PHYSIOLOGICAL CONSEQUENCES OF TRIOSEPHOSPHATE ISOMERASE OVERPRODUCTION IN *Saccharomyces cerevisiae*

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Declaration

I hereby declare that the work presented in this thesis is the result of my own research, unless otherwise stated, and it has been composed by me. It has not been accepted in any previous application for any academic degree.

Nantana Fugtong

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Abbreviations and Symbols

1 Miscellaneous

A	ampere
ADP	adenosine 5' -diphosphate
AMP	adenosine 5' -monophosphate
ATP	adenosine 5' -triphosphate
bp	base pairs
°C	degrees Celsius
C	carbon atom
cm	centimetre
CoA	coenzyme A
conc.	concentrated
CPR	carbon dioxide production rate
Da	dalton
DNase	deoxyribonuclease
DNA	deoxyribonucleic acid
DO	dissolved oxygen
EDTA	ethylenediaminetetra-acetate
EtOH	ethanol
eV	electron volt
g	gram
HPLC	chromatogrophy
h	hour
IPTG	isopropylthio-ß-D-galactoside
kb	kilobases
L	litre
log	logarithm (base 10)
ln	logarithm (base e)

μg	microgram
μl	microlitre
μM	micromolar
μmol	micromol
M	Molar
mA	milliampere
mg	milligram
min	minutes
ml	millilitre
mM	millimolar
mol	mole
ng	nanogram
nm	nanometre
no.	number
OD	optical density
p., pp.	page
PAGE	polyacrylamide gel electrophoresis
PEG	polyethylene glycol
RNAase	ribonuclease
RNA	ribonucleic acid
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
sec	second
sp., spp.	species
TEMED	N, N, N', N'-tetramethylethylenediamine
UV	ultraviolet

2 Amino acids

Ala	alanine
Arg	arginine
Asp	aspartic acid
Asn	asparagine
Cys	cysteine
Gln	glutamine
Glu	glutamic acid
Gly	glycine
His	histidine
Ile	isoleucine
Leu	leucine
Lys	lysine
Met	methionine
Phe	phenylalanine
Pro	proline
Ser	serine
Thr	threonine
Try	tryptophan
Tyr	tyrosine
Ura	uracil
Val	valine

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Abstract

Triosephosphate isomerase was overproduced in yeast *Saccharomyces cerevisiae* by integration of extra copies of the *TPI1* gene into a yeast chromosome. Specific enzyme activity was found to have increased between two and seven fold above the wild-type level depending on the isolate used. The physiological consequences of enzyme overproduction by a factor of two and seven were studied in aerobic batch cultures and aerobic glucose-limited chemostat cultures. Batch cultivation indicated that no significant difference in growth and metabolite production between overproducers and the reference could be detected. However, lower levels of three other glycolytic enzymes (phosphoglycerate kinase, pyruvate kinase and hexokinase) were found in a seven-fold TPI overproducer. Dilution rate profiles of TPI overproducers and the reference were studied in glucose-limited chemostat culture. The seven-fold TPI overproducer showed ethanol formation at $D=0.11\ h^{-1}$ where ethanol could not be detected in the two-fold overproducer and the reference. Increase in dilution rate to $0.32\ h^{-1}$ resulted in increases of ethanol production rate as well as the rates of pyruvate and acetate production. The much more sensitive competitive chemostat cultures between TPI overproducers (two-fold or seven-fold) and the reference strain were studied using *LEU2* gene as a detectable marker. The marker, however, showed strong effects on selection under all competitive chemostat studies. TPI activity was therefore used as a measure to determine the proportion of the strains in competition between the reference and TPI overproducers. Results showed that increase in TPI activity by a factor of two has reduced the relative fitness of the yeast strain but further increase of the TPI activity to seven-fold had no effect on the relative fitness of the yeast strain when compared to the two-fold overproducer. The non-linear relationship between the relative fitness and enzyme concentration indicated that TPI may not be an equilibrium enzyme as it is usually claimed.

Chapter 1

Introduction

1.1 Glucose Metabolism

Metabolism of glucose has received considerable attention from many scientists as several reactions of glucose metabolism can couple many metabolic processes in a wide range of living cells. Glucose metabolism also contributes many precursors for a number of biosynthetic reactions in the living cell. Although the importance of glucose metabolism is recognized, the control over this complicated metabolic process is still not well understood. This is partly due to the fact that glucose metabolism can be affected by number of factors (see below).

Glucose metabolism can be divided into three important metabolic aspects i.e. glucose anabolism, glucose catabolism and glucose transport. Glucose anabolism is the synthesis of glucose when cells are subjected to starvation or to growth on non-carbohydrate substrates. On starvation, cell can synthesize glucose by the breakdown of glycogen, an intracellular preserved carbohydrate. Glucose can also be synthesized from non-carbohydrate substrates such as pyruvate and lactate by the pathway of gluconeogenesis of which several, but not all, reversible steps are shared with glycolysis. In contrast to glucose anabolism, glucose catabolism is a breakdown of glucose. Catabolism of glucose is important to the cell metabolism as it leads to the production of cellular building blocks which involve with the generation of ATP, an essential element of biological energy. Extracellular glucose is unable to freely permeate into the cells, therefore glucose transport acts as a barrier to glucose catabolism. This study is concerned with the glycolytic pathway which is one of the most important glucose catabolic processes. The following sections therefore attempt to give the background and the detail of glucose transport as well as those of glucose catabolism in yeast *S. cerevisiae*, an experimental model used in this study.

Yeast is a suitable model to employ for study of metabolic regulation as yeast cells possess extremely versatile metabolic pathways which are subjected to the same types of control as those of higher eukaryotes. The use of yeast to study metabolism is of advantage as it has been possible to apply the powerful techniques of DNA manipulation with relative ease in this organism. In addition, large quantities of yeast can be easily produced in the laboratory. Yeasts can be classified into three main categories on the basis of the occurrence of alcoholic fermentation when they were grown on glucose as carbon and energy source (van Dijken *et al.*, 1993).

1) Non-Fermentative Yeast

Yeasts in this group e.g. *Rhodotorula glutinis* are unable to ferment glucose to ethanol. These yeasts possibly fail to synthesize key enzymes of the fermentative pathway, therefore, ethanol production can not be achieved.

2) Facultatively Fermentative Yeast

Facultatively fermentative yeasts can be subdivided into two subgroups as shown below:

a) Crabtree-Positive Yeast

Yeasts e.g. *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, can utilize glucose by respiration, alcoholic fermentation or respirofermentation depending on environmental conditions e.g. oxygen concentration and glucose concentration. In the presence of oxygen, oxidative respiration is employed whereas under anaerobic conditions, alcoholic fermentation is employed. A special characteristic of yeasts in this subgroup is that they exhibit the Crabtree effect (see section 1.3.2) when grown in high glucose concentration even in the presence of oxygen.

b) Crabtree-Negative Yeast

As they are named, yeasts in this subgroup do not exhibit the Crabtree effect when grown on glucose as carbon and energy source. Examples of these yeasts are *Candida utilis* and *Kluyveromyces marxianus*.

3) Obligately Fermentative Yeast

By their nature, yeasts in the last group e.g. *Candida slooffi* are unable to undertake the respiration. Energy production is therefore achieved via alcoholic fermentation only.

Among those yeasts classified above, *S. cerevisiae* is the most widely studied species not only because of its attractive molecular biology but also because of its commercial importance in industry. The genetics and physiology of *S. cerevisiae* have been studied in several approaches including glucose metabolism. Being a member of the Crabtree-positive group, *S. cerevisiae* possesses a distinct type of glucose metabolism which has attracted the curiosity of many researchers. It has been reported to have a much higher ethanol tolerance than those yeasts that are able to grow anaerobically (Visser *et al.*, 1990).

1.2 Glucose Transport in Yeast *S. cerevisiae*

The fact that biological membranes restrict the free entry of extracellular nutrients into the cells results in the requirement for specific transport systems for the uptake of these nutrients. Two main types of transport system i.e. channels and transporters (or permeases and carriers, respectively), have been noted (Lagunas, 1993). Channels allow extracellular molecules to penetrate across the membrane by discrimination of substrate molecular size whereas transporters catalyse uptake of substrate as well as conformational changing of substrate molecules (Lagunas, 1993). Some evidence favouring the possibility that channels may be present in yeast has been shown (Serrano, 1991), however, there is still no definitive proof (Lagunas, 1993). For the past three decades, many more studies on transporters have been reported to be involved with the glucose transport system in yeast than those on channels.

The monosaccharide transport system in yeast *S. cerevisiae* consists of two different transporters i.e. glucose transporter and galactose transporter. The glucose transporter deals with the transport of D-glucose, D-fructose and D-mannose into yeast cells, whereas the galactose transporter acts upon D-galactose only. Both α- and β-D-glucose have been reported to be taken up by yeast cells (Heredia *et al.*, 1968). Heredia *et al.* (1968) demonstrated that the basic structural requirement for glucose transport is a pyranose ring of which each carbon atom responds to the structural change differently. A relatively small decrease in affinity was observed as a result of a single modification at C1 or C2 whereas a considerable decrease was observed in the case of C3 and C4 modifications (Heredia *et al.*, 1968).

Mechanism of Glucose Transport in *S. cerevisiae*

The mechanism of glucose transport has been questioned since phosphorylation of sugar was hypothesised to be involved in sugar transport (Rosenberg & Wilbrandt, 1952). The role of sugar phosphorylation in the transport system has therefore been intensively investigated and has attracted many scientists to focus their researches in this field. The controversies on whether sugars are phosphorylated via group translocation systems, i.e. by active transport, during transport or whether they are transported as free sugars, i.e. by facilitated diffusion, and subsequently phosphorylated inside the cell have long been debated (see Romano, 1986; Lagunas, 1993 for reviews).

The transport-associated phosphorylation hypothesis was advanced by Rothstein (1954). The hypothesis was based on the observations that intracellular free glucose was not detectable in actively metabolizing cells which were found to be impermeable to non-metabolizable sugars. In addition, an apparent correlation between substrate specificity and sugar phosphorylation also supported this hypothesis. van Steveninck (1968) investigated glucose uptake in yeast *S. cerevisiae* using pulse-labelling techniques with 2-deoxy-D-glucose (2-DOG), a phosphorylable non-metabolizable glucose analogue which was subsequently used in a number of studies. The author reported that the intracellular concentration of 2-DOG was higher than the extracellular concentration, and 2-deoxyglucose-6-phosphate (2-DOG-P) appeared in the cells before the free sugar. Therefore the conclusion was made in favour of an active transport involving phosphorylation, such that the transport of free 2-DOG into the cells was associated with phosphorylation. However, the higher intracellular concentration of 2-DOG reported by van Steveninck (1968) was subsequently criticized to be an overestimation of 2-DOG accumulation (Kotyk & Michaljanicova, 1974). The criticism was based on the fact that a number of metabolic products inside the cells could have been measured as part of the free 2-DOG. Of those products, the deoxy derivative of the nonreducing disaccharide trehalose in particular of 2,2'-dideoxy-α,α'-trehalose was of special interest since this trehalose derivative was found to accumulate in significant amounts in yeast cells when grown under aerobic conditions (Farkas *et al.*, 1969). Kotyk & Michaljanicova (1974) carried out pulse-labelling experiments with glucose, galactose and α-methyl-D-glucose using the same procedure as van Steveninck (1968), and observed contradictory results, i.e. in all cases, the free 2-DOG appeared in the cells before 2-DOG-P.

To clarify the results observed by van Steveninck (1968) and also to respond to the above criticisms, Jaspers & van Steveninck (1975) repeated the pulse-labelling experiments with yeast *Saccharomyces fragilis*. An enzymic method was used to determine 2-DOG in order to avoid interference by the trehalose derivative previously criticized. Similar results as those reported for *S. cerevisiae* (van Steveninck, 1968) were obtained. However, these results have been suggested to arise from an experimental artifact because in some experiments the reverse order of labelling was observed (Meredith & Romano, 1977).

Similar pulse-labelling experiments as those described by van Steveninck (1968) were carried out using a respiration-deficient petite mutant of *S. cerevisiae* to completely discard the possibility of the "trehalose error" because this mutant would not accumulate the deoxytrehalose derivative under energy-sufficient aerobic conditions (Meredith & Romano, 1977). Results showed that the intracellular concentration of 2-DOG did not exceed the extracellular concentration. Furthermore, results from a modified pulse-labelling technique confirmed the observations of Kotyk & Michaljanicova (1974) in which 2-DOG initially appeared in the cell in the phosphorylated form (2-DOG-P). This conclusion was further confirmed by comparing the wild-type yeast with single kinase mutants i.e. each mutant posssessed only one of the two hexokinase isoenzymes or only the glucokinase (Franzusoff & Cirillo, 1982). They demonstrated that if the phosphorylation was involved with glucose transport, it was not a function of a specific hexokinase or glucokinase. Evidence against the transport-associated phosphorylation of glucose in *S. cerevisiae* has been provided using 6-deoxy-D-glucose (6-DOG), a nonphosphorylable glucose analogue (Romano, 1982). This analogue was found to be transported into the yeast cells via a facilitated diffusion system with affinity equivalent to that shown with glucose.

The role of phosphorylation in glucose transport has been envisaged in a new way since the uptake of glucose in yeast *S. cerevisiae* has been found to be mediated by two components with affinity constants of approximately 1 mM and 20 mM, subsequently referred to as high- and low-affinity transport systems, respectively (Bisson & Fraenkel, 1983a). This evidence was obtained through the use of mutants lacking different kinases i.e. hexokinaseA (P-I), hexokinaseB (P-II) and glucokinase (Bisson & Fraenkel, 1983a). It was initially shown that uptake of 6-DOG in the wild type and both mutants (one lacking hexokinases PI and PII and the other lacking glucokinase) reached a plateau at or below the external concentration (Bisson & Fraenkel, 1983a). Since 6-DOG is a nonphosphorylable analogue of glucose, the possibility that the phosphorylation was involved with glucose uptake could therefore be discarded. Subsequent determinations of kinetic parameters illustrated that the wild type as well as the mutant lacking both hexokinases A and B showed both low- and high-affinity components whereas the mutant lacking all kinases (i.e. glucokinase, in addition) showed only the low-affinity component for glucose uptake (Bisson & Fraenkel, 1983a). These results therefore strongly suggested that the high-affinity component requires the presence of at least one functional kinase to mediate glucose uptake in *S. cerevisiae*, whereas this requirement is unnecessary for the low-affinity component.

Considering the experimental approaches previously used, e.g. use of sugar analogues and the kinase-less mutants, which could influence the interpretation of the results the constitutive transport of hexose in *S. cerevisiae* has been re-examined (Nevado *et al.*, 1994). The study was carried out using a new radioactive experimental approach based on the fact that the label of [2-^{3}H]mannose disappears after phosphorylation because of its conversion to fructose-6-phosphate. Results from this study support the view that substrate phosphorylation is not involved with hexose transport in yeast.

Structure and Genetics of Glucose Transporters

In *S. cerevisiae*, three genes i.e. *SNF3*, *HXT1* and *HXT2*, have been identified to be involved in the high-affinity glucose transport system. The first gene, *SNF3* (sucrose non-fermenting) was identified from the study on glucose repression of invertase (Carlson *et al.*, 1981; Neigeborn & Carlson, 1984). Bisson *et al.* (1987) subsequently demonstrated that the *snf3* mutants did not express the high-affinity glucose transport system, and that increase in copy number of the *SNF3* gene substantially increased glucose uptake in both wild-type and *snf3* mutant strains. The amino acid sequence of the *SNF3* gene of *S. cerevisiae* was found to be homologous to that of the mammalian glucose transporters (Celenza *et al.*, 1988). By complementation of the *snf3* mutation, two additional genes called *HXT1* and *HXT2* encoding for two glucose transporters of high-affinity uptake were cloned (Kruckeberg & Bisson, 1990; Lewis & Bisson, 1991).

The *RAG1* gene has been shown to code for the low-affinity glucose transporter in the yeast *Kluyveromyces lactis* (Wesolowski-Louvel *et al.*, 1992). The *rag1* null mutant which could not grow on high glucose concentrations was used to clone the low-affinity glucose transporter gene (*LGT1*) of *S. cerevisiae* by the means of functional complementation (Prior *et al.*, 1993). The *LGT1* gene was sequenced and analysed by Southern blot analysis, and results showed that several homologous sequences were detected from genomic DNA of *S. cerevisiae* when *LGT1* was used as a DNA probe. This may indicate that multiple genes encode the low-affinity glucose transporter.

Regulation of Glucose Transport

Serrano & DelaFuente (1974) suggested that the transport of glucose may exist in two interconvertible states with different affinities for glucose. These states were once considered to be induced by glycolytic intermediates such as glucose-6-phosphate which had been proposed as a regulator of glucose uptake (Sols, 1967). However, this possibility was rejected in the study of a mutant lacking the enzyme phosphoglucose isomerase (Maitra, 1971). During glucose utilization, the *pgi* mutant showed an accumulation of glucose-6-phosphate inconsistent with the notion that the utilization of glucose is controlled by this metabolite.

Glucose transport in yeast *S. cerevisiae* has been reported to be regulated by several strategies including catabolite repression and catabolite inactivation. Bisson & Fraenkel (1984) reported the effect of culture conditions on the level of high-affinity uptake during batch cultivation of *S. cerevisiae*. The high-affinity component was low at the beginning of batch growth where a high glucose concentration was present, and as glucose was exhausted from the medium, an increase in the synthesis of the high-affinity glucose transporter was found. These findings indicated that the high-affinity component was repressed by a high glucose concentration i.e. catabolite repression, and it was derepressed when the glucose level was low. However, using hexokinase single-gene mutants, the derepression was not clearly shown to be correlated with changes in levels of kinases themselves (Bisson & Fraenkel, 1984). In addition, a ten-fold increase in the level of high-affinity uptake, as a result of a shift from 100 mM to 5 mM glucose, which did not occur in the presence of a protein synthesis inhibitor suggested that protein synthesis was required for high-affinity glucose uptake. The study of *S. cerevisiae* mutants defective in growth on low glucose concentration (*lgn* mutants) has provided information supporting the conclusion that high-affinity glucose uptake in *S. cerevisiae* is controlled by general glucose repression (Bisson, 1988). Using aerobic glucose-limited chemostat cultures, the capacity of glucose transporters of *S. cerevisiae* was also shown to be dependent on glucose concentration (Postma *et al.*, 1989b). At low dilution rate at which glucose was fully respired, residual glucose was constant and independent of glucose concentration in the reservoir, whereas at high dilution rate residual glucose followed Monod kinetics and was dependent on the reservoir glucose concentration. These findings therefore established that synthesis of the glucose transport systems in *S. cerevisiae* is controlled by environmental conditions.

The glucose transport systems were also shown to be regulated by a catabolite-inactivation process (Busturia & Lagunas, 1986). Both high- and low-affinity components of glucose transport were found to be inactivated when protein synthesis was inhibited. According to this study, the inactivation requires the utilization of fermentable substrates by cells. Although the mechanism of the inactivation is still unknown, enzyme cAMP-dependent protein kinase (cAPK) has been suggested to be involved in the inactivation due to the facts that (1) the HXT2 transporter contains two consensus cAPK phosphorylation sites (Kruckeberg & Bisson, 1990) and that (2) glucose transport is not inactivated in cAPK-less mutants (Ramos & Cirillo, 1989).

The rate of glucose uptake may be controlled by the variations in the affinity of glucose transporters. Using data reported by Bisson & Fraenkel (1984), Lagunas (1993) mentioned variations in K_m values calculated for high-affinity glucose transporter during aerobic and anaerobic growth of *S. cerevisiae* at different glucose concentrations but the causes of these variation are still unknown.

A molecular interaction between the three kinases (hexokinase I, hexokinase II and glucokinase) and the glucose transporters (SNF3, HXT1 and HXT2) has been suggested to be involved in the regulation of the glucose transport system (Bisson & Fraenkel, 1983a; Kruckeberg & Bisson, 1990). This suggestion is based on the findings that catabolite repression of the high-affinity component of the glucose uptake system occurred in the wild-type yeast strain as well as the strain carrying *HXK2*, whereas no repression could be observed in strains carrying *HXK1* or *GLK1* genes (McClelland & Bisson, 1988). Mutational analysis of the SNF3 protein indicated that the C-terminal region of this protein may be involved in the interaction between kinases and glucose transporters (Marshall-Carlson *et al.*, 1990). The conserved feature of glucose transporters, the leucine-zipper motif which is the repeats of four or five leucines every seventh amino-acid residue (White & Weber, 1989) was considered. The presence of leucine-zipper motif is found in all three glucose transporters, SNF3 (Celenza *et al.*, 1988), HXT1 and HXT2 (Lewis & Bisson, 1991). Leucine-zippers are able to form coiled structures which result in strong hydrophobic interactions between two protein surfaces. The leucine-zippers of glucose transporter have been proposed to mediate oligomer formation (White & Weber, 1989). Kruckeberg & Bisson (1990) suggested that complexes of membrane transport and cytosolic proteins may occur in yeast cells; and the leucine-zippers of HXT2 and SNF3 may mediate some of protein-protein contacts.

1.3 Glucose Catabolism in Yeast *S. cerevisiae*

Similar to most living organisms, yeasts require a breakdown of carbon substrates to couple other metabolic processes. Although yeasts can grow on a variety of fermentable and non-fermentable carbon sources, glucose appears to be the most favourable. Glucose catabolism in yeast has been investigated from various points of view i.e. genetics, biochemistry and physiology. However, many questions on how metabolic processes of glucose catabolism are regulated still remain to be answered.

1.3.1 Glycolysis

The Pathway

Glycolysis is the sequence of reactions that converts glucose into pyruvate, one of the most important intermediary metabolic compounds, with the concomitant production of ATP (Stryer, 1988). The glycolytic pathway is a central metabolic pathway which is present, at least in part, in both aerobic and anaerobic organisms. An overall view of glycolysis is shown in Figure 1.1 and details are summarized as follows.

The first reaction of the glycolytic pathway, catalyzed by *hexokinase*, is the phosphorylation of glucose to glucose-6-phosphate. The phospho group from ATP is transferred to the hydroxyl group on C-6 of glucose. Glucose-6-phosphate is then isomerized, by *phosphoglucose isomerase*, to fructose-6-phosphate which is further phosphorylated by ATP to fructose-1,6-bisphosphate. This second phosphorylation of the glycolytic pathway is catalyzed by *phosphofructokinase*, an allosterically regulated enzyme. The next step is the cleavage of fructose-1,6-bisphosphate to two molecules of triosephosphate i.e. glyceraldehyde-3-phosphate and dihydroxyacetone phosphate by the enzyme *aldolase*. The remaining steps of the glycolytic pathway therefore involve three-carbon compounds only. *Triosephosphate isomerase* is the enzyme that catalyzes the isomerization between glyceraldehyde-3-phosphate and

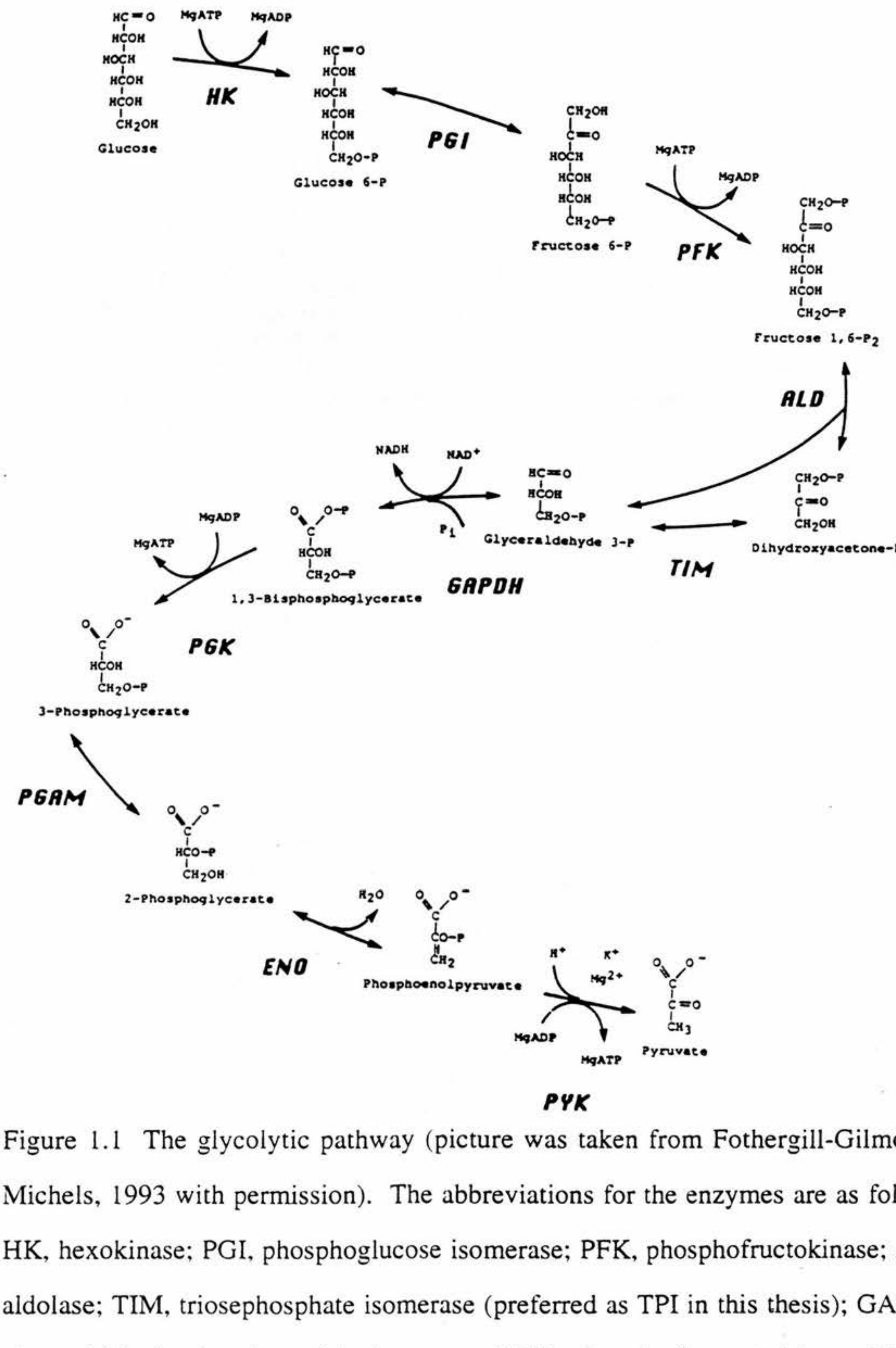


Figure 1.1 The glycolytic pathway (picture was taken from Fothergill-Gilmore & Michels, 1993 with permission). The abbreviations for the enzymes are as follows: HK, hexokinase; PGI, phosphoglucose isomerase; PFK, phosphofructokinase; ALD, aldolase; TIM, triosephosphate isomerase (preferred as TPI in this thesis); GAPDH, glyceraldehyde-phosphate dehydrogenase; PGK, phosphoglycerate kinase; PGAM, phosphoglycerate mutase; ENO, enolase; PYK, pyruvate kinase. The letter "P" in the chemical structure represents a phospho group.

dihydroxyacetone phosphate. Although the reaction is rapid and reversible, the enzyme ensures that the reaction proceeds from dihydroxyacetone phosphate to glyceraldehyde-3-phosphate which is on the direct pathway of glycolysis. Two molecules of glyceraldehyde-3-phosphate are oxidized to 1,3-bisphosphoglycerate with the concomitant reduction of NAD^+ to NADH. The reaction is catalyzed by *glyceraldehyde-phosphate dehydrogenase*. This is thus the first step of the energy production process after two molecules of ATP have been invested (the reactions of hexokinase and phosphofructokinase). 1,3-bisphosphoglycerate is dephosphorylated to 3-phosphoglycerate by the transfer of the phospho group from 1,3-bisphosphoglycerate to ADP. This reaction is catalyzed by *phosphoglycerate kinase* and is the first step in which ATP is produced. The last stage of glycolysis is the conversion of 3-phosphoglycerate to pyruvate. Firstly, the phospho group of 3-phosphoglycerate is shifted from C-3 to C-2 by the enzyme *phosphoglycerate mutase* yielding 2-phosphoglycerate which is further dehydrated to phosphoenolpyruvate by the enzyme *enolase*. In the last step of glycolysis, *pyruvate kinase* catalyzes the formation of pyruvate from phosphoenolpyruvate simultaneously with ATP generation. From all the above reactions two molecules of ATP are consumed and four molecules of ATP are generated. Therefore, in conclusion, the net production of two molecules of ATP is obtained from the conversion of glucose to pyruvate by the glycolytic pathway.

Pyruvate stands in the center of intermediary metabolism (Figure 1.2) and it can be converted to many anabolic products depending on the organism as well as the environmental conditions. In the presence of oxygen, aerobic organisms can further oxidize pyruvate to acetyl CoA by the multienzyme complex pyruvate dehydrogenase. Acetyl CoA can then enter the tricarboxylic acid cycle and electron-transport chain after which glucose is completely oxidized to CO_2 and H_2O. In contrast, if the supply of oxygen is insufficient, pyruvate will be converted in the cytosol into products of fermentation e.g. lactate. This pathway is unique in that it can proceed at very low concentrations of oxygen e.g. in exercising skeletal and cardiac muscles (Smith *et al.*, 1983) or in a complete absence of oxygen. The conversion of pyruvate to lactate could also be seen in homolactic fermentative bacteria, e.g. *Lactobacillus* spp., *Bacillus* spp., *Streptococcus* spp. and *Clostridium* spp., which can convert all pyruvate to lactate by the enzyme lactate dehydrogenase (Dawes & Sutherland, 1992). For some bacteria and yeasts, pyruvate can be oxidized to acetaldehyde by the enzyme pyruvate decarboxylase, acetaldehyde is subsequently reduced to ethanol and CO_2.

