was induced either by galactose addition in exponential phase or by cultivation in glucose + galactose medium throughout the experiment. Samples were withdrawn for biomass, β-galactosidase activity, plasmid stability, glucose, and galactose assays.

Continuous fermentations followed the procedure in Chapter 3. The cells were cultivated to an uninduced steady-state in glucose medium at 30°C. β-galactosidase production was then induced by adding a pulse of galactose to the fermentor and simultaneously switching the feed to a glucose + galactose medium. The induction transient was followed to an induced steady-state. Plasmid stability, β-galactosidase specific activity, and biomass, glucose, and galactose concentrations were determined for the samples.

All of the analytical procedures are described in Chapter 3.

4.4 Results and Discussion

4.4.1 Inducer Metabolism

Galactose, which induces *lacZ* gene expression in the strains, can be utilized as a nutrient by the cells. An experiment was performed to determine when and to what extent galactose is metabolized by strain D603, which has the *reg1* mutation inhibiting catabolite repression. D603:pLGSD5 was cultivated in 0.4% glucose and 0.4% glucose + 0.4% galactose media in shake flasks. Samples taken during the exponential and stationary growth phases were analyzed to determine biomass, glucose, and galactose concentrations.

The concentration profiles are shown in Figure 4.1. The growth curves in the two media illustrate the effects of both galactose induction and galactose metabolism. The stationary phase biomass concentration in the medium containing both sugars is 67% higher than in the glucose medium — indicating galactose utilization as a carbon source. The galactose also induces transcription from the plasmid *GAL10-CYC1* promoter, and the cells in the galactose-containing medium are producing β-galactosidase. The deleterious effects of this expression are reflected in the measured growth rates: μ is 0.30 hr^{-1} in the glucose + galactose medium and 0.33 hr^{-1} in the glucose medium.

The glucose and galactose concentration profiles are also shown in Figure 4.1. The curves in the figure were generated from glucose and galactose assays on samples from the flask containing both sugars. (A similar glucose profile was obtained for the culture in the flask containing only glucose.) By the end of exponential phase, the concentration of glucose has dropped by 62%, while the galactose concentration has remained nearly constant. After 35 hr of growth, the glucose is essentially depleted, but over 70% of the galactose still remains. Therefore, even in the *reg1* strain, glucose is the primary carbon source. Galactose

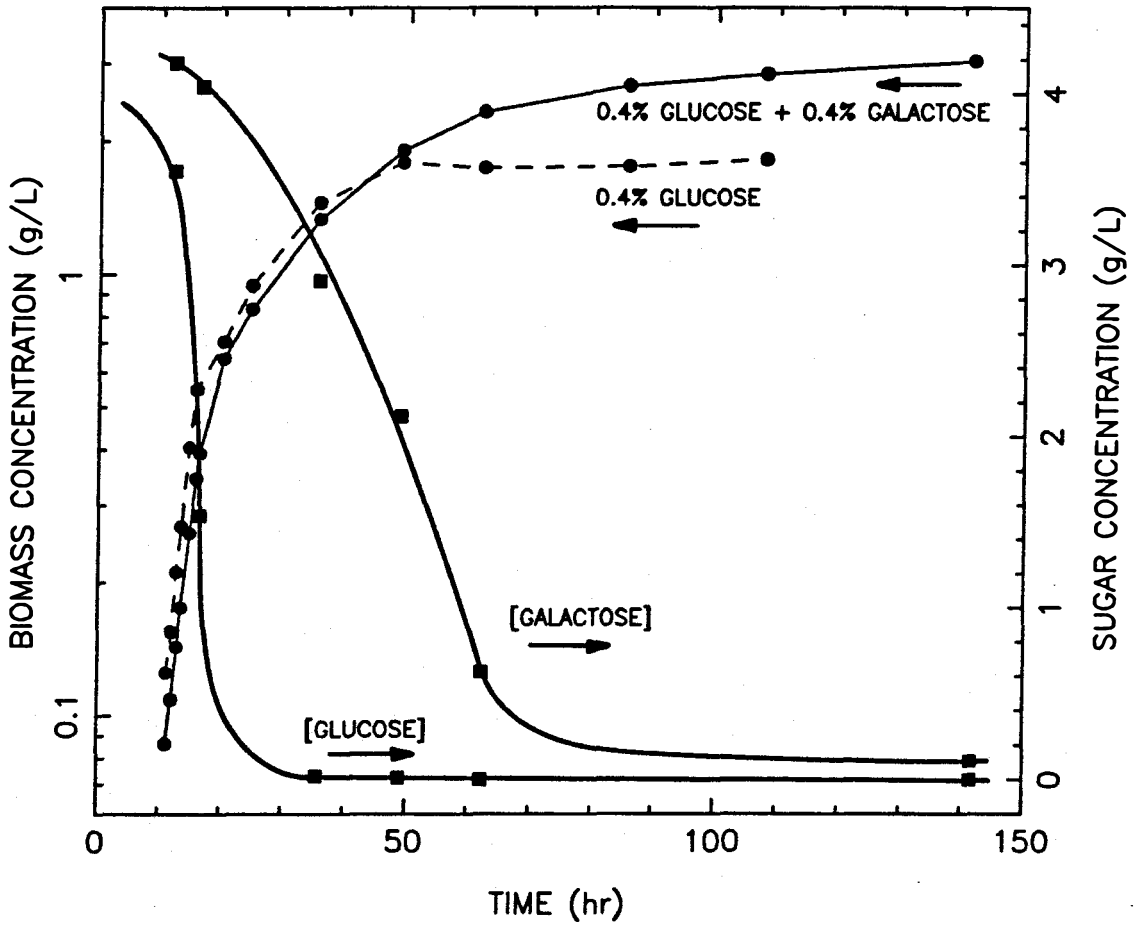

Figure 4.1 Glucose and Galactose Utilization during Batch Culture of D603:pLGSD5

is metabolized, but not preferentially; the galactose concentration drops only after the glucose is nearly depleted.

The ramifications of inducer metabolism on cloned gene expression from *GAL* promoters are important. During exponential phase in batch culture, little galactose is metabolized and galactose concentration can be assumed constant. However, under carbon-limited conditions in continuous culture, glucose levels are very low and a significant amount of galactose may be metabolized (see Chapter 5). Under these conditions, galactose is an additional carbon source, as well as an inducer. Galactose metabolism reduces the absolute inducer concentration which may affect the level of cloned gene expression. This is discussed further in sections 4.4.2 and 4.4.4.

4.4.2 Effects of Inducer Level and Gene Dosage

In the model for the galactose regulatory circuit, galactose binds to the *GAL80* repressor proteins and releases the *GAL4* proteins which then initiate transcription from the *GAL* promoter sites [5]. Therefore, for maximum cloned gene expression from *GAL* promoters, the concentration of galactose must be sufficient to inactivate the *GAL80* repressor proteins.

Batch induction experiments were performed to determine the effect of galactose concentration on the level of β-galactosidase synthesis. Various amounts of galactose were added to exponentially growing cultures of D603-i, D603-2i, and D603:pLGSD5 in 0.4% glucose medium. After 3–4 hr of induction during exponential growth, samples were withdrawn and assayed for β-galactosidase. The β-galactosidase specific activities were compared for the three strains at the various galactose concentrations. After induction during exponential phase, β-galactosidase specific activity increases nearly linearly with time. (Examples are shown in Figures 4.4–4.7.) Therefore β-galactosidase levels along the induction

transient were compared in this experiment — not the final level of β-galactosidase produced. Furthermore, due to various lags in the production of β-galactosidase for the different strains and inducer levels, the scatter in some of the experiments was considerable. Even so, very distinct trends were apparent, as shown by the results for one of the better sets of experiments in Figure 4.2.

β-galactosidase specific activity increases hyperbolically with inducer level, and the curves begin to plateau for the low copy number cells first. Recall that what is compared is essentially the initial rate of β-galactosidase synthesis. Therefore, the "extent of induction" after 3.3 hr increases with galactose concentration, presumably since more galactose is present to prevent GAL80 repression. The required inducer level is lowest for D603-i ("copy number"=1). At low copy number, fewer *GAL4* activator proteins may be required to fully induce cloned *lacZ* transcription. Slightly higher inducer levels are required for D603-2i ("copy number"=2). D603:pLGSD5 has an average copy number of approximately 10 [9–10], and β-galactosidase expression only begins to plateau at the galactose concentration of 4.0 g/L. β-galactosidase specific activity for D603:pLGSD5 continues to increase somewhat when the inducer level is increased to 8.0–10 g/L, while only slight increases are observed for D603-i and D603-2i (data not shown).

The effects of galactose concentration on the ratios of the β-galactosidase specific activities for the three strains are illustrated in Figure 4.3. The values from Figure 4.2 were used to generate this plot. Again, as inducer level increases, the curves begin to plateau. In Figure 4.2, the galactose concentration required for maximum expression decreased with copy number. Therefore, the increase in the ratios for D603:pLGSD5/D603-i and D603:pLGSD5/D603-2i with increasing galactose concentration are expected: the β-galactosidase specific activity for D603:pLGSD5 continues to increase after the specific activities for D603-i and

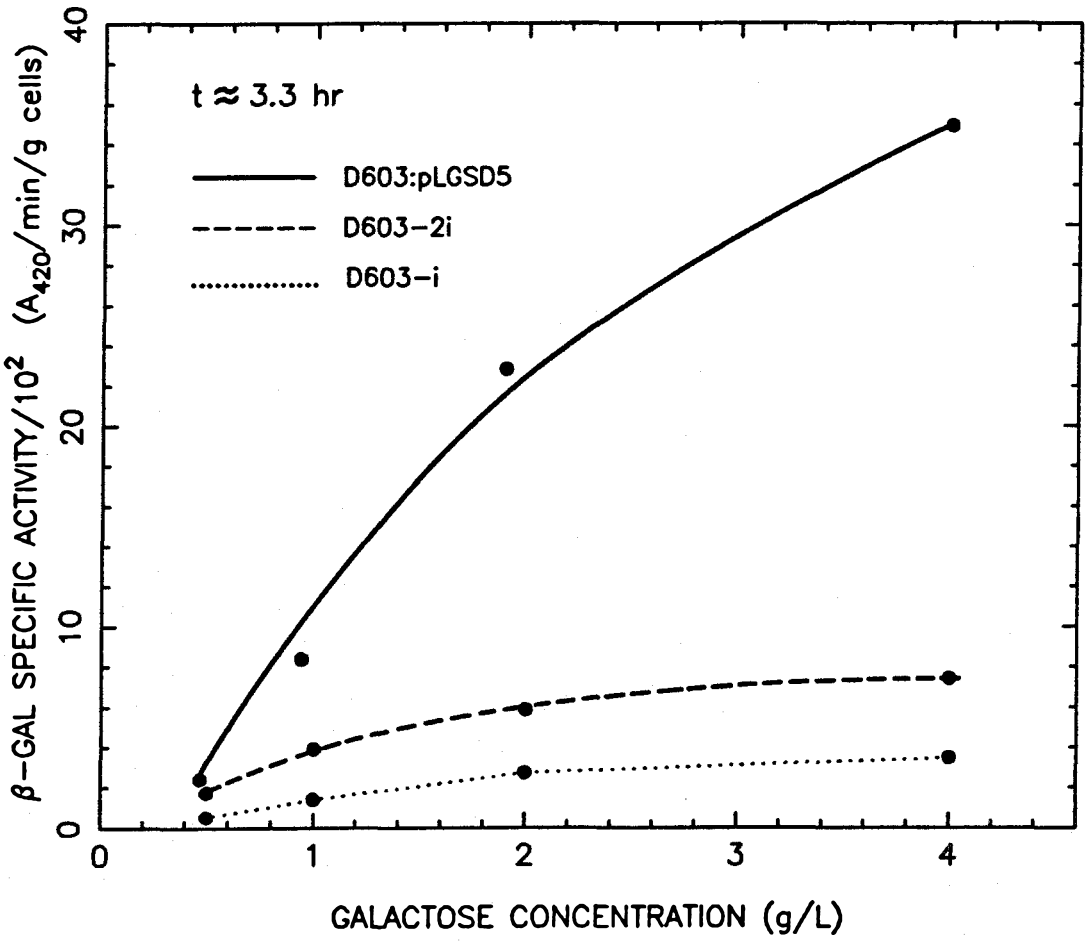

Figure 4.2 Effects of Galactose Concentration and Gene Dosage on Induction of Cloned Gene Expression

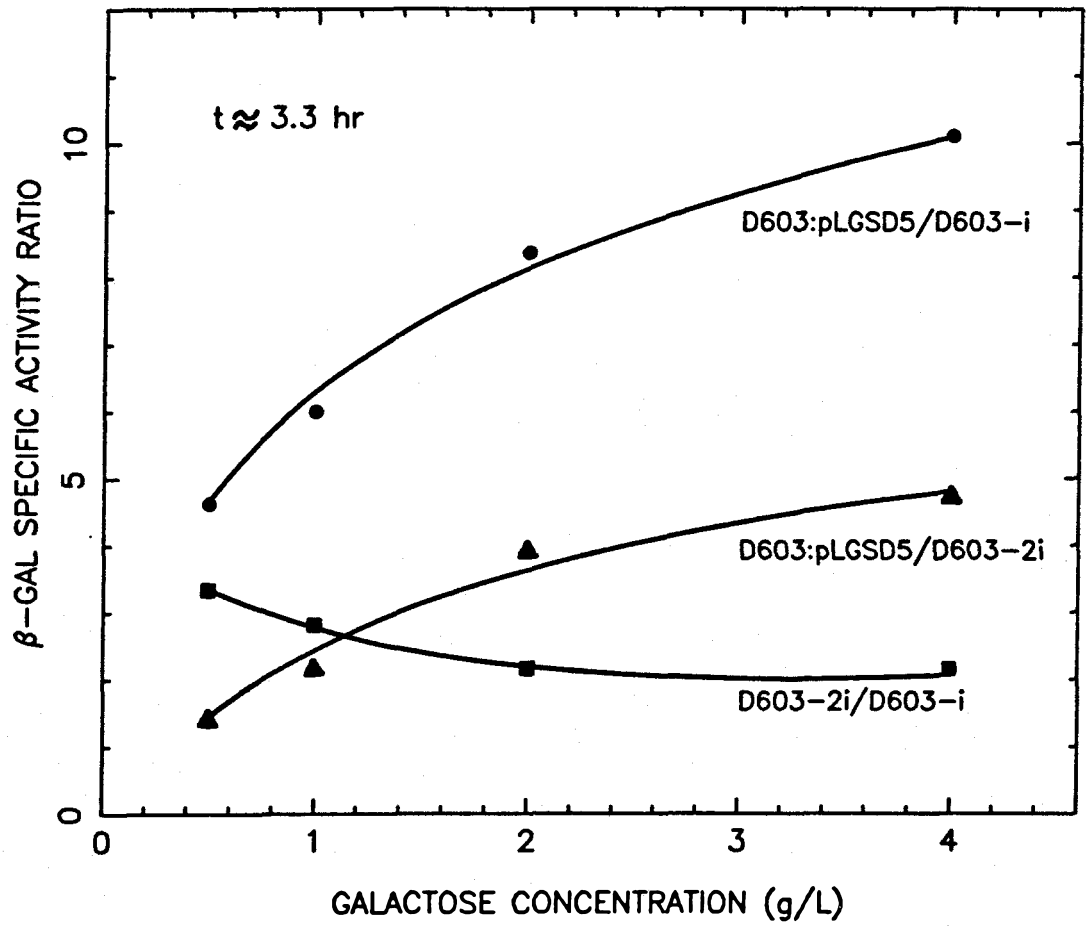

Figure 4.3 Effect of Galactose Concentration on β-galactosidase Specific Activity Ratios for D603-i, D603-2i, and D603:pLGSD5

D603-2i have plateaued. The decrease in the D603-2i/D603-i specific activity ratio with increasing galactose concentration is unexpected. The trend may reflect experimental error since β-galactosidase levels for D603-i at a 0.05% inducer level are barely within the range of the assay and the measurement errors are large. In similar experiments, little change is observed in the D603-2i/D603-i ratio beyond a 0.1% galactose level.

At the galactose concentration of 4.0 g/L, the ratios determined in this experiment were 10, 4.7, and 2.1 for D603:pLGSD5/D603-i, D603:pLGSD5/D603-2i, and D603-2i/D603-i, respectively. The values are essentially equal to the copy number ratios for the three strains. These results and the profiles in Figure 4.2 suggest that a galactose concentration of 4.0 g/L (at a glucose level of 4.0 g/L) may be sufficient to induce β-galactosidase synthesis (from the *GAL10-CYC1* promoter) at a rate proportional to *lacZ* gene dosage.

An additional batch induction experiment to study the relationship between the initial induction behavior and gene dosage was performed. Initial glucose concentration was 4.0 g/L, and β-galactosidase synthesis was induced by galactose addition (4.0 g/L) during exponential phase. Samples were taken every 40 min to 1 hr after induction; therefore, the induction transients were determined, rather than the β-galactosidase level at only one time. The results are shown in Figure 4.4.

The β-galactosidase specific activity profiles reflect the copy number for each strain. The ratios of the slopes of the lines are 2.0, 4.0, and 7.9 for D603-2i/D603-i, D603:pLGSD5/D603-2i, and D603:pLGSD5/D603-i, respectively. The copy number of 7.9–8 for D603:pLGSD5 determined from the ratios is lower than the expected value of 10. Plasmid stability (percent plasmid-containing cells) is approximately 100% for D603-i and D603-2i and 91–95% for D603:pLGSD5 [2,9]. This

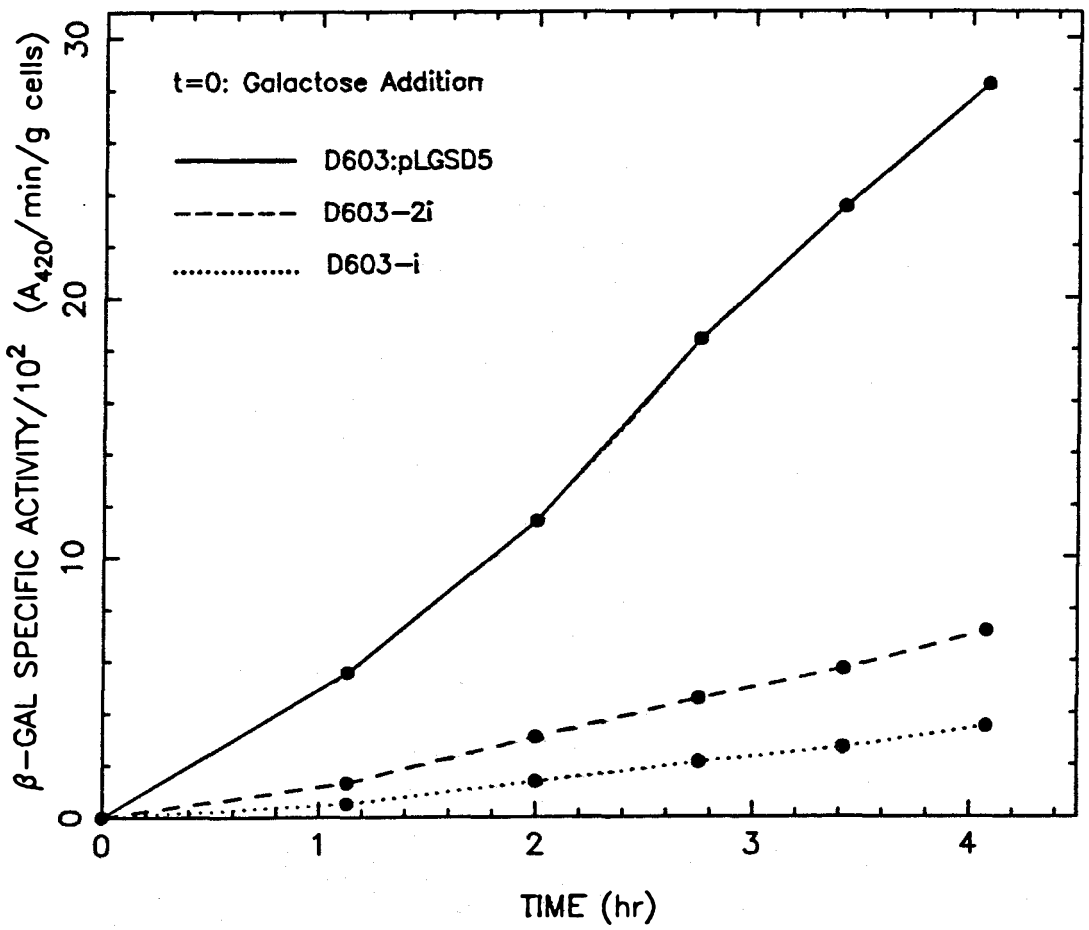

Figure 4.4 β-galactosidase Specific Activity Profiles for D603-i, D603-2i, and D603:pLGSD5 after Induction in Exponential Phase

will account for a 5–10% error in the calculated copy number for D603:pLGSD5 since β-galactosidase specific activities are actually 5–10% higher in this culture. In addition, the galactose concentration might be too low to fully induce transcription from all the promoter sites. The difference may also be due to the levels of expression from integrated versus plasmid genes, or experimental and copy number variations. The ratios fluctuated slightly from one experiment to the next.

In general, however, an inducer concentration of 0.4% (in 0.4% glucose medium) is sufficient for high levels of induction, and provides approximate proportionality between the initial rate of specific β-galactosidase production in batch culture and plasmid number. Galactose concentration has to be increased substantially to obtain significantly higher β-galactosidase levels for D603:pLGSD5. In continuous fermentations under C-limited conditions, higher levels of galactose are not desired. Furthermore, the relative inducer level, or the galactose/glucose ratio, appears to be as important as the absolute inducer level (see section 4.4.4). Glucose levels are very low in C-limited continuous culture (see Chapter 5); therefore, a galactose concentration of 0.4% may be more than sufficient for maximum cloned gene expression.

The growth-rates of D603-i, D603-2i, and D603:pLGSD5 were also measured during the exponential induction period shown in Figure 4.4. Growth rates were approximately the same for all three strains: $\mu = 0.32$–$0.33\ hr^{-1}$. Although, in some experiments, growth rate dropped slightly after induction, μ remained above the value obtained when the cells were cultivated under inducing conditions from the time of inoculation. In this experiment, the growth rate of D603:pLGSD5 is closer to that measured for uninduced cells (see the previous section). Therefore, a certain level or length of induction may be required before growth-rate is significantly reduced by *lacZ* gene expression.

4.4.3 Influence of Plasmid Origin and Promoter

Batch induction experiments were performed to study the effects of plasmid stability on cell growth and cloned gene product synthesis. Galactose (4.0 g/L) was added to exponentially growing cultures of D603:pLGSD5 and D603:pLGARS1 in selective, C-limited medium. Samples were taken every 30 min to 1 hr after induction for biomass concentration and β-galactosidase specific activity measurements. The biomass and β-galactosidase profiles are illustrated in Figures 4.5 and 4.6, respectively.

As illustrated in Figure 4.5, the growth rate for D603:pLGARS1 ($\mu = 0.19$ hr^{-1}) is substantially slower than that for D603:pLGSD5 ($\mu = 0.32\ hr^{-1}$). The average doubling time is 3.65 hr for D603:pLGARS1 versus 2.17 hr for D603:pLGSD5. Another point worth noting is the immediate drop in growth rate for D603: pLGARS1 after induction (at t=0). μ is 0.22 hr^{-1} before and 0.19 hr^{-1} after induction.

The rate of increase in β-galactosidase specific activity is also much lower (by an order of magnitude) for D603:pLGARS1. The slopes of the lines in Figure 4.6 are approximately 0.37 and 3.5 for D603:pLGARS1 and D603:pLGSD5, respectively.

The poor growth and β-galactosidase expression observed for D603:pLGARS1 are primarily due to low plasmid stability. The yeast origin of replication on plasmid pLGARS1 is the chromosomal *ARS1* fragment, while the origin for pLGSD5 is a 2μ fragment containing the 2μ origin and *REP3* site. (Recall that D603 is a [cir^+] strain.) Plasmids containing the *ARS1* fragment are much less stable, and plasmids are lost due to faulty segregation at division [8,11]. (See Chapter 2.) Plasmid stability was determined for the culture of D603:pLGARS1 after 7 hr of induction. The measured value of .075 is within the range of 0.05–0.08

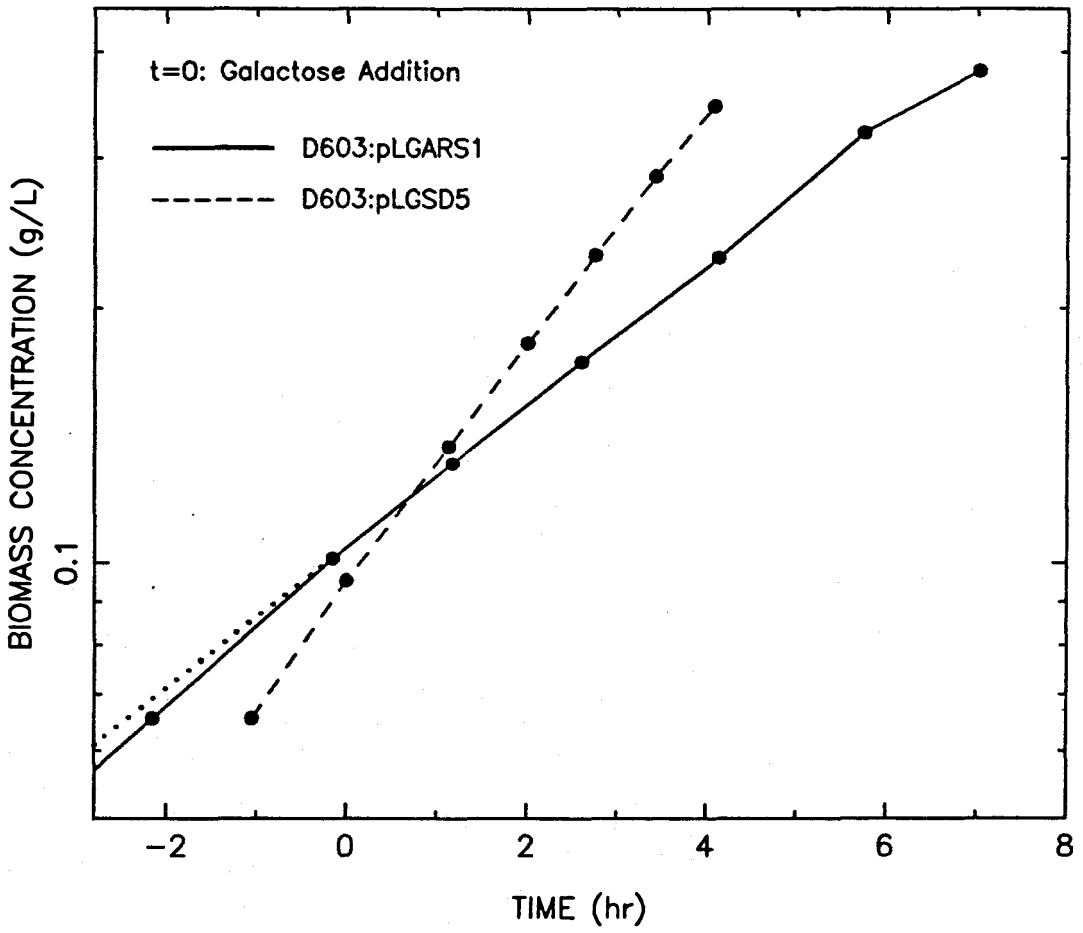

Figure 4.5 Effect of Plasmid Stability on Growth Rate after Induction of Cloned Gene Expression in Batch Culture

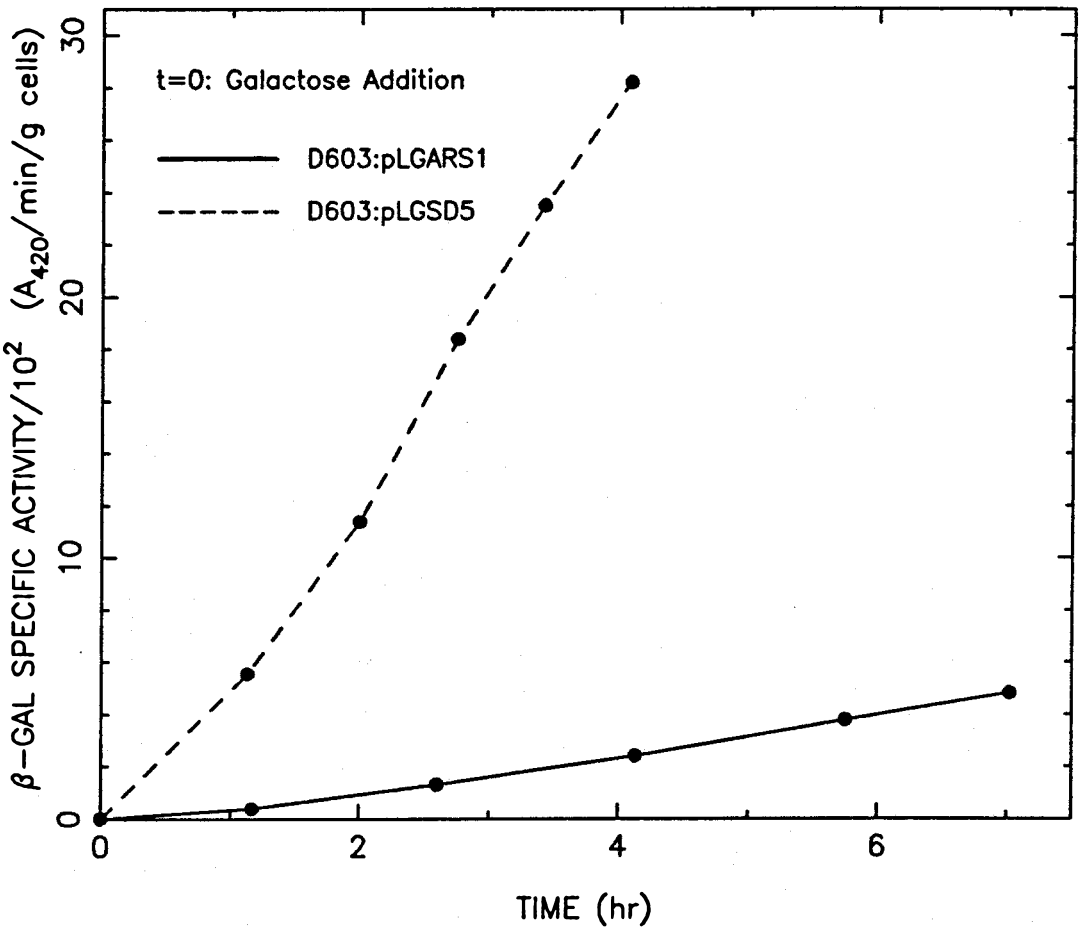

Figure 4.6 Effect of Plasmid Stability on Induction of Cloned Gene Expression in Batch Culture

reported by Srienc et al. [12]. Therefore, only 7.5% of the D603:pLGARS1 population contains plasmids, versus 91–95% for D603:pLGSD5. In selective medium, plasmid-free cells will grow only until the *URA3* gene product is broken down or diluted by growth [8–9]. Therefore, in the culture of D603:pLGARS1, more than 90% of the cells are growing slowly or not at all. Furthermore, the plasmid-free cells no longer produce β-galactosidase. The consequences are the low growth rate and β-galactosidase specific activity observed.