Glycolysis in Yeast *S. cerevisiae*

Similar to the situation found in many prokaryotic and eukaryotic micro-organisms, glycolysis is one of the most important pathways of glucose catabolism in yeast. In *S. cerevisiae,* the mRNAs coding for glycolytic enzymes are the most abundant mRNA species (Holland &Holland, 1978), and as a result, glycolytic genes are among the most highly expressed. Hence the enzymes of glycolysis have been found to be a major fraction, 30-60%, of the soluble protein (Hess *et al.*, 1969; Fraenkel, 1982).

Regulation of Glycolysis

As shown in Figure 1.1, the glycolytic pathway consists of both reversible and irreversible reactions. Three virtually irreversible reactions catalyzed by hexokinase, phosphofructokinase and pyruvate kinase have been qualitatively identified to have regulatory functions on the pathway. The regulatory function of hexokinase on the glycolytic pathway has been thought to be the involvement of this enzyme in the glucose transport system (see section 1.2). The role of phosphofructokinase and pyruvate kinase has been traditionally seen as the control over the major branching points of glycolysis and other metabolic pathways. For example, phosphofructokinase has been thought to control not only the glycolytic flux but also,

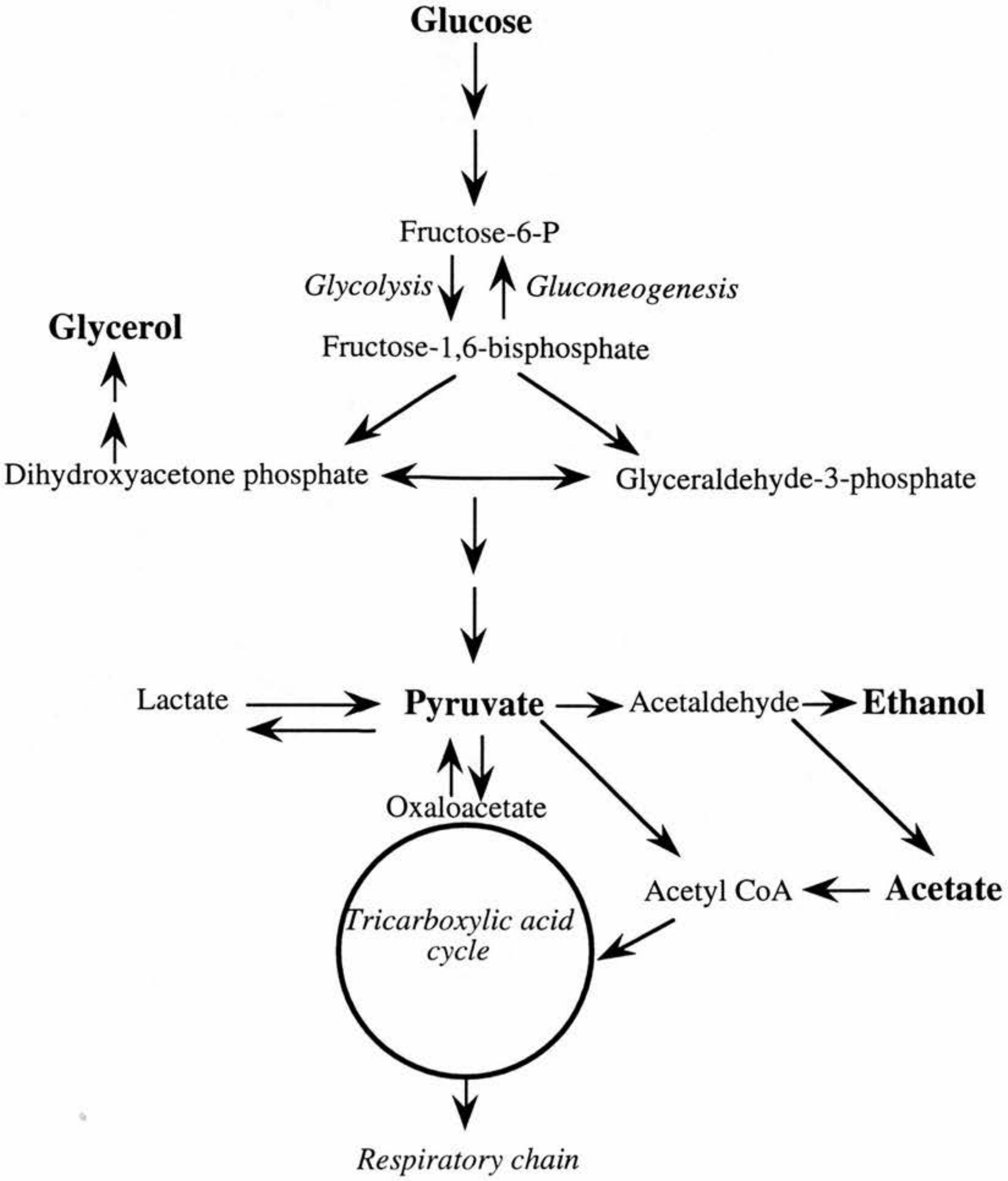


Figure 1.2 Position of pyruvate between major metabolic pathways in yeast.

indirectly, the synthesis of glycerol, lipids and phospholipids via the concentration of dihydroxyacetone phosphate, whilst pyruvate kinase has been considered to influence the synthesis of alanine, serine, ethanolamine and related compounds (Boiteux & Hess, 1981).

Kappeli (1986) described the regulation of glycolysis in a view of feedback regulation based on the limited respiratory capacity observed in respiro-fermentative glucose catabolism of the yeast *S. cerevisiae.* Fructose-2,6-bisphosphate which has been shown to be a positive effector or activator of phosphofructokinase1 (Francois *et al.*, 1984) plays an important part of this concept. Kappeli (1986) discussed that the glycolytic rates and respiratory rate are in balance under normal conditions (feed-controlled conditions). When glucose is present in excess, the formation of fructose-2,6-bisphosphate which activates the activity of phosphofructokinase1 is stimulated resulting in an increase in glycolytic activity. As a result, an imbalance in energy occurs due to the limited respiratory capacity which causes a decrease in ATP content and a decrease in tricarboxylic acid cycle intermediates.

Several glycolysis regulatory (gcr) mutants have been isolated in order to study the regulation of glycolysis (Clifton *et al.*, 1978; Clifton & Fraenkel, 1981). However, it has been difficult to isolate this kind of mutant due to the close link between glycolysis and gluconeogenesis. Mutations in which glycolytic enzymes are strongly repressed may well affect the pathway of gluconeogenesis because some reversible enzymes are shared between these two pathways. It was shown that in a *gcr* mutant, less than 5% of the wild-type levels of glycolytic enzymes were synthesized when grown on gluconeogenic substrates, but 20-50% of the wild-type levels were found when grown on glycolytic substrates. Several attempts, including isolation of the wild type *GCR1*, have been made in order to study the *gcr* phenotype. However, it has been reported that the *GCR1* gene encodes for a DNA-binding protein which is required for high-level expression of glycolytic genes (Baker, 1986; Clifton & Fraenkel, 1981; Clifton *et al.*, 1978; Holland *et al.*, 1987; Huie *et al.*, 1992).

The specific activities of most glycolytic enzymes were mentioned to be relatively independent of growth conditions (Entian & Barnett, 1992). This statement appears to be contradictory to the observations of Maitra & Lobo (1971) in which the carbon source was shown to have significant effects on the level of glycolytic enzymes. Maitra & Lobo (1971) demonstrated that the levels of many glycolytic enzymes of hybrid yeast strain generated by a cross between *Saccharomyces fragilis* and *Saccharomyces dobzhanskii*, were very low when grown in acetate-supplemented rich medium. They also showed that the addition of glucose caused 3- to 100- fold increases in the differential rates of enzyme synthesis over several hours. The steady state levels of all the glycolytic mRNAs of *S. cerevisiae* grown on glucose and lactate were compared, and shown to be differentially regulated in response to the carbon source (Moore *et al.*, 1991). In addition, using *PYK::lacZ* and *PGK::lacZ* fusions independently integrated into the yeast genome, Moore *et al.* (1991) also provided evidence that the regulation of glycolytic mRNA levels is mediated at the transcriptional level.

The promoters of some glycolytic genes in yeast have been found to be activated by high temperature. Phosphoglycerate kinase is one of the approximately 22 heat shock proteins in yeast of which the levels of transcription and translation can be increased with increasing temperature (Piper *et al.*, 1986; Miller *et al.*, 1982; van der Aar *et al.*, 1990a). One of the three glyceraldehyde-3-phosphate dehydrogenase enzymes was also shown to respond similar to phosphoglycerate kinase when the temperature was increased from 25 to 38°C (Piper *et al.*, 1986). Piper *et al.* (1988) studied the upstream sequence of the *PGK1* gene encoding phosphoglycerate kinase, and found that the heat shock response was eliminated after the deletion of the heat shock element upstream sequence. The authors suggested that the incorporation of this element into the promoter may be an adaptation of the cell to an abrupt change in environmental conditions in order to retain its metabolic balance.

1.3.2 Pyruvate Catabolism

Yeast *S. cerevisiae* catabolizes pyruvate by two different metabolic routes, i.e. respiration and alcoholic fermentation, depending on environmental conditions such as oxygen concentration and glucose concentration. In the presence of oxygen and low glucose concentrations where respiration is employed for energy production, *S. cerevisiae* oxidatively decarboxylates pyruvate to acetaldehyde by *pyruvate decarboxylase*. Acetaldehyde is then coupled to acetyl coA which is further oxidized to CO_2 and H_2O via the tricarboxylic acid cycle. Alternatively, under anaerobic conditions, alcoholic fermentation occurs where acetaldehyde is used as an organic electron acceptor and reduced to ethanol by *alcohol dehydrogenase*. Routes of pyruvate catabolism in yeasts are shown in Figure 1.3.

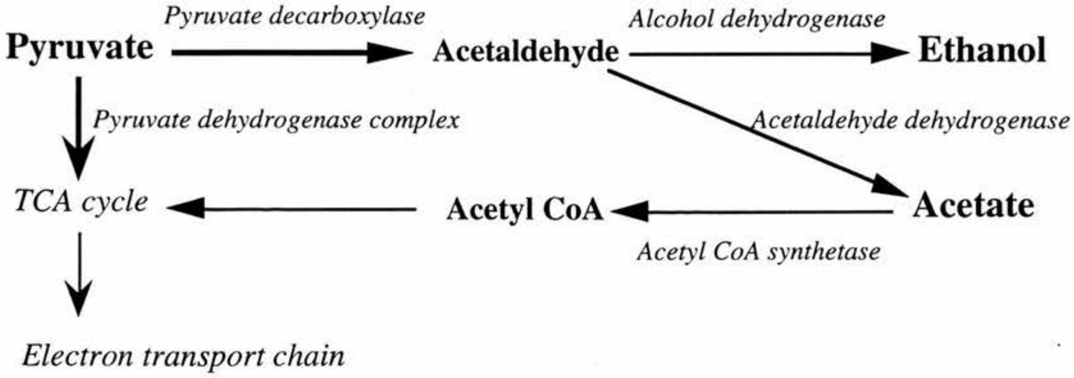


Figure 1.3 Alternative routes of pyruvate catabolism in yeasts.

Alcoholic fermentation in *S. cerevisiae* can be triggered even under aerobic conditions, i.e. an occurrence of the "Crabtree effect", if the glucose concentration in the medium is higher than the critical level (Crabtree, 1929; De Deken, 1966). The Crabtree effect was first described for the results of carbohydrate metabolism of tumour cells. Crabtree (1929) observed that the glycolytic activity of tumour cells inhibited their own respiration. This phenomenon was subsequently found in the yeast *S. cerevisiae* when grown on glucose or fructose as carbon source (De Deken, 1966). Initially, it was concluded that the Crabtree effect in yeasts was a repression of respiration by fermentation. Previously, the Pasteur effect (Warburg, 1926; Burk, 1939) had been reported as a decrease in efficiency of fermentation in the presence of oxygen, following the observation of Pasteur (1861) that oxygen inhibits the fermentation process. Therefore, it can be noted that the Crabtree effect is a contradictory phenomenon to the Pasteur effect.

S. cerevisiae exhibited the Crabtree effect during a high range of dilution rates in glucose-limited chemostat culture. This was originally believed that glucose itself represses respiratory enzymes and the term Crabtree effect is often called the glucose effect (von Meyenburg, 1969). Barford & Hall (1979) criticized this on the grounds that glucose is not the primary cause of repression. They observed that repression by glucose was transient and glucose derepression could be retained after a long adaptation period when respiration was no longer repressed but occurred at its maximal rate. Later, the occurrence of alcoholic fermentation in aerobic glucose-limited cultures of *S. cerevisiae* growing at high dilution rates was viewed as a consequence of the limited capacity of respiration which resulted in an overflow metabolism at the pyruvate level (Kappeli, 1986). Postma *et al.* (1989c) investigated this matter by the means of enzymic analysis at the pyruvate junction. Unexpectedly, during intermediate ranges of dilution rates of the culture, the high rate of respiration was found to be accompanied by an accumulation of weak organic acids e.g. acetic acid and pyruvic acid with the increasing dilution rate. Postma *et al.* (1989c) discussed that the production of these organic acids and their uncoupling effects (Alexander *et al.*, 1987; Baronofsky *et al.*, 1984) could account for the triggering of the Crabtree effect in yeast. Recently, Sierkstra *et al.* (1992) also suggested that the Crabtree effect is not related to glucose repression. The study was carried out by analysis of the transcription and translation of the glycolytic enzymes and glucose-related enzymes in the glucose-limited chemostat culture of *S. cerevisiae*. Glucose-specific repression of enzyme systems was implied to be absent due to the presence of invertase at all dilution rates studied (ranging from 0.05 to 0.315 h^{-1}). Therefore, glucose repression was thought to have no relation to the Crabtree effect.

1.3.3 Regulation of Glucose Catabolism

The utilization of glucose in yeast *S. cerevisiae* is thought to be regulated by several controls to enable the cell to retain its metabolism when environmental conditions, such as substrate concentration, are changed. The regulatory mechanisms of sugar utilization in *S. cerevisiae* have been grouped on the basis of their targets of regulation i.e. enzyme activity or enzyme concentration (Entain & Barnett, 1992).

i Regulation of Enzyme Activity

Activity of one (or more than one) enzyme of one pathway may be regulated by different types of metabolic regulator as described below.

i.1 Regulation by Allosteric Effectors

In this type of regulation, a decrease or increase in enzyme activity depending on type of the effector presents in the system. Effectors which can either be allosteric activators or allosteric deactivators will change the affinity of the enzyme for substrate. As a result, a reversible and rapid (within seconds) loss of enzyme activity occurs.

Phosphofructokinase and pyruvate kinase of the glycolytic pathway are regulated by positive and negative allosteric effectors (Entain & Barnett, 1992). Phosphofructokinase is activated by AMP and fructose-2,6-bisphosphate (F-2,6-BP) and inactivated by ATP (Bar *et al.*, 1990; Reibstein *et al.*, 1986). Unlike bacterial and plant enzymes which were found to be unaffected by F-2,6-BP, yeast phosphofructokinase is reported to be potentially stimulated by F-2,6-BP. This allosteric activator appears to have no other function than that of a regulating ligand (Ottaway, 1988). Pyruvate kinase is found unaffected by F-2,6-BP but activated by fructose-1,6-bisphosphate, the product of phosphofructokinase reaction. The enzyme pyruvate kinase is inactivated by ATP and citrate (Entian & Barnett, 1992). The allosteric regulation of the enzymes phosphofructokinase and pyruvate kinase of the glycolytic pathway is summarized in Figure 1.4.

Sierkstra *et al.* (1992) suggested that the glycolytic flux was not regulated by either transcriptional or translational control. The steady state levels of mRNA and *in vitro* enzyme activities of glycolytic enzymes of *S. cerevisiae* were studied at different dilution rates in glucose-limited chemostat cultures (Sierkstra *et al.*, 1992). Results demonstrated that no clear correlation between the dilution rate and the activity or mRNA level of these enzymes could be observed. The total activity of the three kinases relating to high-affinity glucose transport system (hexokinase I and II and glucokinase) were found constant with increasing dilution rates. The mRNA level of *HXK1* (gene encoding the enzyme hexokinase I) decreased with increasing dilution rates, whereas the transcription of *HXK2* (gene encoding the enzyme hexokinase II) increased.

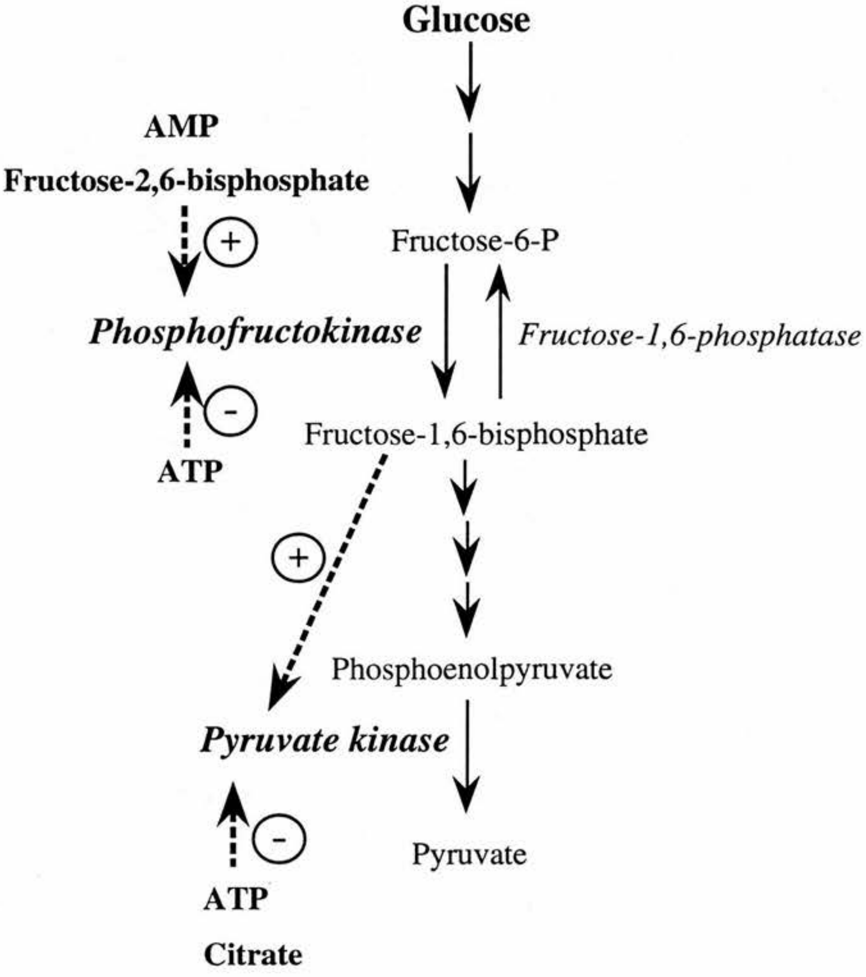


Figure 1.4 Regulation of the enzyme phosphofructokinase and pyruvate kinase by allosteric effectors.

i.2 Regulation by Covalent Modification

Although it has a similar physiological consequence to that of regulation by allosteric effectors, i.e. reversible loss of enzyme activity, it will be seen in this type of regulation that it requires a longer time than allosteric regulation to fulfil the regulatory process (within minutes).

ii Regulation of The Amount of Enzyme

In addition to the regulation of enzyme activity, glucose utilization can also be regulated at the level of enzyme synthesis resulting in changes in enzyme concentrations. The amounts of enzyme can be regulated by the following processes.

ii.1 Regulation by Enzyme Inactivation

Unlike those mechanisms regulating enzyme activity described above, the loss of enzyme activity occurring in this case is irreversible as a result of specific proteolysis of the enzyme.

An addition of glucose to cells grown on non-fermentable carbon source resulted in an inactivation of gluconeogenic enzymes of *S. cerevisiae.* An inactivation of enzyme activity in the presence of glucose is called *catabolite inactivation* which was assigned to the rapid loss of the enzyme fructose-1,6-bisphosphatase of the gluconeogenic pathway as a result of adding glucose to cells grown on non-fermentable carbon source (Gancedo & Gancedo, 1971). Several enzymes of gluconeogenesis have been found to be subjected to the concentration of glucose in the medium (Holzer, 1976). Although it has been clearly shown that the inactivation caused by glucose occurs in the vacuole (Chiang & Schekman, 1991), the mechanism is still unknown.

Catabolite inactivation was also reported to be involved with the control of the glucose transport system (see section 1.2).

ii.2 Regulation by Enzyme Induction

Regulation of the amount of enzyme in this type occurs in the presence of inducers, e.g. an enzyme substrate. In this type of regulation, the transcription of genes encoding the specific enzyme is increased, resulting in an increase in enzyme activity.

Rogers & Stewart (1973) demonstrated the induction of the synthesis of key enzymes of mitochondria and peroxisomes by oxygen in yeast *S. cerevisiae* grown in glucose-limited chemostat culture. An increase in oxidative metabolism which occurred with increasing oxygen concentration was suggested to be related to the development of a functional glyoxylate cycle. In addition the authors also observed that the synthesis of the peroxisomal enzymes occurred at significant higher dissolved oxygen concentration than that of the mitochondrial enzymes.

ii.3 Regulation by Repression and Derepression

In the presence of repressing substrates, transcription of a gene encoding a specific enzyme is inhibited. Once the repressing substrate is removed or lowered, an increase in the specific enzyme activity is obtained, in other words, derepression occurs.

Glucose is known to be an effective repressor in the repression of several enzymes related to other metabolic pathways such as galactose catabolism, gluconeogenesis, tricarboxylic acid cycle and the respiratory chain (Entian & Barnett, 1992). In yeast, the degree of repression caused by glucose is strongly dependent on the enzyme affected and it is strain specific (Gancedo, 1992). Several of the yeast enzymes were found to be effectively repressed by glucose (Table 1.1). The actual mechanism of the repression is however still unknown.

Originally, Holzer (1967) suggested that glucose itself as well as glucose-6-phosphate, ATP, ADP and orthophosphate were the causes of the repression. As the repression was subjected to the presence of glucose, the term glucose effect was therefore used. The glucose effect has come to characterize the Crabtree effect in yeast on the basis of the repression of respiratory enzymes by glucose (see also section 1.3.2 for the Crabtree effect). Beck & von Meyenberg (1968) studied yeast glucose catabolism in a continuous culture and showed that glucose was oxidatively metabolized at low dilution rates. They suggested that the glucose repression was not solely dependent on the presence of glucose but was subjected to metabolic rates of the cells. Barford & Hall (1979) re-evaluated many views associated with the control of glycolysis and respiration of *S. cerevisiae* in continuous culture and observed that the repression of respiration was transient and would not occur after fully adapted growth in continuous culture. After a long adaptation period, independent of the substrate nature, the rate of respiration reached a maximum value at a specific growth rate less than the maximum specific growth rate of the organism (Barford & Hall, 1979). Using continuous cultures of yeast *S. cerevisiae* grown under constant conditions but varying the glucose concentration in the feed-in medium, Rieger *et al.* (1983) found the incomplete oxidation of glucose by *S. cerevisiae*. The results were hypothesized as a consequence of a limited respiratory capacity of the cells. This hypothesis was confirmed by the study of Petrik *et al.* (1983) using the glucose-pulse and dilution rate-shift techniques in continuous cultures of *Saccharomyces uvarum*. Results from pulsing excess glucose into the culture demonstrated the formation of ethanol and acetate in response to the initiation of repressed growth before a decrease in respiration rate or mitochondrial cytochrome content. It was concluded that ethanol and acetate formation was the consequence of the regulation of pyruvate metabolism but not the repression of respiratory activity (Petrik *et al.*, 1983; Kappeli, 1986). The study of the occurrence of alcoholic fermentation in aerobic glucose-limited cultures of *S. cerevisiae* at high dilution rates i.e. the Crabtree effect therefore focussed on the overflow metabolism at the pyruvate level of this yeast (Postma *et al.*, 1989c) (see section 1.3.2).

Table 1.1 List of yeast enzymes which can be repressed by glucose (modified from Gancedo, 1992).

enzyme	repression factor	reference
invertase	750	Estruch & Carlson, 1990
alcohol dehydrogenase	120	Williamson *et al.*, 1981
maltase	90	Entian & Zimmermann, 1982
malate synthase	80	Polakis & Bartley, 1965
isocitrate dehydrogenase (NADP)	80	Polakis & Bartley, 1965
phosphoenolpyruvate carboxykinase	60	Gancedo & Schwerzmann, 1976
fructose-1,6-bisphosphatase	50	Gancedo & Gancedo, 1971
isocitrate lyase	50	Eraso & Gancedo, 1984
galactokinase	40	Torchia *et al.*, 1984
glutamate dehydrogenase (NAD)	25	Polakis & Bartley, 1965
aconitase	5	Polakis & Bartley, 1965
cyst c oxidase	5	Eraso & Gancedo, 1984
isocitrate dehydrogenase (NAD)	4	Polakis & Bartley, 1965

Catabolite repression has also been reported to be involved with the glucose transport system of *S. cerevisiae*. Some component of high-affinity glucose uptake was reported to be repressed by glucose (Bisson & Fraenkel, 1984). The observation was that the level of high-affinity uptake was low during growth in high concentration of glucose (100 mM) and it increased as glucose exhausted from the medium, and decreased again during the stationary phase. In addition, the higher level of uptake was found during growth in a low glucose concentration (0.5 mM) and in normal concentrations of non-glucose carbon substrates (Bisson & Fraenkel, 1984).

1.4 Modulation of Glycolytic Enzymes Production in Yeast

The activity of an enzyme of interest may be up- or down-modulated depending on purposes. In many industrial micro-organism strains of which anabolic pathways have been exploited for the overproduction of commercial compounds e.g amino acids, nucleic acids, antibiotics, vitamins, enzymes and protein (Stephanopoulos & Vallino, 1991), both up- and down-modulations of more than one enzyme may be required to yield a large amount of a desired end product. In some cases e.g. the study of the relationship between metabolic flux and enzyme activity (or concentration), down-modulation may be preferred in order to obtain a larger effect of change in enzyme activity. This is because large increases in enzyme activity have been predicted by metabolic control theory (Small & Kacser, 1993a,b) to have, on average, negligible effects on flux when compared to large decreases (Rosenzweig, 1992).

DNA manipulation has offered a powerful and straightforward method to alter the *in vivo* concentration (or activity) of any particular enzyme in a specific and desired direction. Using this technique, the *in vivo* activity of the enzyme of interest can be directly increased by placing extra copies of the gene specifying that enzyme on a multiple copy plasmid vector (Kawasaki & Fraenkel, 1982; Brindle, 1988; Schaaff *et al.*, 1989; van der Aar *et al.*, 1992; Davies & Brindle, 1992; Niederberger *et al.*, 1992) or, alternatively, the extra copies of the gene can be inserted into homologous chromosomal DNA using an integrative plasmid vector (van der Aar *et al.*, 1990a,b; this thesis). Decreases in enzyme activity can also be achieved by the means of DNA manipulation e.g. gene mutation (Lloyd *et al.*, 1992; Niederberger *et al.*, 1992) or the use of antisense mRNA (Haeuptle *et al.*, 1986; Izant & Weintraub, 1985; van der Krol *et al.*, 1988; Lichtenstein, 1988; Stitt *et al.*, 1991).

Change in one or more enzymes of a pathway is always accompanied by the question concerning whether or not such a change will subsequently alter other parts of the pathway or even the overall metabolism of the cell. Cell metabolism is a complicated operation unit which is composed of many different pathways including branch points, feedbacks, coupling and other kinds of metabolic regulation linked together. Therefore, it is likely that making a change in one part of metabolism will affect other parts of the cell metabolism in some way. Although substantial increases in an enzyme might not change flux through the pathway, they should be informative in other ways (Fraenkel, 1992). However, a compensatory change in one related intermediate metabolite may result in the absence of large effect on another metabolite (Davies & Brindle, 1992). Therefore, an ability to detect the consequences of enzyme alteration is dependent on the resolution of the technique used i.e if only a small subsequent change is present, a very sensitive technique is required.

A number of techniques have been applied to determine the consequences of enzyme alterations in yeast e.g. the use of non-growing cells (Brindle, 1988; Davies & Brindle, 1992; Lloyd *et al.*, 1992; Benevolensky *et al.*, 1994), batch cultivation (Schaaff *et al.*, 1989; van der Aar *et al.*, 1990a; van der Aar *et al.*, 1992;), and chemostat cultivation (van der Aar *et al.*, 1990a,b; van der Aar *et al.*, 1992;). Physiological consequences of changes in enzyme activity which were introduced at the same enzyme of the pathway may be detectable under certain conditions, but may be absent under other conditions. This can be seen (see below) in the use of growing and non-growing yeast cells for the study of PFK1 overproduction (Schaaff *et al.*, 1989; Davies & Brindle, 1992).

The glycolytic enzymes comprise some of the most abundant proteins in wild-type yeast (Fraenkel, 1982). Genes encoding glycolytic enzymes have been intensively studied and cloned (Holland & Holland, 1978; Williamson *et al.*, 1980; Alber & Kawasaki, 1982; Kawasaki & Fraenkel, 1982; Schmitt *et al.*, 1983; Entian *et al.*, 1984; Frohlich *et al.*, 1984; Seehaus *et al.*, 1985; Aguilera & Zimmermann, 1986; Heinisch, 1986; Rodicio & Heinisch, 1987). This information has therefore provided the possibility to isolate these genes and to construct modified yeast strains which possess an increase or decrease in any particular glycolytic enzyme. As a result, the consequences of an alteration in enzyme activity can be intensively studied.

In a number of cases, the presence of multiple copies of a specific gene in yeast does not result in the expected increase in the intracellular concentration of that protein. This has also been found in the expression of some glycolytic genes in yeast *S. cerevisiae*. High level expression of *PYK1*, the gene encoding the enzyme pyruvate kinase has been shown to be subjected to gene dosage limitation at the translational level (Moore *et al.*, 1990).