For specific enzyme activities based on plasmid-containing cells, the slopes in Figure 5.6 would be 4.9 and 3.8 for D603:pLGARS1 and D603:pLGSD5, respectively. These values are much closer, and the plasmid copy number for D603:pLGARS1 can be roughly estimated as 13, assuming a copy number of 10 for D603:pLGSD5. The values for D603:pLGARS1 are only approximate since some β-galactosidase is still present in the plasmid-free cell population. This increases the β-galactosidase and copy number estimates, so actual copy number may be lower.

The effects of plasmid promoter strength on growth and induction of cloned gene expression were also studied in batch induction experiments. Strains D603: pRY121, D603:pRY123, and D603:pLGSD5 were cultivated into exponential phase in 0.4% glucose medium, and *lacZ* gene expression was induced by galactose addition (0.4%). The β-galactosidase profiles after induction are illustrated in Figure 4.7.

Cloned *lacZ* gene expression is regulated by the *GAL1*, *GAL10*, and hybrid *GAL10-CYC1* promoters on plasmids pRY121, pRY123, and pLGSD5, respectively. The initial rates of β-galactosidase synthesis are similar for the *GAL10* and *GAL10-CYC1* promoters. After two hours of induction, however, the curve for *GAL10* regulated expression in Figure 4.7 begins to plateau, indicating a dif-

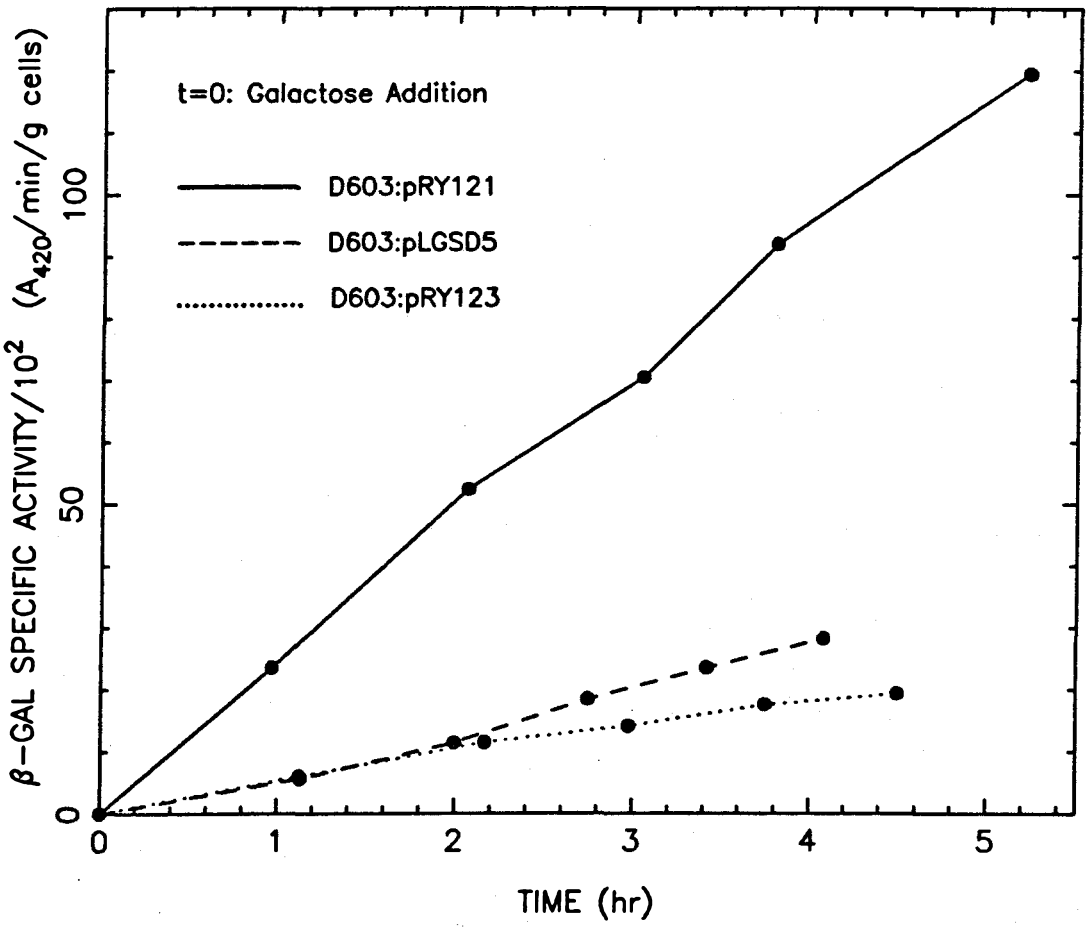

Figure 4.7 Effect of Plasmid Promoter Strength on Induction of Cloned Gene Expression in Batch Culture

ference in strength between the promoters. Both promoters contain the *GAL10* upstream activating site (UAS_G). In the *GAL10-CYC1* hybrid promoter, however, this UAS_G is fused to a fragment containing the *CYC1* TATA box and transcription start site. (See Chapter 3 for details on the plasmid constructions.)

The highest *lacZ* expression levels are observed for D603:pRY121 (containing the *GAL1* promoter). After induction, the rate of increase in β-galactosidase specific activity is ca. 3–5 times greater than with the *GAL10* and *GAL10-CYC1* promoters. The difference correlates well with the strength of the native *GAL1* and *GAL10* promoters in the cell: the level of galactokinase (*GAL1* gene product) synthesis is ca. 4 times that of epimerase (*GAL10* gene product) from the chromosomal genes [6]. In addition, Yocum et al. [6] observed 4.5 times greater expression from *GAL1* (versus *GAL10*) promoters on plasmids similar to pRY121 and pRY123.

Synthesis of large amounts of a cloned gene product useless to the cell negatively affects the growth of the microorganism. The impact of cloned gene expression from the stronger *GAL1* promoter is illustrated by the growth curves in Figure 4.8. The biomass measurements were taken after induction during exponential phase, at the same time as the samples for β-galactosidase specific activity assays (Figure 4.7). Strains D603:pRY123 and D603:pLGSD5 (containing the *GAL10* and *GAL10-CYC1* plasmid promoters, respectively) have the same growth rate: $\mu = 0.32\ hr^{-1}$. The growth rate of D603:pRY121 (*GAL1* promoter), however, drops immediately after induction of cloned gene expression, and between 2 and 4 hr is only $0.27\ hr^{-1}$ (16% lower).

The detrimental effects of plasmid protein synthesis on cell growth were also observed in additional batch fermentations of D603:pRY121 under uninduced and induced conditions. In these experiments, the strain was cultivated in 0.4% glucose

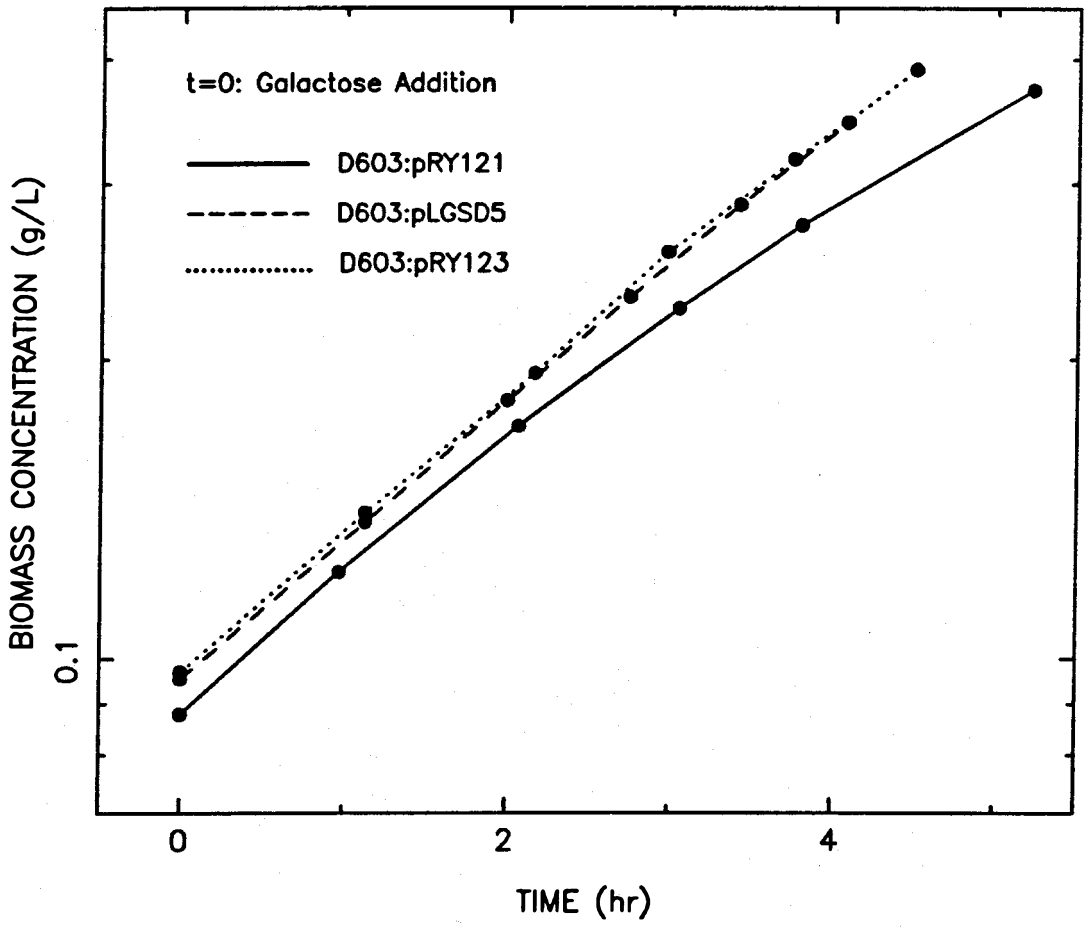

Figure 4.8 Effect of Induction of Cloned Gene Expression on Growth of D603:pLGSD5, D603:pRY121, and D603:pRY123

or 0.4% glucose + 0.4% galactose media from the time of inoculation; therefore, long term induction effects were observed. The resulting growth curves are illustrated in Figure 4.9. The maximum specific growth rate is ca. 15% lower for the cells producing β-galactosidase, and exponential phase is shorter. The specific β-galactosidase activity at 19 hr was 129×10^2 (Abs_{420}/min/g cells). The higher stationary phase biomass concentration is due to galactose metabolism, as observed for D603:pLGSD5 in Figure 4.1. The difference in μ for uninduced and induced cells is significantly greater for D603:pRY121 than for D603:pLGSD5 (see section 4.4.1). This can be attributed to the greater level of cloned gene expression from the *GAL1* promoter.

4.4.4 Effects of Nutrient-Limitation and Relative Inducer Level

Medium optimization and characterization was required to develop the supplemented carbon-limited (C-limited) medium employed in the previous batch induction experiments, and in the continuous fermentations of Chapter 5. In the original SD minimal medium supplemented with the recommended levels of necessary amino acids and purines [13], growth of *S. cerevisiae* D603 was limited by adenine, histidine, lysine, and methionine (see Appendix A). D603 is auxotrophic for these components. The effects of this limitation on cloned gene expression was studied in a continuous fermentation of D603:pLGSD5. The results illustrate the importance of nutrient limitation and relative inducer level.

The medium utilized was the selective amino acid + purine-limited (A-limited) medium described in Chapter 3 and Appendix A. It is identical to supplemented SD minimal medium except for the histidine concentration which is 20% lower. The glucose concentration is 20 g/L. Steady-state growth was established at a dilution rate of 0.2 hr^{-1}. Cloned gene expression was then induced by pulsing the reactor with galactose (to bring the concentration immediately to 4.0 g/L) and

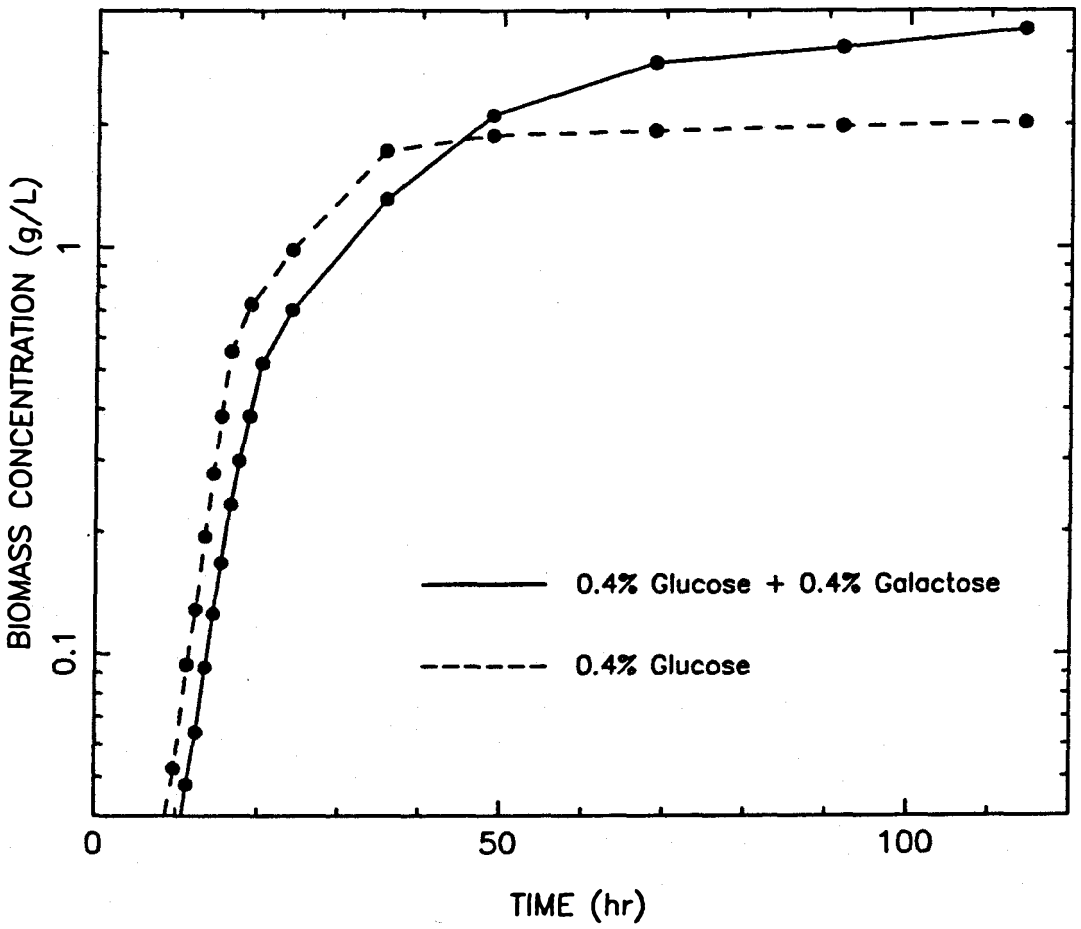

Figure 4.9 Effect of Cloned Gene Expression on Growth of D603:pRY121

simultaneously changing the feed to 20 g/L glucose + 4.0 g/L galactose A-limited medium. This galactose concentration is equivalent to that determined in section 4.4.2. The resulting β-galactosidase and biomass profiles are shown in Figure 4.10.

At time zero, galactose is added and β-galactosidase synthesis begins. Enzyme activity peaks after 8 hr, and then starts to decrease. This trend is similar to those observed in the C-limited continuous fermentations in Chapter 5; however, the magnitudes of the changes are significantly different. The results for A-limited and C-limited fermentations are compared in Figure 4.11. Much higher β-galactosidase and biomass levels are observed in C-limited continuous culture. (The results for the C-limited fermentation are discussed in detail in Chapter 5.) The difference in biomass levels is expected, since growth yields are substantially higher in the optimized C-limited medium (see Appendix A). The very low level of *lacZ* gene expression in the A-limited continuous culture can be attributed to three factors: the stringent growth conditions, selection in the chemostat, and the relative inducer level (galactose/glucose ratio).

After the peak, β-galactosidase specific activity drops rapidly to an extremely low induced steady-state level in the A-limited fermentation. The fraction of the cell population containing plasmids was determined at the uninduced and induced steady-states. Steady-state plasmid stabilities were essentially the same: 96% in the absence of galactose, and 95% in galactose-containing medium. The cell population after induction, however, consisted of two distinct subpopulations. One subpopulation grew quickly on the non-selective plates used in determining plasmid stability, and formed very large colonies. The other subpopulation grew more slowly and formed smaller colonies. The "small" population constituted approximately 70% of the total population, had a plasmid stability of 93%, and retained the ability to produce β-galactosidase. The "large" population, on the

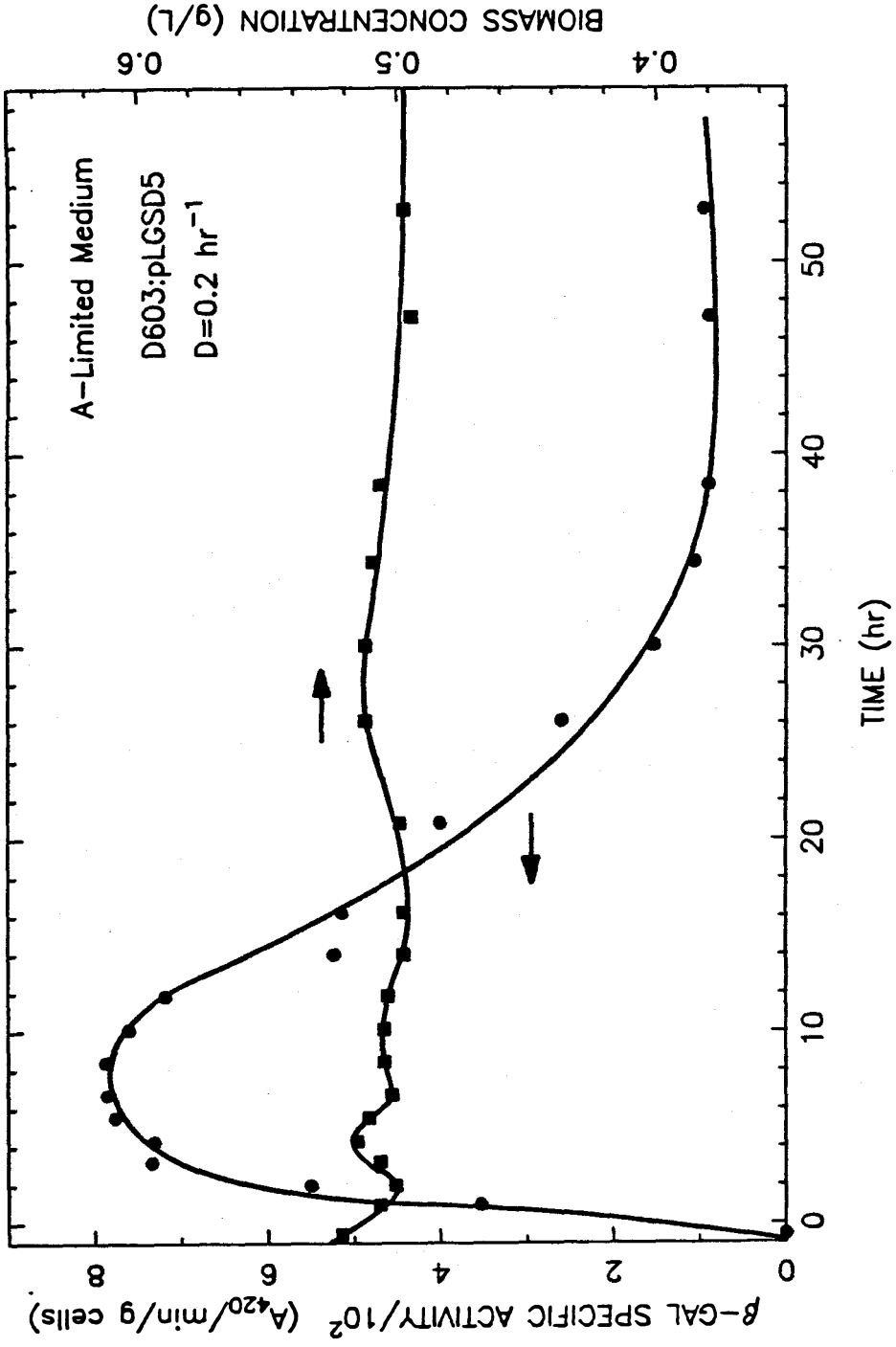

Figure 4.10 β–galactosidase Specific Activity and Biomass Concentration after Induction in Amino Acid + Purine–Limited Continuous Culture

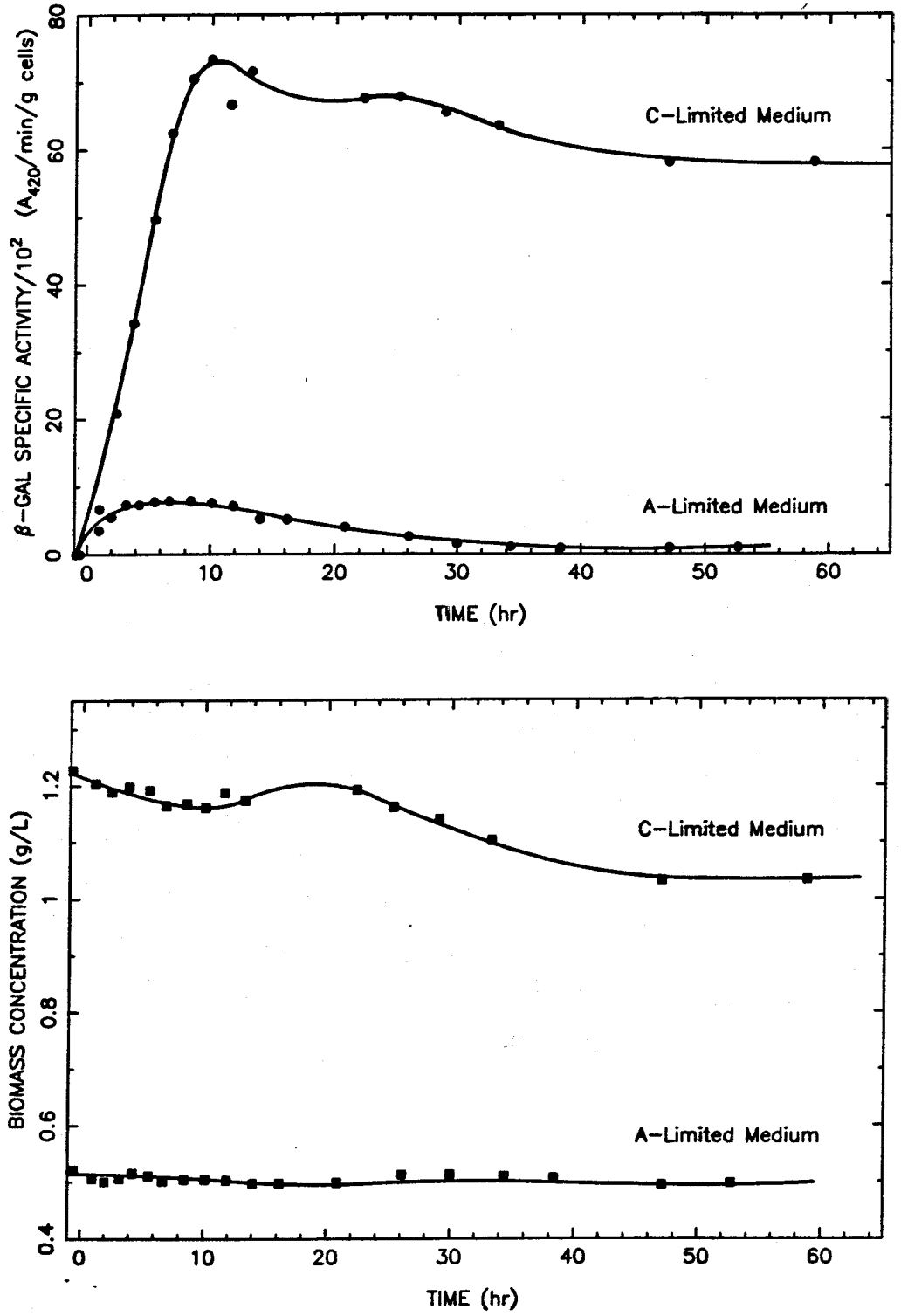

Figure 4.11 Comparison of Biomass and β-galactosidase Levels in C-Limited and A-Limited Continuous Culture

other hand, had a plasmid stability of 100%, and showed no inducible *lacZ* gene expression when tested in a batch induction experiment.

Therefore, ca. 30% of the cells (the "large" population) grew in selective medium, yet produced no β-galactosidase after induction. Contamination by a wild-type yeast is a possibility; however, the nearly constant biomass level observed in the fermentation (Figure 4.10) makes this unlikely. Wild-type yeast grow much more rapidly and have much higher yields in this medium, so biomass would be expected to increase and exceed even that observed in the C-limited fermentation. An alternate explanation might be selection in the chemostat for cells with a plasmid promoter or deletion mutation. If the *GAL10-CYC1* promoter was rendered inactive, or the promoter or *lacZ* gene deleted, the ability to synthesize β-galactosidase would be lost. After induction, cells with these mutations would have a distinct selective advantage under such stringent growth conditions and would eventually dominate in the population. Although the existence of such cells was not apparent at the uninduced steady-state, for selection to occur so rapidly, a fraction of the uninduced population would have to possess the mutation. A slight selective advantage may have existed at the uninduced steady-state as well.