High-level expression of the glycolytic genes have been shown to be controlled by a number of transcriptional activators including the specific DNA binding proteins GCR1 (Baker, 1986; Clifton & Fraenkel, 1981; Clifton *et al.*, 1978; Holland *et al.*, 1987; Huie *et al.*, 1992), RAP1 (Bitter *et al.*, 1991; Brindle *et al.*, 1990; Butler *et al.*, 1990; Chambers *et al.*, 1989; Machida *et al.*, 1989; McNeil *et al.*, 1990; Nishizawa *et al.*, 1989; Scott *et al.*, 1990; Tornow & Santangelo, 1990), REB1 (Chasman *et al.*, 1990) and ABF1 (Brindle *et al.*, 1990; Chambers *et al.*, 1990). For example, the protein RAP1 has been shown to be involved in the high-level expression of *PGK* (Chambers *et al.*, 1989), *ENO1* and *PYK* (Buchman *et al.*, 1988) the genes encoding the glycolytic enzymes phosphoglycerate kinase, enolase and pyruvate kinase respectively. Capieaux *et al.* (1989) suggested that the RAP1 protein may be the same protein as TUF (Huet *et al.*, 1985) which has been proposed to be a component of a generalized transcriptional mechanism for the control of yeast growth. However, the respective roles and the mechanism of how RAP1, as well as other DNA-binding proteins, co-operate with the glycolytic genes in order to promote the high level of gene expression are still unknown.

Eight different glycolytic enzymes, including PFK1 which is usually claimed to be the "rate-limiting step enzyme" of the pathway, were overproduced in yeast *S. cerevisiae* by placing these genes on multicopy vectors (Schaaff *et al.*, 1989). The results obtained from batch cultivations indicated that the overproduction of these enzymes, either in individual or in pairs of enzymes, had no effect on growth rate or ethanol production. The amounts of several glycolytic metabolites were also found to be unaffected. These results were consistent with the study of Heinisch (1986) in which no effect on glycolytic flux could be found from PFK overproduction. However, such a lack of significant effect on glycolytic flux was suggested (Davies & Brindle, 1992) to be a result of the small contribution of respiration to cellular ATP production and the absence of the Pasteur effect in the growing yeast cells (Lagunas, 1979; Lagunas *et al.*, 1982) employed in these two studies. Davies & Brindle (1992) used non-growing yeast cells and noninvasive NMR techniques to study the effects of *PFK1* overexpression on glycolytic flux and glycolytic intermediate levels. They found that the overproduction of enzyme PFK1 had no effect on glycolytic flux under anaerobic conditions, but under aerobic conditions, it increased glycolytic flux up to the anaerobic level. The increased glycolytic flux was found to be accompanied by a compensatory decrease in flux in oxidative phosphorylation. They found only small or insignificant changes in the concentrations of enzyme substrates in the enzyme PFK1 overproducing strain. A study of the physiological consequences of mutations using non-growing yeast cells was also reported by Lloyd *et al.* (1992). The study was carried out using several mutants defective at several part of regulatory subunits of phosphofructokinases. These mutant were studied on the basis of the occurrence of the Pasteur effect. Results indicated that either of the two phosphofructokinases can carry out glycolysis.

Low flux control coefficients for glycolysis were implied for both PGK and GAPDH on the studies of PGK overproduction and GAPDH underproduction in the yeast *S. cerevisiae* (Brindle, 1988). The PGK-overproducing cells and GAPDH-less producing cells were studied using the combination techniques of non-growing cells and ^{31}P NMR magnetization transfer to measure flux between inorganic phosphate and ATP (Brindle, 1988). However, PGK has been shown to have a relatively high flux control coefficient for the ATP<->P_i exchange catalyzed by the two enzymes. With a different experimental approach, the consequences of PGK overproduction in yeast *S. cerevisiae* was investigated in batch and glucose-limited chemostat cultures (van der Aar *et al.*, 1990a,b). The yeast strains carrying 80 and 25 copies of *PGK1* gene, constructed by the means of a multi-copy integration system (Kempers-Veenstra *et al.*, 1984; Lopes *et al.*, 1989), were studied. The PGK overproduction in a medium *PGK1* gene copy integrant (with 25 copies of *PGK1* gene) showed growth benefits over the disadvantage of extra protein synthesis. The strong PGK overproducing strain (with 80 copies of *PGK1* gene) showed unaffected maximal specific growth rate but 40% reduction of the molar growth yield compared to that of the wild-type. The strong overproducer and the wild type were studied in competitive chemostat culture and results indicated the elimination of the overproducer from the culture. The disadvantage of extra protein synthesis was discussed to be the cause of the selection. On the other hand, the higher K_s (0.41 mM) of the strong overproducer than that of the wild type (0.30 mM) was also accounted to be the cause. The authors discussed that cells carrying 80 copies of the *PGK1* gene integrated into the genome were genetically unstable. In subsequent studies (van der Aar *et al.*, 1992), *S. cerevisiae* strains containing extra copies of *PGK1* gene introduced into a multicopy 2µ-based episomal plasmid (Hoekema *et al.*, 1987; van den Heuvel *et al.*, 1989) were constructed to re-analyse the physiological consequences of PGK overproduction.

Results indicated that the overproduction of the PGK was stabilized at the enzyme level about 12% of the total soluble protein. The stabilization was thought to be a result of an equilibrium between normal plasmid loss and the selective pressure due to advantageous enzyme overproduction under glucose-limited chemostat conditions. In addition, results also showed an increased respiration rate and decreased fermentative activity of the PGK overproducer during respiro-fermentative growth compared to the host strain.

The effect of increased PGI on glucose metabolism in yeast *S. cerevisiae* has been studied in a system using substrate labelling techniques and non-growing cells of which protein synthesis was inhibited (Benevolensky *et al.*, 1994). The results from mass action ratios indicated that the PGI reaction is near equilibrium. An increase in PGI activity to a measured factor of 11 was found to have no effect on substrate and product concentrations. However, these results were considered to be an underestimation of intracellular level of labelled glucose-6-phosphate.

1.5 Enzyme Triosephosphate Isomerase

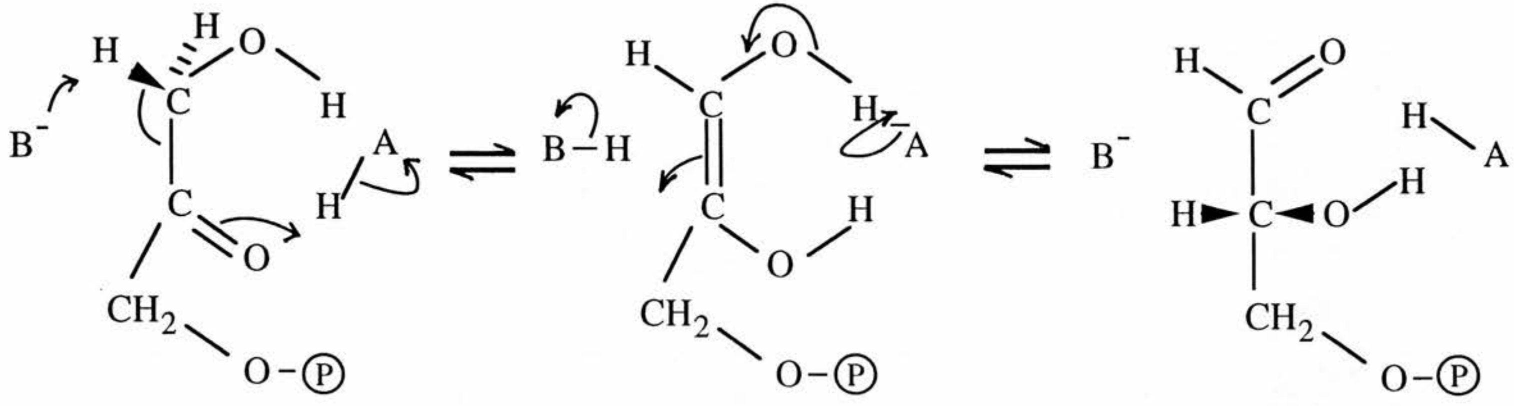
Triosephosphate isomerase, TPI, (EC 5.3.1.1) is the enzyme that catalyzes the interconversion of dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (GAP) (see Figure 1.1) by the stereospecific abstraction of either the pro-R proton from C1 of DHAP or the C2 proton of (R)-GAP by an enzymic base to give a cis-enediol intermediate (Figure 1.5) (Alber *et al.*, 1987; Nickbarg & Knowles, 1988). TPI has been reported to be involved not only with glycolysis but also gluconeogenesis, fatty acid synthesis and the pentose phosphate pathway (Noltmann, 1972). TPI is essential for efficient energy production because, for glycolysis, only glyceraldehyde 3-phosphate continues to proceed along the pathway (Figure 1.1), therefore, TPI ensures that DHAP is converted to GAP and funneled down to pyruvate (Alber *et al.*, 1987). In other words, the enzyme ensures that both halves of the hexose are used for ATP production (Fothergill-Gilmore & Michels, 1993). The enzyme from all sources is a dimer of two identical subunits (Alber *et al.*, 1987). Each monomer is of molecular weight 26,500, and it contains no cofactors, prosthetic groups, or metal atoms (Casal *et al.*, 1987). It has been demonstrated that the active enzyme is always the dimer form (Waley, 1973; Casal *et al.*, 1987).

The amino acid sequences of triosephosphate isomerase from 13 different sources have been reported (Lolis *et al.*, 1990) i.e. yeast *S. cerevisiae* (Alber & Kawasaki, 1982), yeast *Schizosaccharomyces pombe* (Russel, 1985), chicken (Straus & Gilbert, 1985), rabbit muscle (Corran & Waley, 1975), human liver (Maquat *et al.*, 1985), human placenta (Lu *et al.*, 1984), coelacanth (Kolb *et al.*, 1974), maize (Marchionni & Gilbert, 1986), *Aspergillus nidulans* (McKnight *et al.*, 1986), *Trypanosoma brucei* (Swinkels *et al.*, 1986), *E. coli* (Pichersky *et al.*, 1984), *Bacillus stearothermophilus* (Artavanis-Tsakonis & Harris, 1980) and *Macaca mulatta* (Old & Mohrenweiser, 1988). The overall three-dimensional structure of TPI from yeast, chicken and *Trypanosoma* has been found to be very well conserved although their amino acid sequences differ by up to 50% (Fothergill-Gilmore & Michels, 1993).

Triosephosphate Isomerase in Yeast

Triosephosphate isomerase is found in the wild-type yeast strain at approximately 2% of the yeast soluble protein (Norton & Hartman, 1972). Yeast TPI is moderately stable to environmental changes e.g. temperature, pH, and denaturants (Waley, 1973). The nucleotide sequence of the yeast gene coding for TPI (*TPI1*) has been reported and shown to encode a protein with approximately 50% identity to the amino acid sequence of chicken TPI (Alber & Kawasaki, 1982). The enzymes from these two sources have been shown to have essentially identical crystal structures and kinetic properties (Alber *et al.*, 1987).

It is well known, in general, that the nature of the upstream activation sequence (UAS) elements of glycolytic genes is complicated because of multiple binding sites for several different DNA-binding proteins. Similarly to the expression



dihydroxyacetone phosphate

cis-enediol intermediate

(R)-glyceraldehyde phosphate

Figure 1.5 Reaction catalyzed by yeast triosephosphate isomerase. B^- is the enzymic base (Glu-165), HA is an enzymic acid (His-95, or possibly Lys-12). Picture was obtained from Nickbarg & Knowles (1988).

of other glycolytic genes, the *TPI1* gene also requires the specific DNA-binding proteins for its expression. Strong requirements for GCR1 and GCR2 have been illustrated by 17- and 20- fold reductions in enzyme activity as a result of mutations in GCR1 (Clifton & Fraenkel, 1981) and GCR2 (Uemura & Fraenkel, 1990), respectively. In addition, mutation in the GCR3 has also resulted in the reduction in TPI activity. However, the effect of GCR3 mutation was found to be dependent on growth conditions i.e. two-fold reduction in enzyme activity was found only when the yeast strains were grown in a fermentable carbon source, whereas no reduction could be observed when the strains were grown in a non-fermentable carbon source (Uemura & Jigami, 1992). Using the plasmid constructed by Alber & Kawasaki (1982) as an original source of the *TPI1* gene encoding the enzyme triosephosphate isomerase, Scott *et al.* (1990) focussed on a controlling region of this gene and indicated that RAP1 is also necessary for expression of *TPI1*. Scott & Baker (1993) reported that the *TPI* UAS region contains at least four binding sites for DNA-binding proteins (Figure 1.6) i.e. a unique RAP1 site which is surrounded by two GCR1 sites and the fourth site which lies over a near-consensus REB1-binding site in the upstream end of the *TPI* gene. The study of the *TPI* controlling region was extended, and the results suggested a model showing a concerted action of these DNA-binding proteins in the high-level expression of the *TPI* gene (Scott & Baker, 1993).

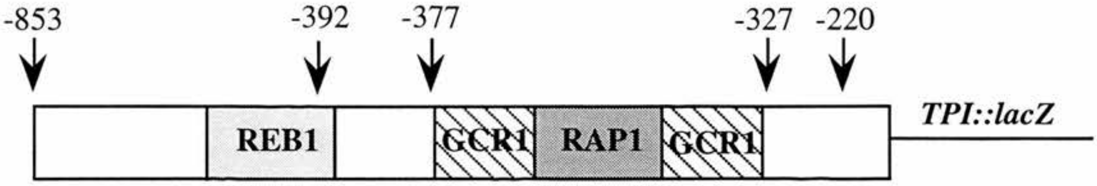


Figure 1.6 The *TPI* UAS region reported by Scott & Baker (1993).

Three-Dimensional Structure and Catalytic Activity

The catalytic site of TPI consists of Glu-165, Cys-126, His-95, Ser-96, Glu-97, Asn-10 and Lys-12 (Figure 1.7). A number of studies have been carried out by crystallography and site-directed mutagenesis to investigate the relationship between the structure and the catalytic activity of the enzyme TPI. The three dimensional structure of yeast TPI has been refined at 1.9 Å (Lolis *et al.,* 1990). In addition to the catalytic subsite, TPI also has a phosphate-binding site which is required for tight binding of the enzyme and its substrate (Alber *et al.*, 1987).

Among the amino acid residues of the TPI catalytic site mentioned above, Glu-165 which is the catalytic base is positioned for the nucleophilic abstraction of the pro-R proton from C1 of DHAP and direct transfer to C2 (see Figure 1.5). The three dimensional structure of the mutant TPI in which the catalytic base Glu-165 is changed to aspartic acid constructed by Straus *et al.* (1985) has been determined by X-ray diffraction at 2 Å and showed that both the distance between the catalytic base Glu-165 and the phospho group, and also orientation factors contribute to the catalytic efficiency of the wild-type enzyme (Joseph-McCarthy *et al.*, 1994).

Cys-126 is found to be invariable in all the TPI sequences observed from 13 different sources (see Lolis *et al.*, 1990 for alignment of amino acid sequences). The invariance of this amino acid residue has suggested that Cys-126 may be required for anchoring Glu-165 without diminishing the base strength of Glu-165 (Alber *et al.*, 1987).

His-95, Lys-12 and Asn-10 are the three amino acid residues which are in contact with the carbonyl and hydroxyl oxygen atoms of the sugar-phosphates (Alber *et al.*, 1987). Among these residues Asn-10 which is uncharged may be purely involved in substrate binding.

The mutant yeast enzyme containing a glutamine residue in place of His-95, has been constructed in order to study the role of His-95 in the catalytic mechanism

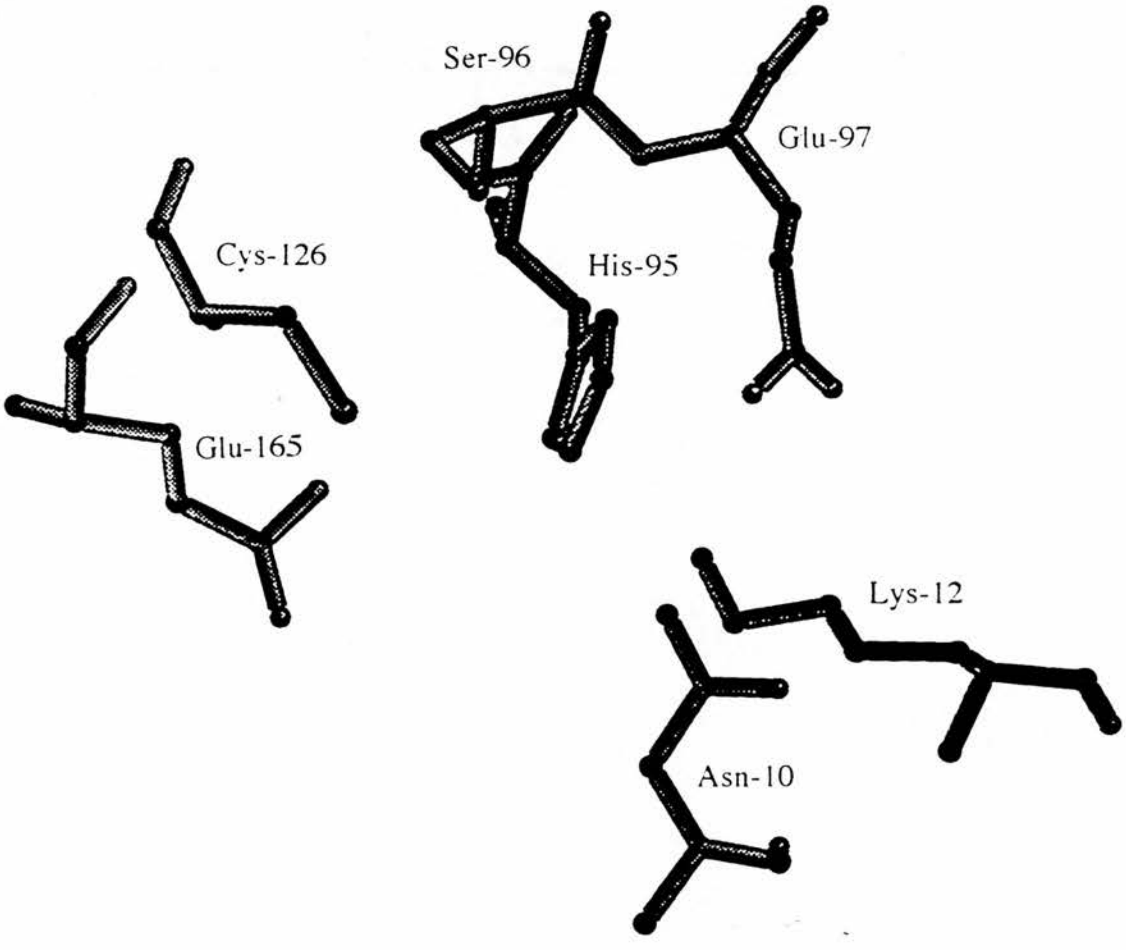


Figure 1.7 Stereo drawing shows amino acid residues of the active site of the enzyme triosephosphate isomerase.

(Alber & Kawasaki, 1982; Petsko *et al.,* 1984). The mutant enzyme was expressed in *E. coli* where it was found to have less specific catalytic activity than that of the wild-type enzyme by a factor of nearly 400 (Nickbarg, *et al.*, 1988)

Using a combination of site-directed mutagenesis, Fourier transform infrared spectroscopy, enzyme kinetics and X-ray crystallography, the mutant forms of TPI in which Lys-12 had been changed to methionine, arginine and histidine was studied and the results showed that a positive charge (provided by Lys-12) is required for substrate binding at the active site of TPI (Lodi *et al.*, 1994).

1.6 Metabolic Control Analysis

Traditionally, the 'rate-limiting step' has been regarded as an ideal mechanism of metabolic regulation. However, the discoveries of feedback inhibition, enzyme cascades and control of gene expression indicated special mechanisms by which metabolic regulation may occur (Porteous, 1990). In contrast to the qualitative assignation of a 'rate-limiting step', Metabolic Control Analysis (MCA) has been proposed (Kacser & Burns, 1973; Heinrich & Rapoport, 1974). MCA has provided the language and framework for quantitative study of control over flux, or over metabolites, by individual enzymes of a pathway (Quant, 1993). A review on MCA has been recently published (Fell, 1992). The main conclusions of MCA can be summarized as follows (Quant, 1993):

1) Control over flux is distributed throughout the component enzymes of the system.

2) More than one enzyme can exert significant control over flux of the system.

3) The distribution of control can shift with a change in metabolic state or external conditions.

Alteration in the *in vivo* enzyme concentration or activity can be achieved by several experimental techniques e.g. genetic manipulation and enzyme modification by inducer, titrater or inhibitor. Among these, genetic manipulation is the most widely used and applied because the amount of enzyme can be changed directly by an increase or decrease in the copy number of the gene encoding the specific enzyme.

MCA has been successfully applied to several cell types e.g. glycolysis in erythrocytes (Rapoport *et al.*, 1976), arginine biosynthesis in *Neurospora* (Flint *et al.*, 1981), respiration in mitochondria from rat liver (Groen *et al.*, 1982) and yeast (Mazat *et al.*, 1986), ethanol metabolism in *Drosophila* (Middleton & Kacser, 1983), and aromatic amino acid catabolism in liver cells (Salter *et al.*, 1986). Originally, MCA was not developed as a predictive theory to measure an effect of 'large' changes such as those generally produced by DNA manipulation and by some physiological experiments. However, the useful approach of MCA has been successfully linked to the 'large' changes (Small & Kacser, 1993a,b). This new approach, in addition to the original concept of MCA, will allow researchers to determine the effect on the metabolic system of all changes in any components.

1.7 Aims of The Thesis

Triosephosphate isomerase (TPI) is one of the glycolytic enzymes which is usually regarded as an equilibrium enzyme. The word "equilibrium" is, however, only a qualitative way to describe the property of the enzyme in the pathway. This description was obtained from a classical study of metabolic regulation where an examination of purified enzymes was achieved *in vitro* and followed by a qualitative conclusion on how the enzymes act in a pathway. This is obviously inadequate to give accurate information on the properties of enzymes *in vivo*. In addition, it is difficult to identify which enzymes are in or near equilibrium. A number of theories have been derived to identify these enzyme and hence to determine which enzymes control the pathway. However, some ambiguity and misunderstanding are still present. A more accurate method to study the influence of a particular enzyme would then be an *in vivo* alteration of enzyme in the pathway and study of the response of the system. This thesis attempts to investigate the physiological consequences of the *in vivo* enzyme overproduction using TPI in yeast *S. cerevisiae* as an experimental model.

1.8 Scope of The Thesis

The project was started with the construction of TPI overproducing strains, as well as a Ura$^+$ reference strain, as described in detail in Chapter 3. This also includes a primary characterisation to determine the level of gene expression in the constructed strains compared to that of the parental strain. Chapter 4 and Chapter 5 describe the individual studies of TPI overproducing strains along with the reference strain in batch and chemostat cultures, respectively. A much more sensitive technique i.e. competitive chemostat culture in which TPI overproducers were grown, pairwise, with the reference strain is described in Chapter 6.

Chapter 2

Materials and Methods

1. Materials

1.1 Strains

E. coli :

pTPIc10 (Alber & Kawasaki, 1982)

XL1-blue was purchased from Stratagene Cloning System

S. cerevisiae :

DBY747 (*MATa his3-Δ1 leu2-3, 112 trp1-289 ura3-52*)

1.2 Plasmid

YIplac211 (Gietz & Sugino, 1988) was obtained from Dr. A. Boyd

pURA3 was obtained from Dr. F. Stuart

pJDB36 was obtained from Dr. J. D. Beggs

1.3 Media

Medium components used in this work came from various suppliers as listed below.

Medium Components	Suppliers
i Bactopeptone, casamino acids, yeast nitrogen base without amino acids	Difco Laboratories Limited
ii Agar bacteriological, yeast extract	Oxoid Limited
iii Ampicillin, Xgal, IPTG, all amino acids, vitamins	Sigma Chemical Company Limited
iv glucose	BDH Laboratory Supplies

1.4 Enzymes and Restriction Enzymes

Enzymes	Suppliers
i enzymes for glycolytic enzyme activity assay	Boehringer Mannheim Biochemica
ii T4 DNA Ligase	Boehringer Mannheim Biochemica
iii restriction enzymes	Sigma Chemical Company Limited and Northumbria Biologicals Limited

1.5 Chemicals

All chemicals used in this thesis came from various companies as shown below. These chemicals were of the highest grade if required.

Chemicals	Suppliers
i AnalaR HCL, Phosphoric acid, $(NH4)_2SO_4$, KH_2PO_4, $MgSO_4.7H_2O$	BDH Laboratory Supplies
ii EDTA, all trace elements	Sigma Chemical Company

1.6 Miscellaneous

Items	Suppliers
i Non-radioactive DNA Labelling Kit, digoxygenin-labelled lambda/*Hin*dIII standard marker	Boehringer Mannheim Biochemica
ii HPLC accessories	
ii.1 ion-exchange column and guard column	Bio-Rad

1.6 Miscellaneous (continued)

Items	Suppliers
ii.2 sample vial	Anachem
ii.3 0.22 μm membrane filter (Acrodisc LC13 PDVF)	Fisons
iii equipment and accessories for fermentation devices	
iii.1 fermentor, pH controller, DO monitor, stirrer controller, pH probe, DO probe	F. T. Applikon Limited
iii.2 CO_2 analyzer	The Analytical Development Company (ADC) Limited
iii.3 peristaltic pump and manifold tubing	Watson-Marlow
iii.4 air flow meter and controller	Platons
iii.5 0.45 μm membrane filter, Millex GS filter	Millipore
iii.6 Bacterial Air Vent	Gelman Science
iii.7 silicone tubing	Altec
iii.8 sampling bottles, glass bottles, clips, connectors and all other accessories	Scotlab
iii.9 0.8% CO_2 in N_2	B.O.C.
iv Polaroid 665 film	Sigma Chemical Company
v Hyperfilm MP X-Ray film	Amersham International plc.

2. Methods

2.1 Growth and Storage of Bacterial Strains

A single colony obtained from an LB plate (Appendix A, Table A.1) was inoculated into fresh LB liquid medium. The culture was incubated overnight at 37°C with constant shaking. The cells were harvested by centrifugation and could be used immediately (e.g. preparation of competent cells) or stored as stock culture for future use (see below).

The long term storage of bacterial strains was done by resuspending cells pellet harvested from the overnight culture described above in sterile 15% (v/v) glycerol. The suspension was quickly frozen and kept at -70°C.

2.2 Plasmid DNA Preparation

2.2.1 Small Scale Plasmid DNA Preparation

The plasmid DNA was isolated from the bacterial strains using the Birnboim-Doly mini-preparation method (Birnboim & Doly, 1979).

Reagents:

Solution I: The solution was comprised of 50 mM glucose, 10 mM Na_2EDTA and 25 mM Tris.Cl, pH 8.0.

Solution II: The solution was comprised of 0.2 M NaOH and 1% (w/v) SDS. This solution was freshly prepared and kept in a plastic bottle.

Solution III: 60 ml of 5 M Potassium acetate was made up prior to the addition of 11.5 ml of glacial acetic acid and 28.5 ml sterile distilled water.

phenol:chloroform:isoamyl alcohol (25:24:1) mixture: The mixture was prepared as described in Sambrook *et al.* (1989)

TE buffer: The buffer was prepared as 10x stock solution and diluted 1:10 before use. The buffer stock solution was comprised of 100 mM Tris.Cl (pH 8.0) and 10 mM EDTA (pH 8.0).

70% ethanol

absolute ethanol

Procedure:

A small culture (5-10 ml) was grown overnight in LB medium at 37°C under selective conditions (eg. 100 $\mu g.ml^{-1}$ ampicillin) with constant shaking. The cells were collected by centrifugation for 1 min in microcentrifuge. The pellet was resuspended in 100 µl of ice-cold Solution I. The cell suspension was incubated at room temperature for 5 min. 200 µl of freshly prepared Solution II was added to achieve cell lysis. The mixture was inverted 4-5 times and stored on ice for 5 min. After adding 150 µl of precooled Solution III, the mixture was mixed gently and further incubated on ice for 5 min. The bacterial genomic DNA and cell debris were spun down at 4°C in a microcentrifuge for 5 min. The supernatant was transferred to a new tube and extracted with 400 µl of phenol:chloroform:isoamyl alcohol mixture. After centrifugation at 12,000 rpm for 5 min, the top layer was carefully transferred to a clean tube. The double stranded DNA was precipitated by adding 2 volumes of absolute ethanol and left for 1 h at -20°C. The plasmid DNA pellet was recovered after centrifugation for 30 min at 4°C. The pellet was washed with 500 µl of 70% ethanol. The plasmid was collected by centrifugation for 5 min and then air dried before redissolving in 20 µl of TE buffer. The suspension was then stored at -20°C.

2.2.2 Large Scale Plasmid DNA Preparation

Reagents:

Solution I, II and III : see section 2.2.1

isopropanol

ethidium bromide solution (10 $mg.ml^{-1}$): 1 g of ethidium bromide was added to 100 ml of distilled water and stirred for several hours. The stock solution was kept in a dark bottle and stored at room temperature.

top-up solution: The solution contains 4.5 g of CsCl in 4.58 ml of TE buffer and 0.243 ml of ethidium bromide solution.