The loss of β-galactosidase synthesis in 30% of the cell population and the stringent growth conditions certainly contribute to the low β-galactosidase specific activity after induction. However, the relative inducer level may be of equal or greater importance. As a control experiment, D603:pLGSD5 was cultivated in C-limited and A-limited media in batch culture. β-galactosidase synthesis was induced by the addition of 0.4 % galactose (t = 0) during exponential phase. The resulting β-galactosidase profiles are illustrated in Figure 4.12. The rate of increase in β-galactosidase specific activity is much greater in the C-limited medium. During exponential phase, however, no nutrient should be limiting. Furthermore, the

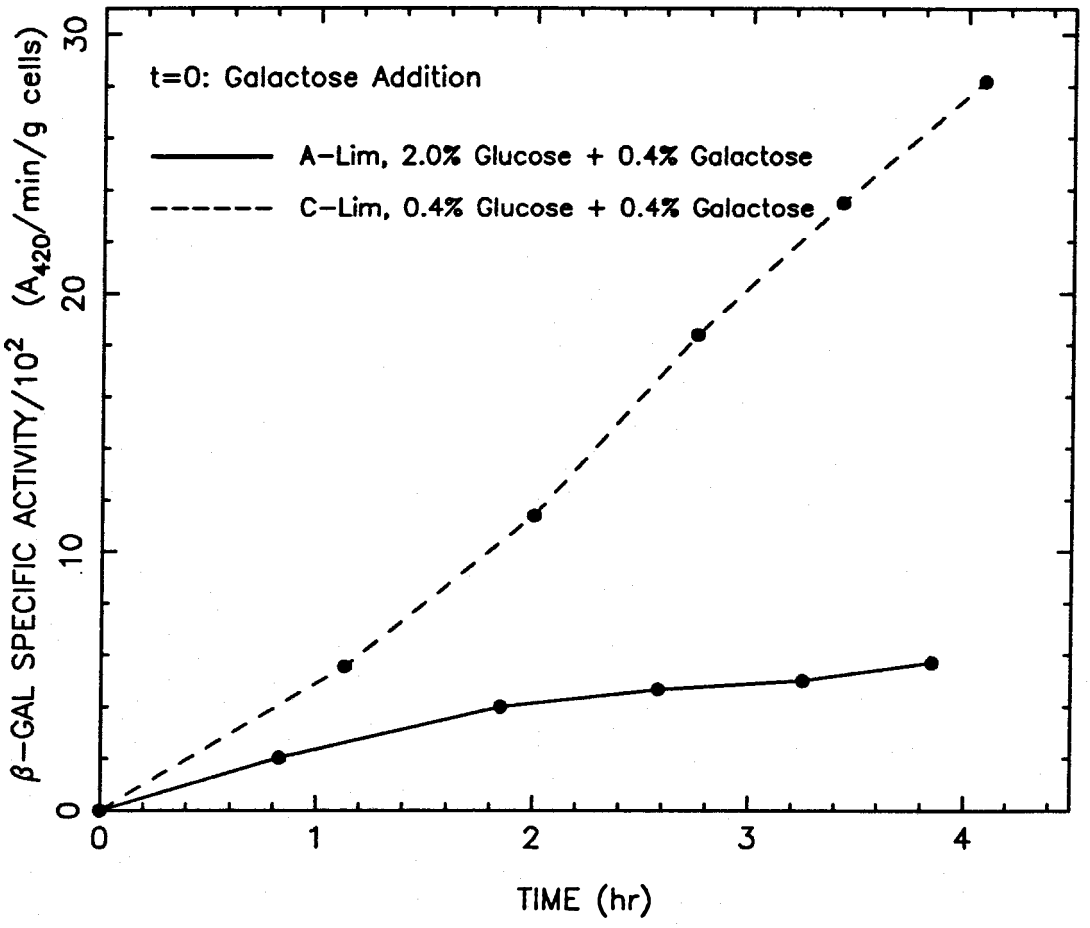

Figure 4.12 Batch Induction Behavior in A-Limited and C-Limited Media

measured growth rates were similar in the two media: μ is 0.32 hr^{-1} in C-limited medium and 0.34 hr^{-1} in A-limited medium. The growth curves after induction are shown in Figure 4.13.

Therefore, the low cloned gene expression observed during exponential phase is probably not a direct result of the effects of the medium on growth. A more likely cause is the relative inducer level, or galactose/glucose ratio. Although absolute galactose concentrations were the same, glucose concentrations are ca. 5 times higher in the A-limited medium. Furthermore, glucose levels drop more dramatically during exponential growth in the C-limited medium (see section 4.4.1). The inhibition of catabolite repression by the *reg1* mutation might depend on the relative levels of glucose and galactose, and inhibition of the galactose uptake system and inducer exclusion may be involved [14–16]. This would explain the low β-galactosidase specific activities observed in batch culture, and may partially explain the low activities during continuous culture. In the C-limited fermentation glucose concentrations were below 0.1 g/L (see Chapter 5). In the A-limited fermentation, the glucose concentration was 13.7 g/L at the induced steady-state. An initial inducer (galactose) concentration of 4.0 g/L was employed in both fermentations.

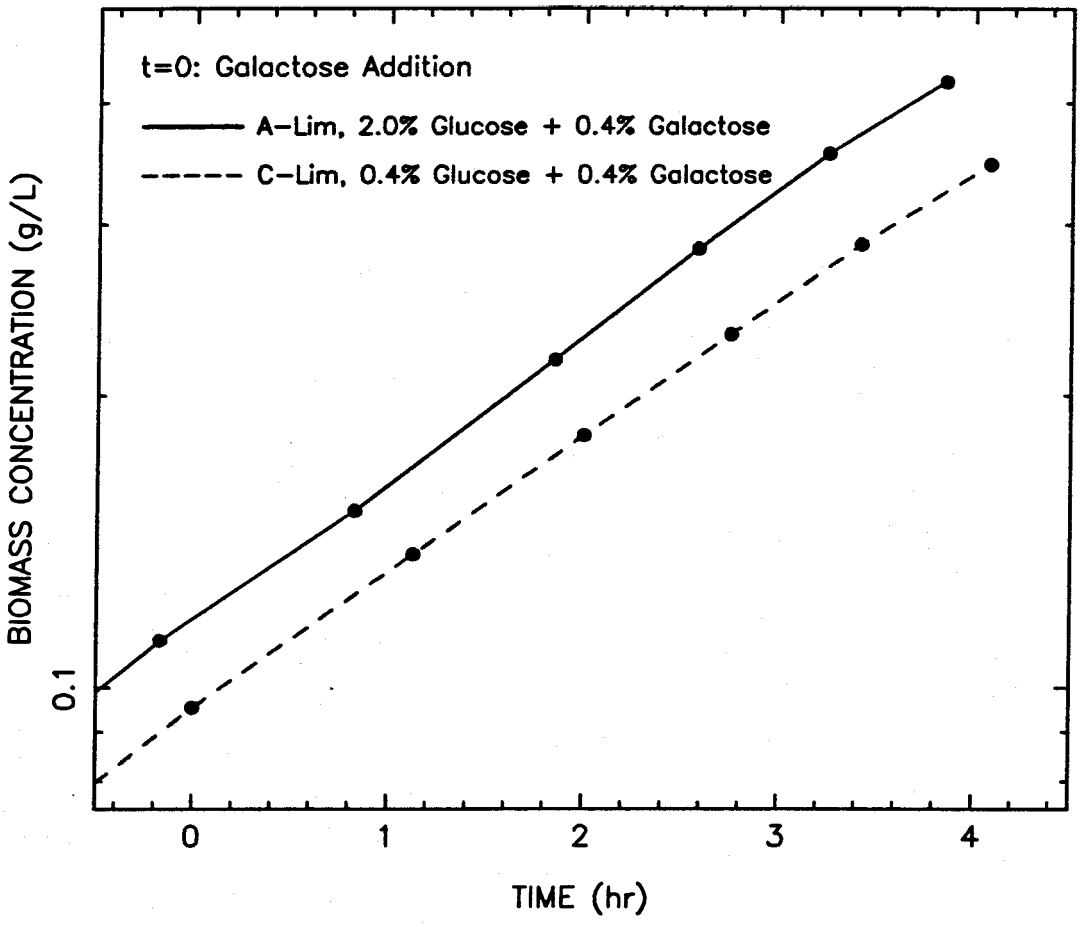

Figure 4.13 Growth Curves after Induction in A-Limited and C-Limited Media

4.5 Conclusions

Several genetic and environmental factors are important in *GAL* regulated cloned gene expression. The plasmid origin and promoter influence plasmid stability and the level of cloned gene product synthesis. As expected, plasmids with the *ARS1* origin exhibited poor growth and β-galactosidase production. The induction kinetics were compared for three different *GAL* promoters. Although at the expense of lower growth rate, β-galactosidase expression in batch culture with the stronger *GAL1* promoter was superior to that with the *GAL10* and *GAL10-CYC1* promoters.

The detrimental effect of cloned gene expression on cell growth was apparent in batch fermentations. Maximum specific growth rates were lower under induced conditions. For strain D603:pRY121 (containing the *GAL1* promoter), μ was 15% lower in galactose-containing medium. In continuous culture under stringent growth conditions (amino acid + purine limitation), induction of cloned gene product synthesis led to selection for cells producing low levels of β-galactosidase. These cells have a selective advantage due to higher maximum specific growth rates. The reverse question of how growth rate affects cloned gene expression will be addressed in the next chapter.

The inducer concentration is also extremely important in determining the level of expression. During batch culture, glucose was metabolized preferentially by the cells. After glucose depletion, however, galactose was metabolized as a carbon source. This inducer metabolism has important ramifications in continuous culture, as seen in the next chapter. In batch culture, the initial specific rate of β-galactosidase synthesis increased with galactose concentration. A galactose concentration of 0.4% (in 0.4% glucose medium) was sufficient for high levels of expression that were approximately proportional to plasmid number (to an average

plasmid number of at least 10). For a galactose concentration of 0.4%, cloned gene expression during exponential phase in 0.4% glucose medium was superior to that in 2.0% glucose medium. These results suggest that the relative inducer level, or galactose/glucose ratio, is very important in determining the extent of expression. This may result from an imperfect inhibition of catabolite repression by the *reg1* mutation.

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CHAPTER 5

INFLUENCE OF DILUTION RATE AND PLASMID PROMOTER STRENGTH IN CONTINUOUS FERMENTATIONS OF RECOMBINANT YEAST

5.1 Abstract

The effects of growth rate and plasmid promoter strength on cloned gene product synthesis in recombinant *Saccharomyces cerevisiae* have been studied in continuous culture. The plasmids employed contain the yeast *GAL1* and *GAL10-CYC1* promoters regulating expression of the *Escherichia coli lacZ* gene. Cloned gene expression was therefore controlled, and the induction process and its effects were studied.

At all growth rates (dilution rates), plasmid stability decreased with induction of *lacZ* gene expression. In some instances, two induced "steady-states" were observed, the first 10–15 τ_R after induction and the second after 40–50 τ_R. The second induced steady-state was characterized by greater biomass concentration, lower β-galactosidase specific activity, and slightly higher plasmid stability relative to the first induced "steady-state."

In the absence of *lacZ* gene expression, plasmid stability increased as dilution rate decreased. Under induced conditions, however, the opposite trend was observed: plasmid stability declined with dilution rate. β-galactosidase specific activity and biomass concentration increased as dilution rate was reduced, and, despite lower flowrate and plasmid stability, overall productivity (activity/L/hr) was substantially higher at low dilution rate. Important factors influencing all of the trends were the glucose and galactose (inducer) concentrations in the vessel, and inducer metabolism.

The influence of plasmid promoter strength on induction behavior and cloned gene expression was studied in continuous fermentations. As expected, higher β-galactosidase production and lower biomass concentration and plasmid stability were observed for the strain bearing the plasmid with the *GAL1* promoter. Despite the decrease in biomass concentration and plasmid stability, overall productivity

using the *GAL1* promoter was three times that obtained with the *GAL10-CYC1* promoter.

5.2 Introduction

The productivity of recombinant cell systems depends on many factors ranging from the interactions between host cell and plasmid to the downstream processing of the product. One important consideration at the cellular level is the influence of plasmid gene expression on the host. High expression levels are desired; however, the synthesis of a cloned gene product places additional stress on the cells. This can result in lower growth rates and yields, and reduce overall productivity [1–2]. To minimize the deleterious effects, inducible plasmid promoters are often employed and the time over which product synthesis occurs is regulated. A typical strategy in batch fermentations is cultivation of the cells to high density, and then initiation of a high level of cloned gene expression by induction of a strong promoter, copy number amplification, or both [2–3]. An analogous strategy in continuous fermentations is use of a multi-stage system, with uninduced cells in the first vessel and cloned gene product synthesis in the second (or later) vessels [4]. In both cases, partial separation of growth and cloned gene expression is achieved.

The yeast galactose regulatory circuit offers one method of control over cloned gene product synthesis in *Saccharomyces cerevisiae*. Expression of genes cloned downstream from *GAL1* or *GAL10* promoters can be induced by galactose addition [5–6]. This system of regulation is subject to catabolite repression, and cells with mutations inhibiting this repression must be utilized for induction by galactose in glucose-containing medium. The galactose regulatory circuit is described in more detail in Chapter 2.

High density, well-characterized continuous fermentations hold great potential for the production of proteins, particularly secreted proteins, from recombinant hosts. In addition, continuous culture is an excellent method for obtaining repro-

ducible cell states, and for studying the effects of growth rate, nutrient limitation, and other factors on the kinetics of growth, induction of cloned gene expression, and product formation. At steady-state, chemostat conditions are well-defined. Biomass and substrate concentrations are time-invariant, and the cells are in a state of balanced growth with average specific growth rate (μ) defined by medium feed rate (F) and reactor volume (V): μ = Dilution Rate (D) = F/V hr^{-1}. In batch culture, balanced growth is only attained (if at all) during the relatively short period of exponential growth. One drawback to continuous operation, however, is the selection for faster growing genetic variants that may occur over time in the chemostat.

The objective of this work was to study the influence of dilution rate, plasmid promoter strength, and induction of cloned gene expression during carbon-limited continuous culture of recombinant yeast. Cloned gene expression was regulated by promoters controlled by the galactose regulatory circuit, and was induced by addition of galactose to the medium. Steady-state behavior under uninduced and induced conditions was studied, as well as the transient induction behavior between the steady-states. This chapter describes the interdependence of biomass, β-galactosidase production, inducer metabolism, and plasmid stability at various dilution rates. Productivities at the dilution rates are also compared. In addition, the effects of increasing plasmid promoter strength on the recombinant system are presented, and the general consequences of cloned gene expression (including selection in the chemostat) are discussed.

5.3 Materials and Methods

5.3.1 Yeast Strains and Plasmids

Saccharomyces cerevisiae diploid strain D603 was utilized in this work. D603 contains *ura3* and *reg1* mutations; the *reg1* mutation inhibits catabolite repression by glucose. This strain is described in more detail in Chapter 3.

Plasmids pLGSD5 and pRY121 were the two plasmids used in this study. Both contain the 2μ origin (and *REP3* site), a *URA3* gene (to complement the *ura3* mutation in D603), and a yeast promoter regulating expression of the *E. coli lacZ* gene (for β-galactosidase). The promoters are a *GAL10-CYC1* hybrid promoter in pLGSD5 and the *GAL1* promoter in pRY121. Additional details on the plasmids can be found in Chapter 3.

5.3.2 Culture Media

Selective carbon-limited medium (supplemented with adenine, histidine, lysine, and methionine) was used in all fermentations. The medium was refrigerated at 4°C until pumped into the fermentor. YPD and ura$^-$ supplemented SD minimal plates were utilized in plasmid stability determinations. All recipes are given in Chapter 3.

5.3.3 Experimental Methods

Continuous fermentations were performed as outlined in Chapter 3. In the chemostat, temperature was maintained at 30°C, pH at 4.5, volume at 1 L, impellor speed at 600 RPM, and air flowrate at ca. 1 L/min.

After reaching the uninduced steady-state in 0.4% glucose medium, *lacZ* gene expression was induced by galactose addition, and the transient was followed to an induced steady-state. To induce the cells, a pulse of galactose was injected into the fermentor to bring galactose concentration immediately to 0.4%. The feed was

simultaneously changed to 0.4% glucose + 0.4% galactose medium.

Samples were assayed for biomass concentration, β-galactosidase specific activity, glucose and galactose concentrations, and plasmid stability. All of the analytical procedures are described in Chapter 3.

5.4 Effects of Dilution Rate

5.4.1 Biomass and Cloned Gene Expression Levels

The maximum specific growth rate of D603:pLGSD5 during batch cultivation in the fermentor was approximately 0.33 hr^{-1} in glucose medium. Under induced conditions, however, μ drops by ca. 10%. The range of dilution rates was chosen to avoid washout, and continuous fermentations were performed at dilution rates of 0.1, 0.2, and 0.26 hr^{-1}.

The results for a dilution rate of 0.1 hr^{-1} are shown in Figure 5.1. At time zero, the cells, at an uninduced steady-state in glucose medium, were induced by galactose addition. The responses in biomass and β-galactosidase production are illustrated. β-galactosidase specific activity rises rapidly, reaches a peak after 2–3 residence times (2–3 τ_R), and then drops. (The mean residence time is the time required for one reactor volume to pass through the system and is equivalent to the reciprocal of dilution rate: τ_R (hr) = V/F = 1/D. In this case, τ_R=10 hr.) After a slight initial drop, biomass concentration also increases steadily for 2 τ_R. Biomass concentration then begins to oscillate, and increases gradually with time. After reaching the maximum level, β-galactosidase specific activity begins to oscillate as well. With few exceptions, the oscillations are matched; as β-galactosidase specific activity increases, biomass decreases, and vice-versa. Recall that specific enzyme activity was determined by dividing activity by the cell density of the permeabilized ONPG test sample. This biomass measurement is independent of that used for the biomass curve. Therefore, the matched oscillations are not explained by experimental measurement error.

Despite the oscillatory behavior, the mean values of biomass concentration and β-galactosidase specific activity remain relatively constant after 90 hr (9 τ_R) of induction, and induced steady-state samples were taken at 207 hr. Biomass

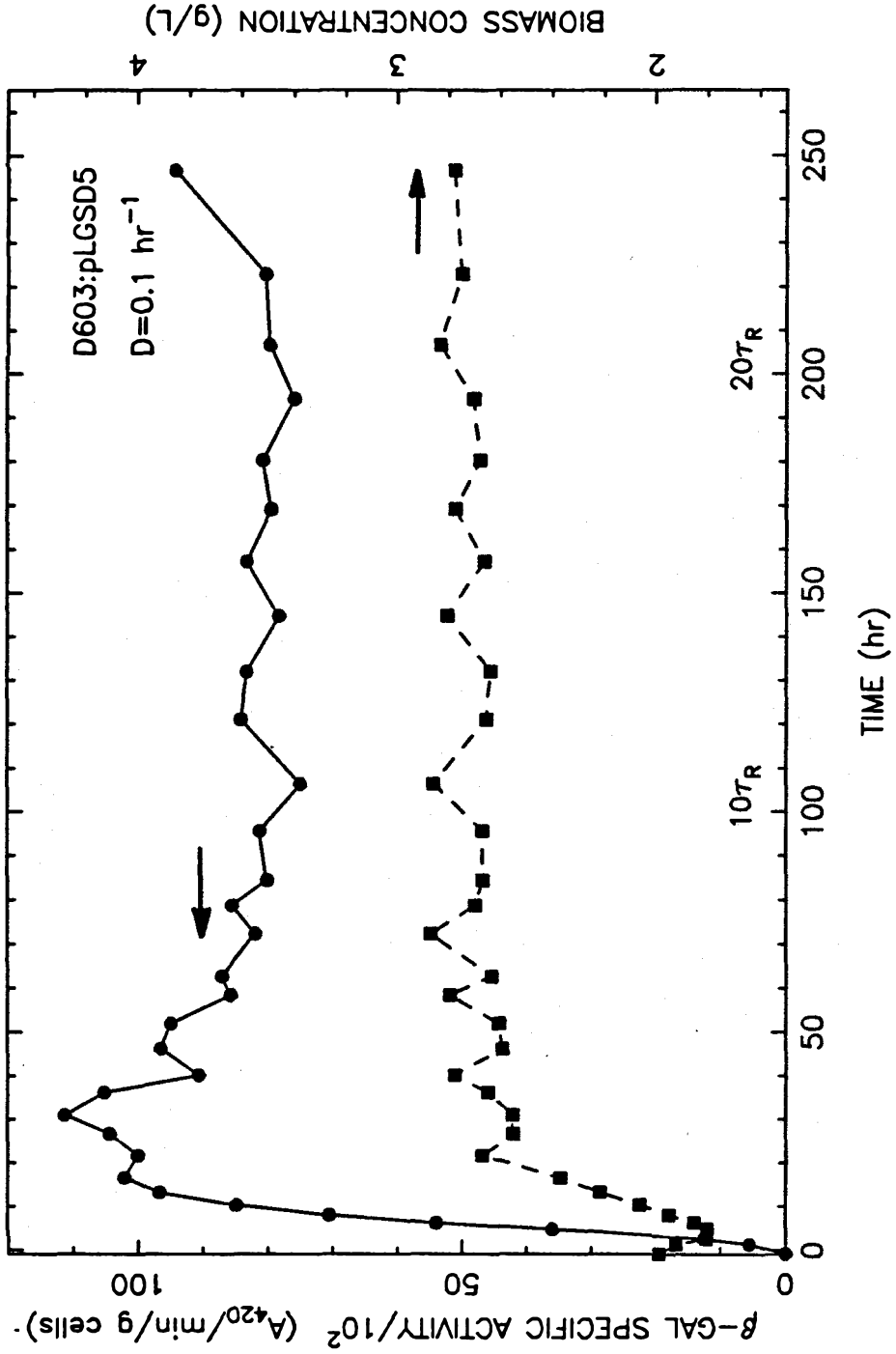

Figure 5.1 β–galactosidase Specific Activity and Biomass Concentration after Induction in Continuous Culture at D=0.1 hr^{-1}

density increased by ca. 38% between the uninduced and induced steady-states.

The biomass and β-galactosidase profiles observed at a dilution rate (specific growth rate) of 0.2 hr^{-1} are shown in Figure 5.2. At time zero, the cells were induced by galactose addition. The trend in β-galactosidase specific activity is similar to that observed at D=0.1 hr^{-1}. Biomass, however, decreases, and no oscillations in biomass and enzyme activity are apparent. Cell density and β-galactosidase specific activity appear to level off after 10 τ_R — similar to the behavior at the lower dilution rate. Cell concentration has decreased by 16% from the uninduced steady-state.

This induced "steady-state" was short-lived, however, and as the experiment continued, β-galactosidase specific activity continued to fall and biomass concentration began to rise. Figure 5.3 shows the biomass and β-galactosidase profiles over the entire course of the fermentation. After ca. 200 hr (40 τ_R), another induced steady-state is reached. Biomass has returned to the uninduced steady-state level, and β-galactosidase specific activity has dropped substantially.

An induced "steady-state" occurring 10–15 τ_R after induction has been observed in all fermentations, including the amino acid+purine-limited fermentation in Chapter 4 and the temperature-sensitive fermentation described in Chapter 6. It will be referred to as the first or short-term induced steady-state. The term "steady-state" as used here refers to a significant time interval (extending over several τ_R) during which all of the monitored culture properties do not change. The second induced steady-state, occurring 40–50 τ_R after induction, will be designated the second or long-term induced steady-state. For the fermentation at D=0.2 hr^{-1}, the short-term induced steady-state was not recognized initially, and no samples were taken. Long-term induced steady-state samples were taken at 267 hr.

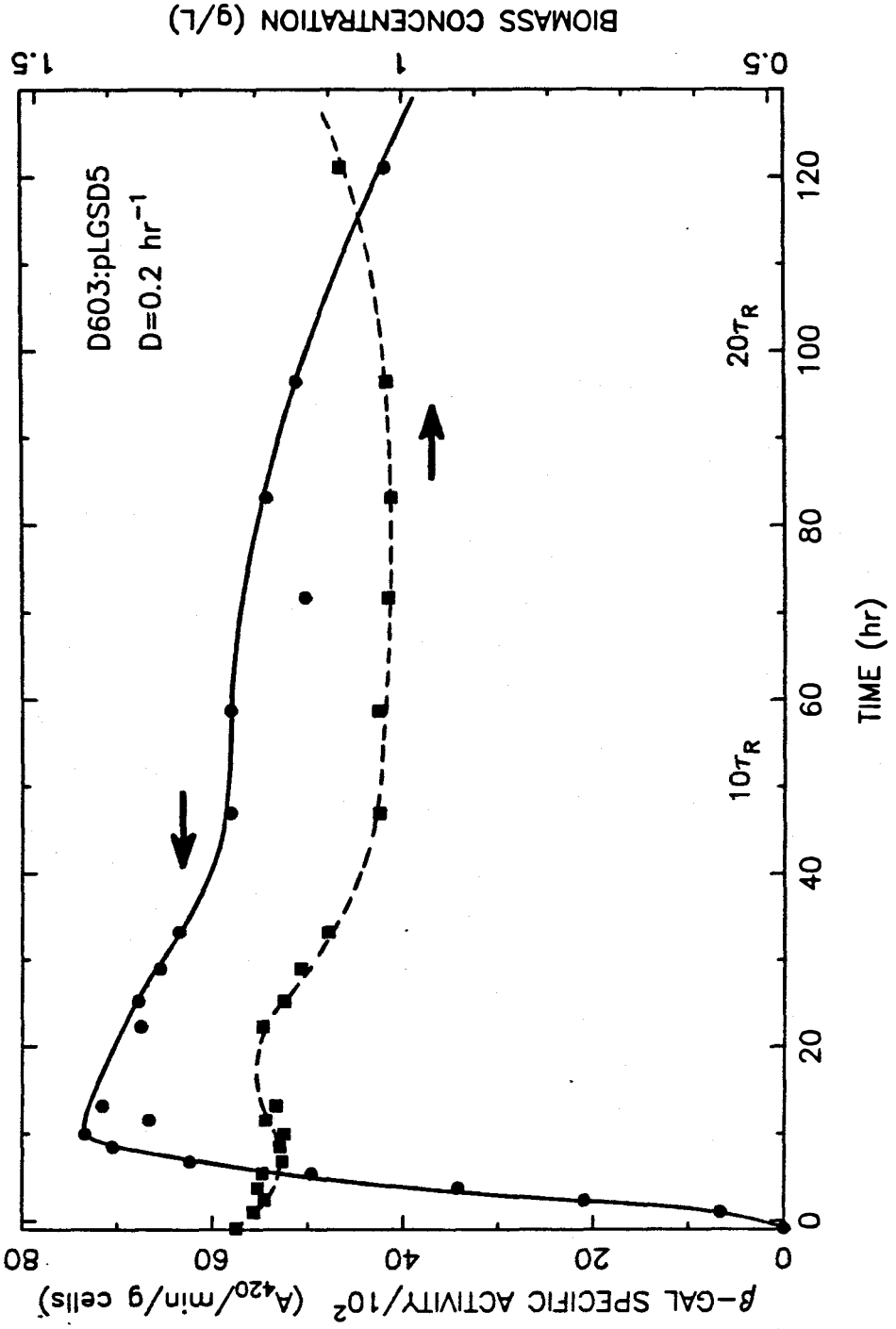

Figure 5.2 β-galactosidase Specific Activity and Biomass Concentration after Induction in Continuous Culture at D=0.2 hr^{-1}

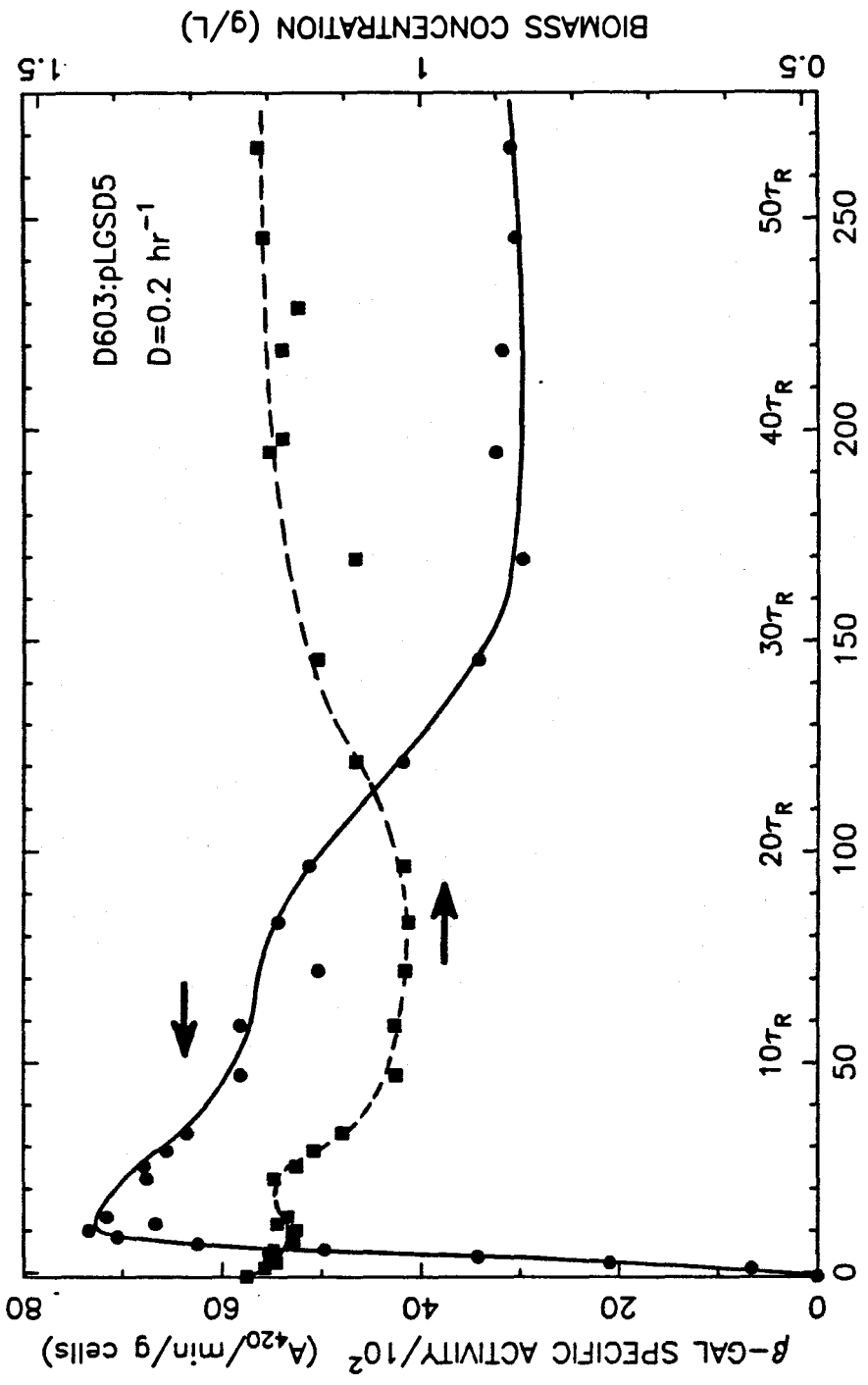

Figure 5.3 β–galactosidase Specific Activity and Biomass Concentration Profiles for $50\tau_R$ after Induction at D=0.2 hr^{-1}

A continuous fermentation at a dilution rate of 0.26 hr^{-1} was also performed. The results are shown in Figure 5.4. The trends in biomass and β-galactosidase are similar to those observed at D=0.2 hr^{-1}. β-galactosidase specific activity peaks after ca. 3.5 τ_R and then drops slightly, while biomass concentration decreases immediately. Both level off approximately 15 τ_R after induction. Short-term induced steady-state samples were taken at 58 hr. Biomass decreases by 34% from the uninduced to the first induced steady-state. The cause of the dip in β-galactosidase activity prior to the first induced steady-state is unknown.

Further drift to a second induced steady-state was also observed at this dilution rate. The biomass and β-galactosidase profiles for the entire fermentation are illustrated in Figure 5.5. β-galactosidase specific activity decreased and biomass increased to long-term induced steady-state levels, and samples were taken at 216 hr.

Despite similarities in trends, the levels of biomass and cloned gene expression were significantly different in the three continuous fermentations. The effects of dilution rate on biomass and β-galactosidase specific activity can be seen in Table 5.1.

Immediately apparent from the biomass results is the failure of the standard Monod chemostat model to describe the growth behavior. Even at the uninduced steady-states (in carbon-limited medium), yield coefficients ($Y_{x/s}$) vary markedly with dilution rate. The yield factor is a measure of the biomass formed per g substrate consumed by the cell, and is defined by $Y_{x/s} = (x - x_f)/(s_f - s)$, where x is biomass, s is limiting substrate (glucose), and f designates the feed stream [7]. In the Monod chemostat model, $Y_{x/s}$ is assumed to be constant with dilution rate. Using the residual glucose measurements in the next section, the yield factors at the uninduced steady-state (in the absence of galactose) are 0.50, 0.31, and

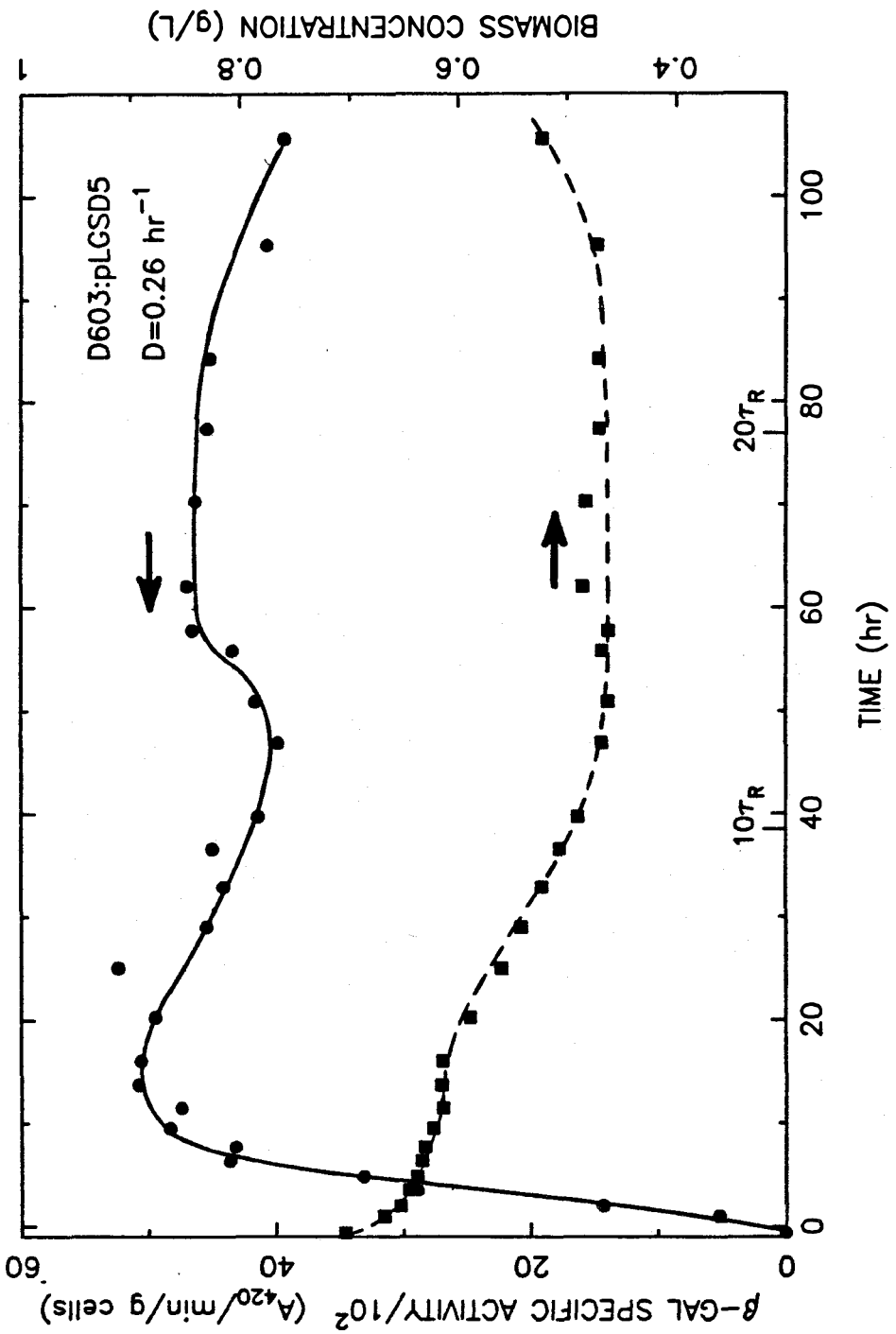

Figure 5.4 β-galactosidase Specific Activity and Biomass Concentration after Induction in Continuous Culture at D=0.26 hr^{-1}

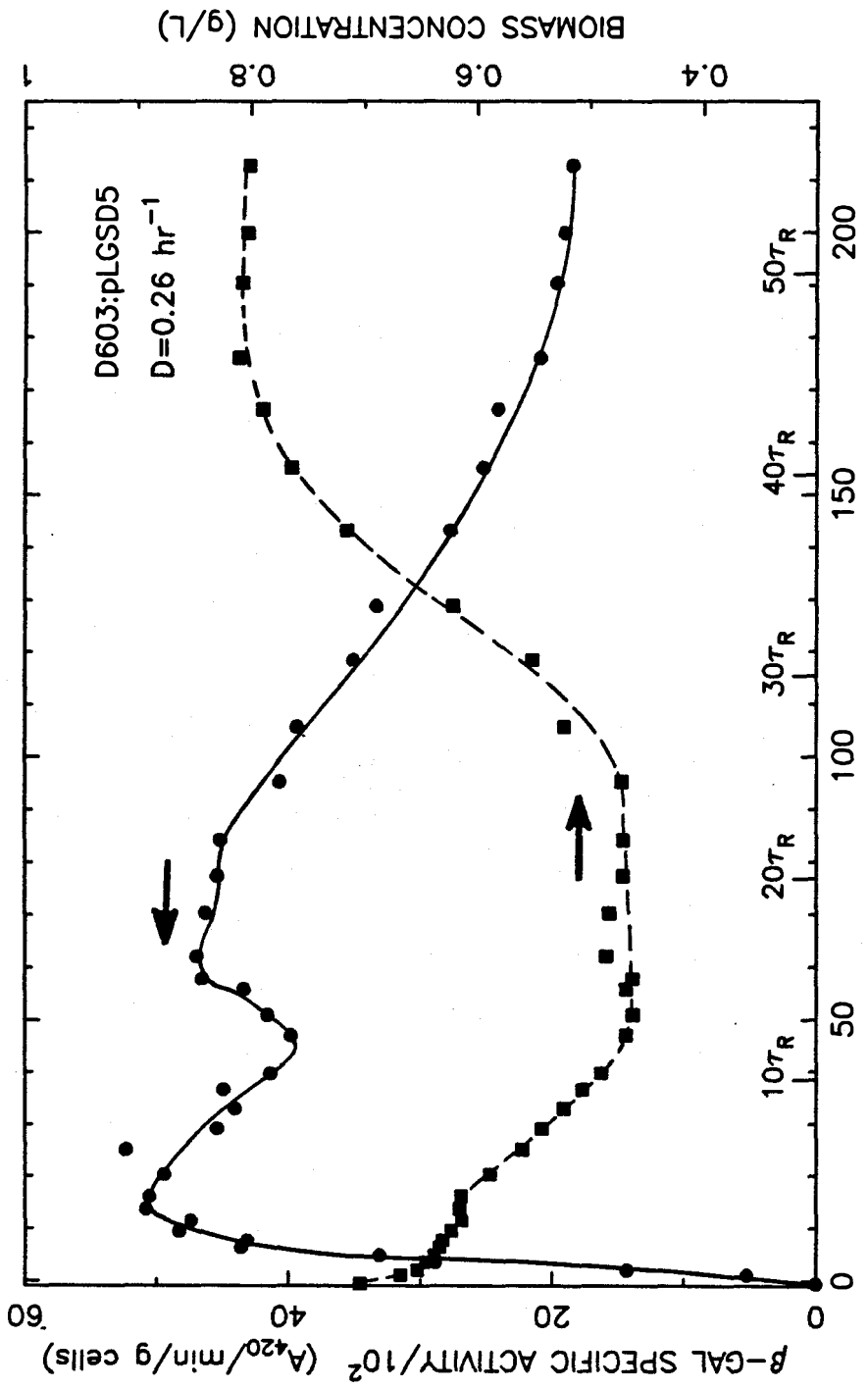

Figure 5.5 β–galactosidase Specific Activity and Biomass Concentration Profiles for $50\tau_R$ after Induction at D=0.26 hr^{-1}

Dilution Rate (hr^{-1})	β-galactosidase Specific Activity/10^2 (Abs_{420}/min/g cells)				Biomass Concentration (g cells/L)		
	U	Peak	I1	I2	U	I1	I2
0.10	0	111	81	----	1.98	2.73	----
0.20	0	73	58	31	1.23	1.03	1.21
0.26	0	51	46	19	0.703	0.465	0.802

U: Uninduced Steady-State
I1: First (short-term) Induced Steady-State
I2: Second (long-term) Induced Steady-State

Table 5.1 Influence of Dilution Rate and Induction on Biomass Concentration and β-galactosidase Specific Activity

0.20 at dilution rates of 0.1, 0.2, and 0.26 hr^{-1}, respectively. This decrease in yield with dilution rate is linear and follows the relation: $Y_{x/s} = 0.69 - 1.9D$ (g cells/g glucose). At higher growth rates, a lower percentage of the glucose is being utilized for biomass. The reasons for this trend are unclear. In galactose$^-$ (non-inducing) medium, plasmid-containing cells had little or no selective disadvantage (see Chapter 6). Furthermore, plasmid stabilities are similar under uninduced conditions for all dilution rates (see Section 5.4.3). Therefore, in the absence of *lacZ* gene expression, it is unlikely that the changes in yield are related to the plasmid.

The uninduced yield factor at D=0.1 hr^{-1} ($Y_{x/s}$ = 0.5 g cells/g glucose) is equivalent to that determined in shake flask cultures during the medium characterization (see Appendix A). At D=0.2 hr^{-1}, $Y_{x/s}$ is significantly lower; however, cell growth is still limited by carbon source (under uninduced conditions). In continuous culture at D=0.2 hr^{-1}, steady-state biomass was proportional to feed glucose concentrations (of 2.0, 3.0, and 4.0 g/L) and yield was constant. Increasing impellor speed from 600 to 900 RPM or altering air flow rate had a negligible effect on the culture at this dilution rate. This suggests that oxygen is not limiting. At D=0.26 hr^{-1}, the fermentor conditions are nearing washout. It is possible that at this dilution rate other components are limiting growth as well [7].

After induction of *lacZ* gene expression (by galactose addition), cell growth may no longer be limited only by carbon source. The trends in biomass differ depending on dilution rate. At D=0.1 hr^{-1}, biomass increases by 38% after induction. At D=0.2 hr^{-1}, however, biomass decreases and is 16% lower at the first induced steady-state. At D=0.26 hr^{-1}, biomass drops by 34% between the uninduced and induced steady-states. Therefore, steady-state biomass concentration also increases with decreasing dilution rate under induced conditions.

These trends might by due in part to a lower efficiency of growth at high dilution rate (as observed at the uninduced steady-state). The induction method and the sugar concentrations in the fermentor, however, likely contribute significantly to the transient and steady-state biomass results. Cloned gene expression places additional stress on the cells, and reduces plasmid stability (section 5.4.3). These result in lower yield and lower maximum specific growth rate. Therefore, steady-state biomass levels should decrease upon induction of cloned gene product synthesis — not increase as observed for D=0.1 hr^{-1}. The increase can be explained by inducer metabolism. At the uninduced steady-states, the cells are growing under carbon-limitation and the glucose levels in the fermentor are very low. Therefore, the galactose (inducer) is immediately utilized as a carbon source and, at a dilution rate of 0.1 hr^{-1}, biomass increases. Galactose metabolism may be an important factor at D=0.2 hr^{-1} and 0.26 hr^{-1} as well, and the drops in biomass (to the first induced steady-state) might have been more pronounced if induction could have occurred in the absence of galactose. The effects of glucose and galactose levels are discussed in more detail in Section 5.4.2.

The effects of dilution rate on β-galactosidase specific activity are also shown in Table 5.1. At all dilution rates, enzyme specific activity reaches a peak in 2–3.5 τ_R, and then decreases to the induced steady-state value. The peak and steady-state β-galactosidase specific activities increase as dilution rate decreases (similar to the trend observed for the biomass). In addition, at dilution rates of 0.2 and 0.26 hr^{-1}, the first induced steady-state is temporary, and biomass increases and β-galactosidase specific activity decreases to a second induced steady-state. If the fermentation at D=0.1 hr^{-1} had been continued, similar behavior might have been observed. This drift to a second steady-state might be due to selection in the chemostat or to galactose and glucose utilization, and will be discussed further

later in the chapter.

The overshoot in β-galactosidase specific activity observed at each dilution rate is likely due to many factors. The detrimental effect of cloned gene product synthesis on yield and growth rate might not be manifested until expression reaches a critical level. This is supported by observations during batch culture: after induction of cloned gene expression during exponential phase, μ remains higher than that measured for cells induced from inoculation (see Chapter 4). The time-scales for change in plasmid stability and copy number may also contribute to the overshoot and subsequent decrease in enzyme activity.

5.4.2 Glucose and Galactose Concentrations

The concentrations of glucose and galactose in the fermentor played an important role in determining the trends observed at each dilution rate. The steady-state sugar concentrations are shown in Table 5.2.

Cell growth was limited by carbon source (primarily glucose) at the uninduced steady-states. Low residual glucose concentrations of 0.019 and 0.057 g/L were measured at dilution rates of 0.1 and 0.2 hr^{-1}, respectively. At D=0.26 hr^{-1}, 12% of the feed glucose is unmetabolized. Although glucose is likely limiting, other components may be limiting as well. Residual glucose concentration at the uninduced steady-state decreases with dilution rate, while biomass concentration and yield increase (Tables 5.1 and 5.2).

At all dilution rates, cloned gene expression was induced by increasing reactor and feed galactose concentrations from 0.0 to 4.0 g/L. Even with the *reg1* mutation (inhibiting catabolite repression), the cells metabolize glucose preferentially (see Chapter 4). As glucose concentration falls, however, the cells begin to utilize galactose as a carbon source (Chapter 4, Figure 4.1). Therefore, at the low residual glucose concentrations of the uninduced steady-states, galactose is metabolized

Dilution Rate (hr^{-1})	Glucose Concentration (g/L)			Galactose [Inducer] Concentration (g/L)		
	U	I1	I2	U	I1	I2
0.10	0.019	0.037	----	0	1.60	----
0.20	0.057	----	0.089	0	----	3.59
0.26	0.46	1.46	0.31	0	3.68	3.65

U: Uninduced Steady-State
I1: First (short-term) Induced Steady-State
I2: Second (long-term) Induced Steady-State

Feed Glucose and Galactose Concentrations: 4.0 g/L

Table 5.2 Influence of Dilution Rate and Induction on Steady-State Glucose and Galactose Concentrations in the Chemostat

upon addition. This is evident from the galactose concentrations measured at the induced steady-states: all are below the 4.0 g/L in the feed.

Galactose utilization increases as dilution rate is reduced. The increase in galactose metabolism at low growth rate may be due to the lower residual glucose concentration (greater carbon-limitation) and to the higher efficiency of cell growth. At D=0.1 hr^{-1}, galactose concentration drops from 4.0 to 1.7 g/L during the first 3 τ_R of induction (the time required to reach the peak in β-galactosidase specific activity). A further slight decrease is observed, and the galactose concentration at the first induced steady-state is ca. 1.6 g/L. The utilization of galactose as a carbon source is reflected in the 38% increase in biomass concentration after induction despite high-level expression (Table 5.1). At D=0.1 hr^{-1}, the residual glucose concentration increases slightly after induction. The increase may be partially due to the high galactose levels and galactose metabolism; however, at such low glucose concentrations experimental error may contribute significantly to the trend.

At a dilution rate of 0.2 hr^{-1}, a much smaller percentage of galactose is utilized as a carbon source. Samples were not taken at the first induced steady-state. At the long-term induced steady-state only 10% of the inducer is metabolized. Again, glucose concentration increases somewhat after induction. At this dilution rate, the degree of galactose metabolism at the second induced steady-state appears to be sufficient to overcome any deleterious effects of β-galactosidase synthesis on biomass concentration, and the uninduced steady-state biomass level is reattained (see Table 5.1 and Figure 5.3).

At D=0.26 hr^{-1}, the galactose concentration is approximately the same at both induced steady-states, and slightly higher than at the dilution rate of 0.2 hr^{-1}. The glucose concentrations show greater variation. The residual glu-

cose level increases substantially from the uninduced to the first induced steady-state, while biomass concentration drops by 34% (Tables 5.1 and 5.2). During the approach to the second induced steady-state, glucose concentration and β-galactosidase specific activity decrease, and biomass concentration increases. These trends may be related in the following manner. After induction, maximum specific growth rate and yield decrease (due to the negative effects of cloned gene expression on the host). According to a Monod-type model, this will increase the residual glucose concentration and decrease biomass concentration (as observed). As β-galactosidase production falls between the two induced steady-states, the host cell growth rate and biomass yield will increase. According to the Monod model, residual glucose will decrease and, due to this and the increase in yield, biomass concentration will increase. The utilization of galactose as a carbon source and the effect of galactose on cell metabolism, however, may also be important in determining these trends.

As observed for D=0.2 hr^{-1}, the low level of galactose metabolism at the long-term steady-state appears to be sufficient to overcome any deleterious effects of the low level of β-galactosidase synthesis on biomass concentration. As shown in Table 5.1, the biomass concentration is higher at this long-term steady-state than under uninduced conditions.

The highest enzyme activities are observed for D=0.1 hr^{-1}, when galactose concentration (and therefore inducer level) is lowest. As discussed in Chapter 4, the galactose/glucose ratio (as well as absolute galactose concentration) appears to be very important in determining the level of induction. It may be that galactose concentrations are so much higher than glucose concentrations at all dilution rates that even significant galactose utilization has little effect on the extent of induction attainable. In addition, the galactose metabolism and increased growth efficiency

at low dilution rate may enhance the ability of the cell to produce cloned gene product. This might be partially responsible for the increase in β-galactosidase synthesis observed in the fermentation at D=0.1 hr^{-1}.

It should also be noted that the overall biomass and β-galactosidase trends in the fermentations could result from the two-substrate (glucose and galactose) metabolism. The *reg1* mutation in the host could significantly influence this growth behavior. The oscillations observed at D=0.1 hr^{-1} might reflect some interaction between population growth and relative glucose and galactose utilization. This interaction might also give rise to the two steady-states observed at the higher dilution rates. Oscillatory systems are likely to have multiple steady-states under different conditions. The drift from the first to the second induced steady-state, however, is more likely due to selection for faster growing cells in the chemostat, as discussed in the next section.

5.4.3 Plasmid Stability and Selection

The fraction of plasmid-containing cells was determined for steady-state samples at the three dilution rates. The results are shown in Table 5.3.

In the absence of *lacZ* gene expression, plasmid stability decreased with increasing dilution rate. This trend is the opposite of that reported in the literature. Kleinman et al. [8] reported increasing plasmid stability with increasing dilution rate in glucose-limited continuous culture. The experiments were performed in the absence of selective pressure, however, and employed a [cir°] strain harboring a recombinant plasmid containing the complete 2μ plasmid. Parker and DiBiasio [9] also reported increasing plasmid stability with dilution rate. The *S. cerevisiae* strain utilized was [cir^+] and the plasmid contained the 2μ origin. The continuous fermentations were not performed under carbon-limitation, however, and cloned gene expression levels were likely quite different from those at the unin-

Dilution Rate (hr^{-1})	Fraction of Plasmid-Containing Cells		
	U	I1	I2
0.10	0.95	0.75	----
0.20	0.93	----	0.86
0.26	0.90	0.84	0.86

U: Uninduced Steady-State
I1: First (short-term) Induced Steady-State
I2: Second (long-term) Induced Steady-State

Table 5.3 Influence of Dilution Rate and Induction on Plasmid Stability

duced steady-state with pLGSD5. High copy number and low expression levels reduce plasmid loss, and could be factors in the trend observed at the uninduced steady-states in the current work. The measured values for plasmid stability at the uninduced steady-states in Table 5.3 have a statistical error of 2–3% (see Chapter 3, section 3.5.6). Taking the error into account, the plasmid-containing fractions may be the same at the three dilution rates. However, an increase in stability with dilution rate is definitely not observed.

At all dilution rates, induction of β-galactosidase synthesis resulted in lower plasmid stability. Furthermore, under induced conditions, plasmid stability increased with increasing dilution rate. These results agree with those of Parker and DiBiasio [9].

In analyzing these trends, it is important to consider the selection occurring in the chemostat. At the uninduced steady-state, the plasmid number will reflect a balance between the negative impact of plasmid replication and gene expression (not *lacZ*) and the positive effects of *URA3* gene expression for *ura3* cells in selective medium. Induction of *lacZ* gene expression upsets this balance by placing additional stress on the cells, and cells with low expression levels have a selective advantage. Therefore, upon induction, plasmid number may drop. This could decrease plasmid stability, since the probability of plasmid loss is greater at low plasmid number [10–11].

Slow growing cells survive better at low dilution rate, and plasmid-free cells (which retain some *URA3* gene product) will not be washed out as rapidly. This may explain the low plasmid stability observed under induced conditions at D=0.1 hr^{-1}. High copy number cells (which grow slowly due to higher expression levels) will also survive better at low dilution rates. Therefore, plasmid number may be higher at low dilution rate, which could account for the high β-galactosidase levels

observed at D=0.1 hr^{-1} despite low plasmid stability. Of course, as previously mentioned, the positive effects of galactose metabolism and increased efficiency of growth at low dilution rate may also be at least partially responsible.

Faster growing cells (e.g., those producing low levels of β-galactosidase) have a selective advantage in continuous culture. Therefore, an alternate explanation for the drift to the second induced steady-state might be selection for low copy number cells. As plasmid number dropped, β-galactosidase specific activity would decrease. Biomass would then increase (as explained in the previous section). These trends were observed between the two induced steady-states. Promoter or other mutations, however, could also be responsible for these results. In either case, the first induced steady-state might be characteristic of the system before long-term selection.

5.4.4 Productivity Comparison

Overall productivity (β-galactosidase activity/L/hr) was calculated for each dilution rate. Productivity depends on specific enzyme activity, biomass concentration, and flowrate. Recall that both biomass concentration and β-galactosidase specific activity increased with decreasing dilution rate (see Table 5.1). The increase in biomass is primarily due to the influence of dilution rate on galactose metabolism and growth efficiency — and may be specific to this induction system. Increasing enzyme specific activity with decreasing dilution rate has also been reported for *S. cerevisiae* by Parker and DiBiasio [9]. It should be noted that, in the work presented here, activities were normalized by total biomass, not only the plasmid-containing fraction. Since plasmid stability dropped with dilution rate under induced conditions, β-galactosidase specific activity based on plasmid-containing fraction would increase even more dramatically as dilution rate was reduced.

The productivity results are shown in Table 5.4. At low dilution rate, the higher biomass and β-galactosidase specific activity more than offset the low flowrate, and productivity is higher. Productivity at D=0.1 hr^{-1} is nearly twice that at D=0.2 hr^{-1}, and productivity at D=0.2 hr^{-1} is approximately twice that at D=0.26 hr^{-1}.

Dilution Rate (hr^{-1})	Overall Productivity/10^2 ([Abs_{420}/min]/L/hr)	
	I1	I2
0.10	22	----
0.20	12	7.5
0.26	5.6	4.0

I1: First (short-term) Induced Steady-State
I2: Second (long-term) Induced Steady-State

Table 5.4 Influence of Dilution Rate on Productivity

5.5 Effects of Promoter Strength

5.5.1 Continuous Fermentation Results

A continuous fermentation utilizing strain D603:pRY121 was performed to study the effects of promoter strength on the behavior of the recombinant system. The results are compared with those for D603:pLGSD5. Plasmid pRY121 contains the *GAL1* promoter and pLGSD5 contains a *GAL10-CYC1* promoter. In the batch induction experiments reported in Chapter 4, cloned gene expression from the *GAL1* promoter was significantly higher (see Figure 4.7).

The biomass and β-galactosidase profiles observed during the continuous fermentation of D603:pRY121 are illustrated in Figure 5.6. At time zero, galactose is added to the uninduced steady-state culture. Dilution rate is 0.2 hr^{-1}. β-galactosidase specific activity increases nearly linearly from 0 to 158 $\times$ 10^2 (Abs_{420}/min/g cells) during the first 10 hr (2 τ_R) of induction, and then decreases. Biomass decreases immediately after induction, levels off when β-galactosidase reaches the peak, and then continues to fall. Both biomass and β-galactosidase specific activity level off after approximately 15 τ_R, although there is a fair amount of scatter in the β-galactosidase measurements. Oscillations in biomass concentration and β-galactosidase specific activity, similar to those observed at D=0.1 hr^{-1} (section 5.4), may be occurring. Steady-state samples were taken at 181 hr.