TE buffer: see section 2.2.1

Procedure:

A 100 ml bacterial culture was grown overnight in LB medium at 37°C under selective conditions (eg. 100 $\mu g.ml^{-1}$ ampicillin) and the cells were harvested by centrifugation at 5,000 rpm for 5 min. 3 ml of Solution I was added prior to incubation at room temperature for 5 min. The tube was then placed on ice before adding 8 ml of Solution II followed by 5 min incubation on ice. A further 5 min incubation was required after adding 6 ml of precooled Solution III. For convenience, the mixture was transferred to a 50 ml centrifuge tube. Cell debris was removed by centrifugation at 15,000 rpm for 30 min at 4°C and the supernatant was collected by filtration through Whatman filter paper no.1. The plasmid DNA was precipitated by adding 12 ml of isopropanol to the supernatant prior to incubating for 15 min at room temperature. The supernatant was discarded after centrifugation at 15,000 rpm (20°C) for 30 min. The pellet was washed with 70% ethanol, dried briefly and resuspended in 4.5 ml of TE buffer.

The plasmid DNA was purified by adding 4.5 g of CsCl followed by 0.243 ml of 10 $mg.ml^{-1}$ ethidium bromide solution. At this step, the density of the mixture should be approximately 1.55 $g.ml^{-1}$. The mixture was transferred to Beckman Quick-Seal centrifuge tubes. The top-up solution was added to fill up the tube before sealing. The tubes were subjected to centrifugation at 50,000 rpm (20°C) for 3 days.

Two DNA bands were visualised by hand-held long wavelength UV lamp. The upper band consists of bacterial chromosomal DNA and nicked circular plasmid DNA, whereas closed circular plasmid DNA was in the lower band. This lower band was then removed by a syringe. The ethidium bromide contamination in plasmid DNA was eliminated by extracting with 1-butanol saturated in water. Further DNA purification was achieved by dialysis of the plasmid DNA in several volumes of TE buffer.

The quality of prepared DNA was determined by measuring the ratio of OD_{260} to OD_{280}. For 'clean' DNA, the ratio should be higher than 1.5. According to the assumption that 1 unit OD_{260} equivalents to 50 $\mu g.ml^{-1}$ of DNA, the concentration of the prepared plasmid DNA can therefore be estimated.

2.3 Bacterial Transformation

2.3.1 Preparation of Competent Cells

Reagents:

$CaCl_2$ solution: The solution contains 100 mM $CaCl_2$ in 10 mM Tris-HCl pH 8.0.

sterile glycerol

Procedure:

A 10 ml bacterial culture was grown overnight in LB medium. 1% of the culture was transferred to a fresh medium in 250 ml flask and shaken vigorously until the OD_{600} of 0.5 for $recA^-$ or 0.25 for $recA^+$ was obtained. The culture was chilled on ice for 10 min. The cells were then collected by centrifugation at 5,000 rpm for 5 min at 4°C. The pellet was gently resuspended in 15 ml of ice cold $CaCl_2$ solution and incubated on ice for 30 min. The cells were spun at 5,000 rpm for 5 min at 4°C and then gently resuspended on ice with 5 ml of ice cold $CaCl_2$ solution. The treated cells can be used immediately or can be frozen at -70°C after adding glycerol to a final concentration of 20% (v/v).

2.3.2 Introduction of DNA into the Competent Cells

Procedure:

10-30 ng of DNA was added to 100 μl of competent cell suspension in a sterile microfuge tube. The mixture was mixed gently and placed on ice for 30 min. The induction of DNA into the competent cells was achieved by incubating the mixture at 42°C for 90 sec and placing immediately on ice. The mixture was diluted with 900 μl of LB medium prior to plating 100 μl aliquots onto LB-Ampicillin plates.

When blue/white selection was required, X-gal and IPTG was added together with the cell suspension. The transformation of control plasmid DNA was carried out to check the transformation efficiency. The transformed colonies appeared after incubating the plates at 37°C for 12 to 16 hours.

2.4 Ligation of DNA Fragments

Reagent:

BRL ligase buffer: A 1x buffer was diluted from 10x stock solution with sterile distilled water.

Procedure:

The linearized DNA fragments were combined to give a 2:1 ratio of insert to vector ends. The mixture was heated at 70°C for 10 min, followed by rapid cooling on ice. The vector was generally used in a minimal amount e.g. 100 ng. BRL ligase buffer was then added to the mixture to obtain a final volume of 20 μl. A 5 μl aliquot was removed as a non-ligated control. 1 unit of T4 DNA ligase was added to the remaining mixture. The ligation mixture was then incubated overnight at 15°C or on the bench for 2-3 h. Vector self-ligations and insert self-ligations were usually set up as controls. The ligation products were analysed by either gel electrophoresis or transformation. For electrophoretic analysis, the mixture was heated at 70°C for 5 mins before loading onto the gel in order to eliminate annealing of compatible ends. A fifth of a five-fold dilution of the ligation mix was used for transformation.

2.5 Storage of Yeast Strains

For temporary storage, yeast strains were maintained on YEPD (Appendix A, Table A.2) slants and stored at 4°C. It was necessary to restreak the cultures from time to time on YEPD plate until single colonies were obtained.

For long-term storage, a single colony obtained from YEPD plate as described above was inoculated into liquid YEPD or YO medium (Appendix A, Table A.3).

This culture was grown until the mid-exponential phase growth was obtained. Cells were harvested, washed, resuspended in sterile 20% (v/v) glycerol and stored at -70°C as stock culture.

2.6 Growth of Yeast Strains in Batch and Chemostat Cultures

A 1 L fermenter vessel was used for study of yeast growth in both batch and chemostat cultivations. The culture system was set up as shown in Figure 4.2 (Chapter 4) for batch cultivation and in Figure 5.1 (Chapter 5) for chemostat cultivation. The culture system used for chemostat culture was an identical system used for batch cultures plus two additional devices i.e. medium feed-in and medium over-flow devices.

2.6.1 Preparation of Working Culture in Fermenter

A 1 L fermenter (Applikon) consists of four main parts i.e. the vessel, the head plate, the stirrer assembly and the stirrer motor. A round bottom glass vessel (model Z81305C001) has the maximum working volume of 900 ml and the minimum working volume of 350 ml. The vessel sat in a water jacket which connected to the temperature controller and cooling system. The stainless steel headplate (model V1M1102003) has six fixed in/outlets, five ports and a fixed thermometer pocket. Four fixed inlets are for water-in from heat exchanger, air-in from air pump, NaOH-in from pH controller and liquid entry pipe for inoculum or additional nutrients. Two fixed outlets are for water-out to heat exchanger and a sample pipe. Only three of five ports were used for batch cultivation, one port is for a glass condensor and another two ports are for pH and dissolved oxygen probes. For chemostat cultivation, two addition ports were used for medium inlet and culture outlet. A magnetically coupled stirrer assembly (model Z81315MG02) and the P100 stirrer moter of which a speed range is 0-1,250 rpm were used in this thesis. The stirrer motor was controlled by the ADI1012 stirrer speed controller.

The vessel was filled with 700 ml working medium (defined medium described in Appendix A, Table A.7). The headplate was fastened to the vessel by screwing the six mill nuts crosswise by hand. pH and dissolved oxygen probes were fastened onto the headplate after the calibration with standard buffers for pH probe and the membrane replacement for the dissolved oxygen probe. The connections for air and liquid to the vessel were made by silicone tubing the size of which was varied depending on ports and connections. For air inlet and air outlet, autoclavable membrane filters were fitted at the open end of the tubing. The tubing between the headplate and the filters were closed to avoid the wetting of the filter by liquid from the vessel during sterilization using autoclavable clamps. All connections were closed except the air outlet to maintain pressure equilibrium inside and outside the vessel during sterilization. The sterilization was carried out at 121°C for 20 min. After sterilization completed, the sterile vessel should be allowed to cool down inside the autoclave until the temperature of the autoclave has dropped below 90°C to avoid underpressure in any part of the vessel in particular of all the tubings.

After the vessel has cooled down to room temperature, all probes were connected to the control equipments. Additional medium components which had been sterilised separately were added. The sterile medium was usually left overnight with aeration and constant stirring on before inoculation to ensure the sterility of the medium.

2.6.2 Preparation of Medium Reservoir

10 L of defined medium was prepared in a glass bottle which was capped with an autoclavable silicone bung. The medium was autoclaved at 121°C for 45 min. After it has cooled down to room temperature, all additional nutrients e.g. glucose, vitamins and amino acids were added before connection to the vessel.

2.6.3 Preparation of Waste Bottle

20 L glass bottle was used to collect the overflow culture from the vessel. The bottle was capped with an autoclavable silicone bung and autoclaved at 121°C for 20 min.

2.6.4 Preparation of Inoculum

Inoculum for growth in the fermenter was prepared from the -70°C stock culture. As the -70°C stock culture may have lost its viability during storage, enrichment of the culture may be required prior to inoculum preparation. Small amount of stock culture was inoculated into YEPD liquid medium and grown in a shake flask culture until an approximate exponential phase growth was obtained. The culture was then sub-cultured into the working medium in 250 ml Erlenmeyer flask. The shake flask cultured until the precise mid-exponential phase growth, determined by OD_{600} measurement, was obtained. Cells were harvested, washed and resuspended in fresh medium. This cell suspension was therefore ready to be inoculated into the working medium in the fermenter (prepared as decribed in section 2.6.1). The working culture was usually inoculated to obtain an initial OD_{600} of 0.1 unit.

2.6.5 Cultivation Procedure

In both batch and chemostat cultures, the following constant parameters were used unless otherwise stated. All cultivations were performed at 30°C with a working volume of 700 ml. The pH of the culture was automatically controlled at 5.0±0.1 through out the run by the automatic addition of 1N NaOH. The dissolved oxygen tension in the medium was measured to assure that higher than 50% air saturation was obtained as a result of an agitation speed of 750 rpm simultaneously with an airflow of 1.2 L.min^{-1}.

Batch Culture

After inoculation, the OD_{600} of the culture was measured by taking small samples hourly from the sampling unit. The OD_{600} measurement was carried out hourly throughout the batch growth. Because of low cells density during an early growth the culture was allowed to grow for 5 h before the sample for dry weight measurement was taken. The dry weight measurement (section 2.17) was carried out hourly from 5-10 h where the exponential phase was obtained.

Chemostat Culture

After inoculation, the culture was allowed to grow without any disturbance until steady state was established. The measurement of cells density could be used as simple means to determine whether or not steady state had been reached. Small samples (5 ml) were obtained by removing culture fluid directly from the chemostat via a sampling port and measured for culture density (by OD_{600} measurement). When OD_{600} of the culture was stable, steady state conditions were assumed and a larger volume of sample was taken for the study of growth and metabolite production.

Growth in chemostat is assessed in terms of biomass yield which is defined as the amount of biomass produced from one gram of carbon substrate used. The amount of biomass can be determined as dry weight or wet weight of cells. In this project, dry weight measurement (section 2.16) was preferred. To obtain an accurate reading of biomass dry weight, a large sample size is required. This causes little problem with chemostat cultures because, unlike batch culture, the culture volume soon re-adjusts after the samples have been taken due to the fresh medium which is continuously being introduced into the culture. However, the culture working volume should be disturbed as little as possible because the larger the sample taken out the longer the period of time taken to replace that volume. In addition, dramatic changes of the culture working volume results in an alteration of dilution rate and could therefore affect the present steady state. To avoid this possible effect, sampling from the effluent line was considered. Dry weight measurement of samples taken directly from the culture vessel and from the effluent line were compared. No variation between those two sources could be found (data not shown). As a result, samples for dry weight measurements would be taken from effluent line throughout this project. However, samples taken directly from the culture vessel were still required for analysis of the glycolytic metabolites and for analysis of residual glucose concentrations because glucose could be continuously catabolized even in the non-growing yeast cell. This would continue to occur in the effluent line where the yeast cells were still exposed to the spent medium and, probably, the residual glucose in that medium. Samples for metabolites and glucose determinations were therefore taken by direct transfer of the culture from the vessel into liquid nitrogen (Postma *et al*, 1989c) in order to stop metabolic activity instantaneously. Several steady state samples were collected at each growth condition to analyze for growth and metabolite concentration. After sampling (or any disturbance to the culture), the culture was allowed to grow without any disturbance until steady state (previous or new condition) was resumed prior to the next sampling. When increasing the dilution rate to establish a new steady state condition, a stepwise procedure was followed, for example an increase of 0.02 h^{-1} at 2 h interval until the desired dilution rate was reached because a sudden increase could interfere and alter the actual metabolic state of the culture.

2.7 Yeast Transformation

Yeast transformation was carried out by the lithium acetate method as described by Rose *et. al.* (1990)

Reagents:

lithium acetate solution: The solution contains 0.1 M lithium acetate in TE buffer (10 mM Tris.Cl pH 7.5, 1mM EDTA).

PEG solution: 40% (w/v) PEG 3000 was dissolved in lithium acetate solution.

Procedure:

A 10 ml yeast culture was grown in YEPD medium at 30°C to a cell density of approximately $2x10^7$ cell.ml^{-1}. The cells were harvested by centrifugation at 2,000 rpm for 5 min in a microcentrifuge and washed with 5 ml of lithium acetate solution. The pellet was then resuspended in 1 ml of lithium acetate solution and transferred to a 10 ml tube and placed on a roller drum at 30°C for 1 h. The suspension was dispensed into 0.1 ml aliquots in microcentrifuge tubes. Approximately 1 μg of plasmid DNA was added into 0.1 ml of concentrated cells, followed by 10 μl of carrier DNA (5 mg.ml^{-1} sheared, deproteinized calf thymus or chicken blood or herring sperm DNA). The mixture was incubated at 30°C for 30 min prior to addition of 0.7 ml of PEG solution. The tube was inverted gently and incubated for a further hour at 30°C then heat shocked at 42°C for 5 min. The cells were collected by centrifugation for 5 sec in microfuge and washed with 1 ml of TE buffer. The pellet was then resuspended in 0.2-0.4 ml TE buffer or sterile distilled water. 0.2 ml of cell suspension was spread onto the plate containing selective medium. The plates were then incubated at 30°C. The transformed colonies appeared within 2-4 days depending on the strains, the vector and the selective marker.

2.8 Preparation of Yeast Genomic DNA

High molecular weight genomic DNA was isolated from yeast using a method described by Hoffman & Winston (1987).

Reagents:

smash buffer: The buffer was comprised of 2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-Cl (pH 8) and 1 mM Na_2EDTA.

phenol:chloroform:isoamyl alcohol (25:24:1) mixture: see section 2.2.1

TE buffer : see section 2.2.1

4 M ammonium acetate

absolute ethanol

Procedure:

Yeast cells were harvested from the exponential phase (10-15 ml) by centrifugation for 2 mins in a centrifuge and were resuspended in 0.5 ml of water. The cells were transferred to a 1.5-ml microfuge tube and were collected by centrifugation for 5 sec in a microcentrifuge. The pellet was resuspended in the residual liquid after decanting of the supernatant. 0.2 ml of the "smash buffer" was added, followed by 0.2 ml of the phenol:chloroform:isoamyl alcohol mixture and 0.3 g of acid-washed glass beads were added, before vortexing for 3-4 mins. The cell lysate was diluted with 0.2 ml of TE buffer (pH 8) and cell debris was removed by centrifugation for 5 min in a microcentrifuge. The aqueous layer was carefully transferred to a new tube. The DNA was precipitated by adding 1 ml of absolute ethanol and mixing thoroughly by inversion. The DNA pellet was collected after centrifugation for 2 min and resuspended 0.4 ml of TE buffer plus 3 μl of a 10 $mg.ml^{-1}$ RNase A solution. The mixture was incubated at 37°C for 5 min. 10 μl of 4 M ammonium acetate and 1 ml of absolute ethanol were then added, mixed by inversion. The DNA pellet was recovered after centrifugation for 2 min in microcentrifuge, air dried and redissolved in 50 μl of TE buffer and stored at -20°C. By this preparation, approximately 2-4 μg of the DNA was obtained.

2.9 Digestion of DNA by Restriction Enzymes

Reagent:

KGB buffer: The buffer contains 200 mM potassium glutamate, 50 mM Tris acetate (pH 7.6), 20 mM magnesium acetate, 100$\mu g.ml^{-1}$ bovine serum albumin and 1 mM 2-Mercaptoethanol (McClelland *et al.*, 1988). The buffer was filter sterilised and stored at 4°C.

Procedure:

The desired amount of DNA was digested with 1 μl of diluted restriction enzyme (corresponding to at least 1 $unit.\mu g^{-1}$ of DNA) and 1 μl of KGB buffer. The reaction was made up to 10 µl with sterile distilled water and incubated at 37°C for 2-3 hours. In case of a double enzymes digestion, the total volume of the reaction is increased to 20 µl and the same amount of another restriction enzyme and buffer was added at the same time. After the digestion was completed, the reaction mixture was inactivated by incubation at 65°C. The reaction mixture was then subjected to agarose gel electrophoresis in order to analyse the result.

2.10 Agarose Gel Electrophoresis

Reagents:

TBE buffer: The buffer was prepared as 10x stock solution which was comprised of (per litre) 10 g of Tris base, 55 g of boric acid and 9.3 g of Na_2EDTA.

gel loading buffer: The buffer was prepared as 5x stock buffer which was comprised of 0.025% (w/v) Bromophenol blue, 0.25% (w/v) Xylene cyanol, 25% (w/v) Ficoll, 0.5% (w/v) SDS and 50 mM Na_2EDTA.

ethidium bromide solution: see section 2.2.2

Procedure:

0.8 g of agarose was melted in 100 ml of TBE buffer. In order to visualize the DNA by UV light, ethidium bromide was added to the final concentration of 0.5 µg/ml. The molten gel was cast in a BRL mini-gel system. For convenience, multiple gels can be prepared and stored at 4°C before use. Samples (2-500 ng of DNA) were mixed in 1x gel loading buffer and then heated at 65°C for 5 min prior to loading into each well alongside the standard Lambda DNA cut with *Hin*dIII (250 ng of total DNA). A voltage of 80 V was applied to the electrophoresis tank until the bromophenol blue and xylene cyanol have migrated to the appropriate distance through the gel. Bands of DNA were then examined by UV light on a 312 nm transilluminator or a hand-held long wavelength UV lamp.

2.11 Recovery of DNA from Agarose Gel

After the separation of restriction fragments by agarose gel electrophoresis, the DNA band of interest was located using a hand-held long wavelength UV lamp. The band was cut from the gel as small as possible by using a sharp scalpel. From this step, the DNA can be recovered from agarose gel by a simple dialysis bag technique (Sambrook *et al.*,1989) or the method described by Pai & Bird (1991).

2.11.1 Electroelution into Dialysis Bags

Reagents:

TBE buffer: see section 2.10.

ethidium bromide solution: see section 2.2.2

Procedure:

A dialysis bag, prepared as described in Sambrook *et al.* (1989), was sealed at one end by a plastic clip before being filled with TBE. The agarose gel slice containing the DNA fragment was placed into the bag and allowed to sink to the bottom of the bag. Only a small amount of buffer was retained in order to keep the gel wet. The bag was then sealed at the other end by a plastic clip before immersed into an electrophoresis tank filled with TBE. An electric field of 4-5 V was applied for approximately 2-3 h or until the DNA is electroeluted out of the gel and onto the inner wall of the bag. This can be determined by using a hand-held, long wavelength UV lamp. In order to release the DNA from the wall of the bag, the polarity of the current was reversed for 1 min, thus the DNA was suspended into the buffer surrounding the gel slice. The DNA solution was then carefully transferred to a clean tube. The complete electroelution of DNA from the gel slice was checked by staining the gel slice with ethidium bromide (0.5 $\mu g.ml^{-1}$) and examining by UV transilluminator. The DNA was extracted with phenol:chloroform:isoamyl alcohol mixture, followed by ethanol precipitation for further purification.

2.11.2 Electroelution of DNA by Hand-made Device (Pai & Bird, 1991)

Materials and Reagents:

a strip of plastic screen (2x7 cm^2): The screen was cut to fit in an electrophoresis chamber. One or more holes were punched into a plastic screen depending on the number of simultaneous elutions carried out.

disposable pipette tip filled with agarose: The tip was cut and sealed at the tapered end by parafilm. 0.4 ml of molten 0.6% agarose was loaded into the tip and allowed to set.

a piece of dialysis tubing

TAE buffer pH 7.8: The buffer was comprised of 40mM Tris-acetate and 2 mM EDTA, and adjusted pH to 7.8.

Procedure:

The slice of agarose gel containing the desired DNA fragment was carefully transferred into the disposable pipette tip filled with agarose and overlaid by the remaining molten agarose. After the over layer had set, the tip was cut at a distance of 2 cm from the tapered end. The over layer was filled with TAE buffer and covered with a small piece of dialysis tubing. Another pipette tip was cut at a distance of 3.2 cm from the narrow end and pushed into the first tip in order to hold the dialysis membrane firmly. The cut end of the agarose containing tip was fitted into the hole of the plastic screen. The entire apparatus was then placed into the electrophoresis chamber containing TAE buffer, in the direction of the cathode. A current of 0.15 A was applied to the electrophoresis tank. The completed electroelution of the DNA fragment from agarose gel slice into the buffer could be confirmed by illumination with a longwave UV lamp. After the DNA had been eluted and accumulated onto the dialysis membrane, the polarity was reversed for 20 sec to release the DNA from the dialysis membrane. The DNA which was resuspended in the buffer was then collected, concentrated and further purified if required.

2.12 Southern Analysis

2.12.1 Transfer of DNA from Agarose Gel to Nylon Membrane

Transfer of DNA from agarose gel to nylon membrane was performed in order to localize particular DNA sequences by Southern analysis (Southern, 1975).

Reagents:

0.25 NMHCl

Denaturing Buffer: The buffer contains 1.5 M NaCl and 0.5 M NaOH.

Neutralising Buffer: The buffer contains 1 M Tris.Cl pH 8.0 and 1.5 M NaCl.

20x SSC: The solution contains 3 M NaCl and 0.3 M Trisodium citrate.

Procedure:

After agarose gel electrophoresis, the gel was soaked in 0.25 M HCl with shaking for 15 min to ensure transfer of the larger DNA fragments (>10 kb). Denaturation of DNA was achieved by soaking the gel in Denaturing Buffer for 1 hour with constant shaking. The buffer was then replaced by Neutralizing Buffer, shaking well for at least an hour. Transfer of DNA from the agarose gel to a nylon membrane was carried out by the capillary transfer method as described in Sambrook *et al.* (1989).

The gel was placed on wet 3MM paper which covered the support and surrounded by 20x SSC. A piece of nylon membrane was cut to the size of the gel and soaked in 20x SSC solution. The membrane was carefully placed on the top of the gel so that no air was trapped. Three sheets wet 3MM paper, cut to the size of the gel, were laid over the nylon membrane. The capillary transfer was achieved by placing a stack of gel size paper towels, on the top of 3MM paper prior to a glass plate. A 1 kg weight was placed on the top of the glass plate. The transfer of DNA was allowed to proceed at room temperature overnight. After blotting, the membrane was air dried for 1 h. A series of positive control DNA concentrations were spotted and dried onto the bottom of the dried membrane in order to check the efficiency of hybridisation procedures.

The dried membrane, DNA side down, was exposed to the UV light on a UV transilluminator for 3-5 min to fix the DNA to the membrane. The membrane was then ready for hybridisation.

2.12.2 DNA Labelling

Random primed incorporation of digoxigenin-labelled deoxyuridine-triphosphate was used as nonradioactive DNA labelling. This technique was performed by using a DNA labelling and detection kit from Boehringer Mannheim (catalogue no.1093657). The following method is described as set out in the kit instruction manual.

Reagents:

EDTA solution: The solution was comprised of 0.2 M EDTA, pH 8.0.

Lithium chloride solution: The solution was comprised of 4 M Lithium chloride.

70% ethanol

TE buffer: see section 2.2.1

Procedure:

10 ng to 3 µg of purified linearized DNA was recommended to be used per standard reaction. The DNA was incubated in boiling water bath for 10 min to separate double strand DNA and then quickly chilled on ice/NaCl for complete denaturation. The following components were added to 1 µg of freshly denaturated DNA or to 5 µl of denatured control-DNA (vial 2) in an microfuge tube tube on ice; 2 µl of hexanucleotide mixture (vial 5) and 2 µl of dNTP labelling mixture (vial 6). The reaction mixture was made up to final volume of 19 µl with sterile redistilled water prior to adding 1 µl of Klenow enzyme (vial 7). The reaction was incubated at 37°C for at least 1 h. However, the longer incubation (up to 20 h) can increase the amount of labelled DNA. The reaction was stopped by adding 2 µl of EDTA solution. The labelled DNA was then precipitated by mixing with 2.5 µl of LiCl solution and 75 µl of prechilled ethanol. The precipitation was allowed to proceed at

least 30 min at -70°C (or 2 h at -20°C). The labelled DNA probe was collected by centrifugation at 12,000g for 15 min. The pellet was then washed with cold 70% ethanol and dried under vacuum. The suspension of DNA probe in 50 µl of TE buffer could be used straight away or stored in -20°C until the hybridisation was performed.

2.12.3 DNA Hybridisation

Reagents:

10% (w/v) N-Lauroylsarcosine

10% (w/v) SDS

Blocking reagent

20x SSC: see section 2.12.1

Hybridization solution: The solution contains 5x SSC, 0.5% (w/v) blocking reagent, 0.1% (w/v) N-Lauroylsarcosine and 0.02% (w/v) SDS.

sterile 2x SSC and 0.1% (w/v) SDS solution: The solution was prepared from 10% (w/v) SDS and 20x SSC.

sterile 0.1x SSC and 0.1% (w/v) SDS solution: The solution was prepared from 10% (w/v) SDS and 20x SSC.

Procedure:

The dilution series of the unlabelled control DNA (vial 1) in the dilution buffer (vial 3) was made from 10^{-4} (10 pg.μl^{-1}) to 10^{-7} (10 fg.μl^{-1}). The dilutions were heated in boiling bath for 10 min and chilled quickly on ice/NaCl. 1 µl of each of the diluted denatured control DNAs were then spotted onto a sheet of nylon membrane and fixed by UV cross linking with a transilluminator for 3-5 min. It should be noted that the following volume of solution or DNA concentration are calculated for a membrane size of 100 cm^2. The control membrane and the membrane carrying denatured target DNA were prehybridised in a hybridisation plastic bag at 42°C for 2-3 h with at least 20 ml of hybridisation solution. For the control membrane, the hybridisation solution was replaced by 2.5 ml of hybridisation solution containing 5 μl (26 ng) of freshly denaturated labelled control DNA (vial 4) per ml. For the membrane containing target DNA, the hybridisation solution was replaced with 2.5 ml of hybridisation solution containing freshly denaturated labelled DNA probe. The hybridisation was carried out overnight at 42°C with gentle shaking. The hybridisation solution was then removed and the membrane was washed twice at room temperature with at least 50 ml of 2x SSC; 0.1% (w/v)SDS for 5 min. The membrane was further washed twice with 0.1xSSC; 0.1% (w/v) SDS for 15 min at 68°C. The membrane was now ready to be used directly for detection or stored air-dried for later detection.

2.12.4 DNA Detection

The hybridised DNA can be detected by chemiluminescent detection. The procedures described below is the detection of hybridised DNA using 3-(2'-Spiroadamantane)-4-methoxy-4-(3''-phosphoryloxy)-phenyl-1,2-dioxetane);AMPPD, a chemiluminescent substrate for alkaline phosphatase.

Reagents:

Buffer 1: The buffer was comprised of 0.1 M maleic acid and 0.15 M NaCl. The pH of the buffer was adjusted to 7.5 with concentrated NaOH before sterilisation.

Washing Buffer: The buffer was comprised of 0.3% (v/v) of Tween 20 in Buffer 1.

Buffer 2: The buffer was comprised of 1% (w/v) Blocking reagent in Buffer 1.

Buffer 3: The buffer was comprised of 0.1 M Tris.HCl, 0.1 M NaCl and 50 mM $MgCl_2$ (pH 9.5).

AMPPD solution: AMPPD was freshly diluted (from the 10 $mg.ml^{-1}$ stock solution) 1:100 in Buffer 3.

Procedure:

All incubations were performed with constant shaking at room temperature unless otherwise stated. The membrane was briefly washed in Washing Buffer and then transferred to 100 ml of Buffer 2 for 30 min. Anti-DIG-AP-conjugate stock solution was diluted to 75 mU.ml^{-1} (1:10^{5}) in Buffer 2. The membrane was then subjected to the incubation with 20 ml diluted antibody conjugate solution for 30 min. Two 15 min washes with 100 ml Washing Buffer were carried out before the membrane was washed in 20 ml of Buffer 3 for 2-5 min. The membrane was incubated in 10 ml of AMPPD solution for 5 min. After blotting on 3MM paper to let the excess liquid drip off for a few seconds but not to complete dryness, the damp membrane was sealed in a hybridisation bag. Preincubation of the sealed membrane was carried out at 37°C for 5-15 min. The sealed membrane was then exposed to X-ray film (Hyperfilm-ECL, Amersham) in a Dupont Cronex cassette. The exposure time depended on strength of the fluorescent signal and on background.

2.13 Preparation of Crude Cell Extract

Reagent:

Extraction Buffer: The buffer contains 50 mM K_2HPO_4, 2 mM EDTA and 3 mM 2-mercaptoethanol (Kawasaki & Fraenkel, 1982). pH of the buffer was adjusted to 7.4.

Procedure:

Cells were harvested by centrifugation at 3000 rpm for 5 min (room temperature) and washed twice in Extraction Buffer. After discarding of the supernatant, the pellet was resuspended in 100 µl of the same buffer containing the protease inhibitors in the final concentration as described below. The cell suspension could be stored at -20°C. The enzyme assay required the addition of acid washed glass beads to the cell suspension prior to vigorous vortexing for 2 min. The base of the microfuge tube was pierced with a needle and the cell free extract was collected by centrifugation of the microfuge tube inside a 9 ml tube at 3,000 rpm for 5 min. The clear liquid crude cell extract was then transferred to a new microfuge tube and spun in a microcentrifuge at 4°C for 10 min. This crude extract was assayed immediately, for enzyme activity.

Protease Inhibitors

The following protease inhibitors were added to cell suspensions prior to breakage in order to prevent enzyme degradation.

i) 3,4-Dichloroisocoumarin (DCIC) -inhibits serine protease

A 50 times concentrated stock solution was prepared as a final concentration of 5 mM DCIC in DMSO.

ii) Trans-Epoxysuccinyl-L-Leucyl Amino(4-Guanidino)butane (E64C) -inhibits cysteine protease

A 50 times concentrated stock solution was prepared as a final concentration of 1 mM E64C in distilled water.

iii) 1,10-Phenathroline -inhibits metallo enzymes

A 1,000 times concentrated stock solution was prepared as a final concentration of 100 mM 1,10-phenathroline in DMSO.

2.14 Enzyme Activity Assay

2.14.1 Triosephosphate isomerase Assay

The activity of enzyme triosephosphate isomerase was determined using the enzyme-linked spectrophotometric assay modified from the method of Hartman & Norton (1975). The assay is based on monitoring the decrease of absorbance at 340 nm due to the oxidation of NADH at 25°C.

Assay Components:

i) assay buffer: 24 mM Triethanolamine-HCl, pH 7.9
6 mM EDTA

ii) 2.26 mM NADH

iii) DL-Glyceraldehyde-3-phosphate (3-PGA)

iv) α-Glycerophosphate dehydrogenase (α-GDH)

v) sterile distilled water

Assay Procedure:

The assay was carried out in a 1 ml cuvette which contained:

825 µl of assay buffer
5 µl of 3-PGA
70 µl of 2.26 mM NADH
69 µl of dH_2O
1 µl of α-GDH
30 µl of sample

The sample was diluted to the appropriate enzyme concentration i.e. the dilution at which the rate of change in absorbance between 0.01-0.02 was achieved. The reaction was started by adding the diluted sample to the reaction mixture shown above. The oxidation of NADH was followed by the decrease in absorbance at 340 nm. Enzyme activity was calculated from the rate of decrease in absorbance which was linear with respect to time for several minutes. One unit of activity was defined as giving a rate of decrease in A_{340} of 1 unit.min^{-1}.

2.14.2 Phosphoglycerate kinase Assay

Phosphoglycerate kinase activity was assayed in the gluconeogenic direction by coupling the reaction with glyceraldehyde-3-phosphate dehydrogenase (Scopes, 1975). The reaction was followed by monitoring the rate of decrease in absorbance at 340 nm due to the oxidation of NADH.

Assay Components:

i) assay buffer: 30 mM triethanolamine.HCl, pH 7.5
50 mM KCl
0.2 mM $Na_2EDTA.2H_2O$
5 mM $MgSO_4$

ii) 1 M 3-phosphoglycerate (3-PGA)

iii) 28 mM NADH

iv) 50 mM ATP

v) glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

Assay Procedures:

A standard 1 ml assay mixture contained:

835 μl of assay buffer

10 μl of 1 M 3-PGA

5 μl of 28 mM NADH

100 μl of 50 mM ATP

2 μl of GAPDH

50 μl of sample

The reaction mixture was mixed thoroughly before the reaction rate was followed in a recording spectrophotometer. The assay was carried out at 25°C. Enzyme activity was calculated from the rate of decrease in absorbance which was linear with respect to time for several minutes. One unit activity was defined as giving a rate of decrease in A_{340} of 1 unit.min^{-1}.

2.14.3 Pyruvate kinase Assay

Pyruvate kinase activity was assayed using the coupling reaction with lactate dehydrogenase (Bergmeyer, 1974).

Assay Components:

i) assay buffer: 100 mM MES, pH 6.3
100 mM KCl
15 mM $MgCl_2$

ii) 100 mg.ml^{-1} phosphoenolpyruvate (PEP)

iii) 100 mg.ml^{-1} ADP

iv) 28 mM NADH

v) lactate dehydrogenase (LDH)

Assay Procedure:

A 1 ml reaction mixture contained:

950 μl of assay buffer

15 μl of PEP

15 μl of ADP

5 μl of 28 mM NADH

10 μl of LDH (5 unit)

5 μl of sample

The reaction was initiated by the addition of appropriate dilution of sample to the reaction mixture shown above. The rate of decrease in A_{340} was monitored at 25°C. One unit of enzyme activity was defined as a decrease in absorbance of 1 unit.min^{-1}.

2.14.4 Hexokinase Assay

Hexokinase activity was assayed by coupling glucose-6-phosphate to 6-phosphogluconolactone production with the enzyme glucose-6-phosphate dehydrogenase (Bergmeyer, 1974).

Assay Components:

i) assay buffer: 100 mM triethanolamine.HCl, pH 7.6

ii) 10 mM ATP

iii) 10 mM glucose

iv) 110 mM NAD$^+$

v) 100 mM $MgCl_2$

vi) glucose-6-phosphate dehydrogenase (G6PDH)

Assay Procedure:

A 1 ml standard assay mixture contained:

500 μl of assay buffer

100 μl of 10 mM ATP

100 μl of 10 mM glucose

100 μl of 100 mM $MgCl_2$

10 μl of 110 mM NAD^+

1 μl of G6PDH (0.6 unit)

150 μl of sterile distilled water

40 μl of sample

The assay was carried out at 25°C. Enzyme activity was calculated from the rate of increase in absorbance which was linear with respect to time for several minutes. One unit of enzyme activity was defined as giving a rate of increase in absorbance at 340 nm of 1 unit.min^{-1}.

2.15 Protein Determination

2.15.1 Bradford Mini Assay

Protein concentration in the crude extract was estimated as described by Bradford (1976).

Reagent:

A reagent was prepared by dissolving 100 mg of Coomassie Brilliant Blue G-250 in 50 ml of 95% ethanol followed by adding 100 ml of 85% (w/v) phosphoric acid. The solution was made up to a final volume of 1 litre with distilled water and then filtered through filter paper.

Procedure:

0.1 ml of crude extract (containing approximately 1-10 μg of protein) was added to 1 ml of reagent. The absorbance at 595 nm was measured after incubating for 2 min (but not longer than 1 hour) at room temperature. A standard curve was constructed using bovine albumin (0-100 μg/ml) as the standard protein.

2.15.2 Sodium dodecyl sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Polyacrylamide gel electrophoresis used in this work was carried out using the method outlined by Laemmli (1970).

Reagents:

30% acrylamide stock solution: The solution contained (per 200 ml) 58 g of acrylamide and 2 g of bisacrylamide. The stock solution was filtered and stored at 4°C.

separating gel buffer: The solution contained 1.5 M Tris.Cl (pH 8.8) and 0.5% (w/v) SDS.

stacking gel buffer: The buffer contained 0.5 M Tris.Cl (pH 6.8) and 0.5% (w/v) SDS.

loading buffer: The buffer contained (per 100 ml) 10 ml of stacking gel buffer, 2 g of SDS, 5 ml of mercaptoethanol, 10 ml of glycerol and 5 mg of bromophenol blue.

electrode buffer: The electrode buffer was prepared as 10 time concentrated stock solution. The solution contains 126 g of Tris, 80 g of glycine and 20 g of SDS. The pH of the solution should be approximately 8.3 and need not be adjusted.

Coomassie blue dye: The dye contains 2.5 g of Coomassie brilliant blue R250, 454 ml of methanol, 454 ml of distilled water and 92 ml of acetic acid.

destaining solution: The solution contains 70 ml of acetic acid. 50 ml of methanol and 880 ml of distilled water.

1% (w/v) ammonium persulphate (APS): This component must be prepared freshly each time.

Procedure:

Gel Preparation

The glass plates were assembled as recommended by the manufacturers. The separating and stacking gels were prepared as described below. The separating gel could be prepared to required acrylamide concentration as follows:

separating gel components:

final acrylamide concentration (%)	30% acrylamide (ml)	separating buffer (ml)	distilled water (ml)
6	7.5	9.25	20.3
9	11.2	9.25	16.6
12	14.9	9.25	12.9
15	18.6	9.25	9.2
18	22.4	9.25	5.4

In order to achieve complete and reproducible polymerization, the gel solution was degassed for 10 mins at room temperature. The gel was polymerised by the addition of 2 ml of 1% APS and 20 µl of TEMED (N, N, N', N'-tetramethylenediamine) followed by thoroughly mixing. The gel was then poured immediately and gently overlayed with distilled water. The gel was allowed to set for approximately 30 mins at room temperature.

After the separating gel was set, the stacking gel was prepared. The stacking gel solution (4.5% stacking gel) was comprised of:

0.75 ml of 30% acrylamide stock solution
1.25 ml of stacking gel buffer
2.85 ml of distilled water

The gel solution was thoroughly mixed and degassed for 10 min at room temperature. 0.15 ml of 1% APS and 5 µl of TEMED were added and mixed with the stacking gel solution. The overlay was completely removed and the stacking gel was poured directly onto the surface of the separating gel. The comb which was rinsed with alcohol and dried immediately prior to use, was carefully inserted into the stacking gel solution. The gel was placed in a vertical position and allowed to set for approximately 30 min at room temperature.

Sample Preparation

In this work, the crude extracts which were used for enzyme activity assay were also used as SDS-PAGE sample. The sample was added to an equal volume of the loading buffer. The mixture was then boiled for 10 min in a boiling bath prior to loading onto the gel.

Electrophoresis

The comb was gently removed from the stacking gel. The upper and lower chamber were filled with the electrode buffer. The samples containing approximately 10 µg of protein were loaded into the wells using a microsyringe. The syringe was thoroughly rinsed with 70% ethanol between loading of each sample. The standard molecular weight markers were also loaded alongside the samples. The gel was run at 50 mA until the dye front reached 0.5 cm from the bottom of the gel.

Staining and Drying of the Gel

After electrophoresis, the protein bands were stained with Coomassie blue dye. The gel was submerged in the staining solution with constant shaking at room temperature until the gel became dark blue in colour. The background stain was removed by soaking the gel in destaining solution until the background dye disappeared. Once the background colour had cleared, the gel was dried under vacuum and stored.

2.16 HPLC (High Performance Liquid Chromatography) Analysis

This technique was used to determine the concentration of metabolites in the culture fluid. The ion exchange column (Aminex HPX-87H, BIO-RAD) and the guard cartridge (Micro-Guard IG cation H^+ cartridge, BIO-RAD) were employed throughout this thesis. The column was connected to the guard cartridge, the HPLC machine and the detector as recommended by manufacturer. The IBM computer was connected to the HPLC machine to control the system operation and also to analyze the data obtained from the chromatography. In all cases, 0.22 µm membrane filtered 10 mM H_2SO_4 was used as a single mobile phase. The flow rate of the mobile phase was 0.4 $ml.min^{-1}$. Sample size injected into the machine was 20 µl. Running time and integration time were dependent on metabolites analyzed (see below).

Sample Preparation

Because the working pH of the Aminex column must be kept below 3, 10 µl of 1 M H_2SO_4 was added to 990 µl of the cell-free culture fluid to acidify the sample. The mixture was then filtered through a membrane filter (Acrodisc LC13 PDVF).

2.16.1 Analysis of Glucose, Glycerol and Ethanol Concentrations

The refractive index detector (refractoMonitor III, model 1109, LDC/Milton Roy) was used to determine glucose, glycerol and ethanol concentrations. 20 µl of each sample was analyzed along with standard concentration of glucose (range from 1-20 mM), glycerol (range from 0.14-13.69 mM) and ethanol (range from 1.74-171.48 mM). Total running time was 35 min and peak integration started at 11 to 24 min after sample injection. A glucose peak appeared at approximately 13.3 min whereas glycerol and ethanol peaks appeared at approximately 20 and 31 min, respectively. The peak area of these metabolites obtained from samples were compared to peaks obtained from standard metabolite concentrations.

2.16.2 Analysis of Pyruvate and Acetate Concentrations

Pyruvate and acetate concentrations in samples were analyzed using the ultra violet detector (Applied Biosystem model 759A Absorbance Detector). The analysis was carried out at 210 nm. Sample size was 20 µl. Sensitivity of the detector was set as appropriate depending on metabolite concentration of the samples. Samples were analyzed along with standard pyruvate (range from 0.05-0.8 mM) and acetate (range from 0.5-8.0 mM) concentrations. Total analysis time was 28 min and peak integration started at 11 to 27 min after sample injection. The pyruvate peak appeared at approximately 14 min after sample injection whereas the acetate peak appeared at approximately 23 min after sample injection.

2.17 Biomass Determination

Nitrocellulose membrane filters, pore size 0.45 μm, (Millipore) were used for dry weight measurement. An appropriate amount of cultures were pipetted onto pre-dried, pre-weighed filters. After removal of the medium by filtration, the filters were washed twice with distilled water and dried at 105°C overnight. All samples were weighed in triplicate and expressed as gram of biomass per litre of the culture.

2.18 Carbon Dioxide Determination

The carbon dioxide concentration in the effluent air was measured in the carbon dioxide analyzer (typeSB100-2, The Analytical Development Company Limited). The gaseous sample to be measured flew through the analysis cell of the instrument which was situated between the source and the infra-red detector. The percentage of CO_2 was shown at the reading panel of the analyzer. The analyzer was calibrated with 0.8% CO_2 in N_2 (B.O.C.) as recommended by manufacturer.

2.19 Dissolved Oxygen Determination

Dissolved oxygen in the culture was measured by the dissolved oxygen probe (Uniprobe type G-2) which was connected to the dissolved oxygen meter (Uniprobe model 703/5). The probe was inserted and sealed in to the fermenter before sterilisation. After sterilisation, the probe was connected to the dissolved oxygen meter by the probe lead. Calibration was performed before inoculation. The zero point was set after saturation the sterile medium with oxygen-free nitrogen. The full scale point (i.e. 100% dissolved oxygen) was set after extensive passing the fermenter with sterile air, with agitation and at the temperature of operation. The maintainance of dissolved oxygen probe was performed as recommended by manufacturer.

2.20 pH Measurement and Control

pH of the culture was measured by the pH probe (Uniprobe) which connected to the pH meter (Uniprobe model 706). During an experiment, the pH was maintained at 5.0±0.1 by addition of 1 M NaOH via a peristaltic pump which responded to the signal from the pH meter. The pH probe was calibrated with standard buffers before sterilisation. The maintenance of pH probe was done as recommended by manufacturer.

Chapter 3

Strain Construction and Characterisation

Introduction

The alteration of enzyme activity or enzyme concentration *in vivo* provides the data necessary for the study of metabolic regulation. A number of methods are available to vary the level of intracellular enzymes e.g. genetic manipulation or DNA recombination (Schaaff *et al.*, 1989; Cremer *et al.*, 1991; Haeuptle *et al.*, 1986; Izant & Weintraub, 1985; Lichtenstein, 1988; van der Krol *et al.*, 1988; Stitt *et al.*, 1991), enzyme induction (Stuart *et al.*, 1986; Salter *et al.*, 1986), enzyme titration (Groen *et al.*, 1982; Torres *et al.*, 1986) or specific inhibitor titration (Wander *et al.*, 1984; Groen *et al.*, 1986; Dyszynski *et al.*, 1982; Rigoulet *et al.*, 1987; Pryor *et al.*, 1987). Of these, genetic manipulation is found to be an effective and straightforward technique because it allows the possibility of altering a particular gene in specific ways. Using this technique, the regulatory mechanisms of an organism can be probed in remarkable detail (Nimmo & Cohen, 1987).

There have been a number of studies involving the overexpression of glycolytic genes in yeast *S. cerevisiae* using recombinant DNA techniques (see Chapter 1 for details). Schaaff *et al.* (1989) and van der Aar *et al.* (1992) studied the overproduction of glycolytic enzymes in yeast, and the variation in enzyme activity reported there was achieved by introducing extra copies of the particular glycolytic gene of interest into the yeast cell using a yeast episomal plasmid (YEp) vector. Alternatively, a multi-copy integration system was used in the study of phosphoglycerate kinase (PGK) overproduction in yeast (van der Aar *et al.*, 1990a,b). Although this technique resulted in a strong PGK overproducer carrying 80 copies of the *PGK1* gene in the yeast genome, it was subsequently shown (van der Aar *et al.*, 1992) to be inferior, in terms of stability of the recombinant DNA, to the use of a multi-copy 2μ-based episomal plasmid described by van den Heuvel *et al.* (1989) and

Hoekema *et al.* (1987).

It is well known that YEps have the highest transformation frequency (between 10^3 and 10^5 transformed cells per μg of DNA) compared to other types of yeast cloning vectors and also yield high copy numbers of recombinant plasmids per cell. However, variation in the plasmid copy number per cell might occur due to unequal plasmid segregation during cell division or a spontaneous loss of the recombinant plasmid particularly when cells are grown without selective pressure (Futcher & Cox, 1984; DiBiasio & Sardonni, 1986; Parker & DiBiasio, 1987). This variation would lead to a heterogeneous yeast population which can make results more difficult to interpret, as well as creating problems of reproducibility. In contrast, yeast transformants generated using yeast integrative plasmid (YIp) vectors are extremely stable and show less variability in gene copy number.

The YIps are basically bacterial plasmids carrying a yeast gene. As it does not contain any parts of the 2μ plasmid, it will integrate into a yeast chromosome by homologous recombination instead of replicating as a plasmid. An additional advantage of a YIp vector is that the variation in gene copy number during cell division, seen in YEps, can be avoided since copies of the gene will be replicated simultaneously with the chromosomal DNA. The copies of the gene of interest are thus inherited in a Mendelian fashion. In this project, a YIp vector was employed as a vector for the cloning strategy.

This chapter describes the construction and characterisation of yeast strains overexpressing the triosephosphate isomerase gene (*TPI*). It also describes the construction of the Ura^+ reference strain.

Results

3.1 Construction of Yeast Strains Overproducing the Enzyme Triosephosphate Isomerase

The plasmid pTPIc10 (constructed by Nasmyth & Tatchell, 1980; Figure 3.1), carrying the yeast *TPI1* gene encoding the enzyme triosephosphate isomerase, was isolated from the bacterial strain *E. coli* pTPIc10 (Alber & Kawasaki, 1982). After *Pst*I-*Eco*RI digestion, a 3.7 kb *TPI* fragment, containing both an upstream activator sequence and the entire coding region of the *TPI1* gene, was recovered and purified as described in section 2.11. This DNA was then subcloned into a yeast-*E. coli* shuttle vector. The integrative plasmid vector, YIplac211, (Figure 3.2) constructed by Gietz & Sugino (1988) was chosen because it allows not only the integration of the *TPI* gene into the yeast chromosome after crossing-over but also the simple screening for plasmid containing insert by the colour assay i.e. blue/white selection (section 2.3). The subcloning strategy is summarised in Figure 3.3. The plasmid vector was digested with the restriction enzymes *Pst*I and *Eco*RI before ligation to the 3.7 kb fragment from pTPIc10. An expected recombinant plasmid DNA is shown in Figure 3.4. After DNA ligation, bacterial transformation was carried out using the *E. coli* strain XL1-blue as the host strain. The transformants which contained recombinant DNA were identified as white colonies.

The transformants were grown in LB medium (Appendix A, Table A.1), and the recombinant plasmid DNA was isolated and purified as described in section 2.2.1. The DNA was then cut at a unique *Kpn*I restriction site within the *TPI* gene (Figure 3.4). The linearized plasmid DNA was then introduced into the yeast *S. cerevisiae* strain DBY747 which is Ura$^-$ strain by the lithium acetate yeast transformation method (section 2.7). The recombinant DNA was expected to integrate into the yeast chromosome, as a result of a crossing over between the chromosomal *TPI* locus and the *TPI* gene carried on the plasmid (Figure 3.5).

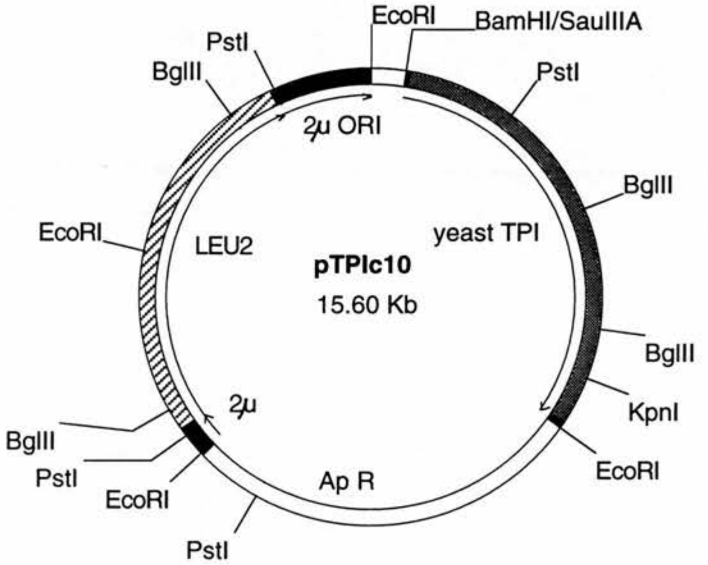


Figure 3.1 Plasmid pTPIc10 constructed by Nasmyth & Tatchell (1980) (figure is modified from the restriction map reported by Alber & Kawasaki (1982)). The grey bar represents 5 kb of yeast *TPI1* insert, open bars represent *E. coli* plasmid DNA (pBR322), and hatch bar and solid bars represent *LEU2* gene and 2µ DNA from YEp13, respectively.

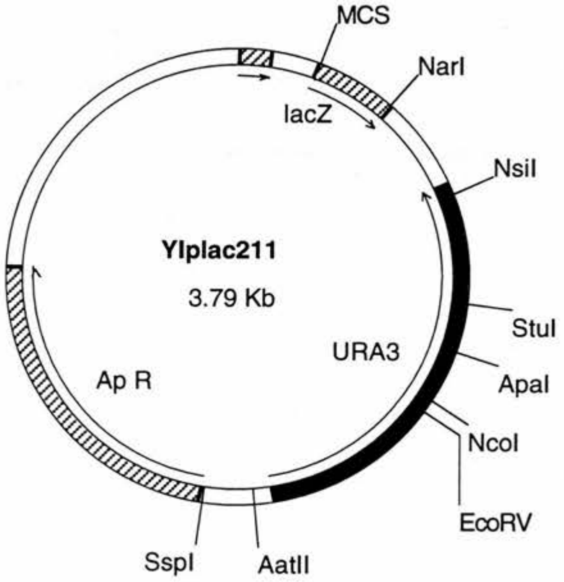


Figure 3.2 Plasmid vector YIplac211 (drawn from Gietz & Sugino, 1988). The multicloning sites (MCS) is pUC19 MCS: *Hin*dIII, *Sph*I, *Pst*I, *Sal*I, *Xba*I, *Bam*HI, *Sma*I, *Kpn*I, *Sst*I, *Eco*RI (Yanisch-Perron et al., 1985). The open bars represent *E. coli* plasmid nucleotide sequences, the solid bar represents yeast *URA3* gene, the hatch bars represent the *lacZ* and ampicillin resistance genes of pUC19, and the unlabelled hatch bar represents the *lacI* gene of pUC19.

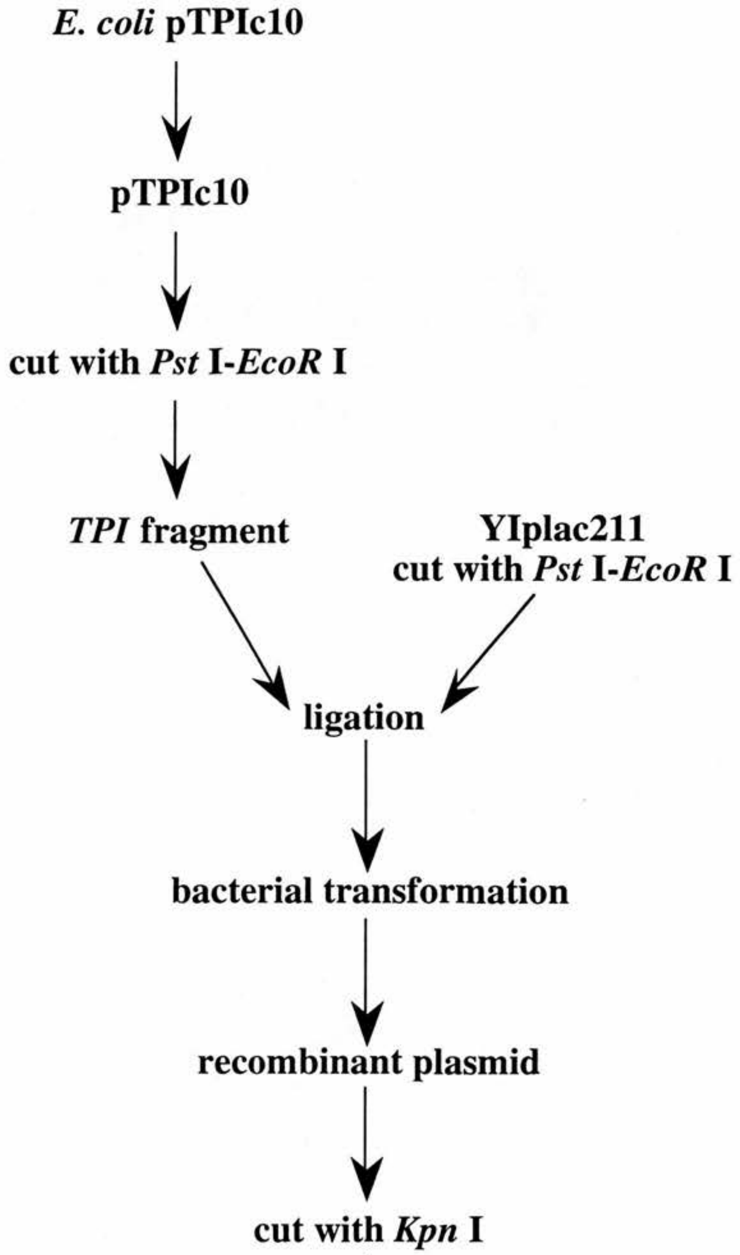


Figure 3.3 The subcloning strategy used for the construction of TPI overproducing strains.

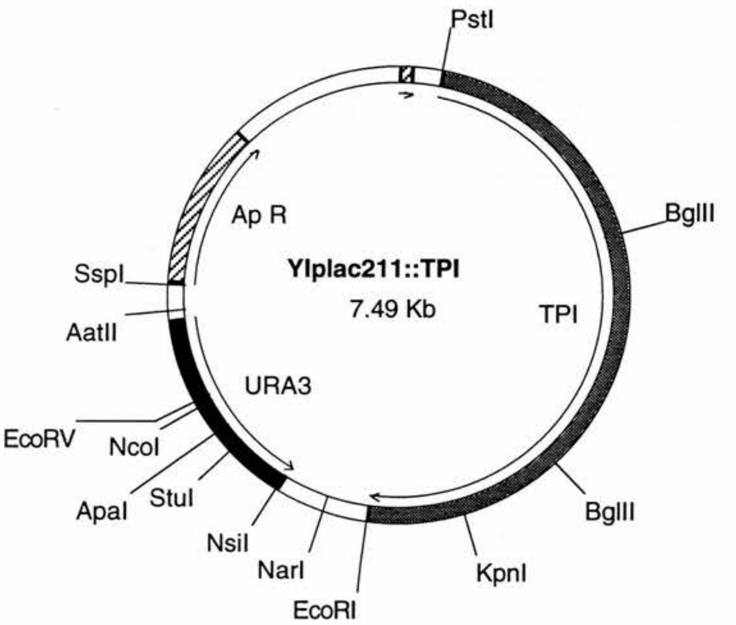


Figure 3.4 Recombinant plasmid DNA YIplac211::*TPI*. A 3.7 kb *Pst*I/*Eco*RI TPI fragment was expected to insert into the YIplac211 plasmid vector at *Pst*I/*Eco*RI sites of MCS on the vector. The gray bar represents a 3.7 kb *Pst*I/*Eco*RI *TPI* fragment isolated from the plasmid pTPIc10 (Figure 3.1). The following nucleotide sequences were from plasmid vector YIplac211 (Figure 3.2). The open bars represent *E. coli* plasmid nucleotide sequences, the solid bar represents yeast *URA3* gene, the hatch bar represents the ampicillin resistance gene from pUC19 and the unlabelled hatch bar represents the *lacI* gene from pUC19.