At the uninduced and induced steady-states, biomass concentration, β-galactosidase specific activity, sugar concentrations, and plasmid stability were determined. The values for biomass and β-galactosidase were averaged over the steady-state period. At time zero, the reactor and feed galactose concentrations were brought to 4.0 g/L. As observed for D603:pLGSD5, the inducer was metabolized as a carbon source upon addition. At the induced steady-state, galactose concentration was 3.36 g/L. The residual glucose concentration increased between

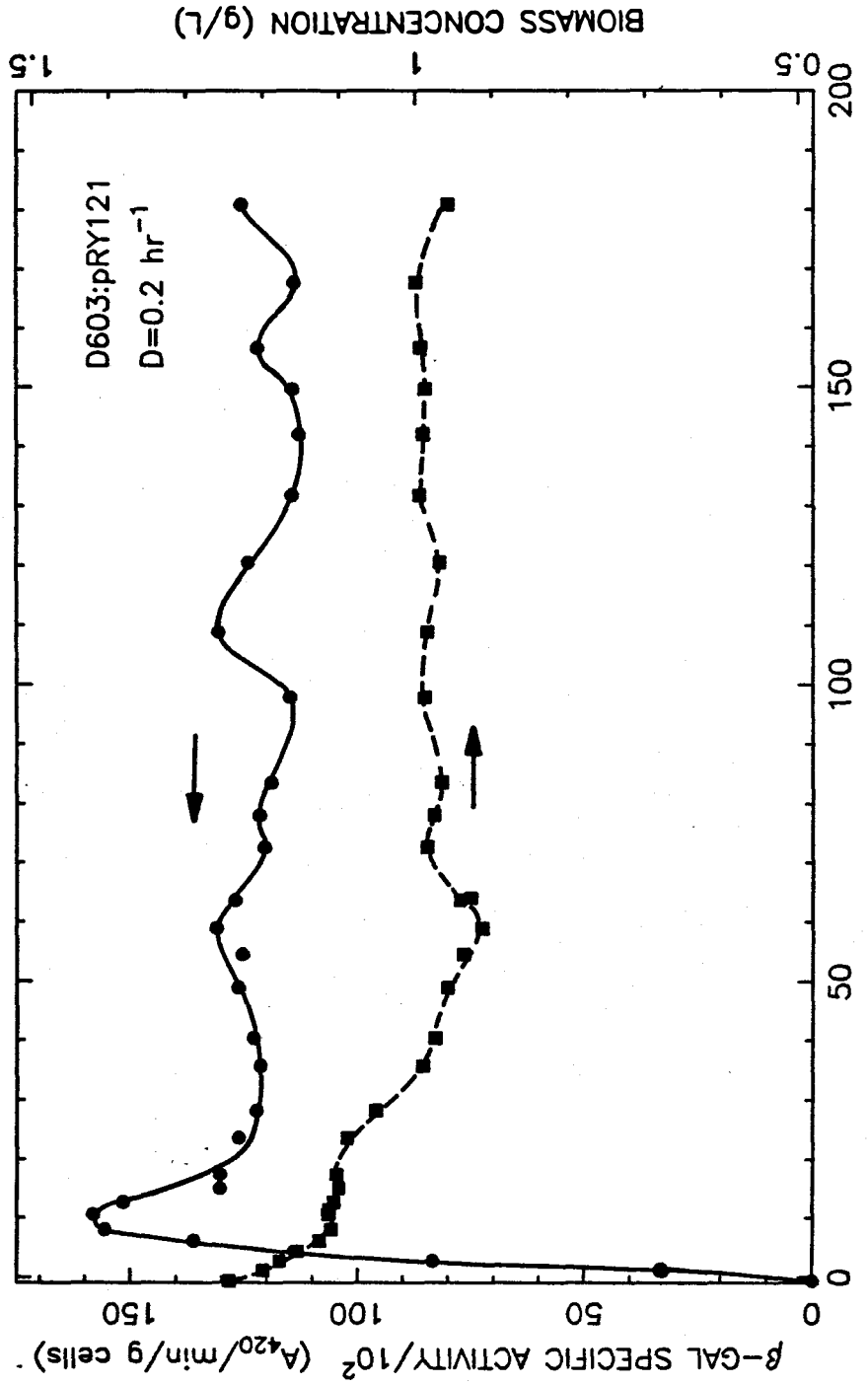

Figure 5.6 β-galactosidase Specific Activity and Biomass Concentration after Induction in Continuous Culture of D603:pRY121

the uninduced and induced steady-states, and biomass concentration decreased by 21%. As expected, plasmid stability decreased after induction: only 72% of the cells contain plasmids at the induced steady-state versus 92% at the uninduced steady-state.

5.5.2 Promoter Strength Comparison

The biomass and β-galactosidase profiles after induction of *lacZ* gene expression in continuous fermentations of D603:pRY121 (*GAL1* promoter) and D603:pLGSD5 (*GAL10-CYC1* promoter) are compared in Figure 5.7. Dilution rate was 0.2 hr^{-1} (τ_R=5 hr) in both experiments. Although the general trends are similar, the magnitudes of the values are significantly different. In both cases, the peak β-galactosidase level is attained in 2 τ_R; however, the peak and steady-state β-galactosidase specific activities are much higher for strain D603:pRY121. In addition, only one induced steady-state is observed in the fermentation of D603:pRY121.

Steady-state values for the two fermentations are shown in Table 5.5. At the uninduced steady-state, nearly identical results are obtained for the two strains. Residual glucose concentrations are different; but both are low. The only genetic difference between the strains is the promoter governing *lacZ* transcription (see Chapter 3); therefore, growth behavior should be the same in the absence of galactose. After induction, however, very different values are obtained for the two strains. β-galactosidase specific activity is 2–4 times higher and productivity is 2—3 times higher when transcription is controlled by the stronger (*GAL1*) promoter. The higher β-galactosidase expression level is accompanied by lower plasmid stability. Biomass is 5–20% lower for D603:pRY121, depending on which steady-states are compared, and glucose concentration is higher. These differences may be due to the greater level of cloned gene expression with D603:pRY121.

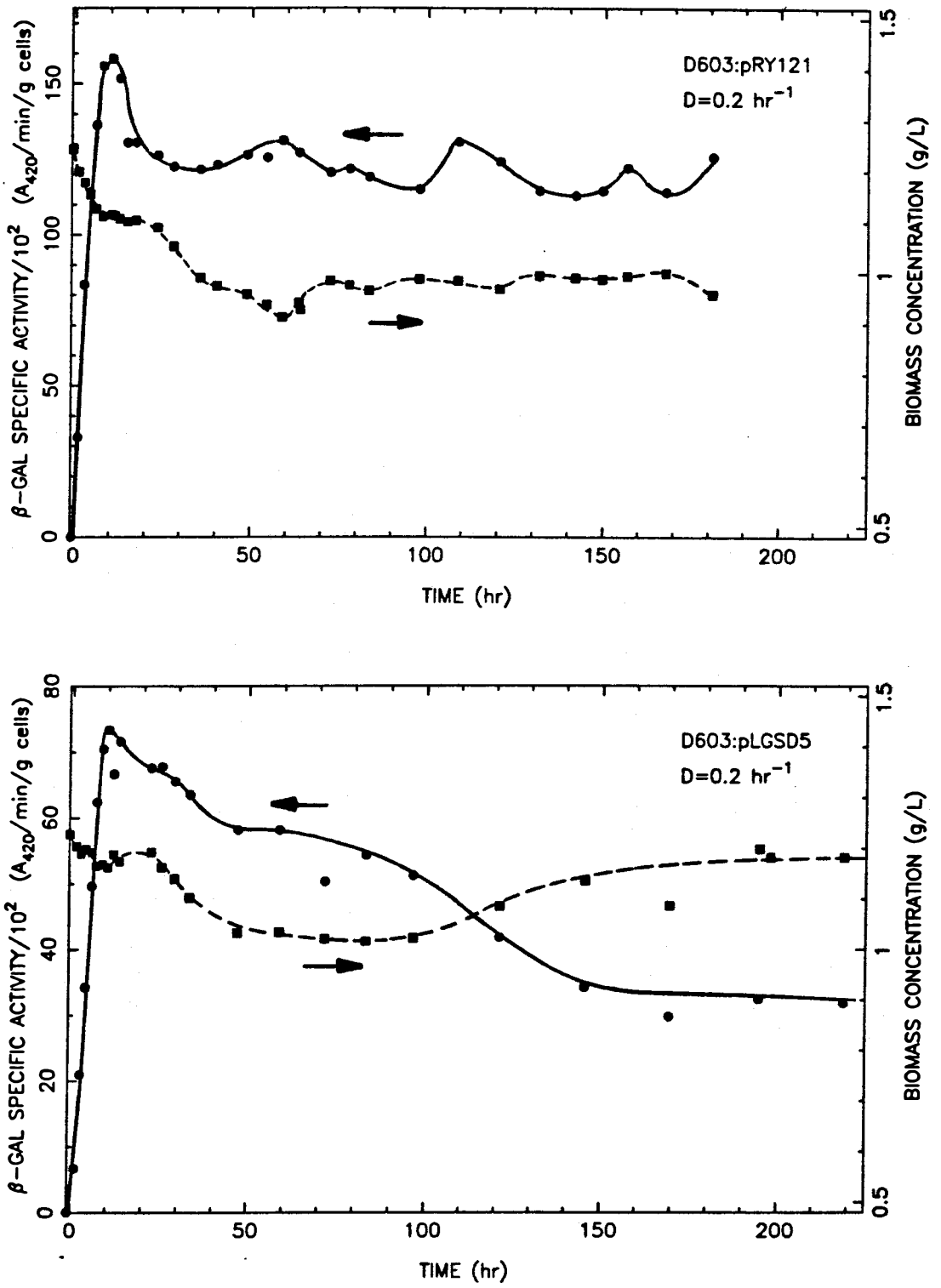

Figure 5.7 Comparison of Biomass and β-galactosidase Levels in Continuous Fermentations of D603:pLGSD5 and D603:pRY121

	D603:pRY121 (*GAL1* Promoter)		D603:pLGSD5 (*GAL10-CYC1* Promoter)		
	U	I (~35τ_r)	U	I1 (~10τ_r)	I2 (~50τ_r)
Biomass (g/L) :	1.24	0.981	1.23	1.03	1.21
β-gal Specific Activity/10^2 (Abs_{420}/min/g cells) :	0	120	0	58	31
β-gal Productivity/10^2 ([Abs_{420}/min]/L/hr) :	0	24	0	12	7.5
Glucose (g/L) :	0.098	0.40	0.057	----	0.089
Galactose (g/L) :	0	3.36	0	----	3.59
Plasmid Stability :	92%	72%	93%	----	86%

U: Uninduced Steady-State
I : Induced Steady-State

Table 5.5 Influence of Plasmid Promoter Strength on Steady-State Results at D=0.2 hr^{-1}

The absence of a second induced steady-state in the *GAL1* fermentation is interesting. It was hypothesized earlier that the long-term steady-states may be due to the two-substrate metabolism or to selection for cells producing low levels of β-galactosidase — in particular, cells with low plasmid number. In this fermentation, oscillations in the levels of biomass and β-galactosidase specific activity may be occurring (as observed for D603:pLGSD5 at D=0.1 hr^{-1}). In both cases, β-galactosidase specific activities were high. Alternatively, selection for faster growing cells could be the important factor determining the observed behavior. In the fermentations of D603:pLGSD5 at D=0.2 and 0.26 hr^{-1}, the first induced steady-state occurred 10–15 τ_R after induction. After 60 hr (12 τ_R) in the fermentation of D603:pRY121, a slight maximum and minimum occur in the β-galactosidase and biomass curves, respectively. Beyond 12 τ_R, biomass increases and β-galactosidase specific activity decreases (see Figure 5.6). It may be that, due to the high expression level and additional stress on the cells, selection occurs faster and cells with a high plasmid number are washed out sooner. The state of the system at 12 τ_R would then correspond to the short-term steady-state observed for D603:pLGSD5; however, in this case, the steady-state is not established. Selection continues and the biomass increases while β-galactosidase specific activity decreases. The induced steady-state values for D603:pRY121 in Table 5.5 would then correspond to long-term steady-state values, and should be compared with column I2 (the second induced steady-state) for D603:pLGSD5.

5.6 Conclusions

The goal of this work was to study the influence of growth rate, plasmid promoter strength, and induction of cloned gene expression in continuous fermentations of recombinant *S. cerevisiae*. Several factors were important in producing the observed trends, including inducer metabolism, plasmid stability fluctuations, and the efficiency of growth at different dilution rates. Although the interdependence of the variables complicates the analysis, several trends are apparent.

β-galactosidase productivity (β-galactosidase activity/L/hr) increases with decreasing dilution rate. This is due to an increase in both biomass concentration and β-galactosidase specific activity which more than compensate for the lower flowrate. This trend in specific enzyme activity has been observed by other researchers [9]; however, the biomass trend (particularly the magnitudes) may be specific to this induction system. The increase in biomass with decreasing dilution rate is primarily due to two factors: variable yield and inducer metabolism. Even under uninduced conditions, yield increased with decreasing dilution rate. Upon induction, galactose was metabolized as an additional carbon source, and the amount of inducer utilized increased at low dilution rate when glucose concentration in the chemostat was very low. The observed trends in biomass and β-galactosidase specific activity may depend on the relative inducer level (galactose/glucose ratio) and on the *reg1* mutation which inhibits catabolite repression. The influence of the *reg1* mutation on cell metabolism at different growth rates and sugar concentrations is unknown.

Plasmid stability varied only slightly with dilution rate under uninduced conditions. After induction, however, plasmid stability dropped significantly, and the highest fraction of plasmid-free cells was found at low dilution rate.

In some cases, two induced "steady-states" were observed. The second steady-

state was characterized by higher biomass and lower β-galactosidase levels, and may reflect the effects of selection in the chemostat. Plasmid number, for example, may have decreased.

Many of the same results were observed when plasmid promoter strength was increased. Higher levels of β-galactosidase were obtained, with concomitant drops in biomass and plasmid stability. Productivity increased with promoter strength. Therefore, for this system (plasmid gene expression controlled by the galactose regulatory circuit), strong promoters and low dilution rates should be employed. The drops in plasmid stability and flowrate are more than offset by the increase in specific enzyme activity (and biomass concentration at low dilution rate), and productivity increases.

5.7 References

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CHAPTER 6

CONSTRUCTION AND CHARACTERIZATION OF A TEMPERATURE-SENSITIVE EXPRESSION SYSTEM IN RECOMBINANT YEAST

6.1 Abstract

A temperature-regulated system for the expression of cloned genes has been developed and studied in recombinant yeast. A temperature-sensitive (ts) strain of *Saccharomyces cerevisiae* was constructed which, in conjunction with plasmid promoters controlled by the yeast galactose regulatory circuit, allows induction of cloned gene product synthesis by temperature-shift.

Induction of cloned gene expression by temperature-shift in the ts strain was compared with induction by galactose addition in a non-ts, but otherwise identical, strain. In batch fermentations at 35°C, the two induction methods produced comparable levels of product protein during exponential phase. However, higher levels were formed by galactose induction at the preferred temperature of 30°C. At either temperature, β-galactosidase synthesis was greatest with the ts strain in galactose-containing medium. Continuous culture experiments were performed to further characterize and compare the two induction methods. The results illustrate the importance of temperature and galactose metabolism on cell growth and cloned gene expression.

6.2 Introduction

The control of cloned gene expression can be of great importance, especially when the synthesized product is harmful to the host cell or the level of plasmid gene expression significantly reduces cell growth or plasmid stability. In such cases, it is desirable to control the time over which product synthesis occurs, and plasmids with inducible promoters controlling cloned gene expression are an attractive means of implementing this regulation.

The galactose regulatory circuit in yeast offers one method of control over plasmid protein synthesis. This system of regulation (in the wild-type, nontransformed yeast) is involved in the uptake and catabolism of galactose, and many of the important genes (and proteins) have been identified. The *GAL4* gene codes for an activator protein required for transcription of the genes for the Leloir pathway enzymes and galactose permease (*GAL7*, *GAL1*, *GAL10*, and *GAL2*). The *GAL80* gene encodes a repressor protein which, in the absence of galactose, prevents transcriptional activation by the *GAL4* protein [1]. In one model for the interaction [2], binding of the *GAL80* repressor to the *GAL4* protein prevents activation of transcription at the promoter sites. Galactose interacts with the repressor, releasing the *GAL4* protein, which is then free to activate transcription. (Further details on the galactose regulatory circuit can be found in Chapter 2.) Therefore, a plasmid gene cloned downstream from a *GAL* promoter can be "turned on" by galactose addition. This system of regulation is subject to catabolite repression [1–2], and, in the wild-type yeast, induction of transcription will not occur in the presence of glucose. Although this repression is not yet fully understood, several mutations inhibiting catabolite repression are known [2–3]. In strains with these mutations, induction can occur by galactose addition to glucose-containing medium.

This method of control requires galactose — an additional, and relatively

expensive, carbon source. An alternative is the use of temperature-controlled promoters. Such systems have proved successful in *Escherichia coli* for both plasmid gene expression regulation and copy number amplification [4–5]. By utilizing temperature-controlled promoters, cloned gene expression can be controlled by temperature-shift (T-shift) rather than metabolite addition or reduction. The main disadvantage is the possible deleterious effects of T-shift on the host cell. Often the temperature is increased to one less preferred by the host. Side effects may include increased proteolysis and an overall lower efficiency of growth (lower growth-rate, lower yield, and increased maintenance requirements) [4–5]. The trade-offs between these disadvantages and the advantages of T-shift as a method of induction depends on the system and microorganism utilized.

The objective of this work was to construct and characterize a temperature-inducible expression system in recombinant *Saccharomyces cerevisiae.* The intent was to develop a control system based on the galactose regulatory circuit, but with expression dependent on temperature rather than the presence of galactose. Previous work by Matsumoto et al. [6] and the model of interaction for the *GAL80* repressor and *GAL4* activator proteins [2] suggested isolating strains with ts mutations in the *GAL4* or *GAL80* genes. A ts mutation in the *GAL80* gene could result in a temperature-sensitive repressor protein. At 25°C, the repressor would actively bind the *GAL4* protein preventing transcription from the promoters sites. At 35°C, however, the repressor would become inactive, allowing induction of transcription to occur. This mutation would be a thermo-labile (TL) mutation [6] in which the repressor protein, or at least the *GAL4* binding site, changes conformation at increased temperatures. Alternatively, the mutation might be a temperature-sensitive synthesis (TSS) mutation [6], in which case, the repressor protein is no longer synthesized at 35°C. A ts*GAL4* mutation could produce similar

behavior. At 35°C, the activator protein might alter conformation, preventing the binding of repressor protein while retaining the ability to activate the promoter sites.

This chapter describes a novel method for isolating ts strains. Unlike previous methods [6], the initial strain contains the wild-type *GAL80* and *GAL4* genes; thus, only a single mutation in the affected gene is expected in the resulting strains. It was hoped this might reduce the "leakiness" of the mutation and provide stricter temperature-control over the induction process. In these strains, transformed with a plasmid containing a *GAL* promoter, cloned gene expression can be induced by either T-shift or galactose addition. The performance of the ts strain was characterized in both batch and continuous fermentations. Experiments with a non-ts, but otherwise identical, strain allowed a direct comparison between the two methods of induction. Batch fermentation results illustrate the effect of temperature on growth behavior, the initial kinetics of cloned gene induction, and the level of cloned gene expression for both the ts and non-ts strains. Finally, the two induction methods are compared under continuous culture conditions.

6.3 Materials and Methods

6.3.1 Yeast Strains and Plasmids

Saccharomyces cerevisiae strain YM603 was utilized in the isolation of the ts strain. D603, a homozygous diploid version of YM603, was the control (non-ts) strain used throughout this study. Both YM603 and D603 were described previously in Chapter 3; important features include *ura3*, *his3*, and *reg1* mutations. The *reg1* mutation inhibits catabolite repression by glucose. *S. cerevisiae* strain YM623 (*MATα ade2-101 ura3-52 his3 lys2-801 tyr1⁻ reg1-501*) was used in determining the ts mutation site. YM623 was provided by M. Johnston.

Plasmids pLGSD5 and pNN219 were utilized in this work. The general plasmid map for pLGSD5 is shown in Chapter 3, Figure 3.1. The plasmid contains the 2μ origin of replication (and *REP3* site), a *URA3* selection gene, and a *GAL10-CYC1* hybrid promoter controlling transcription of the *E. coli lacZ* gene. The plasmid map for pNN219 (gift of S. Scherer) is show in Figure 6.1. This plasmid was constructed from YCp50 [7], and includes *CEN4* and *ARS1* fragments, a *URA3* selection gene, and the *GAL1* promoter controlling transcription of the *HIS3* gene (S. Scherer, personal communication).

6.3.2 Culture Media

Selective growth media included SD minimal medium (supplemented with adenine, histidine, lysine, and methionine), optimized C-limited minimal medium (supplemented with adenine, histidine, lysine, and methionine), and YPD medium. For plasmid-free strains, uracil was added to the minimal medium. The compositions of these media are described in Chapter 3. For batch growth, all media were buffered to pH 4.4 with 0.05M citrate buffer. Under induced conditions, the initial galactose concentration in the minimal medium was 4.0 g/L.

SD minimal plates supplemented with a combination of adenine, histidine,

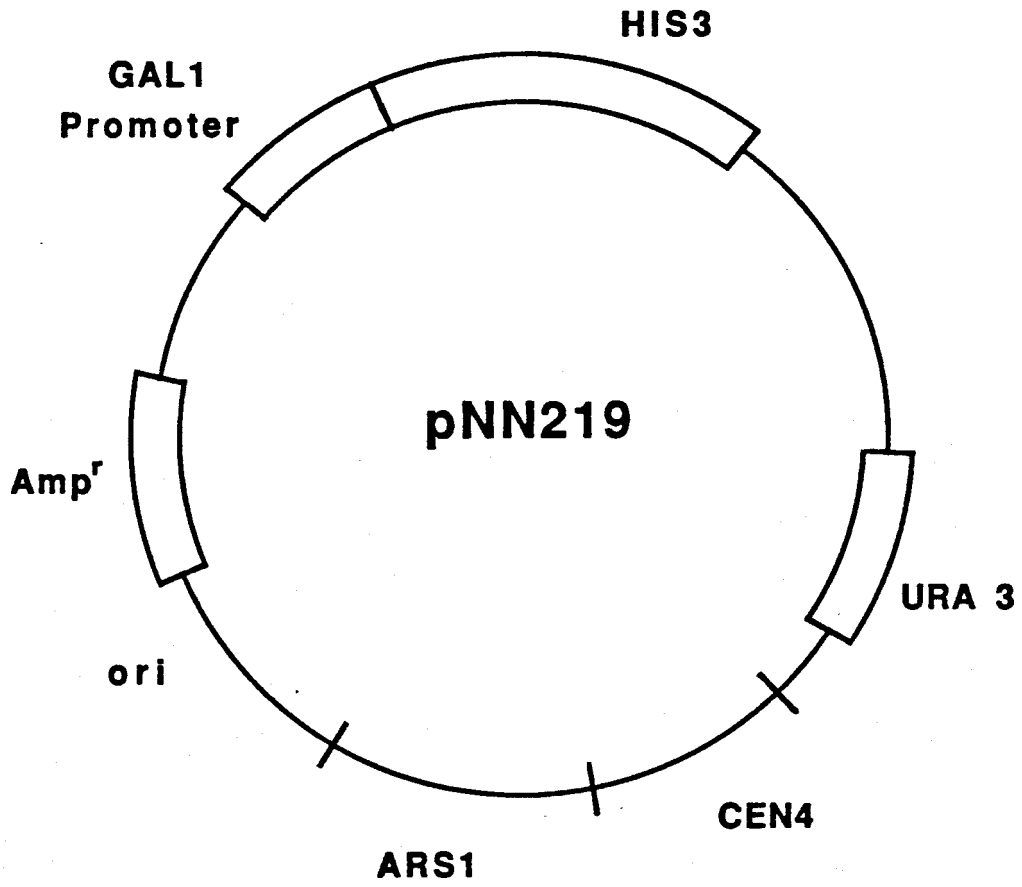

Figure 6.1 Plasmid pNN219

lysine, methionine, and uracil were used as selective plates throughout the work. Glycerol minimal plates and YPG plates were used to select against petite mutants. In addition, YPD, presporulation, and sporulation plates were utilized. The recipes for all plates are given in Chapter 3.

6.3.3 Experimental Methods

Batch experiments were performed in shake flasks as described in Chapter 3. The cells were either induced (by galactose addition and/or T-shift) in exponential phase or cultivated under inducing conditions throughout the experiment. Samples were withdrawn for biomass and β-galactosidase specific activity measurements.

Continuous fermentations followed the procedure outlined in Chapter 3. After an uninduced steady-state was attained, *lacZ* gene expression was induced (by galactose addition or T-shift), and the transient followed to an induced steady-state. Steady-states were determined by optical density (biomass) and β-galactosidase specific activity measurements.

Yeast transformations, biomass determinations, and ONPG tests (for β-galactosidase) followed the procedures in Chapter 3. Mating and sporulation of yeast strains also proceeded as previously described. A forced mating (the crossing of two strains with the same mating type, i.e., *MAT*a) was required in the ts strain construction. A successful cross depends on a spontaneous change in the mating type of one of the cells [8]. Due to the low frequency of diploids that arise after such a cross, resuspending the cells in water before transferring to selective plates helped in the identification of diploids cells (the colonies were not lost in the background growth of streaked cells). Colonies took ca. 18 days to form on the selective plates. To verify that diploid strains had been obtained, the cells were sporulated.

6.4 Temperature-Sensitive Strain Construction

The goal of the isolation procedure was to select cells containing ts mutations allowing temperature-dependent induction of the galactose regulatory circuit. In the absence of galactose, transcription from the *GAL* promoters would be repressed at 25°C and constitutive at 35°C. To detect the ts cells, an easily selectable phenotype had to be associated with *GAL* regulated gene expression. Strain YM603 (*his3*) harboring plasmid pNN219 (*HIS3* under *GAL* control) provided a simple selection system: cell growth on his$^-$ plates only occurs when the *GAL1* plasmid promoter is "on". Therefore, the strategy was to select cells with mutations allowing growth at 35°C, but not at 25°C. The cells could then be tested more rigorously and a diploid strain constructed.

The first step in the isolation was to transform haploid strain YM603 with plasmid pNN219. Following transformation, independent colonies of the plasmid-containing strain were used to inoculate culture tubes containing 5 mL ura$^-$ supplemented SD-minimal medium. The tubes were placed in a shaker at 30°C and 250 RPM for approximately 24 hr. New tubes were inoculated from these "overnight" cultures, and cultivated for an additional ca. 24 hr. The cells were then centrifuged, washed three times, resuspended in deionized water, spread onto his$^-$ plates at ca. 1×10^8 cells/plate, and placed in an incubator at 35°C. As described previously, YM603 has a *his3* mutation and pNN219 contains the *HIS3* gene under the control of the *GAL1* promoter. Therefore, YM603 cannot grow on the his$^-$ plates and YM603:pNN219 will not grow unless the *GAL1* promoter is "on" and the *HIS3* gene expressed. Therefore, plating on his$^-$ plates selects only those cells with a mutation allowing transcription of the *HIS3* plasmid gene. (The mutation in the chromosomal *HIS3* gene is a deletion mutation preventing reversion at this site.) The spontaneous mutation rate in yeast is 10^{-5}–10^{-6}.

Therefore, plating 1×10^8 cells/plate should produce ca. 100 colonies per plate. In practice, somewhat fewer colonies are obtained.

A total of 34 independent test-tube cultures were plated to obtain the mutants. The colonies which arose on the his^- plates were then screened for growth at 25°C to select for cells containing temperature-sensitive mutations. The cells were transferred to his^- or his^-, ura^- grid plates and placed at 35°C and room temperature (approximately 25°C). Those exhibiting significantly less growth at 25°C were rechecked at both temperatures. Over 1000 cells were initially checked for growth at 35°C and 25°C. Of these, only four demonstrated the ts phenotype: growth at 35°C and little or no growth at 25°C.