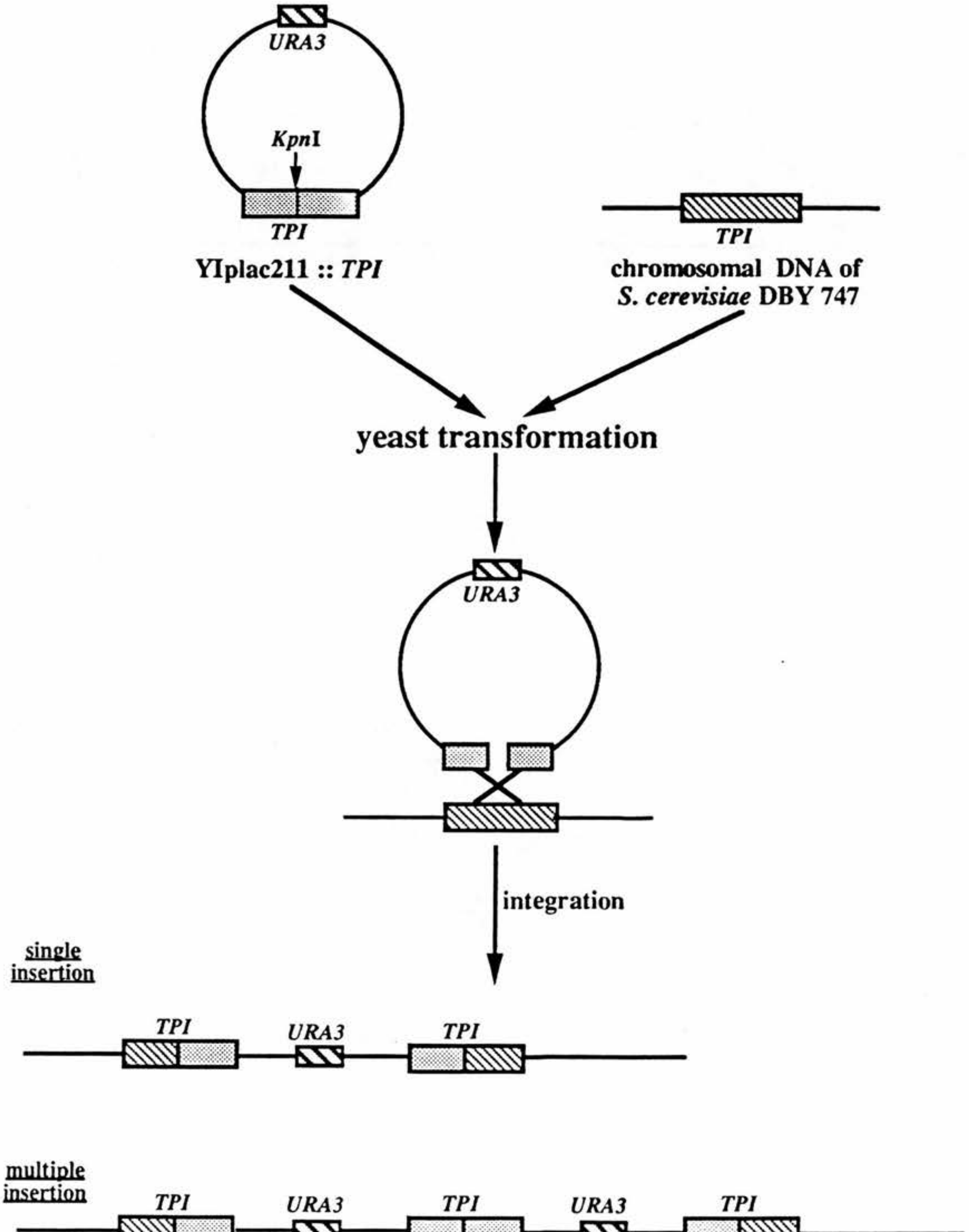


Figure 3.5 Integration of the YIplac211::*TPI* into the yeast chromosomal DNA after crossing-over at the *TPI* locus.

As a consequence of co-integration of the *URA3* gene carried on the plasmid vector YIplac211, the transformants could be selected as uracil prototrophs by plating on selective medium, the minimal medium without uracil (Appendix A, Table A.3). Ten transformants (DBY747::TPI) designated as DT1-DT10 were collected after 4 days growth on selective medium. The expression of the *TPI* gene in these ten transformants was further examined.

3.2 Study of *TPI* Gene Overexpression in Transformed Strains

The transformed strains were studied in parallel with a reference strain to assess the expression of the additional copy (or copies) of the *TPI* gene. It should be emphasized that for this part of the study, the yeast *S. cerevisiae* DBY747, the Ura$^-$ parental strain, was used as a reference instead of a Ura$^+$ reference strain (see section 3.3).

Transformants were grown in 50 ml of liquid minimal medium, YO-uracil, whereas the parental strain DBY747 was grown in YO+uracil (Appendix A, Table A.3), in 250 ml Erlenmeyer flasks at 30°C with constant shaking. To obtain the mid-exponential phase cells, the cultures were harvested after 7.5 h (established by following the OD_{600} with time). Cell suspensions for cell crude extract preparation were collected from 10 ml of each culture (section 2.13). The rest of the culture was grown to stationary phase in order to extract chromosomal DNA (section 2.8) for Southern analysis.

3.2.1 Southern Analysis

Southern analysis was carried out to determine whether single or multiple copies of the *TPI* gene had integrated into the chromosome of those ten transformants. The genomic DNA of the transformants was prepared and digested (sections 2.8 and 2.9) with *Nco*I, the restriction enzyme which cuts within the *URA3* gene of the YIplac211::*TPI* (Figure 3.4). The digested genomic DNAs were transferred onto the nylon membrane as described in section 2.12.1 and probed with the 3.7 kb *Pst*I-*Eco*RI fragment (Figure 3.1) from pTPIc10 which contains the *TPI* gene which was labelled with digoxygenin (section 2.12.2). The results of this experiment are given in Figure 3.6 and the schematic diagrams describing the results are shown in Figure 3.7. The strain DBY747 generated a unique DNA band, with the size dependent on the position of the *Nco*I restriction sites on the chromosomal DNA. This band, corresponding to band A in Figures 3.6 and 3.7, disappeared in transformants since this part of the DNA had been disrupted by a single copy or a tandem number of copies of YIplac211::*TPI* depending on the number of integrations which occurred. In the single integration event which led to a direct repeat of the *TPI* gene flanking the plasmid, two hybridisation bands corresponding to band B and band C (Figures 3.6 and 3.7) were detected. In the transformants which possessed multiple integrations, a third band (7.5 kb) was also observed. These results revealed that most of the transformants collected had obtained only a single copy of YIplac211::*TPI* integrated into their chromosomal DNA. As a result of having double copies of the *TPI* gene per cell, these strains were then expected to show an expression of the *TPI* gene of at least double the reference strain level. Multiple integration was observed in the transformants DT1 and DT9, these strains were therefore expected to express the enzyme activity at a higher level, perhaps greater than double that of the reference strain level.

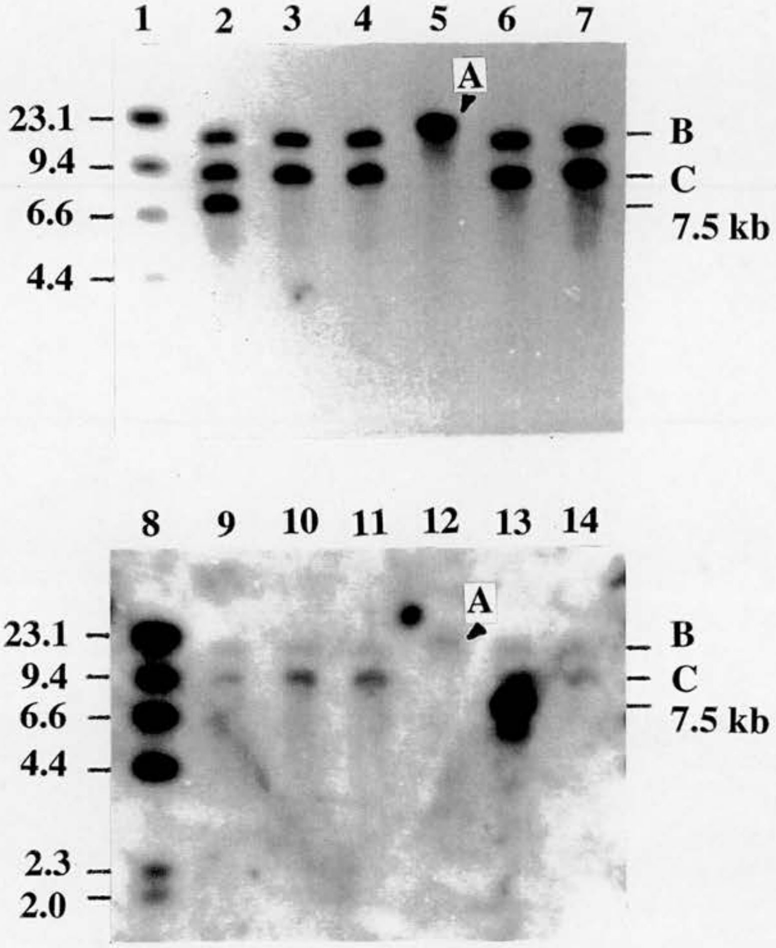


Figure 3.6 The Southern blots of the genomic DNAs of the DBY747 and the transformants DT1-DT10 probed with the TPI probe (see section 3.2.1 and Figure 3.7 for details of bands A, B and C). Upper blot, lane: 1) λ/*Hin*dIII marker, 2) DT1, 3) DT2, 4) DT3, 5) DBY747, 6) DT4, 7) DT5; lower blot, lane: 8) λ/*Hin*dIII marker, 9) DT6, 10) DT7, 11) DT8, 12) DBY747, 13) DT9, 14) DT10.

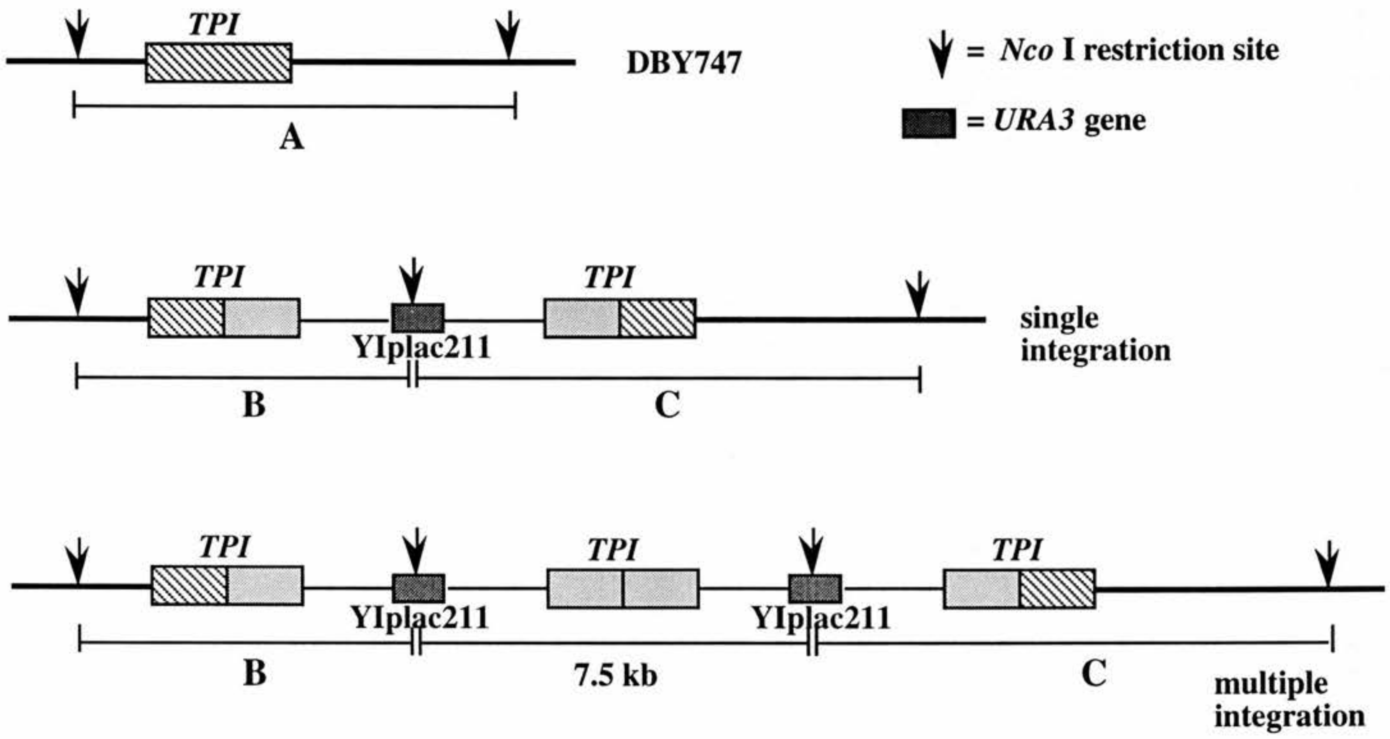


Figure 3.7 Schematic diagram describe the results from Southern analysis of strain DBY747 and the transformants DT1-DT10.

3.2.2 Measurement of Specific TPI Activity

Assays of TPI activity as well as total protein assays were carried out as described in sections 2.14.1 and 2.15.1, respectively. The specific TPI activity of each strain was measured, and the relative expression of the *TPI* gene calculated from the specific activity of the transformants when compared to the reference strain, DBY747. The results, including those from Southern analysis, are summarized in Table 3.1. The highest specific TPI activity was obtained in transformant DT9 for which the relative level of overexpression was 4.9 fold compared to the reference. An increase in specific enzyme activity by approximately a factor of two was observed in all the rest of the transformants studied. Surprisingly, multiple integration was observed in the transformant DT1 (section 3.2.1), however, the relative level of overexpression was only double that of the reference level.

3.2.3 SDS-PAGE

TPI gene overexpression was further assessed by SDS-PAGE. Cell crude extracts used for enzyme and protein assay were run on 9% SDS-polyacrylamide gels (section 2.15.2). The TPI monomer band, molecular mass of 26,500 Da, was identified by comparing with a standard mixture of the purified TPI and α-glycerophosphate dehydrogenase (α-GDH). The results are shown in Figure 3.8. Consistent with the result obtained from specific TPI activity measurement shown in Table 3.1, the highest intensity of the TPI band was observed in the DT9 sample. Although not very clear, overproduction of TPI in the rest of the transformants can, however, be visualized when compared with the reference strain.

Table 3.1 Analysis of TPI-overexpressing strains.

strain[a]	specific TPI activity[b] (unit.mg protein^{-1})	relative expression[c]	estimate of copy number of *TPI* gene[d]
DBY747	24.6±3.7	1.0	1
DT1	53.3±9.1	2.2	>2
DT2	51.9±2.1	2.1	2
DT3	54.8±3.1	2.2	2
DT4	53.9±4.4	2.2	2
DT5	51.2±2.0	2.1	2
DT6	46.0±2.7	1.9	2
DT7	49.6±6.8	2.0	2
DT8	42.4±5.4	1.7	2
DT9	121.1±9.3	4.9	>2
DT10	46.4±4.7	1.9	2

[a] These strains were grown under the conditions described in section 3.2.

[b] Results are given as the mean ± standard deviation of triplicate assays.

[c] Relative expression is defined as specific TPI activity of transformant divided by specific TPI activity of the reference.

[d] Results are estimated from Southern analysis described in section 3.2.1.

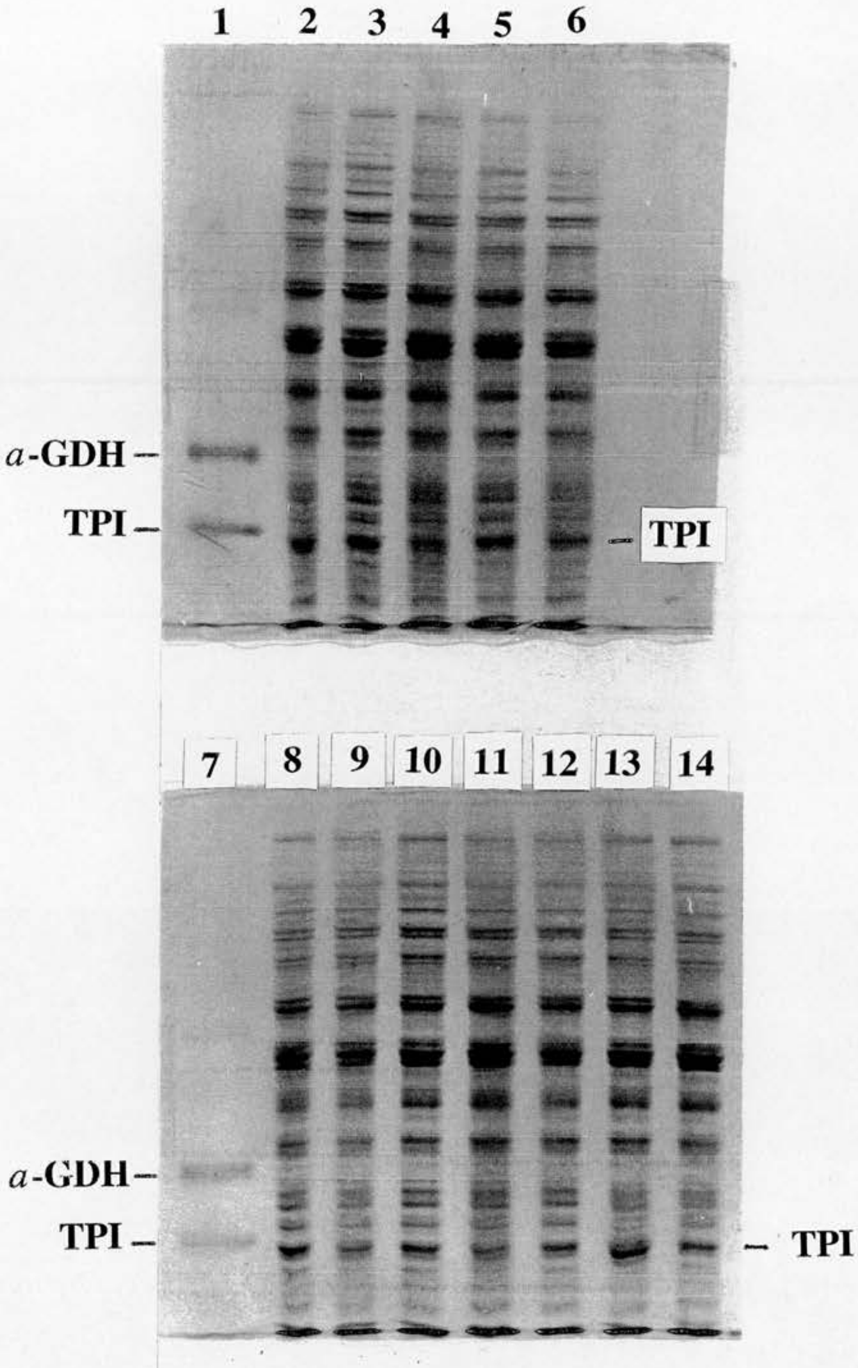


Figure 3.8 SDS-PAGE of the DBY747 and the transformants DT1-DT10. Cell crude extracts of these strains were run on 9% SDS-PAGE gel and were stained with Coomassie blue. Upper gel, lane: 1) purified mixture of TPI+α-GDH, 2) DT1, 3) DT2, 4) DBY747, 5) DT3, 6) DT4; lower gel, lane: 7) purified mixture of TPI+α-GDH, 8) DT5, 9) DT6, 10) DT7, 11) DBY747, 12) DT8, 13) DT9, 14) DT10

3.3 Construction of Ura^+ Reference Strain

The parental strain has been considered to be an acceptable and comparable reference in some publications or even in the section of strain characterisation in this thesis. However, a reference strain which is more nearly identical in genetic background would be preferable for further studies. According to the *URA3* gene carried on the plasmid vector, all the constructed TPI overproducers are uracil prototrophs while the parental strain DBY747 still requires uracil for growth. In order to eliminate the difference between the transformants and the reference in terms of the uracil requirement, a strategy for the construction of a new reference strain was then designed.

The strain DBY747 fails to synthesize its own uracil because it carries *ura3-52* mutation (Rose & Winston, 1984) on the chromosome. This mutation is a result of a Ty insertion within the *URA3* gene coding sequence with no other associated rearrangements. The presence of Ty almost completely eliminates *URA3* replacement events (reversion frequency $< 10^{-9}$), which require two crossovers, and reduces but does not eliminate the integration events which require a single crossover only (Rose & Winston, 1984).

To construct a Ura^+ strain, the strain DBY747 was transformed with YIplac211 in the same manner as in the construction of TPI overproducers except that the ligation of the *TPI* gene was omitted. The plasmid vector YIplac211 was linearized with restriction enzyme *Nco*I prior to introducing into the yeast strain DBY747. The integration, after crossing over, was expected to occur at the *Nco*I site in the chromosomal DNA (Figure 3.9). The transformants were selected on minimal medium without uracil, and five Ura^+ transformants (DBY747::YIplac211) called DY1-DY5 were collected after 4 days. Southern analysis was carried out to check whether the integration of YIplac211 had occurred on the chromosomal DNA of these transformants.

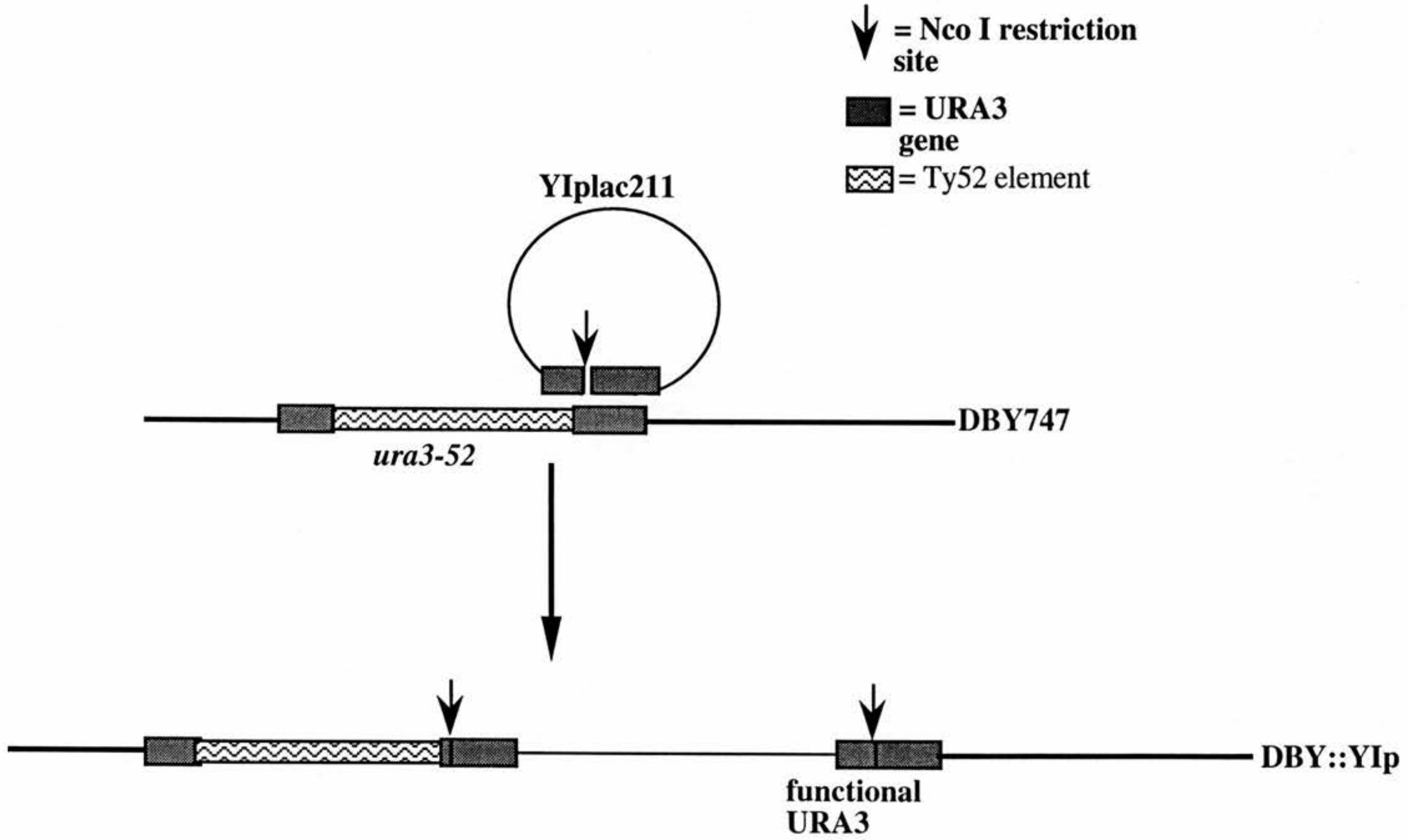


Figure 3.9 Integration of the plasmid vector YIplac211 into yeast chromosomal DNA after crossing-over at the *ura3-52* locus.

The precise number of YIplac211 copies integrated into the yeast chromosome could be determined by the measurement of the increase in DNA fragment size from the size of the parental fragment after the partial digestion of the chromosomal DNA with a restriction enzyme which fails to cut anywhere within YIplac211. In order to decide on which restriction enzyme to be used, a preliminary experiment was carried out. Of four different enzymes i.e. *Sal*I, *Bgl*II, *Cla*I and *Xho*I, the restriction sites of which are absent in the *URA3* gene (data obtained from GCG7 database), only *Sal*I can cut YIplac211 (see the restriction map in Gietz & Sugino, 1988). To confirm this, the genomic DNA of the strain DBY747 and the YIplac211 vector were digested with these restriction enzymes and as expected that all of them, except *Sal*I, could be employed for genomic DNA digestion. These results also suggested that the genomic DNA of the DBY747 was cut more frequently by *Bgl*II and *Cla*I than by *Xho*I because there were many more smaller DNA fragments from *Bgl*II and *Cla*I digestion (Figure 3.10).

Genomic DNAs of the transformants, as well as that of DBY747, were prepared and purified (section 2.8). The DNAs were cut with the restriction enzyme *Bgl*II and separated by electrophoresis in an agarose gel. The digested genomic DNAs were transferred onto the nylon membrane as described in section 2.12.1 and probed with a digoxygenin-labelled 1.16 kb *URA3*/*Hind*III fragment (isolated from pURA3). The results of Southern analysis are shown in Figure 3.11 as well as a schematic diagram of expected results in Figure 3.12.

As three restriction sites for *Bgl*II are present on the Ty52 inserted in *ura3-52* (Rose & Winston, 1984), two bands designated as A and B in Figure 3.12, were obtained from the DBY747 track. The size of these bands depends on the positions of the *Bgl*II restriction sites on the yeast chromosome. According to the standard DNA size marker, the size of bands A and B could be estimated as 4 and 2.5 kb, respectively. In transformants which had a single copy of YIplac211 integrated into the chromosome, band B obtained from DBY747 was replaced by band C. The size

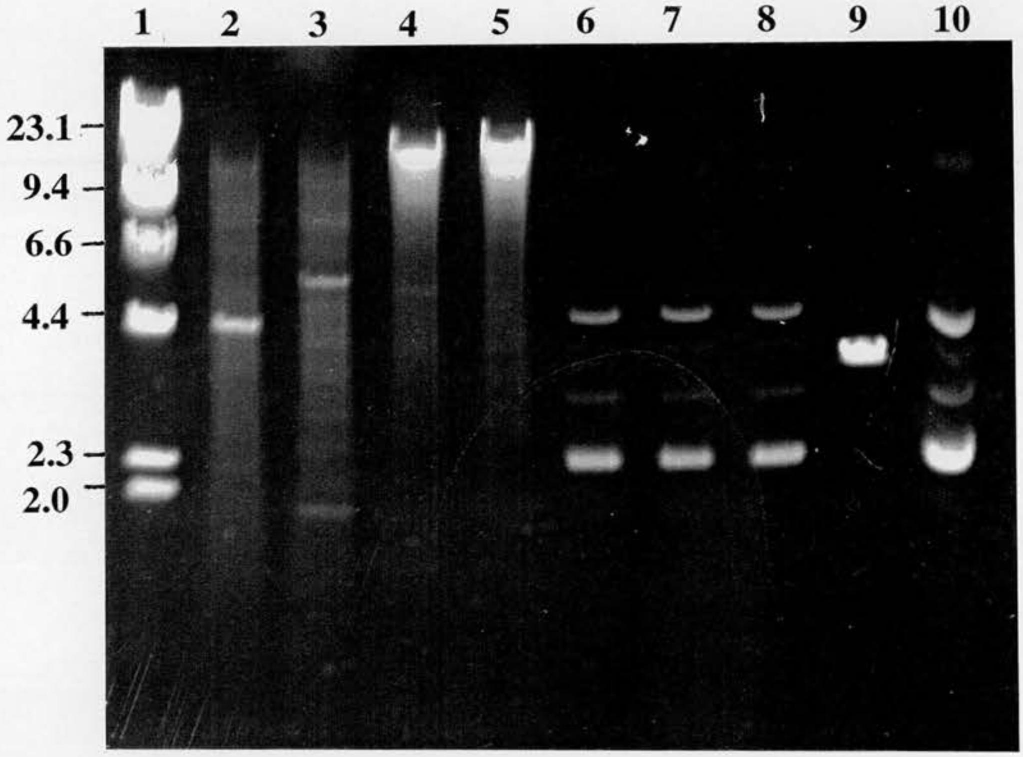


Figure 3.10 Restriction analysis of the DBY747 genomic DNA and YIplac211. Lane 1) λ/*Hin*dIII marker, 2-5) DBY747 cut with *Bgl*II, *Cla*I, *Xho*I and *Sal*I, respectively, 6-9) YIplac211 cut with *Bgl*II, *Cla*I, *Xho*I and *Sal*I, respectively, 10) uncut YIplac211.

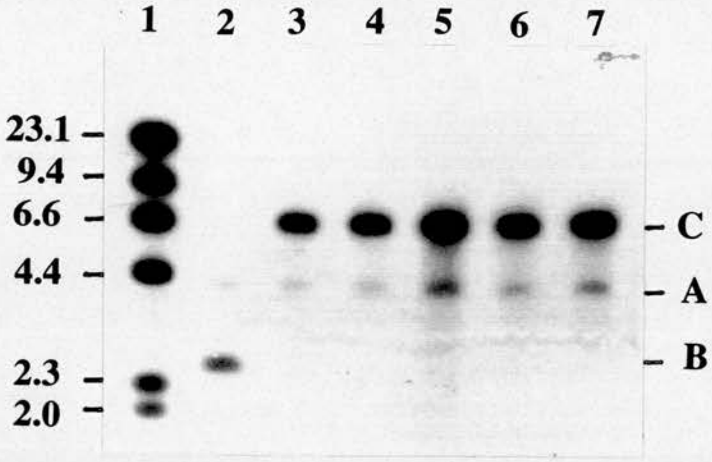


Figure 3.11 The Southern blots of the genomic DNAs of the DBY747 and the transformants DY1-DY5 probed with the *URA3* probe (see section 3.3 and Figure 3.12 for details of bands A, B and C). Lane 1) λ/*Hind*III marker, 2) DBY747, 3-7) DY1-DY5 respectively.

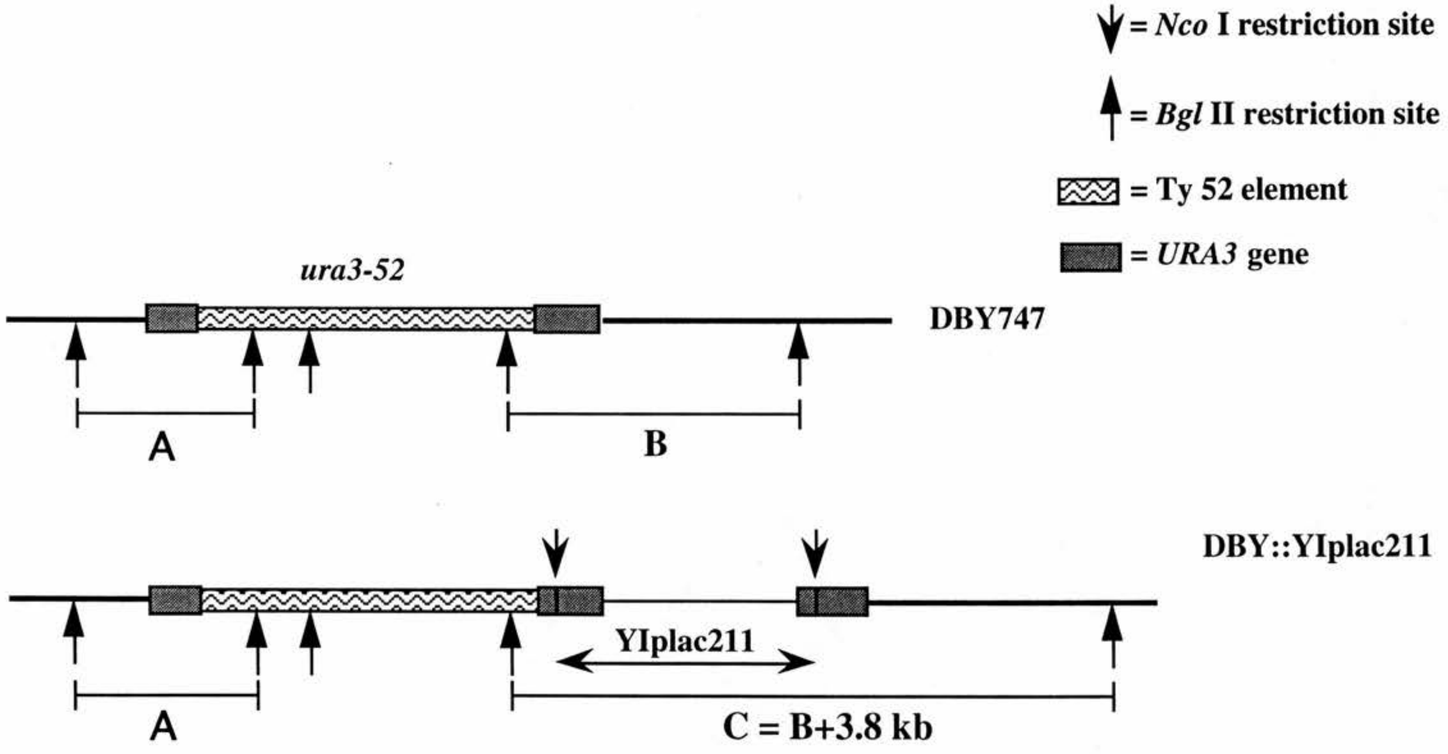


Figure 3.12 Schematic diagram describe the results from Southern analysis of DBY747 and the transformants DY1-DY5.

of band C (6.3 kb) was band B (2.5 kb) plus the size of YIplac211 (3.8 kb). Multiple integration of YIplac211 could also occasionally occur. As a result, a third band which was bigger than 6.3 kb would be detected e.g. a band which is 10.1 kb in size would be seen for the case of triple integration. An increase in size of this band depended on the copy number of YIplac211 which would form a tandem repeat within a *ura3-52* gene. It appeared from Figure 3.11 that all the transformants studied in this experiment obtained only a single copy of YIplac211 integrated into their chromosomes because none of them generated a band of around 10 kb. This suggested that any of the transformants collected could be used as a reference for further studies. Strain DY4 was chosen as a new reference strain.

Discussion

The results of the construction and characterisation of the TPI overproducing strains have shown that most of the transformants studied, except DT9, possessed an increase in TPI activity by approximately a factor of two compared to the reference. The consistent results obtained from strain DT5 resulted in this strain being chosen as a representative of the two-fold increase of the *in vivo* TPI activity.

The physiological consequences of a larger increase of the TPI activity was also of interest to study in detail. The results of Southern analysis suggested that two transformants, DT1 and DT9, contained multiple extra copies of the *TPI* gene integrated into the chromosome. These results were confirmed, in the case of the DT9, by the measurement of the specific enzyme activity. An increase in specific TPI activity by only a factor of two was, however, observed in the strain DT1 although the multiple extra copies of the *TPI* gene were seen in Southern blot results. In addition, the level of *TPI* gene overexpression of this strain determined by SDS-PAGE was very similar to that of the other transformants which had obtained only a single extra copy of the *TPI* gene. According to the obscure nature of this strain, DT1 was therefore not used in the subsequent study. Strain DT9 was preferred as a candidate for studying an effect of the large change of the *in vivo* TPI activity i.e. approximately five-fold compared to the reference DBY747 strain under the culture conditions used in this chapter.

The Ura$^+$ reference strain, DY4, was constructed by transformation of the parental strain with the *URA3* gene carried on the plasmid vector YIplac211. As a result, a similar background between this new reference and the TPI transformants, in term of uracil requirement, was obtained. However, some dissimilarities between these strains relevant to the *URA3* gene still exist and should be discussed. Although only a single copy of the *URA3* gene was present in the chromosome of the DY4 and the DT5 strains, they were integrated into the chromosome at a different locus i.e. at the homologous *URA3* gene for the DY4 (Figure 3.9) but within the *TPI* gene for the DT5 (Figure 3.5). In addition, there is a difference in the functional *URA3* copy number between a transformant which has obtained only a single copy of the *TPI* gene and one which has obtained multiple copies of the *TPI* gene. As a consequence of the multiple integration of the *TPI* gene, the *URA3* gene is also present in multiple copies (Figure 3.5). This resulted in greater copy numbers of the functional *URA3* gene than the strains DY4 and DT5 which have only single copy of this gene. However, the differences discussed above are not expected to have any effect on the yeast glycolytic pathway.

In the following chapters the three strains chosen will be re-named. The Ura$^+$ reference strain (DY4) will be referred to as REF1 strain and the TPI overproducers (DT5 and DT9) as OP2 and OP7, respectively (the DT9 was shown to be an approximate seven-fold TPI overproducer compared to the reference in batch experiments carried out in Chapter 4). These strains were subjected to physiological study in batch and chemostat cultures described in the following chapters.

Chapter 4

Study of the TPI-overproducing Strains in Batch Culture

Introduction

Batch culture is a traditional method of studying microbial physiology. With this technique, microbial cells are inoculated into a closed system supplied with an initial excess amount of essential substrates. The inoculated culture will pass through a growth cycle which is well characterized as illustrated in Figure 4.1. The characteristics of phases of the growth cycle can be described as follows:

1. Lag phase

After inoculation, batch growth starts with a *lag phase* during which no significant increase in biomass can be observed. This period is considered as the time required for the cells to adapt to the new physiological environment. The adaptation process includes the degradation and synthesis of mRNA and proteins, which enables the cells to grow successfully in the new environmental conditions. This phase is generally observed when stationary phase cells are inoculated into a fresh medium. As a result, such cells will require an adaptation period before the initiation of growth. In addition, the lag phase could also occur when cells are transferred from a complex medium to a minimal medium. This process is known as nutritional down-shift and is associated with differential changes in macromolecular content (Davidson, 1992).

2. Exponential phase

After successful adaptation, the culture enters the *log phase* (or *exponential phase*) during which the growth of the cells increases. As all essential nutrients are present in excess, the cell mass produced during this phase therefore increases in an exponential fashion and is accompanied by an exponential increase in the concentration of biochemical metabolites in the vessel. Cells under these conditions have a constant doubling time, this results in the maintenance of a constant growth

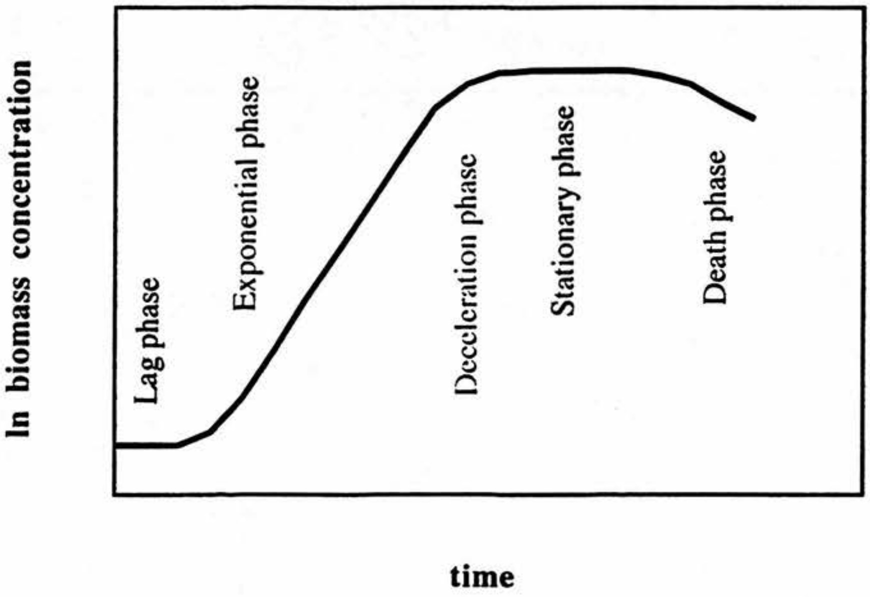


Figure 4.1 Typical growth curve obtained in batch cultivation.

rate i.e. steady state of growth and metabolite production. A steady state metabolism can be obtained and studied during this phase of growth. An exponential growth phase is described by the following equation :

$$\frac{dx}{dt} = \mu x$$

where x is the concentration of microbial biomass ($g.L^{-1}$)

t is time (h)

μ is the specific growth rate (h^{-1})

At this stage, under optimal conditions, the cells will grow at their maximum specific growth rates (μ_{max}) the level of which is determined by the components of the medium and environmental factors (Schaechter *et al.*, 1958). The exponential growth phase would continue indefinitely as long as the nutrients essential for growth are available in excess. However, as the nutrient consumption proceeds, the excretion of microbial products e.g. proteins, cell-wall associated molecules, overflow products and lytic products, also occurs (Wanner & Egli, 1990). These metabolites can affect the growth of the organism particularly when they accumulate to a considerable degree and may be toxic to the cells in high concentration.

3. Deceleration phase

Following the exponential phase, after a certain period of time, the culture enters a short phase called *deceleration phase* where the cellular biochemical components are synthesised at unequal rates. During this phase, the concentration of one (or more) of the essential nutrients becomes limiting and falls to a low level which is not enough to support the maximum growth. As a result, the growth rate of the culture is gradually decreased. The additional effect of toxicity of the accumulated metabolites may also be taken into account as the cause of the cessation of growth.

If the medium contains a mixture of substrates, some organisms can control the utilisation of those substrates by using each substrate individually. At this stage, the culture which has ceased growing due to the depletion of one substrate can now use another substrate which is still available in the medium. This could be observed when *E. coli* is grown in the mixture of glucose and lactose as the substrates. Glucose has a simpler structure than lactose, and would initially be consumed followed by the utilisation of lactose. In addition, this *diauxic growth* phenomenon, can also be observed in the presence of a single carbon source. In this case, the metabolites produced during the exponential growth will be utilized after an exhaustion of the first substrate. This could be found when the yeast *S. cerevisiae* is grown under an excess of glucose in batch culture, the ethanol produced during glucose oxidation in the exponential phase as a product of the fermentation process serves as a second carbon source soon after the glucose is exhausted. *Klebsiella pneumoniae* grown on glucose was found to utilize acetate which was produced during exponential growth as a substrate after glucose becomes exhausted (Malmcrona-Friberg *et al.*, 1986). Identical results have been reported for both *E. coli* (Anderson & Von Meyenburg, 1980) and *Bacillus natriegens* (Niven *et al.*, 1977).

4. Stationary phase

As a result of nutrient deprivation and/or accumulation of inhibitory products of metabolism, the culture is then subjected to *stationary phase* at which the specific growth rate (μ) approaches zero. At the beginning, "unnecessary" components are slowly degraded and endogenous reserves are used ensuring the viability of the cell. The cells maintain viability for an extended period without added nutrients retaining the ability to resume growth promptly when appropriate nutrients become available once again. However, the ability to survive varies between the species, some are capable of surviving for an extensive period e.g. weeks or more without any loss of viability, while others begin to die almost as soon as the nutrients become exhausted (Dawes & Sutherland, 1992).

For the yeast *S. cerevisiae*, the process of entry into stationary phase is a complex, poorly understood part of the life cycle. A review on the physiology and genetics of stationary phase in this yeast has recently been published (Werner-Washburne *et al.*, 1993).

5. Death phase

After the stationary phase, cells eventually enter a *death phase* which occurs at a variable time after the onset of the stationary phase. As the consequence of either the accumulation of toxic metabolites or cell lysis by autolytic enzymes, the number of actively growing cells is diminished and eventually becomes zero. In complex morphological species, a survival metabolism i.e. sporulation can be observed during this phase.

This chapter describes the physiological study of the consequences of TPI overproduction in the yeast strains constructed in Chapter 3. The study was carried out in batch conditions. In experimental practice, batch cultivation can be simply achieved in shake flask culture where, however, there may be a limit to the amount of aeration within an individual flask and/or variation in environmental parameters between sample flasks may be a problem. To overcome these restrictions, all the batch experiments described in this chapter were carried out using the culture system prepared for future chemostat experiments, except that the continuous supply of fresh medium was omitted. The aim of these studies was to obtain data on batch growth and also to achieve preliminary data for future chemostat experiments in addition to the practical purpose of assembling and testing the equipment. These studies involved the measurement of growth as well as the production of metabolites in the TPI overproducing strains. One strain studied had only a single extra copy of the *TPI* gene (OP2) and the other had multiple extra copies of the *TPI* gene (OP7), compared to a reference strain (REF1). In addition, the production of glycolytic enzymes at steady state metabolism (exponential phase growth) was also investigated and will be discussed.

Results

4.1 Optimization of Medium

Low level of growth was occasionally observed in yeast strains constructed in Chapter 3 (data not shown) when grown in the medium supplemented with an amino acid mixture which had previously been found to give a reasonable growth to the PGK mutant studied by Wilson (1985). As there may be differences in essential nutrient requirements between the yeast strains particularly those which possess a different genetic background, a small preliminary experiment was carried out to improve the level of growth by varying the source of amino acids supplied. The yeast strain REF1 was grown in the defined medium (Appendix A, Table A.7) with 2% (w/v) glucose in the shake flask culture. Two alternative sources of amino acids, an amino acid mixture which had previously been used by Wilson (1985) and 1% (w/v) casamino acids (Difco), were used. Amino acid compositions in defined medium supplemented with these two alternative sources are shown in Appendix A, Table A.6.

The level of growth was assessed by measuring the OD_{600} of the cultures with time. The results showed that a stationary phase OD_{600} of only 1-1.5 units was obtained for the culture grown with an amino acid mixture supplement. The yeast strain grown with 1% (w/v) casamino acids supplement grew better, determined by the stationary phase OD_{600} of approximately 7 units (Figure 4.2). The higher level of growth observed in the latter case was considered to be due to the higher amino acid content of the casamino acid mixture, and in addition, other vitamins and growth factors contained within the casamino acid mixture could also promote the better growth. The 1% casamino acid was therefore preferred for the future experiments.

The reference strain (REF1) and the TPI overproducers (OP2 and OP7) were grown individually on the defined medium with 2%(w/v) glucose and 1%(w/v) casamino acids. The culture was cultivated in a 1 L glass fermenter vessel which was connected to system controllers i.e. the controller of aeration, agitation, temperature,

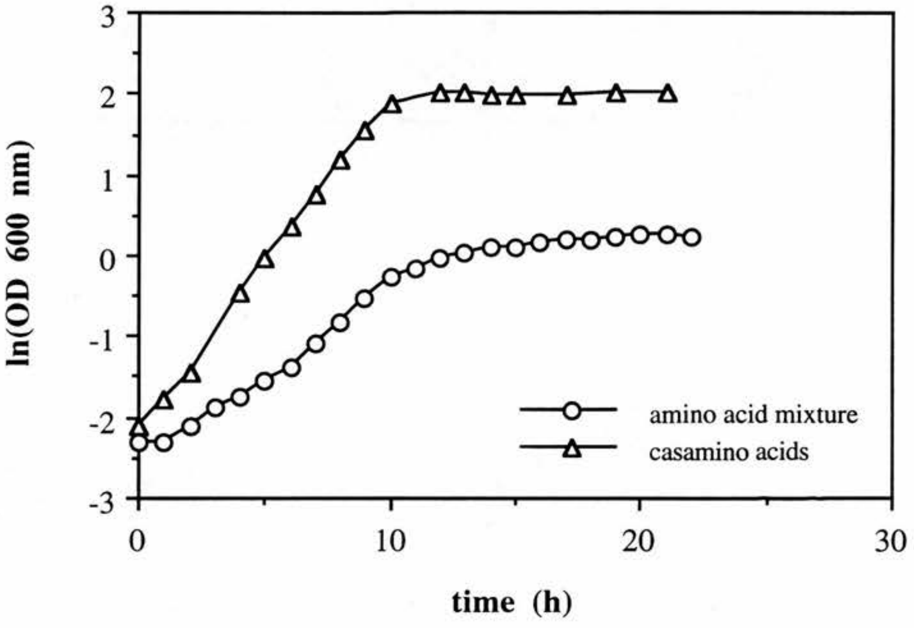


Figure 4.2 Growth curves of the REF1 strain in defined medium supplemented with 2%(w/v) glucose. Amino acids were supplied by amino acid mixture or casamino acids.

pH and dissolved oxygen concentration, as shown diagramatically in Figure 4.3. All batch cultivations were carried out under the conditions described in section 2.6.5. A number of samples were taken during the cultivation to measure growth by OD_{600} and dry weight measurements (section 2.17) and also to keep the cell suspensions for enzyme and metabolite assays. The sample size was restricted to be not larger than 70 ml i.e 10% of the working volume, to disturb the working volume of the culture as little as possible.

4.2 Steady State Growth

The batch growth of yeast can be studied simply by measuring the change in OD_{600} with time. Alternatively, dry weight measurement can be done. However, the latter method takes much longer time to achieve constant dry weight before the results could be interpreted. In this part of the study, growth of the TPI overproducing strains and the reference strain were measured by following both the OD_{600} and the cell dry weight with time. As the OD_{600} measurement requires only a small size of sample, therefore it could be measured as often as at hourly intervals after inoculation until the stationary phase which was seen by a deceleration of the increase in OD_{600} of the culture. Unlike the OD_{600} measurement, the dry weight measurement requires a greater volume of sample particularly in the early part of growth where cell density is low in order to obtain an accurate measurement. As a result, the cell dry weight was followed only at hourly intervals from 5 h after inoculation up to 10 h where the stationary phase which was determined by the OD_{600} measurement was observed. To obtain a curve describing an exponential growth, the results of both OD_{600} and dry weight measurements of the strains REF1, OP2 and OP7 obtained from individual batch experiments are plotted on a semi-logarithmic plot against the incubation time in Figure 4.4.

The specific growth rate (μ) of the TPI-overproducing strains could be compared with that of the reference strain simply by comparing the slopes of each

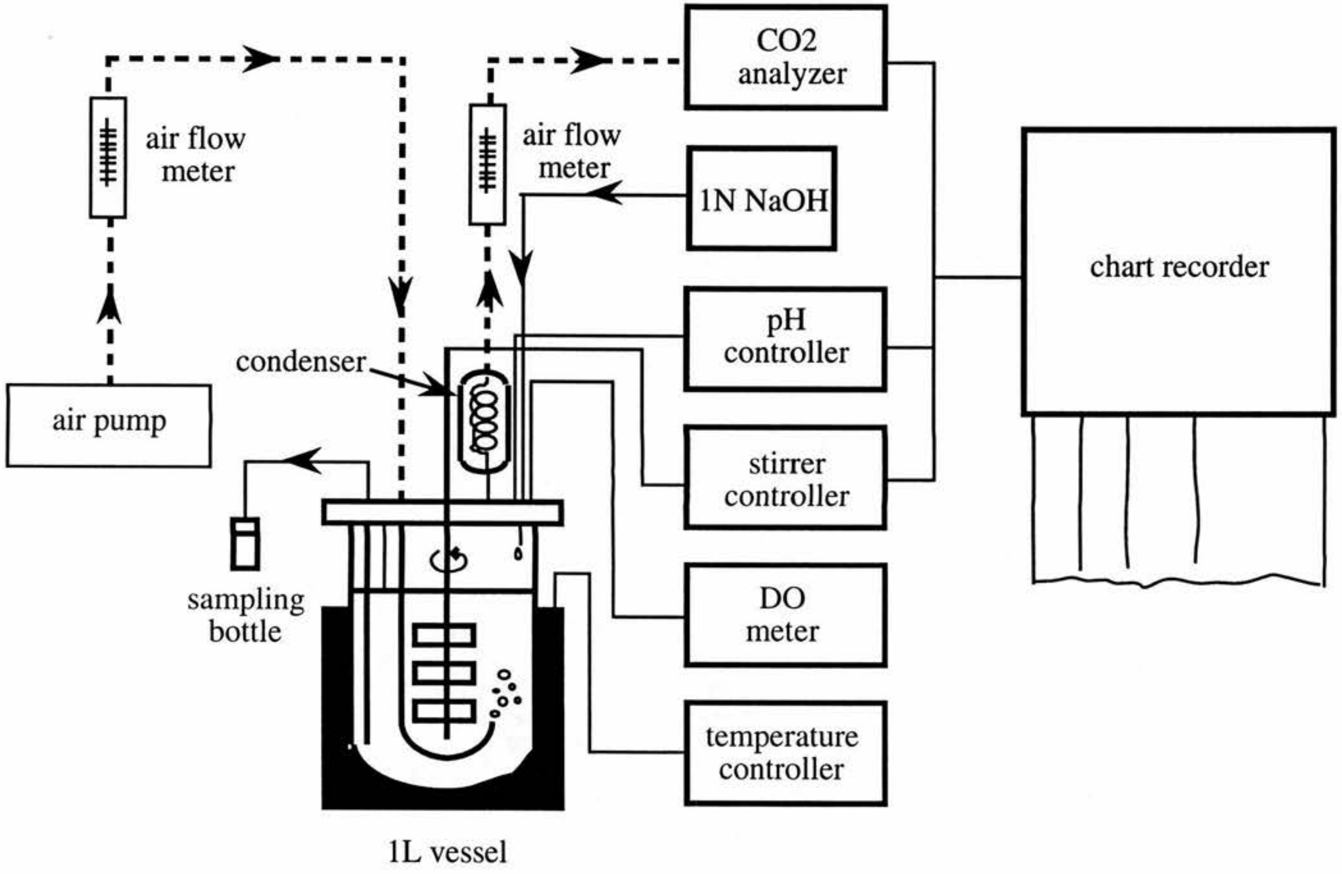


Figure 4.3 The culture system used in yeast batch culture.

regression line of these plots. The linear regression was computed by the Cricket Graph program for the Macintosh computer and all the equations obtained are summarized in Appendix B, section 1.1, Table B.1. The μ values of 0.376, 0.400 and 0.390 were obtained from the OD_{600} curves of the strains REF1, OP2 and OP7 respectively. This program was also applied to the dry weight curves and the slopes of 0.330, 0.268 and 0.414 were obtained for the corresponding strains respectively. The similarity of the slopes obtained from the three strains studied has been assessed using a statistical test of homogeneity of the regression coefficients (Appendix B, section 2.1, Table B.3). The results showed that no significant difference in growth rates was observed between the three slopes obtained from the OD_{600} curves (F= 0.52 with 2 and 12 degrees of freedom). This implies that, within the conditions used in this experiment, the three strains do not significantly differ in growth rate. However, with the dry weight measurements, the slopes obtained from strains REF1 and OP2 were different from that obtained from strain OP7. Test for the homogeneity of the regression coefficients (Appendix B, section 2.1, Table B.4) was therefore applied to those values and a significant difference between the three strains was found (F= 3.96 * with 2 and 12 degrees of freedom). These conflicting results obtained from dry weight curves and the OD_{600} curves could be due to a systematic error in the measurement of cell dry weight particularly during the early exponential phase of growth where cell density was low. Dry weight measurements, in these studies, were therefore considered less reliable than the measurement of OD_{600}.

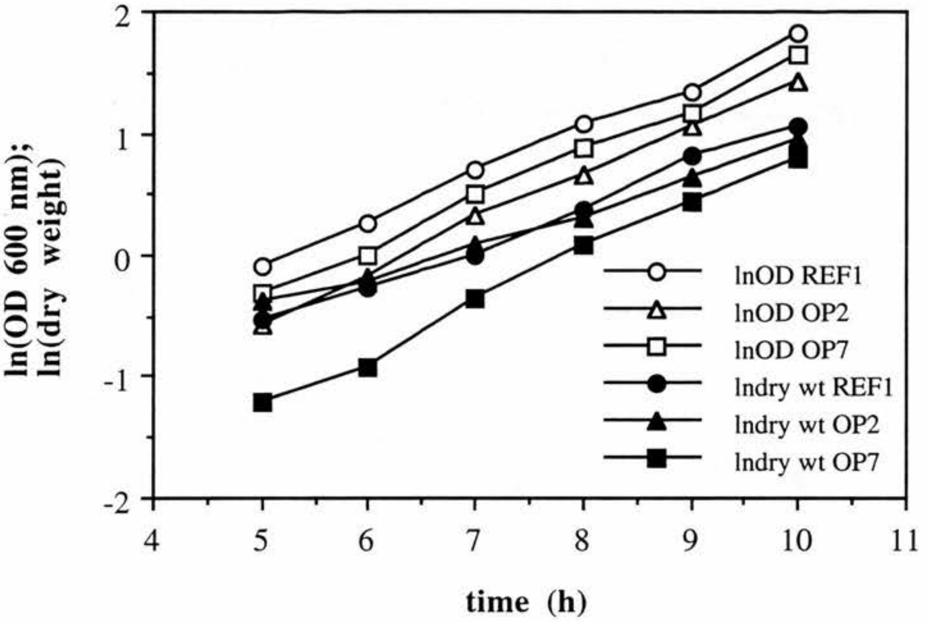


Figure 4.4 Growth curves of the strains REF1, OP2 and OP7 in defined medium supplemented with 2%(w/v) glucose and 1%(w/v) casamino acids. Growth was followed by the measurement of optical density of the cell culture at 600nm (open symbols) and dry weight (closed symbols).

4.3 Carbon Dioxide Production

The biomass production by yeast cells is accompanied by the release of CO_2 gas into the atmosphere. The percentage of CO_2 produced by yeast cells was analysed from the out-flow air by the carbon dioxide analyser (section 2.18) and the production rate was calculated using the following equation (Parton & Willis, 1990):

$$\mathrm{CPR} = \left(\frac{\%\mathrm{CO_2}}{100}\right) \times 0.0416 \times \frac{G_f}{v}$$

where G_f is the gas flow rate ($\mathrm{ml.min^{-1}}$)

v is the working volume (L)

The CO_2 production rates ($\mathrm{mmol\ CO_2.L^{-1}.min^{-1}}$) obtained during the exponential phase growth (from 5 to 10 hours after inoculation) for the three strains studied were plotted with time as shown in Figure 4.5, and the linear regression was carried out similarly to that done for the growth curves. Slopes of 0.346, 0.406 and 0.407 were obtained for the strains REF1, OP2 and OP7 respectively (Appendix B, section 1.1, Table B.1). The test of homogeneity of the regression coefficients (Appendix B, section 2.1, Table B.5) showed no statistical difference between these three strains studied (F= 1.09 with 2 and 12 degrees of freedom). This indicated that the TPI-overproducing yeast strains produced CO_2 at the same rate as the reference strain during exponential growth.

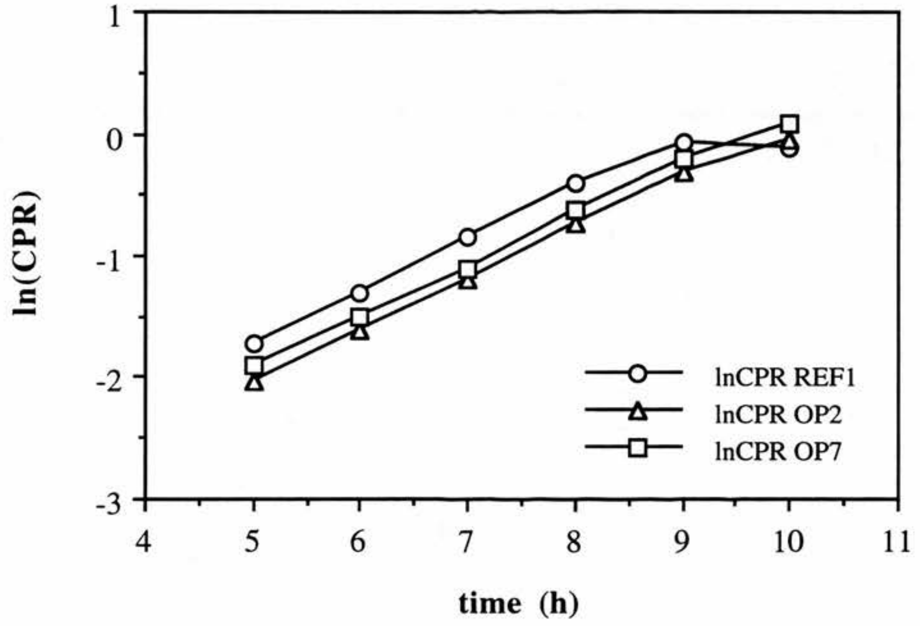


Figure 4.5 Carbon dioxide production rate (CPR) of the strains REF1, OP2 and OP7 in defined medium supplemented with 2%(w/v) glucose and 1%(w/v) casamino acids.