The four ts possibilities were cured of pNN219 by cultivation in non-selective YPD medium for several generations. The cells were then plated on YPD plates and individual colonies tested for growth on ura^- plates. Those exhibiting growth only in the presence of uracil were rechecked and assumed to be plasmid-free.

The four potential ts strains, cured of pNN219, were transformed with plasmid pLGSD5. The temperature-sensitivity of the mutations could now be tested more rigorously using β-galactosidase assays. Each of the strains was inoculated into tubes containing 5 mL ura^- supplemented C-limited medium. After 24–36 hr of growth on a rotator at room temperature (approximately 25°C), each culture was used to inoculate four new tubes. The new test-tube cultures were placed on the rotator at 25°C and cultivated into exponential phase. At this time, one tube of each strain was kept at 25°C, one supplemented with galactose and kept at 25°C, one transferred to a 35°C shaker, and one supplemented with galactose and transferred to a 35°C shaker. After 5–10 hr, the cells were harvested, permeabilized, and assayed for β-galactosidase (ONPG tests). One of the four strains showed no temperature-inducibility: β-galactosidase was formed

only in the galactose-containing tubes. The other three strains did exhibit the ts phenotype. β-galactosidase production was induced by either galactose addition or temperature-shift. For these strains, β-galactosidase was formed in all tubes except the ones kept at 25°C without galactose.

The three strains were further tested in a batch induction experiment. In early exponential phase, the flasks were transferred from 25°C to 35°C. Samples taken each hour were permeabilized and assayed for β-galactosidase. Two of the strains behaved identically; after a lag of approximately 2 hr, β-galactosidase activity increased sharply with time. These two strains came from the same plate in the initial isolation experiment, and likely possess the same ts mutation. The third strain exhibited very low β-galactosidase activity even after 5 hr at 35°C. One of the two more productive strains was used for the remaining steps in the ts strain construction. It is designated as tsYM603.

A diploid version of tsYM603 was obtained by a forced mating of tsYM603 (without pLGSD5) and a petite mutant of tsYM603 harboring pLGSD5. Diploid cells were selected on ura^- glycerol minimal plates and checked by sporulation. The diploid strain is designated tsD603 and should be identical to D603 with the exception of the ts mutation. (D603 was constructed from haploid strain YM603 in the same manner [9].)

The temperature-sensitivity of the diploid strain was verified by β-galactosidase assays using the same procedure employed for the haploid strains. Again, either T-shift or galactose addition induced β-galactosidase production, and only the culture at 25°C (without galactose) failed to produce the enzyme.

The ts mutation may disrupt the interaction between the *GAL80* and *GAL4* proteins. As previously discussed, a mutation in the *GAL80* gene could result in a thermo-labile (TL) repressor which is unable to bind the *GAL4* protein and allows

induction at 35°C. This ts*gal80* mutation would be recessive; wild-type *GAL80* repressor would prevent induction at 35°C. A ts synthesis (TSS) mutation (repressor no longer synthesized at 35°C) would also allow induction at 35°C. Alternatively, the ts mutation could lie in the *GAL4* gene; at 35°C, the *GAL80* binding site on the activator protein might alter conformation and prevent repression. This ts*GAL4* mutation would be dominant.

To determine the site of the mutation, haploid strain tsYM603:pLGSD5 was crossed with YM623 and diploids were selected on ura$^-$, met$^-$, tyr$^-$ minimal plates. Four of the resulting diploid cells (obtained from independent crosses) were tested by β-galactosidase assays in the manner previously described. The ts phenotype was lost in all four diploids; β-galactosidase was formed only in the presence of galactose. The diploids have wild-type *GAL80* and *GAL4* genes from YM623, and possibly a ts*gal80* or ts*GAL4* gene from tsYM603:pLGSD5. The loss of the ts phenotype indicates that the mutation is recessive, and suggests that the ts mutation lies in the *GAL80* gene or in the synthesis of the *GAL80* repressor protein.

Further experiments to differentiate between TL (ts*gal80*) and TSS mutations proved inconclusive. However, some indirect evidence comes from batch induction experiments with the ts strain (see section 6.5.2). After increasing temperature to 35°C, long lags (relative to those for galactose induction) were observed before the production of β-galactosidase. Since the timescales for repressor turnover and dilution by growth (required for induction in a strain with a TSS mutation) are greater than that for denaturation (TL), the long lag may indicate a mutation in the synthesis of the *GAL80* protein. Alternate explanations, however, may exist for the increased lag.

6.5 Results

6.5.1 Growth Behavior

To determine the influence of temperature and cloned gene expression on the ts and non-ts strains, growth-rates were determined during batch cultivation. Strains D603 and tsD603 (harboring pLGSD5 and plasmid-free) were cultivated at various temperatures. Galactose was included as an inducer in some of the flasks. To facilitate heat transfer and provide stricter temperature control, a water bath shaker was utilized. Growth rates were generally higher and more consistent than those obtained in an air shaker.

The results are shown in Table 6.1. The values for D603 (column 1) illustrate the general effect of temperature on growth-rate in the absence of plasmid gene expression. Growth-rate is low at 25°C, reaches a maximum around 30°C–32.5°C, and falls off slightly at 35°C. The values for tsD603 (column 2) and D603:pLGSD5 (column 3) are nearly identical to those for D603. Therefore, the ts mutation and *URA3* expression (from the selection gene on the plasmid) have little or no effect on growth rate.

Columns 4–6 illustrate the effect of cloned gene expression (and temperature) on growth-rate. The measured growth-rates for the plasmid-containing strains in the presence of galactose (columns 5–6) are generally lower at all temperatures. The observed drop in growth-rate is due to β-galactosidase production induced by galactose.

β-galactosidase production can be induced by T-shift (as well as by galactose addition) in the ts stain. The growth-rate of tsD603:pLGSD5 (column 4) at 30°C is equivalent to that of tsD603 and significantly higher than that of tsD603:pLGSD5 (+ galactose). It is only at 35°C that μ for tsD603:pLGSD5 falls below that of tsD603 and equals that of the strain in galactose-containing medium (see columns

Growth Rate (hr^{-1})

	D603	tsD603	D603:pLGSD5	tsD603:pLGSD5	D603:pLGSD5 (+ galactose)	tsD603:pLGSD5 (+ galactose)
25°C :	0.26	0.28	0.25	0.27	0.23	-----
30°C :	0.36	0.37	-----	0.37	0.32	0.33
32.5°C :	0.38	0.38	0.38	0.40	0.36	-----
35°C :	0.34	0.35	0.35	0.32	0.34	0.32

Table 6.1 Influence of Temperature and Cloned Gene Expression on Growth Rate

2, 4, 6). These results indicate that significant β-galactosidase production (induced by temperature alone) occurs only after a T-shift to 35°C.

For all strains, growth rate at 25°C is significantly below that at 30°C–35°C. The value for tsD603:pLGSD5 at 25°C (μ = .27 hr^{-1}) is low for both batch and continuous fermentations. At 30°C, there is some background expression of β-galactosidase in tsD603:pLGSD5 (see section 6.5.2); however, the level is low compared to that at 35°C. Therefore, a T-shift from 30°C to 35°C appears to be best for T-shift induction of the ts strain.

6.5.2 Batch Induction Results

The performance of the ts strain was characterized in batch induction experiments. The initial kinetics of cloned gene induction and the level of cloned gene expression were determined for the ts and non-ts strain. A direct comparison could then be made between the two methods of induction: T-shift and galactose addition.

In the first set of experiments, exponentially growing cultures of D603: pLGSD5 and tsD603:pLGSD5 at 30°C were induced by the addition of galactose, raising the temperature to 35°C, or both.

Figure 6.2 illustrates the induction behavior of both strains at 35°C. At t = 0 on the figure, all cultures were shifted to 35°C, and galactose was added to one flask of each strain. The curves show the increase in β-galactosidase with time after induction. tsD603:pLGSD5 produces a low level of β-galactosidase at 30°C. This is evident from the non-zero y-intercept for the ts curves.

Two important results should be noted. First, the lag before induction of *lacZ* expression is much longer for the ts strain induced by T-shift alone. β-galactosidase specific activity increases only slightly during the first two hours of induction. This observation might be explained by a *GAL80* TSS mutation: the

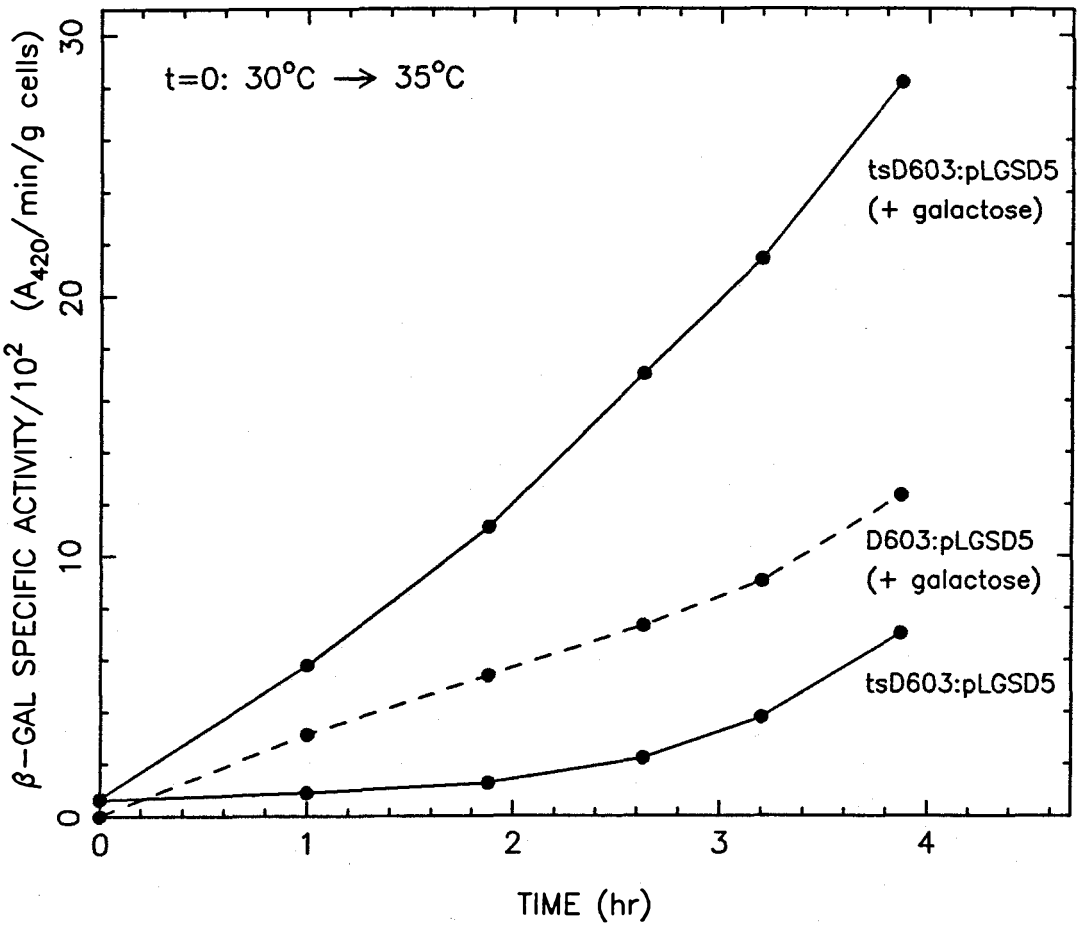

Figure 6.2 Induction Behavior at 35°C in Batch Culture

GAL80 repressor already present in the cell must be turned over or diluted by growth before significant induction occurs.

The second important feature is the high expression level observed for the ts strain induced by both T-shift and galactose addition. After ca. 4 hr of induction, the β-galactosidase produced by the ts strain is more than twice that produced by D603:pLGSD5 under identical conditions. This may be due to some unknown effect of the ts mutation. It is more likely, however, that the greater level of *lacZ* expression is due to "increased inducer level." Although 0.4% galactose (in 0.4% glucose medium) is sufficient for a linear correlation between copy number and expression, raising galactose concentration beyond 0.4% does increase induction of β-galactosidase expression somewhat (see Chapter 4). Therefore, the combination of 0.4% galactose and T-shift may deactivate the *GAL80* repressor more completely than galactose alone, resulting in more transcription from the *GAL10-CYC1* promoter of pLGSD5.

Due to the importance of temperature on the growth behavior of the cells, a comparison of induction behavior at 30°C and 35°C is worthwhile. Figure 6.3 illustrates the induction behavior during exponential phase of both strains after galactose addition (at 30°C) and after galactose addition and T-shift (30°C to 35°C). The curves for the latter case are the same as those in Figure 6.2. For both the ts and non-ts strains, β-galactosidase production is significantly higher for the cultures kept at 30°C. This is especially true for the non-ts strain. The lower expression observed at 35°C is not surprising; the cells grow less efficiently (growth-rate and yield are lower) at 35°C. Note also that, at both temperatures, higher expression levels are again observed for galactose induction in the ts versus the non-ts strain.

The next set of batch induction experiments was designed to study long-term

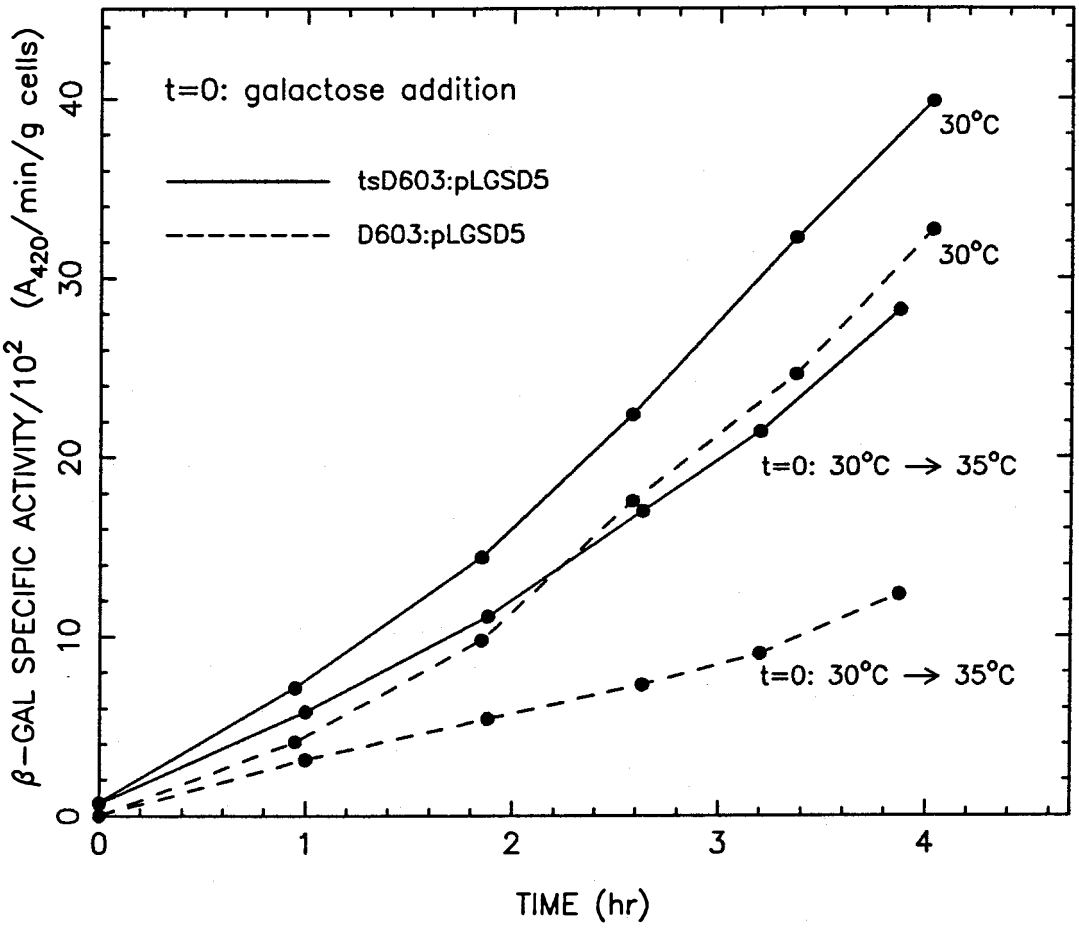

Figure 6.3 Comparison of Induction Behavior at 30°C and 35°C in Batch Culture

expression levels rather than initial induction behavior in the two strains. Rather than inducing in exponential phase, tsD603:pLGSD5 and D603:pLGSD5 were cultivated under constant conditions for the entire experiment. This eliminates the complication of the longer lag observed for T-shift induction and provides a better comparison of the two induction methods. Growth and expression were studied at 30°C and 35°C, with and without galactose. Growth-rates were measured during exponential phase. In 0.4% glucose medium, stationary phase is reached by ca. 60 hr after inoculation. In 0.4% glucose + 0.4% galactose medium, however, the cells utilize the galactose as a carbon source, and the attainment of stationary phase requires more time. (See Figure 4.1 in Chapter 4 for representative growth curves in the two media.) Therefore, cell biomass (g cells/L) was determined after 65 and 90 hr of cultivation, and β-galactosidase specific activity was determined in late exponential phase (at a biomass concentration of 0.4 g cells/L) and after both 65 and 90 hr of growth.

The results are shown in Table 6.2. The trends in growth-rate are the same as those observed in section 6.5.1. At 30°C, strain tsD603:pLGSD5 (without galactose and therefore producing little β-galactosidase) has the highest growth-rate. At 35°C, all strains produce significant amounts of enzyme and have similar values of μ.

The cell densities obtained for all three strains are lower at 35°C than at 30°C. This is due to less efficient growth at 35°C. The low biomass concentrations for tsD603:pLGSD5 at both temperatures reflect the lack of galactose (as a carbon source) in the medium. Biomass does not increase after 65 hr in this case.

The β-galactosidase specific activity results in Table 6.2 are quite interesting. The trends observed in the previous section hold true: expression in glucose + galactose medium is better at 30°C, and the highest levels of β-galactosidase

			D603:pLGSD5 (+ galactose)	tsD603:pLGSD5	tsD603:pLGSD5 (+ galactose)
Growth Rate (hr^{-1}) :					
	30°C		0.32	0.37	0.33
	35°C		0.34	0.32	0.32
Biomass (g cells/L) :					
	30°C	**65 hr**	2.57	1.80	2.46
		90 hr	2.82	1.80	2.89
	35°C	**65 hr**	2.06	1.31	1.86
		90 hr	2.40	1.35	2.22
β-gal Specific Activity/10^2 (A_{420}/min/g cells) :					
	30°C	**Late exp.**	65.3	2.04	75.4
		65 hr	53.0	11.1	81.2
		90 hr	37.4	10.7	52.8
	35°C	**Late exp.**	29.8	25.7	45.2
		65 hr	27.7	38.5	25.5
		90 hr	12.3	36.0	17.0

Table 6.2 Growth and β-galactosidase Specific Activity during Batch Cultivation at 30°C and 35°C

are produced by the ts strain in galactose-containing medium. In this set of experiments, however, the cells have been cultivated under inducing (or non-inducing) conditions from the start, including the "overnight" test-tube cultures. The results, therefore, reflect the effects of long-term induction. For example, β-galactosidase levels at the end of exponential phase in these experiments are greater than those observed when induction occurs during exponential phase (compare with Figures 6.2 and 6.3).

The change in β-galactosidase specific activity with time in batch culture is worth noting (Table 6.2). In general, the strains cultivated in galactose-containing medium exhibit maximum enzyme specific activity in late exponential phase (relative to measured activities at 65 and 90 hr). Specific activities are lower at 65 hr, and drop even further by 90 hr. For the ts strain without galactose, however, the minimum β-galactosidase specific activity is observed in late exponential phase and significantly higher activity is found at 65 hr. This level is maintained into far stationary phase. In fact, at 35°C, the specific enzyme level in the ts strain induced by T-shift is comparable (late exponential phase) or better (stationary phase) than that observed for galactose induction in the non-ts strain. Of course, cell densities are greater in galactose-containing medium at stationary phase, so overall productivities are similar.

The decreasing enzyme activity observed in the two galactose-containing cultures is likely due, in part, to inducer metabolism. If the cause were enzyme deactivation or attack by proteases, the same effect would be expected in the culture of tsD603:pLGSD5 induced by T-shift. As the cells leave exponential phase and glucose concentration drops, galactose is utilized as a carbon source (see Chapter 4). As inducer level drops, *lacZ* gene expression may also fall for the remaining generations of growth. However, the specific activity of the ts strain (+ galac-

tose) at 35°C falls below that of the cells induced by temperature alone (Table 6.2). This suggests that inducer metabolism is not the sole cause of the loss in specific activity with time. Decreasing copy number and many of the other factors influencing cloned gene expression in this system may be involved.

6.5.3 Continuous Fermentation Results

To further characterize the ts expression system, β-galactosidase synthesis was induced during a continuous fermentation of tsD603:pLGSD5. The response of the system to induction by T-shift is illustrated in Figure 6.4.

At time zero, biomass and β-galactosidase specific activity were at an "uninduced" steady-state at 30°C and a dilution rate of 0.2 hr^{-1} (τ_R = 5 hr). Both biomass and enzyme activity had been relatively constant for several residence times (τ_R). At t = 0, temperature was shifted to 35°C, and the resulting transients in β-galactosidase expression and biomass are illustrated. Specific activity rapidly increased, remained at the maximum level for 2–3 τ_R, and then fell off to an induced steady-state. (This is the same general trend observed for all continuous fermentations in the preceding chapter.) Biomass decreased continuously to the induced steady-state; the slight humps in the data at 60 hr were caused by a leak in the feed line.

The T-shift of 30°C to 35°C affects the fermentation in two direct ways: by changing the growth properties of the cells and by inducing higher levels of cloned gene expression. The combined effect of these changes influences the enzyme and biomass profiles. The decrease in biomass likely results from the drop in growth rate and yield caused by both temperature change and cloned gene product synthesis. Approximately 2 τ_R (2 doubling times) are required for the cells to reach peak β-galactosidase expression. As biomass continues to fall, however, β-galactosidase specific activity also begins to decrease. Selection is likely the

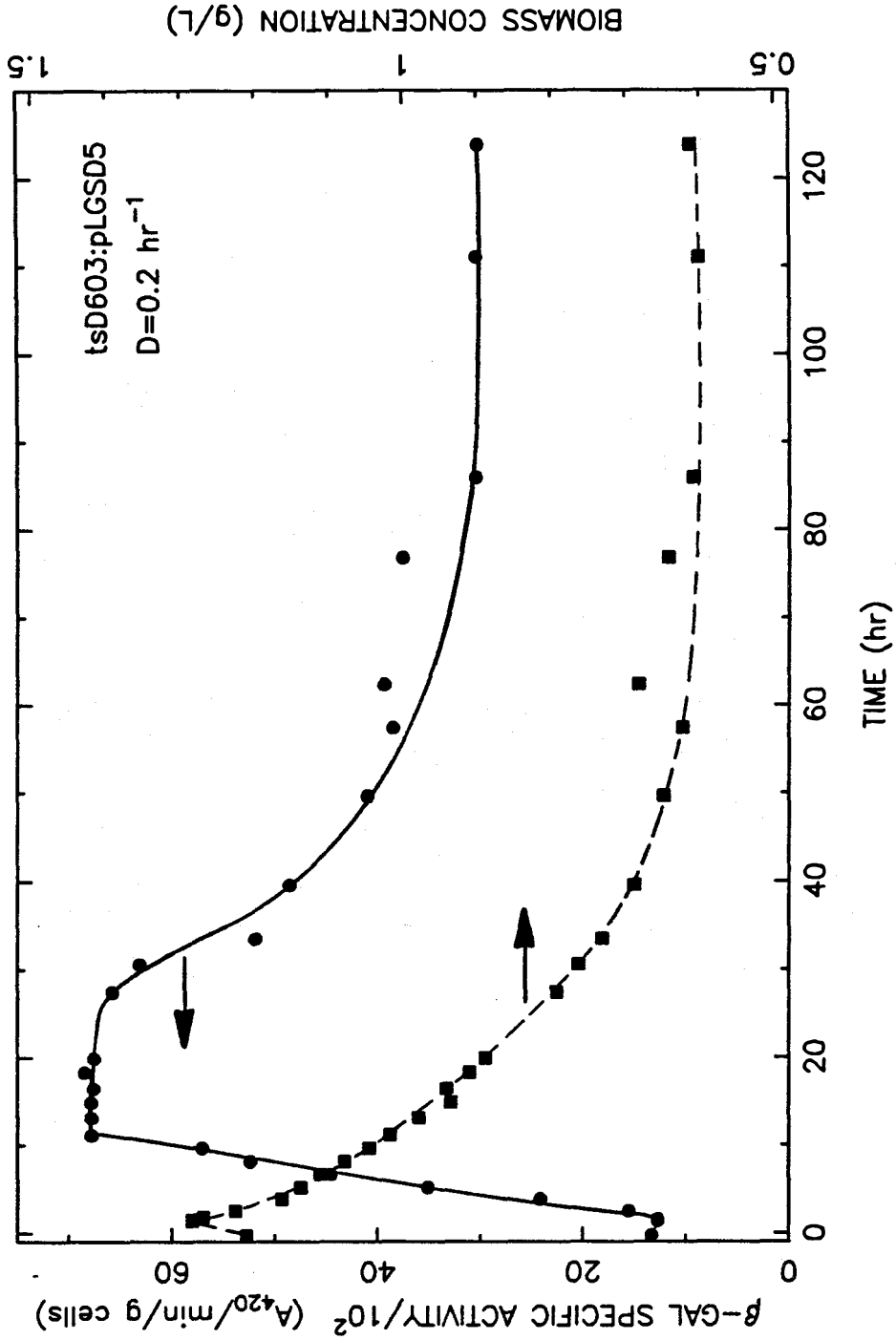

Figure 6.4 β–galactosidase Specific Activity and Biomass Concentration after Induction by T–Shift (30°C to 35°C) in Continuous Culture

important factor. The slower growing cells in the population (including the cells expressing the highest levels of β-galactosidase) may be washed out. The overshoot in β-galactosidase activity is likely due to several factors. The effect of cloned gene expression on growth-rate and yield may not be totally manifested until the cells are producing high levels of β-galactosidase. This has been observed in shake flask culture: during the two generations after induction in exponential phase, μ drops only slightly (if at all) and remains above the growth-rate measured for cells induced from inoculation. An additional factor contributing to the overshoot and decrease in β-galactosidase production in the ts fermentation might be a drop in plasmid stability after induction (see Chapter 5).

Induction by T-shift and galactose addition in continuous culture are compared in Figure 6.5. The upper plot (same as Figure 6.4) shows the response of tsD603:pLGSD5 to induction by T-shift (30°C to 35°C). The lower plot is for D603:pLGSD5 induced by galactose addition at $t = 0$. The latter fermentation is described in more detail in Chapter 5. Only the transients leading to the first induced steady-state are shown.

At the uninduced steady-state, the biomass concentrations are the same in both fermentations — 1.2 g/L. Therefore, as observed in batch culture, the level of β-galactosidase production at 30°C has little effect on the growth of the ts strain. The trends in β-galactosidase specific activity after induction are similar, as are the peak levels attained. In the ts strain, however, the initial rate of β-galactosidase production is ca. 30% slower, and the β-galactosidase specific activity at the first uninduced steady-state is much lower: 30×10^2 (Abs_{420}/min/g cells) in the ts fermentation versus 58×10^2 in the galactose-induced fermentation. It is of interest to note, however, that the β-galactosidase specific activity for the ts strain is identical to that observed at the long-term steady-state for D603:pLGSD5 (see

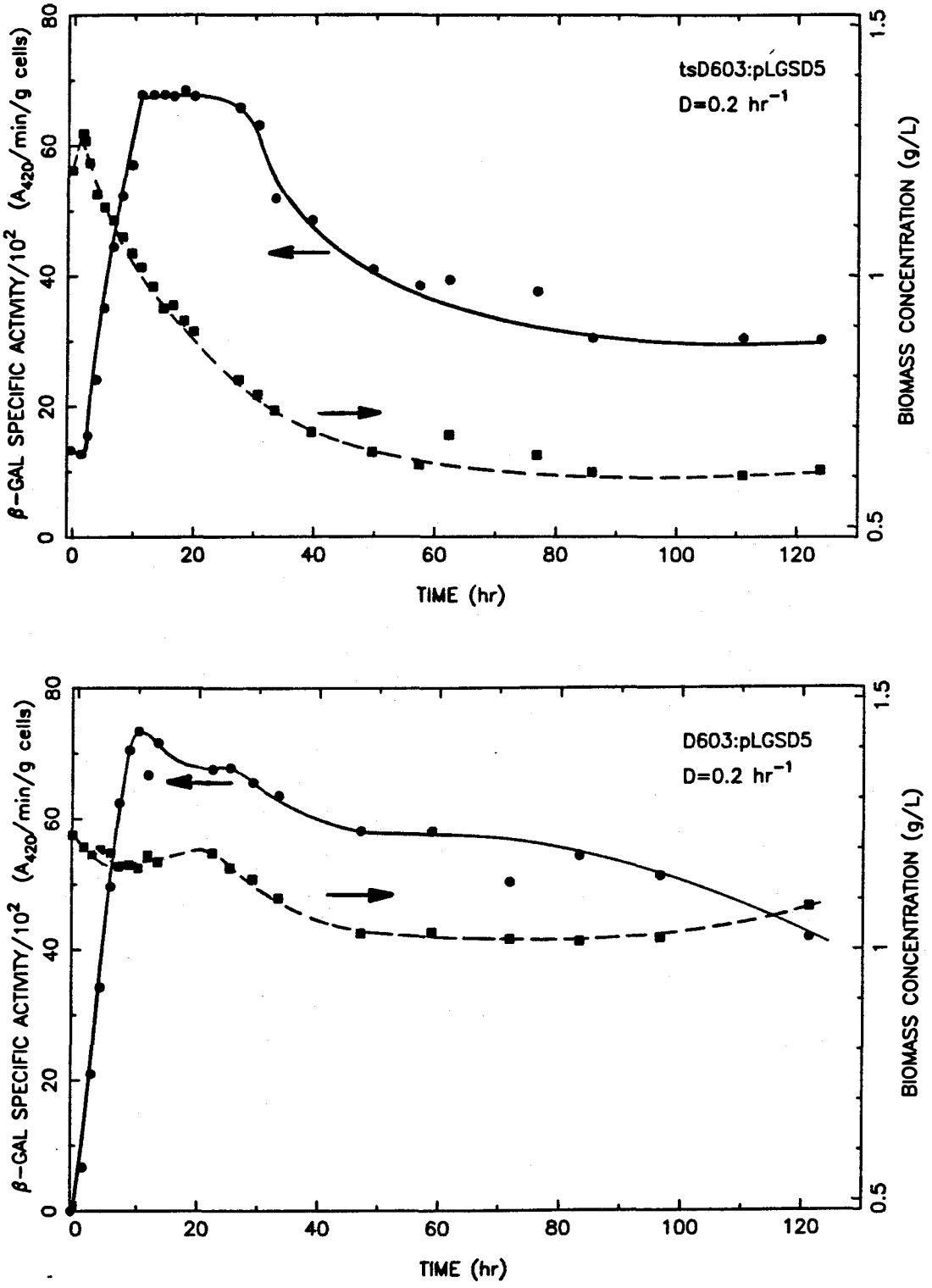

Figure 6.5 Comparison of Induction Methods in Continuous Culture

Chapter 5, Table 5.1). The ts fermentation was terminated after attainment of the first uninduced steady-state. Whether or not further drift would have occurred is not known.

The biomass profiles for the two fermentations are quite different. For D603: pLGSD5 induced by galactose addition, biomass decreases only slightly before the peak in β-galactosidase, and then drops gradually to the induced "steady-state". Biomass in the ts fermentation, however, falls sharply once induced. Cell density in the ts fermentation drops by 50% (1.2 g/L to 0.61 g/L) after induction, while only a 20% decrease is observed for the non-ts strain (1.2 g/L to 1.0 g/L).

As discussed in Chapter 5, an important factor in the galactose-induced fermentations is galactose metabolism. Under carbon-limited conditions, glucose concentrations are very low. Therefore, the inducer is utilized as an additional carbon source which may help keep biomass high. In addition, the temperature remains at 30°C, so the deleterious effects of temperature change are not observed. These factors may also influence cloned *lacZ* gene expression. In the fermentation at 30°C with additional carbon source (galactose), the cells may be under less stress. Therefore, selection for cells producing low levels of β-galactosidase might not be as great.

6.6 Discussion

The aim of this work was to develop a temperature-sensitive yeast strain in which *GAL* regulated cloned gene expression is controlled by temperature, and to compare the results of induction by T-shift and galactose addition. In the resulting strain, T-shifts of 25°C to 35°C and 30°C to 35°C provided excellent and good "off/on" control, respectively.

In continuous fermentations, galactose addition was the superior induction method. Both biomass and cloned gene product synthesis were higher than those in the ts fermentation. The primary reasons appear to be the beneficial effects of additional carbon source (galactose) under carbon-limited conditions, and the negative impact of increased temperature on growth. It is likely that the performance of the ts expression system could be improved by enriching the medium. In addition, more comparable levels might have been observed for growth under alternate limitations, where additional carbon source is not metabolized.

The advantages of T-shift induction were more apparent in batch fermentations. Although longer lags were observed for induction by T-shift during exponential phase, the ts strain performed well. When the cells were cultivated at 35°C throughout the experiment, cloned gene expression induced by T-shift was comparable or better than that observed for galactose induction under the same conditions. Higher levels were observed, however, for galactose induction at the preferred temperature of 30°C. At either temperature, the best levels of cloned gene expression were obtained with the ts strain in galactose-containing medium. The enhanced β-galactosidase production (relative to that for the non-ts strain) effectively reduces the level of galactose required for a given expression level.

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CHAPTER 7

THEORETICAL GROWTH YIELD ESTIMATES FOR RECOMBINANT CELLS

7.1 Introduction

The presence of plasmids in recombinant cells can be viewed as a form of intracellular parasitism. Plasmids may affect the host cell allocation of precursors, energy reserves, catalysts (e.g., RNA polymerase and ribosomes), activators, and repressors (e.g., of replication). Consideration of all of these effects requires a complete kinetic picture of metabolism including regulatory phenomena. This is approximately accessible for *Escherichia coli*, but not for other genetically engineered hosts.

Part of the intracellular parasitic phenomenon can be analyzed in the absence of kinetic and regulatory information. The synthesis of plasmids and their products requires that nutrients be utilized to satisfy the additional material and energy demands of the cell. Thus the metabolic stoichiometry is altered. Such stoichiometric perturbations can be analyzed independently of cell kinetics.

Following Stouthamer's approach, theoretical yield factors have been estimated for recombinant cells. These reflect the additional burden plasmids can place on the host cell purely in terms of energetic and material requirements. The microorganism chosen for the calculations is *E. coli*. However, the method can be readily extended to other organisms for which ATP use in metabolic activities is known or can be reasonably estimated.

7.2 Framework for $Y_{ATP}{}^{max}$ Yield Factor Calculations

The values of the theoretical yield coefficient $Y_{ATP}{}^{max}$ (g cells/mol ATP) illustrate, in part, the effect plasmids can have on their host cell. The calculations for $Y_{ATP}{}^{max}$ are based on those of Stouthamer [1–4], but extend his work to recombinant *E. coli*.

To determine $Y_{ATP}{}^{max}$, Stouthamer calculated the number of moles of ATP required, based upon known biosynthetic pathways, to form one gram of biomass in defined media. The monomer composition of *E. coli* cells determined by Morowitz [5] was taken as a base and used to estimate the percentage of polysaccharide (16.6%), protein (52.4%), lipid (9.4%), RNA (15.7%), and DNA (3.2%) in an average *E. coli* cell. The moles of ATP required for biosynthesis of the various macromolecules and nutrient transport were carefully counted to determine the total ATP required per gram of cells synthesized [1]. The reciprocal of this is $Y_{ATP}{}^{max}$. The theoretical yield factor calculations for plasmid-containing cells are performed in a similar manner. The ATP required for the additional plasmid DNA and product protein, however, must now be considered. It should be emphasized that the calculations give the theoretical maximum Y_{ATP} values. Actual Y_{ATP} values may be significantly lower due to maintenance and other processes described below.

A number of approximations are necessary for the calculations. Stouthamer's assumptions have been adopted and can be found in the references [1–2]. They primarily include transport approximations and simplifying assumptions about the composition of the cell (e.g., polysaccharide content calculated as polyglucose, lipid content calculated as phosphatidylethanolamine with two C16 chains).

In order to extend the $Y_{ATP}{}^{max}$ calculations to recombinant cells, the following additional assumptions were invoked:

1. The product protein has the same average composition as the normal cell protein.

2. A cell with plasmids is the same in composition as a normal cell except for the additional product protein and plasmid DNA (i.e., the polysaccharide, lipid, RNA, chromosomal DNA, and cell protein content is considered the same).

3. A cell with plasmids utilizes the same metabolic pathways and methods of transport as a normal cell. Therefore, the amount of ATP required for biosynthesis of any cell component is presumed to be identical for recombinant and plasmid-free cells.

The first assumption was made to simplify the calculations and may be readily eliminated for a specific protein product. The second assumption was necessary because the composition of recombinant *E. coli* cells has not been reported. Changes in microbial cell composition, however, have a relatively small effect on $Y_{ATP}{}^{max}$ unless large amounts of storage materials are synthesized [2]. Neither of these assumptions affects the calculations significantly. The third assumption was made since detailed information on the metabolism of plasmid-containing cells is unavailable. Corrections can be made once any metabolic changes are known.

An average *E. coli* cell is 52.4% protein and 3.2% chromosomal DNA by weight. Its chromosome is approximately 4.7×10^6 base pairs in length [6]. Using this information the following equations for P and D can be derived:

$$P = \frac{52.4\delta}{1-\delta} \tag{1}$$

$$D = \frac{3.2n\beta}{4.7 \times 10^6} \tag{2}$$

where

P = Additional protein resulting from plasmid gene expression

D = Additional DNA (plasmid DNA)

δ = Fraction of total cell protein that is gene product P
(i.e., δ(Cell Protein+P)= P)

n = Copy number (number of plasmids per chromosome equivalent)

β = Number of base pairs in the plasmid

The basis for the calculations is now $100 + P + D$ grams of cells; that is, there are 52.4 grams of cell protein and P grams of plasmid product protein in $100+P+D$ grams of biomass rather than in 100 grams. Therefore, a new percent composition for the *E. coli* cells was calculated. The growth medium was assumed to be glucose and inorganic salts with NH_3 as the nitrogen source. Using the same method as Stouthamer, the ATP requirements for formation, polymerization, and transport in recombinant cells were determined. The equations for these values are summarized in Table 7.1. The sum of the ATP requirements can be used to determine $Y_{ATP}{}^{max}$:

$$Y_{ATP}{}^{max} = \frac{1}{(\text{Total ATP requirement in moles/g cells})} \qquad (3)$$

An analysis of yield on other growth media may be accomplished after appropriate adjustments in the ATP requirements for biosynthesis and transport.

Table 7.1 Summary of Equations: ATP Requirements for Synthesis and Transport

	Moles ATP Required/g Cells
Synthesis of:	
Polysaccharide	$\frac{2(16.6)}{161.8(100+P+D)}$
Protein (total)	
formation	$\left\{1+\frac{P}{52.4}\right\}\left\{\frac{13.55\times10^{-2}}{100+P+D}\right\}$
polymerization	$\frac{4(52.4+P)}{109.5(100+P+D)}$
Lipid	$\frac{9.4}{671.4(100+P+D)}$
RNA	
formation	$\frac{34.50\times10^{-2}}{100+P+D}$
polymerization	$\frac{2(15.7)}{341.3(100+P+D)}$
DNA (total)	
formation	$\left\{1+\frac{D}{3.2}\right\}\left\{\frac{8.64\times10^{-2}}{100+P+D}\right\}$
polymerization	$\frac{2(3.2+D)}{333.3(100+P+D)}$
mRNA Turnover	$\frac{2(1.5)(15.7)}{341.3(100+P+D)}$
Transport of:	
Phosphorous	$\frac{2.1+0.3(1+\frac{D}{3.2})}{30.97(100+P+D)}$
K^+ Ions	$\frac{0.5(1.5)}{39.10(100+P+D)}$
NH_4^+ Ions	$\frac{(64.0\times10^{-2})(1+\frac{P}{52.4})+(3.6\times10^{-2})(1+\frac{D}{3.2})+(17.25\times10^{-2})}{2(100+P+D)}$

7.3 Theoretical $Y_{ATP}{}^{max}$ Estimates

The effect of varying levels of plasmid gene expression and copy number on the ATP requirements and $Y_{ATP}{}^{max}$ were determined. It is presumed that these characteristic stoichiometric parameters of the recombinant cell vary independently, since plasmid constructions with different origins of replication and different promoters and ribosome binding sites provide a broad spectrum of copy number-expression level combinations. The influence of any correlation that might exist between copy number and expression level for a particular class of host-vector systems may easily be analyzed using the general equations in Table 7.1. Sample results are shown in columns 2, 3, and 4 of Table 7.2. The plasmid was assumed to be the size of pBR322, which contains 4362 base pairs [7]. Therefore, D is $2.97 \times 10^{-3}n$ in the equations in Table 7.1. The δ values considered here, 0.2 and 0.5, correspond to cloned-gene product expression at 20 and 50% of total cell protein. These values, at the high end of the range reported for recombinant *E. coli*, are used repeatedly here to show, in the extreme, the extent to which plasmid-directed activity alters energetic and material stoichiometry. Yield factors for other cases can be evaluated from the general equations. As illustrated in the table and in other calculations (not shown), plasmid copy number (n) has little or no effect on $Y_{ATP}{}^{max}$. The ATP requirement for the synthesis of the additional plasmid DNA is minimal compared to that required for the excess product protein. The level of expression, however, does influence the $Y_{ATP}{}^{max}$ values (compare columns 1–3).

Neither copy number nor expression level, however, greatly changes $Y_{ATP}{}^{max}$ as can be seen by comparing any of these columns (2, 3, or 4) with column 1. The maximum difference is only approximately 10% for the cases shown. This insensitivity to plasmid metabolic perturbations is a consequence of basing yield

Table 7.2 ATP Requirements (moles $\times 10^{-4}$/g cells) and $Y_{ATP}{}^{max}$ Estimates

ATP Requirement for Synthesis of	Normal Cell	Recombinant Cell					
		Product Retained			Product Secreted		
		δ=0.2 n=50	δ=0.5 n=50	δ=0.5 n=100	δ=0.2 n=50	δ=0.5 n=50	δ=0.5 n=100
Polysaccharide	20.52	18.12	13.45	13.44	20.49	20.49	20.46
Protein (f)	13.55	11.96	8.88	8.87	13.53	13.53	13.51
(p)	191.42	169.02	125.48	125.36	191.13	191.13	190.85
Lipid	1.40	1.24	0.92	0.92	1.40	1.40	1.40
RNA (f)	34.50	30.46	22.62	22.59	34.45	34.45	34.40
(p)	9.20	8.12	6.03	6.03	9.19	9.19	9.17
DNA (f)	8.64	7.63	5.66	5.66	8.63	8.63	8.61
(p)	1.92	1.70	1.26	1.26	1.92	1.92	1.91
Product (f)	0.00	2.99	8.88	8.87	3.38	13.53	13.51
(p)	0.00	42.26	125.48	125.36	47.78	191.13	190.85
Plasmid DNA (f)	0.00	0.35	0.26	0.53	0.40	0.40	0.80
(p)	0.00	0.08	0.06	0.12	0.09	0.09	0.18
mRNA Turnover	13.80	12.19	9.05	9.04	13.78	13.78	13.76
Sub-total	294.95	306.12	328.03	328.03	346.16	499.66	499.41
Transport of							
NH_4^+	42.42	44.60	48.84	48.85	50.43	74.40	74.37
K^+	1.92	1.69	1.26	1.26	1.92	1.92	1.91
P	7.75	6.88	5.11	5.13	7.78	7.78	7.82
Total ATP Requirement	347.04	359.30	383.24	383.27	406.29	583.76	583.51
$Y_{ATP}{}^{max}$ (g cells/mol ATP)	28.8	27.8	26.1	26.1	24.6	17.1	17.1

on the mass of biomass produced rather than on the number of cells obtained. As mentioned previously, Stouthamer found that cell composition does not greatly affect Y_{ATP}^{max} [2]. The slight decrease that is observed in Y_{ATP}^{max} is due to the significant increase in protein content in the recombinant cell.

To better appreciate the detrimental effect which plasmid-directed synthesis can have on the cell, the results should be viewed on a per cell basis. While the ATP required per gram of cells is not drastically increased, the amount per cell is. Assuming the product protein remains in the cell, each cell now weighs significantly more. A large proportion of the cell is not active host cell biomass, but product protein. For a δ of 0.5 and a copy number of 100, one gram of recombinant *E. coli* cells contains 52.7% fewer cells than one gram of plasmid-free cells. These theoretical yield calculations indicate that, *on a per cell basis*, those cells with plasmids require approximately 68% more ATP. This shows more clearly the increased biosynthetic, and hence energetic, burden on the host cell when substantial inactive biomass must be synthesized.

Another way to appreciate the additional demands on the microorganism is to consider product secretion. If the *E. coli* cell secretes all of the cloned-gene protein product, the theoretical yield factors listed in columns 4, 5, and 6 of Table 7.2 are obtained. (No ATP requirement for secretion is included.) In this case, the mass of recombinant cells is essentially the same as the plasmid-free host cell mass, since the additional plasmid mass is negligible. Accordingly, calculated yield factor values for secreted product on a cell mass basis are exactly the same as those for intracellular product accumulation on a per cell basis. Again, for δ=0.5 and n=100, 68% more ATP is required.

In a recombinant cell, turnover of mRNA may increase. Assuming that additional mRNA turnover is proportional to the amount of protein product synthe-

sized, the theoretical estimate for $Y_{ATP}{}^{max}$ (g cells/mol ATP) in a recombinant cell is 25.5 for δ=0.5, n=100, and an intracellular product. The effect of this possible increase in mRNA turnover on $Y_{ATP}{}^{max}$ is a modest 2.3% decrease.

7.4 Extension to Y_{glu}^{max} and $Y_{O_2}^{max}$ Calculations

The values of the yield factors Y_s (g cells/mol substrate) and, in the case of aerobic growth, Y_{O_2} (g cells/mol O_2) are often desired. The actual values of these quantities cannot be calculated since they depend on maintenance and other kinetic phenomena which are outside the scope of stoichiometric analysis. However, the maximum theoretical values Y_s^{max} and $Y_{O_2}^{max}$ can be estimated from the Y_{ATP}^{max} values if the substrate assimilation equation and the number of phosphorylation sites in the microorganism's respiratory chain are known. Again, a comparison of the results for normal and recombinant cells shows the extent to which synthesis of plasmids and plasmid products perturbs cellular stoichiometry.

The calculations of Y_s^{max} (in this case Y_{glu}^{max}) and $Y_{O_2}^{max}$ are based on those of Stouthamer's in reference [2], p. 11. The same procedure is followed, but is extended to recombinant systems. The calculations presented here are for aerobic growth. Y_{glu}^{max} for anaerobic growth may be determined by similar methods.

As in Stouthamer's work, the normal cell formula was taken as $CH_{1.807}N_{0.23}O_{0.512}$ and "H_2" refers to reducing equivalents. This cell formula has been altered for the recombinant cells; both the plasmid DNA and product protein (when retained in the cell) are included. Thus, recombinant cell composition $CH_aN_bO_c$ depends on plasmid content, the level of gene expression directed by plasmid genes, and the extent to which cloned gene product is secreted.

Glucose is both assimilated into biomass and dissimilated for energy. The glucose assimilation equation is

$$C_6H_{12}O_6 + \alpha NH_3 + \epsilon \text{“}H_2\text{”} \rightarrow \gamma CH_aN_bO_c + \eta H_2O + \omega CH_{1.63}N_{0.277}O_{0.296} \quad (4)$$

for secreted protein product which is presumed to have the chemical formula $CH_{1.63}N_{0.277}O_{0.296}$ based on average cell protein composition. If cloned-gene

product is retained in the cell, ω is zero and γ is 6 in equation (4). For given δ and n, the assimilation equation is completely determined.

For every mole of glucose dissimilated by *E. coli* under aerobic conditions, 4 moles of ATP (substrate-level phosphorylation), 10 moles of NADH, and 2 moles of $FADH_2$ are formed. The assimilation equation (4) shows that ϵ/γ moles of NADH are required per mole of biomass synthesized. The balance of the NADH produced from glucose dissimilation is oxidized via the respiratory chain, consuming one-half mole of O_2 per mole of NADH oxidized. Simultaneously, S moles of ATP are generated per mole NADH oxidized, where S denotes the number of phosphorylation sites in the respiratory chain. Each mole of $FADH_2$ oxidized produces $S - 1$ moles of ATP and consumes one-half mole O_2.

For every mole of glucose assimilated, γ moles of cells (with the formula $CH_aN_bO_c$ are formed. Therefore, denoting ${Y_{glu}}^{max}$ by χ and the molecular weight of the cells by MW, χ/γMW moles of glucose are assimilated per mole of glucose and $1 - \chi/\gamma$MW moles are dissimilated. Considering this metabolic partitioning of the glucose consumed and the ATP and oxygen stoichiometry of dissimilatory metabolism, the formulae for ATP production and oxygen consumption outlined in Table 7.3 follow. These results may then be employed with ${Y_{ATP}}^{max}$ estimates to obtain

$$ {Y_{glu}}^{max} = \chi = {Y_{ATP}}^{max} \times \text{ATP Production (mol ATP/mol glu)} \qquad (5) $$

$$ {Y_{O_2}}^{max} = {Y_{glu}}^{max}/\text{Oxygen Uptake (mol } O_2\text{/mol glu)} \qquad (6) $$

7.5 Y_{glu}^{max} and $Y_{O_2}^{max}$ Estimates

Sample values of Y_{glu}^{max} and $Y_{O_2}^{max}$ have also been determined for recombinant *E. coli*. The base case was again taken to be δ=0.5 and n=100. The resulting glucose assimilation equations for product retention and secretion are, respectively.

$$C_6H_{12}O_6 + 1.50NH_3 + 0.36\text{“}H_2\text{”} \rightarrow 6CH_{1.73}N_{0.25}O_{0.43} + 3.41H_2O \quad (7)$$

$$C_6H_{12}O_6 + 1.50NH_3 + 0.36\text{“}H_2\text{”} \rightarrow 3.76CH_{1.80}N_{0.23}O_{0.512} + 3.41H_2O$$
$$+2.24CH_{1.63}N_{0.277}O_{0.296} \quad (8)$$

The secretion equation shows that even at a copy number of 100 the cell formula is the same as that for a normal *E. coli* cell. Using the equations described above for Y_{glu}^{max} and $Y_{O_2}^{max}$, these theoretical yield values were evaluated for 2 or 3 phosphorylation sites in the respiratory chain (Table 7.4). As before, the presence of plasmids decreases the yield factors slightly when the protein is retained in the cell and drastically reduces yields when product is secreted. In these calculations, as in those for Y_{ATP}^{max}, the results for product secretion are essentially identical to those for intracellular product accumulation on a per cell basis.

It is interesting to examine the fate of each mole of glucose used by the recombinant cell for δ=0.5, n=100, and S=2. While 80.6% of the total glucose consumed is employed in assimilatory metabolism by recombinant cells, only 50.5% of the glucose carbon consumed is converted into normal host cell biomass.

Table 7.3 ATP Production and Oxygen Uptake during Aerobic Growth

	ATP Production (mol ATP/mol glu)	Oxygen Uptake (mol O_2/mol glu)
Substrate Phosphorylation	$4\left\{1-\frac{\chi}{\gamma(MW)}\right\}$	---
NADH Oxidation	$(S)(10)\left\{1-\frac{\chi}{\gamma(MW)}\right\}-(S)(\varepsilon)\left\{\frac{\chi}{\gamma(MW)}\right\}$	$5\left\{1-\frac{\chi}{\gamma(MW)}\right\}-0.5(\varepsilon)\left\{\frac{\chi}{\gamma(MW)}\right\}$
$FADH_2$ Oxidation	$(S-1)(2)\left\{1-\frac{\chi}{\gamma(MW)}\right\}$	$1\left\{1-\frac{\chi}{\gamma(MW)}\right\}$
Total	$2+12(S)-\left\{2+(S)(12+\varepsilon)\right\}\left\{\frac{\chi}{\gamma(MW)}\right\}$	$6-\left\{6+0.5(\varepsilon)\right\}\left\{\frac{\chi}{\gamma(MW)}\right\}$

Table 7.4 Estimated Values of Y_{glu}^{max}(g cells/mol glu) and $Y_{O_2}^{max}$(g cells/mol O_2)

Yield Factor	Normal Cell Y_{ATP}^{max}=28.8	Recombinant Cell δ=0.5, n=100	
		Product Retained Y_{ATP}^{max}=26.1	Product Secreted Y_{ATP}^{max}=17.1
Y_{glu}^{max}(2 sites)	123	116	75.9
(3 sites)	130	123	80.1
$Y_{O_2}^{max}$(2 sites)	126	114	75.2
(3 sites)	188	171	109

7.6 Discussion

The calculated values of $Y_{ATP}{}^{max}$, $Y_{glu}{}^{max}$, and $Y_{O_2}{}^{max}$ illustrate the detrimental effect, in terms of metabolic stoichiometry, of plasmid protein production on recombinant *E. coli* cells. This is especially obvious when the product is secreted or the results are viewed on a per cell basis. It is, however, important to stress the limitations of these estimates. The monomer composition and metabolic pathways of the recombinant cells were assumed, as stated earlier, to be the same as those of a normal cell. Once the detailed changes in recombinant cell composition are known, the calculations can be easily corrected. Of more importance is the assumption that the metabolic pathways are the same. Certain reaction pathways of the cell are quite important in determining $Y_{ATP}{}^{max}$. If these pathways are altered considerably in the recombinant cell, the calculated estimates in Tables 7.2 and 7.4 may be incorrect.

It must also be remembered that the calculated values are only the maximum theoretical values of Y_{ATP}, Y_{glu}, and Y_{O_2}. Processes such as maintenance have not been taken into account. True Y_{ATP} values depend on the organism, growth conditions, and growth-rate. Stouthamer [2,8] proposed the equation

$$q_{ATP} = \frac{\mu}{Y_{ATP}} = \frac{\mu}{(Y_{ATP}{}^{max})_{exp}} + m_e = \frac{\mu}{(Y_{ATP}{}^{max})_{theor}} + m_g\mu + m_e \qquad (9)$$

since usually $(Y_{ATP}{}^{max})_{experimental} < (Y_{ATP}{}^{max})_{theoretical}$. This is a variation of Pirt's equation [9]. The parameter q_{ATP} is the specific rate of ATP production (mol ATP/g cells hr) and μ is the specific growth rate (hr^{-1}). The theoretical $Y_{ATP}{}^{max}$ is the calculated value and m_g is a growth-rate-dependent energy term (for processes other than biomass synthesis). Both m_g and m_e include energy required for true maintenance (turnover of constituents in cell, maintenance of correct intracellular pH, etc.) and processes other than true maintenance (energy-

spilling mechanisms, formation of storage materials, respiratory chain branching, etc. during uncoupled growth). It is likely that the ATP requirement for these processes is greater in plasmid-containing cells. Therefore, the difference in Y_{ATP} between normal and recombinant cells may be even greater than the difference in $Y_{ATP}{}^{max}$.

The calculations only investigate the effect of plasmid-directed synthesis on metabolic stoichiometry. The kinetic effects (such as the partitioning of biocatalysts between metabolism and product synthesis) have not been addressed.

Despite the limitations, however, the estimates obtained do illustrate the general stoichiometric effects. The level of expression of the plasmid is very important since the energy cost of protein synthesis is high. The copy number and plasmid chosen, however, have little or no effect from a stoichiometric perspective (except in their relation to the level of expression). The large difference in ATP requirements for the normal and recombinant *E. coli* on a per cell basis is also significant. When the protein is retained, a portion of the biomass is product. The cell can be thought of as containing active host cell biomass (normal cell constituents) and inactive biomass (product). To appreciate the additional material and energy demands placed on the recombinant *E. coli*, the results should be viewed per gram of active biomass, not per gram of cells. This is equivalent to the per cell basis or the secreted case.

Additional knowledge about recombinant cells will improve the theoretical estimates of the yield factors. In particular, changes in the metabolic pathways should be incorporated in such calculations. A great deal must be learned, however, about the kinetic effects of plasmids (as on growth and protein synthesis rates) as well as the changes in maintenance and other requirements before the calculations can be extended from theoretical to actual yield factors.

7.7 References

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CHAPTER 8

CONCLUSIONS

Several methods have been utilized to study host-plasmid interactions and the regulation of cloned gene expression in recombinant cells. Batch and continuous fermentations were employed to examine the effects of several genetic and environmental factors on *GAL* regulated cloned gene expression in *Saccharomyces cerevisiae*. The impact of plasmid-directed synthesis on the host cell was studied experimentally with this system, and theoretically by estimating yield factors for recombinant *Escherichia coli*. A temperature-sensitive (ts) yeast strain was constructed in which *GAL* regulated cloned gene product synthesis is controlled by temperature, and the induction methods of temperature-shift and galactose addition were compared. Specific conclusions from each avenue of study are presented at the end of the corresponding chapter.

Some general conclusions and observations can be made from the work in the preceding four chapters. Inducible promoters, such as the yeast *GAL* promoters, are valuable for controlling cloned gene expression, and for studying the effects of cloned gene product synthesis on the recombinant system. Furthermore, if the molecular mechanism of the regulation is understood, various aspects can be manipulated to improve or alter the system of control. For example, the construction of the ts strain exploited the interaction between the *GAL80* and *GAL4* regulatory proteins, so that an additional carbon source was no longer required for induction of *GAL* regulated gene expression.

Two conclusions from the results in Chapter 5 were that (for this system) a drop in dilution rate or an increase in *GAL* promoter strength increases overall productivity in continuous culture. In these cases, the high β-galactosidase specific activities (and high biomass concentration at low dilution rate) more than

compensated for decreases in plasmid stability and flowrate. At low dilution rate, galactose metabolism and higher growth efficiency increased biomass concentration, which helped boost productivity. Although some of the observed trends (such as the drop in plasmid stability with cloned gene expression) agree with those of previous researchers and are likely true for many recombinant yeast systems, some may be system specific. In this work, inducer metabolism, variable biomass yield with dilution rate, and the *reg1* mutation in the host strain, influenced the behavior observed during continuous culture. In addition, the response to induction of cloned gene expression will depend to a large degree on the plasmid stability, promoter strength, and other characteristics of the particular system employed.

Many factors interact to produce the observed results. This was especially apparent in the continuous fermentations where nearly every measurable quantity changed significantly during the transient period between steady-states. β-galactosidase expression, biomass concentration, plasmid stability, glucose and galactose concentrations, and other variables are all interdependent. At each steady-state, the results must be analyzed keeping all factors in mind. Despite the complex interrelations, some of the trends may be separable and amenable to modeling. Two possibilities are the two-substrate growth kinetics and the plasmid stability and copy number trends.

The batch induction experiments were useful for examining the effects of inducer concentration, plasmid stability, and promoter strength on the induction of cloned gene expression. During the relatively short period of exponential growth, inducer is not metabolized and, except at high expression levels, other quantities such as growth rate and copy number might not change substantially. To study long-term selection or the interdependence of the variables, however, a fed batch or continuous process is necessary. For research and production, each culture method

has its merits and disadvantages.

Appendix A

Medium Optimization and Characterization

Supplemented SD minimal medium was optimized to improve the biomass yields in batch and carbon-limited (C-limited) continuous culture.

Strain D603 is auxotrophic for adenine, histidine, lysine, methionine, and uracil. SD minimal medium, supplemented with these components at the recommended levels (Chapter 3: ref. [9]), contains glucose (20 g/L), nitrogen base without amino acids (6.7 g/L, Difco), adenine-sulfate (20 mg/L), L-histidine-HCl (20 mg/L), L-lysine-HCl (30 mg/L), L-methionine (20 mg/L), and uracil (20 mg/L). To determine the glucose concentration to utilize for C-limited growth, D603 was cultivated in supplemented SD minimal medium at various glucose concentrations in batch culture. Stationary phase biomass concentrations were determined for each glucose level.

The results are illustrated in Figure A.1. The arrow indicates the concentration of biomass obtained for the basic recipe. At this high glucose concentration, biomass concentration is only 1.3 g/L. Furthermore, to ensure C-limited growth, the concentration of glucose must be reduced below 1.0 g/L. The upper limit on biomass concentration in C-limited continuous culture is then below 0.8 g/L.

To improve both batch and continuous growth yields, a series of medium optimization experiments was performed. Figure A.1 shows that glucose is not the limiting component in SD minimal medium, and other experiments indicated that the nitrogen base, adenine, histidine, lysine, methionine, adenine, and uracil were not independently limiting growth. When the concentrations of amino acids and purines were increased simultaneously, however, the results in Figure A.2 were obtained. Again, D603 was cultivated into stationary phase, and the arrow (at the relative concentration of 1) indicates the biomass concentration for the

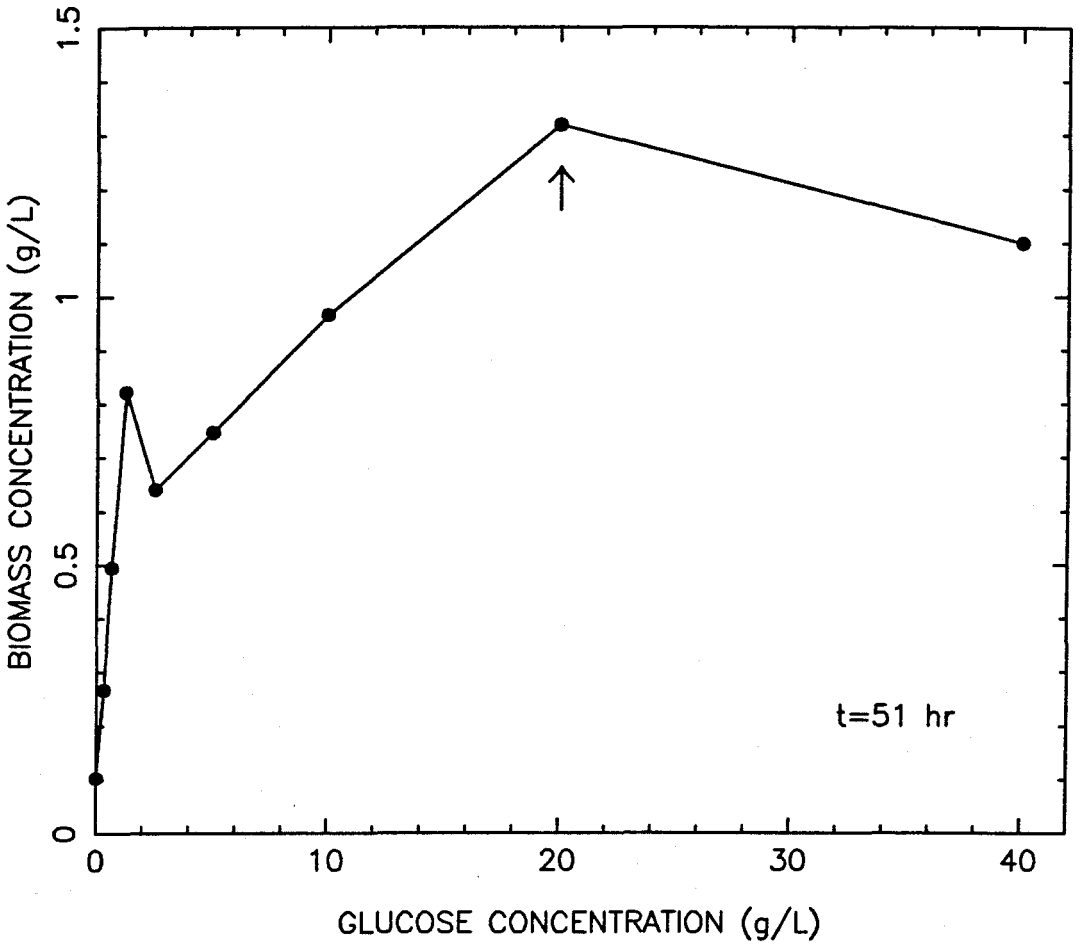

Figure A.1 Dependence of Biomass Concentration on Glucose Concentration in Supplemented SD Minimal Medium

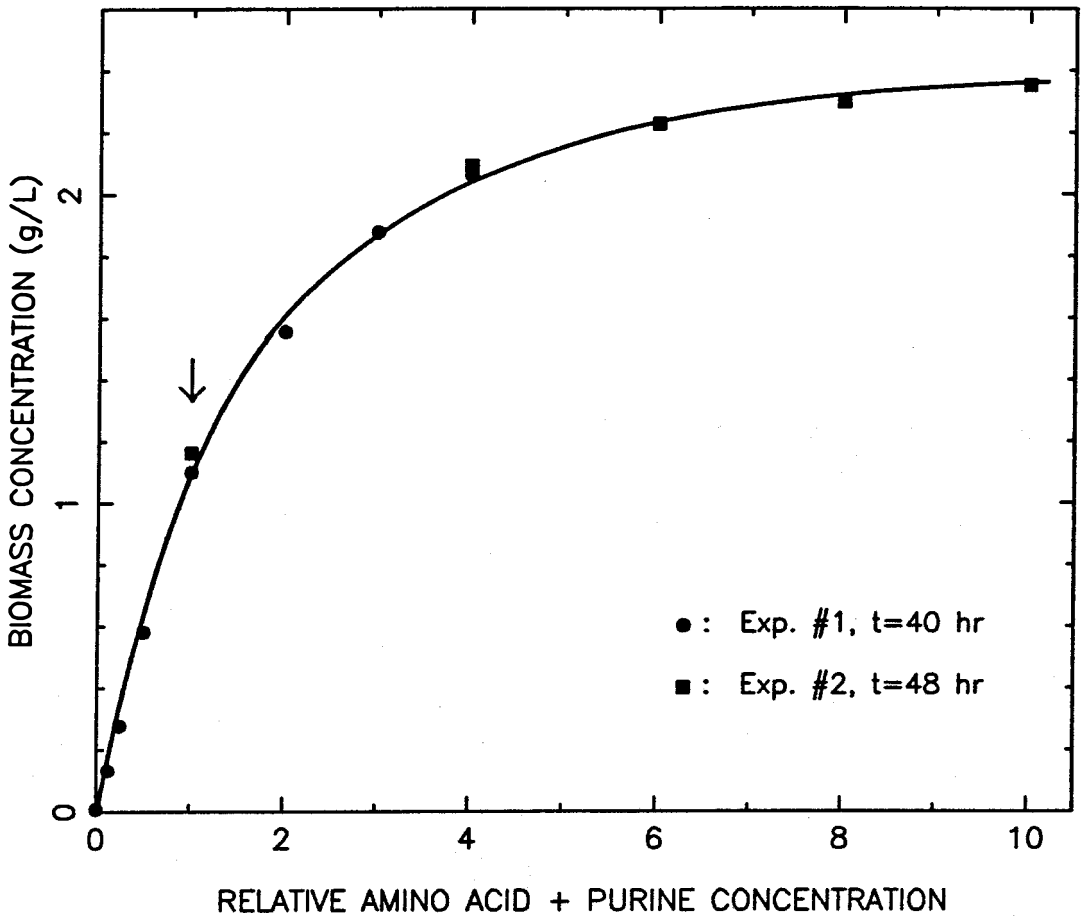

Figure A.2 Dependence of Biomass Concentration on Lumped Amino Acid and Purine Concentration in Batch Culture
(Relative to Levels in Supplemented SD Minimal Medium)

basic supplemented SD minimal medium. These results clearly indicate that some combination of amino acids and purines (for which the strain is auxotrophic) is limiting growth in the original medium.

At a relative concentration of 6 in Figure A.2, this limitation is essentially removed. The concentrations of the amino acids and purines were individually reduced to determine the optimum concentration of each. The results showed that increasing adenine, histidine, lysine, methionine, and uracil concentrations by factors of 5, 4, 5, 5, and 1, respectively, gave the same result as increasing all by a factor of 6: ca. 2.1 g/L biomass. Furthermore, the yield of D603 in this medium is approximately equal to that of a wild-type strain in the original medium. This further indicates the the amino acid + purine limitation has been removed.

To determine the glucose concentration for C-limitation in this new medium, D603 was cultivated into stationary phase at various glucose concentrations. The results are shown in Figure A.3. C-limitation can be achieved at significantly higher glucose and biomass concentrations than were possible in the original medium (compare Figures A.1 and A.3). Therefore, the maximum chemostat biomass concentration can be improved by more than a factor of 2. The results of an additional experiment focusing on glucose concentrations below 10 g/L are illustrated in Figure A.4. A linear correlation between biomass and glucose is obtained for glucose concentrations below 5.0 g/L, and a straight line fit to the date gives a yield coefficient ($Y_{x/s}$) of 0.49 g cells/g glucose. A concentration of 4.0 g/L glucose was chosen for the C-limited growth medium.

Therefore, the recipe for supplemented C-limited medium is: glucose (4 g/L), nitrogen base (6.7 g/L, Difco), adenine-sulfate (100 mg/L), L-histidine-HCl (80 mg/L), L-lysine-HCl (150 mg/L), L-methionine (100 mg/L), and uracil (20 mg/L).

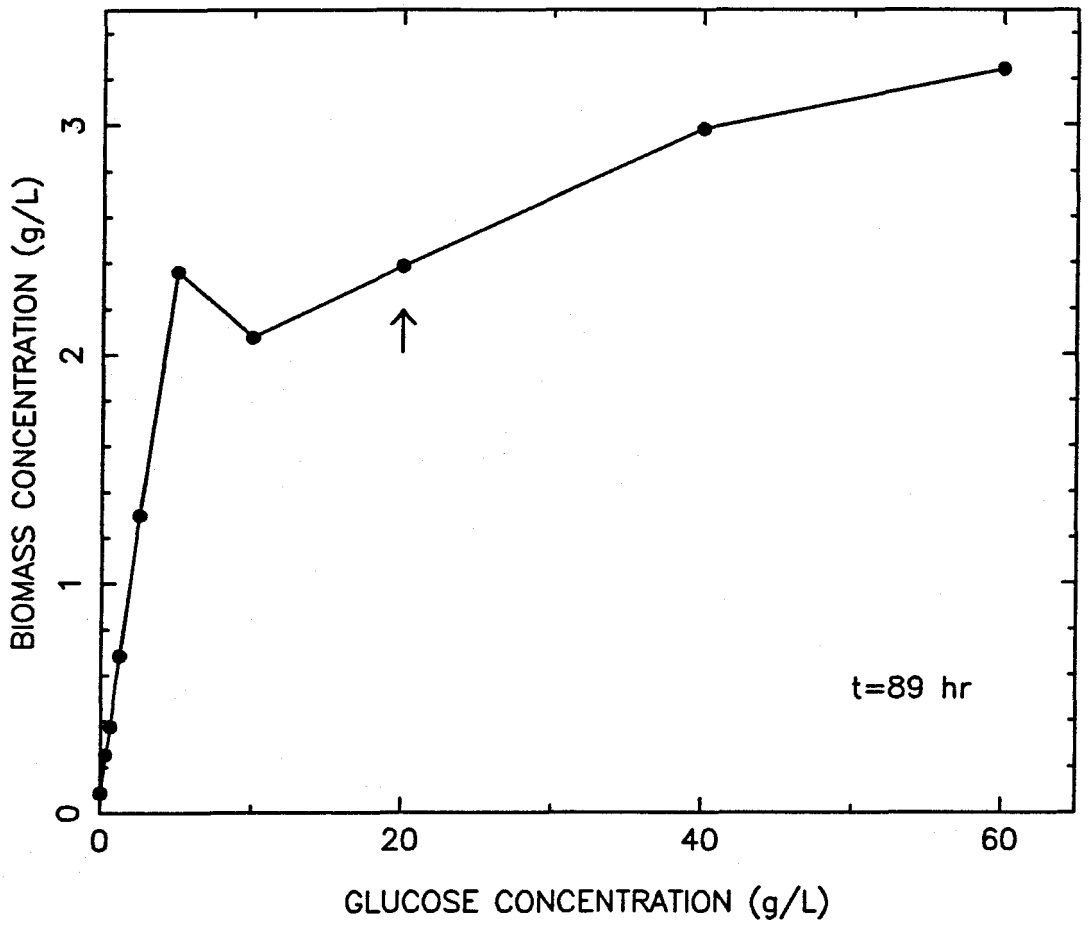

Figure A.3 Dependence of Biomass Concentration on Glucose Concentration in Optimized SD Minimal Medium

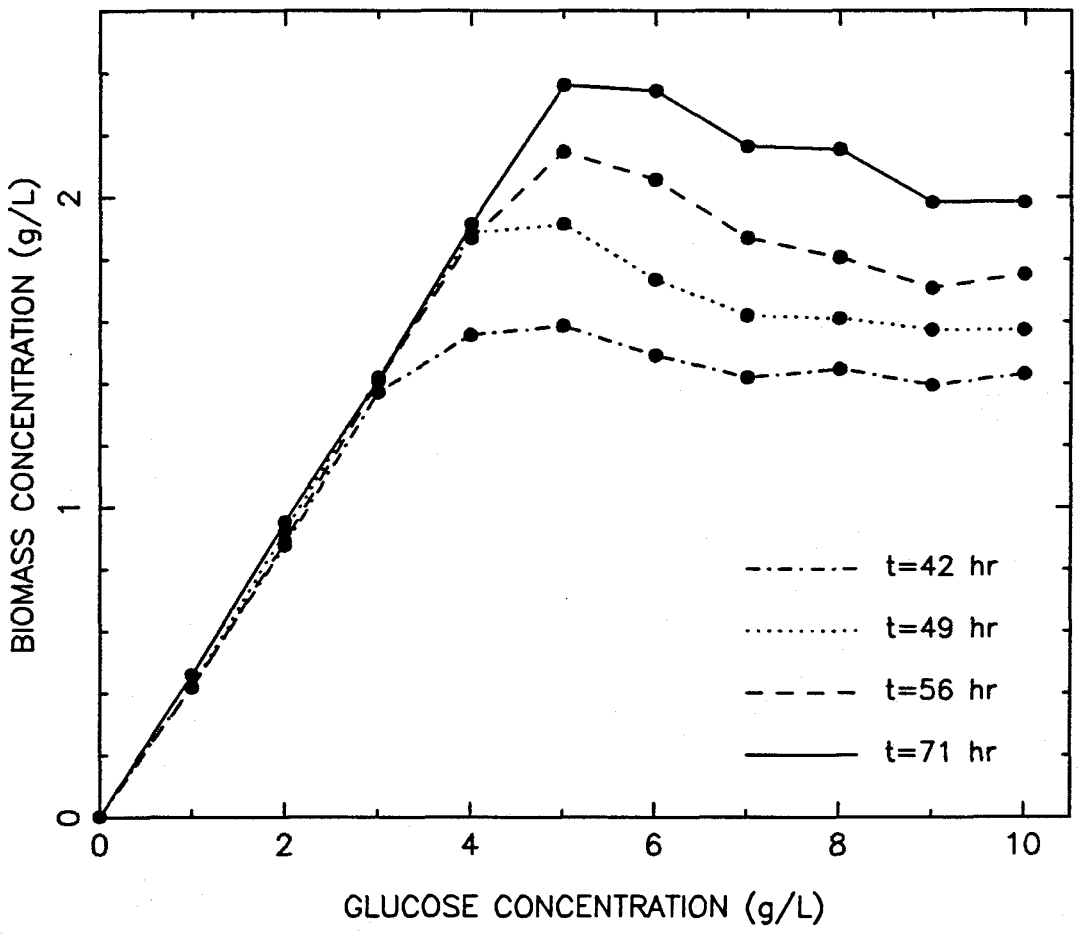

Figure A.4 Biomass Concentration Versus Glucose Concentration (to 10 g/L) in Optimized SD Minimal Medium

A few important features were noted for this optimized medium. The nitrogen base is in excess and is not limiting growth. Even at a glucose concentration of 20 g/L, an increase in nitrogen base concentration did not increase biomass yield. Also, uracil is not limiting in either the original or optimized medium, and its concentration was not increased. For plasmid-containing strains, uracil is not added to the medium. In the supplemented C-limited medium, the stationary phase biomass concentration for D603:pLGSD5 was the same as that for D603 with uracil. Therefore, the extent of plasmid *URA3* gene expression is not limiting growth in the plasmid-containing strain. Finally, the supplemented C-limited medium is not stable at room temperature and must be refrigerated at 4°C until used (see Appendix B). The stabilities of the other media were not checked.

In Chapter 4, selective amino acid + purine-limited (A-limited) medium was utilized. In this medium, cell growth is limited by adenine, histidine, lysine, and methionine — the amino acids and purines for which strain D603 is auxotrophic. The original supplemented SD minimal medium is A-limited (see Figure A.2). However, in order to keep the ratios of amino acids and purines the same in the C-limited and A-limited media, the histidine concentration must be reduced by 20% to 16 mg/L. Therefore, the composition of the A-limited medium is: glucose (20 g/L), nitrogen base without amino acids (6.7 g/L, Difco), adenine-sulfate (20 mg/L), L-histidine-HCl (16 mg/L), L-lysine-HCl (30 mg/L), and L-methionine (20 mg/L). This medium was also refrigerated until used.

Appendix B

Medium Stability

During the initial continuous fermentations, problems were encountered in maintaining stable steady-states. Even under uninduced conditions, biomass concentration fluctuated continuously. This observation prompted a detailed chemostat experiment in which every variable was monitored carefully.

Figure B.1 shows the biomass curve during the approach to "steady-state" in this experiment. The dips in the curve correlated with only one variable: the times that fresh medium was pumped into the feed bottle (indicated by the arrows on the plot). The results suggest that the medium is not stable. An essential component may be breaking down over time, or a toxic substance might be formed by a reaction between the medium components or by a light-mediated reaction.

The stability problem was solved by refrigerating the medium at 4°C. This is graphically illustrated in Figure B.2, in which biomass curves are compared for continuous fermentations with the medium reservoir placed at room temperature and 4°C. A stable steady-state is achieved with the refrigerated medium. The medium remained stable at 4°C for approximately one week; longer times were not investigated.

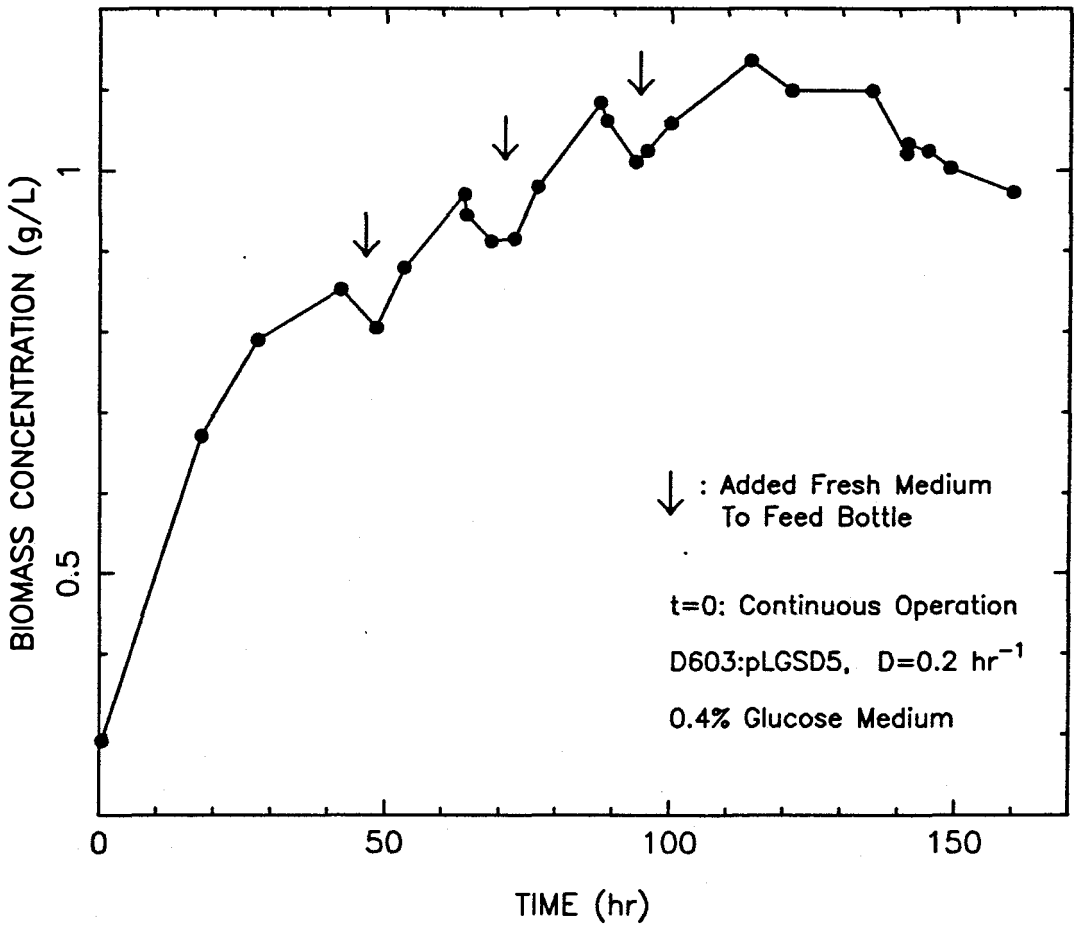

Figure B.1 Biomass Concentration and Medium Additions after Switch to Continuous Operation

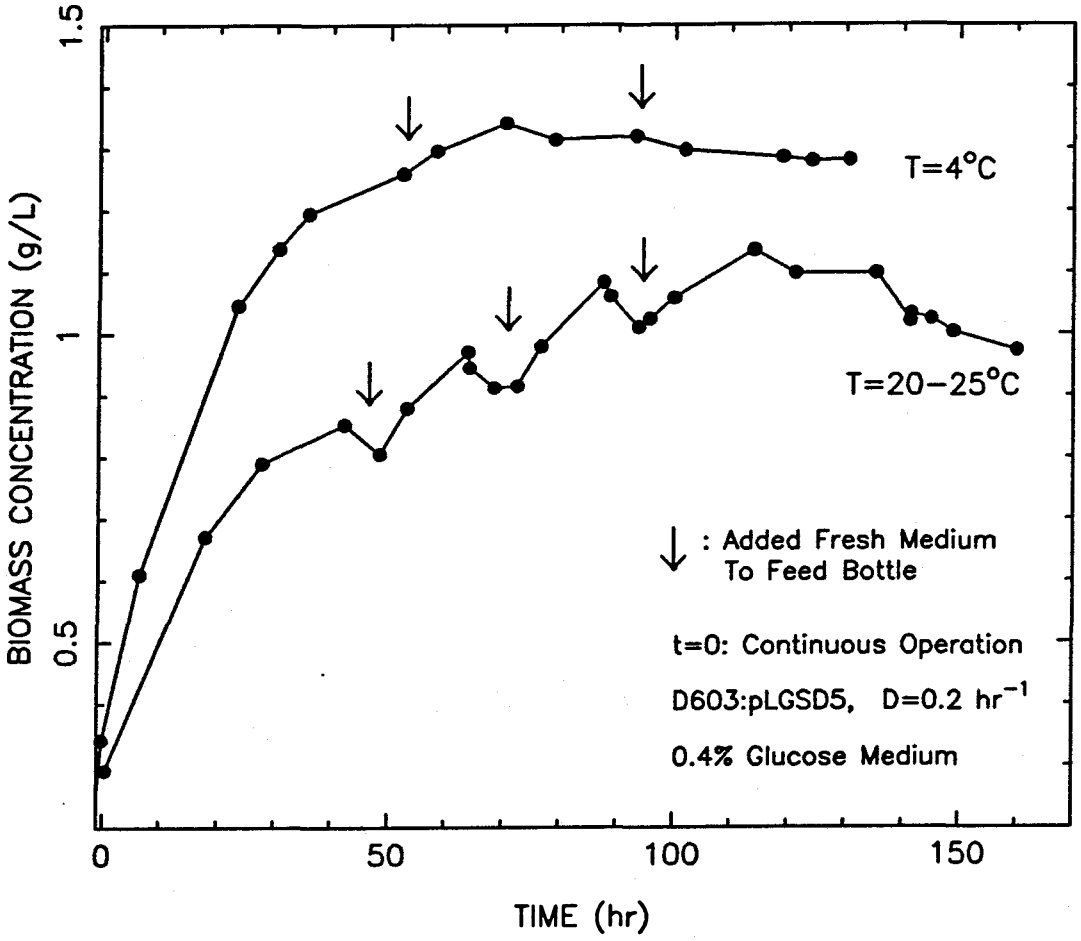

Figure B.2 Biomass Concentrations after Switch to Continuous Operation (Media Stored at Room Temperature and 4°C)