4.4 Production of Glycolytic Metabolites

The formation of ethanol is one of the main characteristics of an aerobic batch fermentation of the yeast *S. cerevisiae* when grown with glucose as a carbon source. Glycerol is also produced as a minor fermentation product during the process. The production of these two metabolites at the steady state growth of the TPI-overproducing strains and the reference strain were determined.

Extracellular ethanol and glycerol concentrations were analysed using an ion-exchange HPLC column connected to a RI detector (section 2.16.1). Steady state extracellular concentrations of these metabolites were plotted with time for the three strains studied. The results of the steady state ethanol concentration are shown in Figure 4.6 and the steady state glycerol concentration of the corresponding strains are shown in Figure 4.7. The data were subjected to a similar statistical test as that carried out in section 4.2, and no difference in the ethanol production rates, the slopes of 0.389, 0.394 and 0.413 (Appendix B, section 1.1, Table B.1) for the strains REF1, OP2 and OP7 respectively, was observed between these strains (F= 2.78 with 2 and 12 degrees of freedom) (Appendix B, section 2.1, Table B.6). By visual inspection of the curves, slightly different glycerol production was observed in the strain OP7 when compared with that of the other two strains (the slopes of 0.303, 0.303 and 0.345 for the strains REF1, OP2 and OP7 respectively from Appendix B, section 1.1, Table B.1). However, a test for the homogeneity of the regression coefficients showed that there is no statistically significant difference in the rate of glycerol production between the three strains studied (F= 3.24 with 2 and 12 degrees of freedom) (Appendix B, section 2.1, Table B.7).

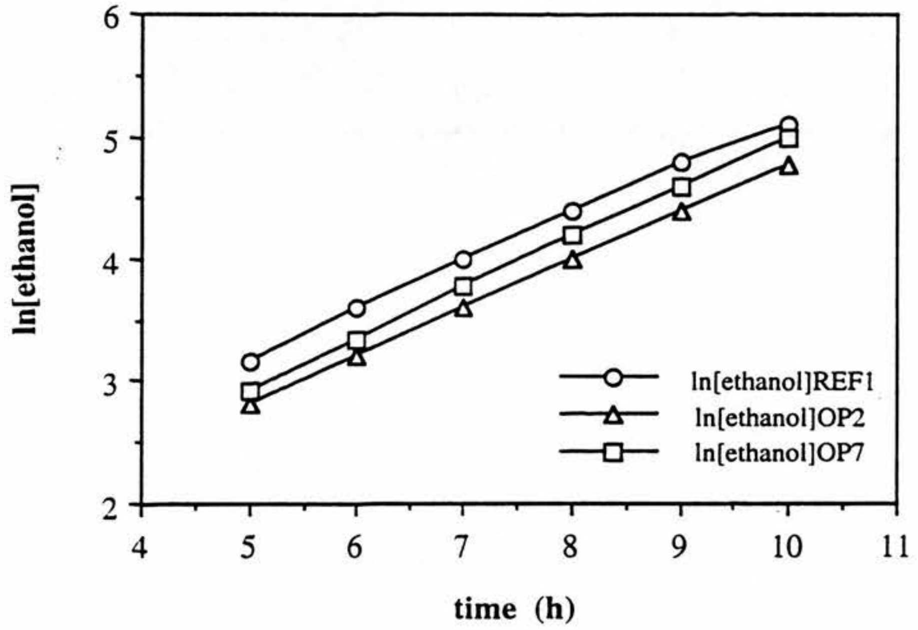


Figure 4.6 Steady state ethanol concentration of REF1, OP2 and OP7 in defined medium supplemented with 2%(w/v) glucose and 1%(w/v) casamino acids.

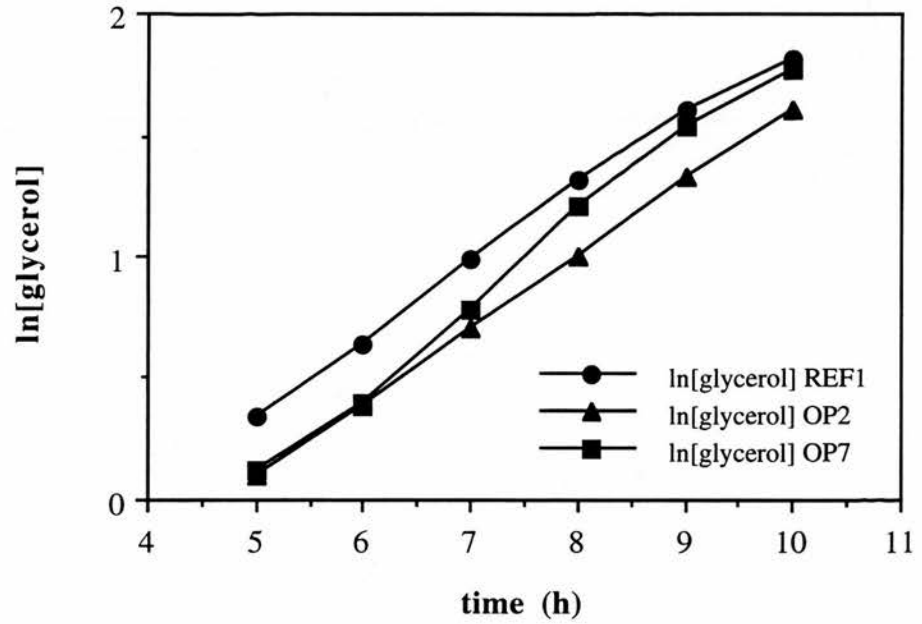


Figure 4.7 Steady state glycerol concentration of the strains REF1, OP2 and OP7 in defined medium supplemented with 2%(w/v) glucose and 1%(w/v) casamino acids.

4.5 Stability of Glycolytic Enzymes

To study the consequences of TPI overproduction in the yeast glycolytic pathway, it was important to establish that the rest of the pathway was not altered. Three other glycolytic enzymes (phosphoglycerate kinase, pyruvate kinase and hexokinase) were assayed along with the TPI in each batch experiment. In practice, the assay of four enzymes was difficult to accomplish within a limit of time when many samples were assayed due to interference of enzyme instability. In addition, only a small sample size can be taken from the culture vessel at each sampling time in order not to disturb the working volume of the culture. It was considered to be more sensible if the same crude cell extract could be used for all the assays of enzyme activity allowing the sample size taken at each sampling time to be reduced. However, some intracellular enzymes may lose their activities during storage. It was therefore necessary to examine the stability of the enzymes of interest i.e. TPI, HK, PGK and PYK.

The following experiment was carried out to establish evidence that the crude cell extracts could be stored and re-used for other enzyme assays after the initial assay for TPI was accomplished. The sample used in this part of the study was the OP2 cell pellet collected from the batch culture at 10 h after inoculation. The crude cell extract was prepared as described in section 2.13. After the fresh crude cell extracts were prepared, the enzyme assays of TPI, HK, PGK and PYK were carried out immediately. The extracts were kept at 4°C and re-assayed for the activity of the four enzymes at 24 h intervals along with the fresh sample prepared daily. After 4 days, the total protein was assayed and the specific enzyme activities were calculated.

The stabilities of the enzyme TPI as well as that of HK, PGK and PYK are shown in Figure 4.8. The results suggested that the enzyme TPI (Figure 4.8a) and HK (Figure 4.8b) were stable at 4°C throughout the period of study. The enzyme PYK (Figure 4.8d) was less stable when stored under the same conditions, whereas PGK was shown to be the most unstable enzyme in this study (Figure 4.8c). Large

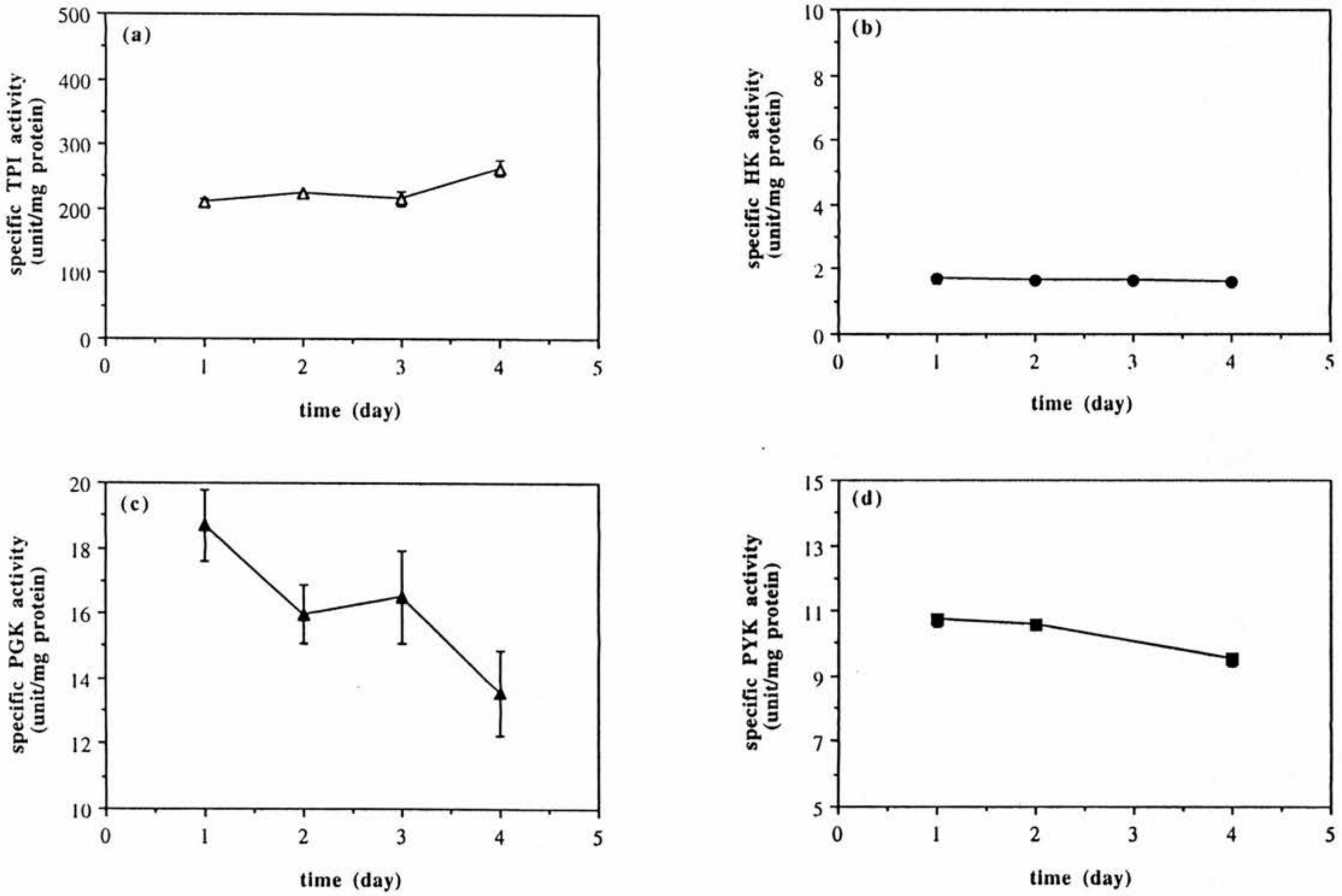


Figure 4.8 Stability of glycolytic enzymes: (a) TPI (b) HK (c) PGK and (d) PYK, studied in the cell crude extract of the strain OP2.

errors were observed throughout the pattern of PGK stability. The experimental error could not be due to the assay for protein concentration because all samples were assayed for protein concentration at the same time, and results of the protein assay were applied to all the calculations for other glycolytic enzyme specific activities (TPI, HK and PYK) and no such large error could be found in those three enzymes. These large errors were therefore considered to be the experimental errors occuring from the PGK activity assay which may reflect the instability of PGK during an assay.

According to all the results, the assay for the TPI activity would therefore be carried out initially followed by the assay for PGK, PYK and HK as soon as possible but not longer than 4 days after sampling.

4.6 Production of Glycolytic Enzymes

Glycolytic enzyme production was studied during exponential growth. The assays of TPI activity and total protein concentration of the reference strain (REF1) and the overproducers (OP2 and OP7) were carried out in the samples taken from 5-10 h after inoculation during which time steady state growth was expected. The specific enzyme activity of the three strains studied is shown in Figure 4.9. To ensure that the production of the enzymes of interest were at steady state levels, a statistical test for evidence of a zero slope of each curve was done using the Minitab program for the Macintosh computer (Appendix B, section 3.1). The results indicated no significant deviation from a zero slope in all cases (p-values are shown in Appendix B, section 3.1) and implied that the enzymes were at the steady state levels.

The results of the TPI production shown in Figure 4.9a indicated that the strain OP2 possessed a specific TPI activity 1.9 times higher than the reference strain level (an average of 85.8±7.6 and 162.7±13.0 unit.mg protein^{-1} for the strains REF1 and OP2 respectively) whereas the strain OP7 showed a value 6.9 times higher than that of the reference (an average of 590.9±41.9 unit.mg protein^{-1}). It may be

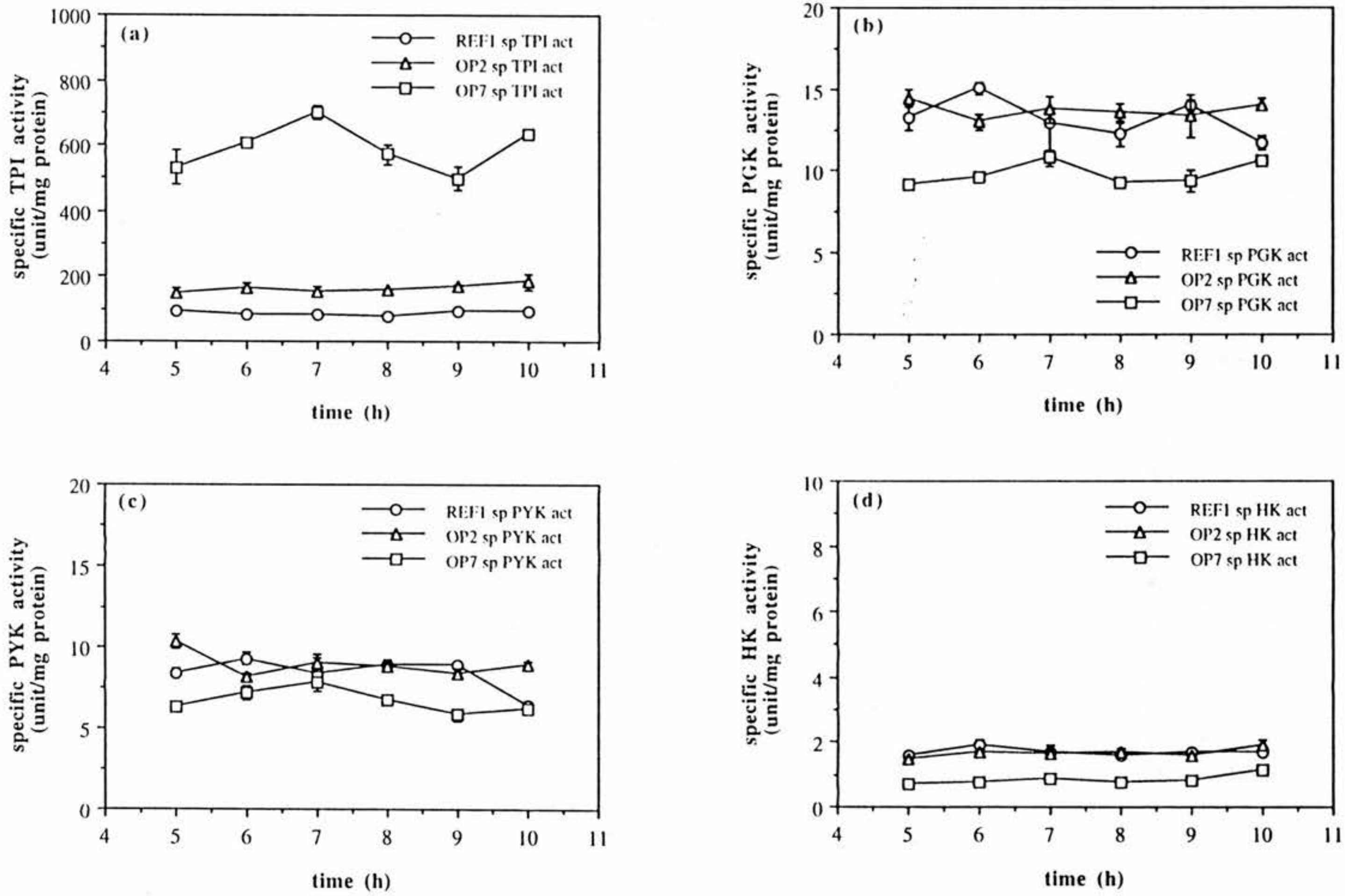


Figure 4.9 Glycolytic enzyme production: (a) TPI (b) PGK (c) PYK and (d) HK, at steady state of the strains REF1, OP2 and OP7 in defined medium supplemented with 2%(w/v) glucose and 1%(w/v) casamino acids.

noticed that the specific TPI activity of the strain OP7 fluctuated throughout the experiment. If this observation was a real phenomenon, i.e. not an experimental error due to serial dilution or crude extract preparation, this fluctuation may not be simply explained because cell growth in batch culture may have been affected by continuously changing environments.

The assay of other glycolytic enzyme activities i.e. HK, PGK and PYK was carried out to investigate the influence of the overproduction of TPI, if there was any, upon the rest of the glycolytic pathway. Crude cell extracts which were initially used for the TPI assay, kept at 4°C, and were re-assayed for the specific activities of the enzymes PGK, PYK and HK respectively. The comparative results of three strains for each enzyme are respectively shown in Figures 4.9b to 4.9d. There was no statistically significant difference the level of any of the glycolytic enzymes studied in the strains REF1 and OP2 according to Tukey's pairwise comparisons (Tukey, 1953) carried out using the Minitab program for the Macintosh computer (Appendix B, section 4.1). Using the same method of statistical test, the OP7 strain was shown to have statistically significant lower levels of specific activities for all the other glycolytic enzymes assayed when compared to the other two strains.

Discussion

Although many of the disadvantages of batch cultivation in shake flask culture have been avoided by using a much more controllable culture system, batch experiments carried out in this chapter still possess inherent difficulties. Batch culture is characterised as a closed system in which metabolic products (or by-products) produced by the culture could be accumulated and may be simultaneously utilised by the cells. The culture was therefore subjected to a continuously changing environment as a result of an excretion of metabolic products into the medium. Non-significant differences in steady state growth rates (with respect to OD_{600} measurement) observed in both constructed yeast strains when compared to the reference strain could have been altered by such environmental changes, particularly during the late exponential phase when metabolic products e.g. ethanol and glycerol were accumulated to considerable levels. These metabolites may well be utilized as growth substrates during the late exponential phase of growth where glucose was no longer present.

The results of the assay for specific TPI activity at steady state revealed that the yeast strains, OP2 and OP7, which have single and multiple extra copies of the *TPI* gene, respectively (see section 3.2.1) showed a level of expression 1.9 and 6.9 times higher than the reference strain level. These values were dissimilar to those obtained from the shake flask culture in the primary screening experiment (Chapter 3) because the yeast strains were subjected to different growth conditions e.g. culture conditions, growth medium. From the results presented in Figure 4.9a, Southern analysis and SDS-PAGE (Chapter 3), it can be concluded that the strains REF1, OP2 and OP7 contain 1, 2 and more than 2 copies of the *TPI* gene.

The assay for specific activity of the other glycolytic enzymes (PGK, PYK and HK) of the corresponding samples at steady state suggest that a two-fold increase in TPI specific activity from the reference level does not affect the specific activity of the other glycolytic enzymes. However, a further increase in TPI specific activity to 7 fold compared to the reference level resulted in a significant decrease in steady state specific activity of these enzymes, although the glycolytic flux, in terms of the rate of ethanol and glycerol production, was unchanged. The explanation for these results is still unclear but will be re-examined in the following chapter.

The study of yeast metabolism carried out under batch conditions may have limited accuracy for a comparative study of the yeast strains, particularly when there was only a small difference between those strains. In addition, the exponential phase of growth in batch culture could not be sustained indefinitely although the cultures were initiated at a high level of glucose content because the substrate would gradually be diminished with time. To overcome these possible disadvantages of batch cultivation and to further investigate the evidence of decreases in other glycolytic enzymes discussed above, the yeast strains were then subjected to continuous culture in which the culture conditions are more controllable in views of substrate utilization and, in addition, the steady state growth could be obtained as long as the culture is continuously supplied with fresh medium. The three constructed yeast strains studied here, were re-analysed in glucose-limited chemostat culture described in the following chapters.

Chapter 5

Study of the TPI-overproducing Strains in Glucose-Limited Chemostat Culture

Introduction

The technique of continuous culture is used extensively in the study of micro-organisms. The basic principle of continuous culture is to prolong the exponential phase of growth by the addition of fresh medium into the culture at the same time as the removal of spent medium plus cells from the vessel. If the medium is continuously fed to the culture at a suitable rate, a steady state is achieved and it can be maintained indefinitely. At steady state, the formation of new biomass is therefore balanced by the loss of cells from the vessel.

A *chemostat* is one type of the continuous culture arrangement. It is termed a chemostat because the growth rate of the culture is controlled by its chemical environment. In chemostats, the feed medium contains excess amounts of all nutrients essential for growth, except one component which is designated as the growth-limiting substrate. If glucose is the growth-limiting substrate, the term *glucose-limited chemostat* is used.

The Kinetics of the Chemostat

The flow of fresh medium into the vessel is related to the volume of the vessel by the term *dilution rate*. The dilution rate (D) is an important parameter and is defined as:

$$D = \frac{F}{V} \qquad (1)$$

where F is the flow rate of the fresh medium into the vessel (ml.h^{-1})

and V is the volume of the culture (ml)

The dilution rate is thus expressed in the units h^{-1} (per hour). As the fresh medium is pumped into the culture vessel at the same rate as the spent medium plus cells is removed, the dilution rate therefore sets the rate of supply of nutrients and the rate of removal of residual substrate, metabolic products and biomass. In addition, it also 'sets' the growth rate of the micro-organisms, at steady state conditions (see below).

Biomass and Substrate Concentrations at Steady State

The change in biomass over a time period in a chemostat culture is determined by the amount of biomass formed in the culture vessel and the amount of biomass removed by dilution. This can be described as :

rate of change in biomass = growth - removal

or

$$\frac{dx}{dt} = \mu x - Dx$$

$$\frac{dx}{dt} = (\mu - D)x \qquad (2)$$

where x is cell concentration ($g.L^{-1}$)

μ is the specific growth rate (h^{-1})

D is the dilution rate (h^{-1})

Under steady state conditions, the biomass concentration in the vessel remains constant i.e. $\frac{dx}{dt} = 0$, therefore it follows that:

$$\mu = D \qquad (3)$$

Equation (3) shows that, at steady state, the growth rate of an organism is controlled by the dilution rate of the culture. This relationship is derived assuming that the steady state condition has been reached. However, under some conditions the steady state can not be achieved. For example when the dilution rate is set to be greater than μ_{max}, the maximum specific growth rate of the organism for the limiting substrate used, cells will be removed from the growth vessel at a greater rate than the rate they can grow. As a result, cell concentration in the culture will decrease until eventually it becomes zero and the growth-limiting substrate concentration in the vessel will be approaching its concentration in the reservoir. This is a so-called *wash out phenomenon.* A culture which has just been inoculated also best illustrates non-steady state conditions. After inoculation, the cell concentration is very small, and the growth-limiting substrate concentration is nearly equal to its concentration in the reservoir. During this stage, cells grow at μ greater than D. Cell concentration will increase, but owing to the resulting fall in substrate concentration there will be a decrease in μ, until μ eventually becomes equal to D and the steady state condition is obtained (Herbert *et al.*, 1956).

The balance of substrate concentration in chemostat can be explained as follows:

rate of change of substrate concentration = input from the reservoir
- output in effluent
- consumption by cells

or

$$\frac{ds}{dt} = Ds_r - Ds - \frac{\mu x}{Y}$$

$$\frac{ds}{dt} = D(s_r - s) - \frac{\mu x}{Y} \qquad (4)$$

where s_r is substrate concentration in the reservoir (g.L^{-1})

s is residual substrate concentration in the culture vessel (g.L^{-1})

x is cell concentration (g.L^{-1})

Y is yield of cells on substrate consumed (g biomass.g substrate^{-1})

At steady state, there is no change in substrate concentration, i.e. $\frac{ds}{dt} = 0$ therefore:

$$\tilde{x} = (s_r - \tilde{s})Y \qquad (5)$$

where $\tilde{x}$ is the cell concentration at steady state ($g.L^{-1}$)

$\tilde{s}$ is the substrate concentration at steady state ($g.L^{-1}$)

The steady state concentration of substrate can be obtained by substituting $\mu = D$ in (3) into Monod equation (Monod, 1942).

$$\mu = \frac{\mu_{max} s}{K_s + s} \tag{6}$$

where μ_{max} is the maximal specific growth rate of organism (h^{-1})

K_s is the half saturation constant

s is the substrate concentration ($g.L^{-1}$)

After substitution,

$$\tilde{s} = \frac{K_s D}{\mu_{max} - D} \tag{7}$$

Equations (5) and (7) indicate that at steady state, the concentrations of cells and substrate in the culture are constant and dependent on physiological parameters e.g. μ_{max}, K_s and dilution rate of the culture. However, the steady state metabolism can also be affected by the metabolic regulation of organism. For the yeast *S. cerevisiae*, unpredictable changes in biomass yield can be observed during growth in glucose-limited chemostat culture due to its dual glucose catabolism.

Glucose catabolism of the yeast *S. cerevisiae* grown under glucose as carbon and energy source may involve respiration, alcoholic fermentation, or a combination of these two processes. A number of factors, including the strain and culture conditions e.g. oxygen availability and glucose concentration, determine which pathway is dominant. Under aerobic conditions, glucose catabolism is accompanied by respiration yielding biomass and carbon dioxide. On the other hand, when oxygen is absent, energy production from glucose occurs by alcoholic fermentation yielding ethanol and carbon dioxide. However, alcoholic fermentation can occur even under fully aerobic condition i.e. the *Crabtree effect*. A number of studies have been carried out to clarify the mechanism of this metabolic regulation (see Chapter 1, section

1.3.2). When glucose is used as the growth limiting substrate in a chemostat, glucose catabolism of the yeast *S. cerevisiae* is correlated with dilution rate of the culture. At the low range of dilution rates, respirative glucose catabolism and constant biomass yields will be observed with increasing dilution rate. If dilution rate of the culture surpasses D_C, the dilution rate at which ethanol production is triggered, respiro-fermentative glucose catabolism occurs i.e. the respiration is accompanied by the ethanol production. Due to this switch in metabolism, different biomass yields will be observed at higher dilution rates when compared to those obtained at the dilution rates in the range below D_C.

Respiro-fermentative glucose catabolism of the yeast *S. cerevisiae* also occurs in aerobic batch growth where glucose is supplied in excess and ethanol is produced simultaneously with biomass formation. It proceeds during most of exponential growth until the glucose concentration becomes lower than the critical level and purely oxidative glucose catabolism via respiratory processes occurs. However, this stage lasts only a short period of time since cell growth is diminished as a result of substrate depletion.

As discussed in Chapter 4, the steady state conditions obtained in batch culture could only be studied within a defined period of time and could be affected by a continuously changing environment e.g. ethanol excretion and/or ethanol utilization. The study of the constructed yeast strains under conditions where glucose is utilized by purely oxidative respiration would be impossible in batch experiment. However it can be achieved using chemostat cultivation where steady state conditions can be obtained indefinitely with glucose as the limiting substrate.

If possible, it would be of interest to study differences between the constructed yeast strains under two different glucose catabolisms i.e. respirative and respiro-fermentative glucose catabolisms. This chapter describes a study of the constructed yeast strains in a glucose-limited chemostat culture at different steady state conditions (dilution rate profile studies) and the optimization of the culture medium. The consequences of TPI overproduction in the constructed yeast strains was examined under the optimized conditions.

Results

5.1 Optimisation of Medium

Evidence of a high biomass yield (0.66 g biomass.g glucose^{-1}, see Appendix C, Table C.4) was observed when the yeast strain REF1 was grown in the defined medium (Appendix A, Table A.7) supplemented with 0.5% (w/v) glucose and 1% (w/v) casamino acids in aerobic glucose-limited chemostat culture. This result was considered to reflect the utilization of an additional carbon source because, theoretically, a biomass yield of approximately 0.55 g biomass.g glucose^{-1} is expected when the yeast *S. cerevisiae* respires glucose as a sole carbon source (Fiechter *et al.*, 1987). Among all of the nutrient components present in the defined medium, casamino acids were considered as possible additional carbon sources partly due to their complicated composition (Appendix A, Table A.5). To allow glucose to be a sole carbon source in the culture, the level of casamino acids which will be used by the yeast strain as a single source of amino acids only was minimized in the defined medium.

A chemostat culture of the strain REF1 was prepared as described in section 2.6. The yeast strain was cultivated in the culture system previously used for batch experiments described in Chapter 4, except that a continuous supply of fresh medium was connected to the system as well as an overflow device to remove the spent medium plus cells from the vessel (Figure 5.1). This culture system was used for all the chemostat experiments described in this chapter and also in the following chapter. The cultivation techniques described in section 2.6.5 were used e.g. cultural conditions, sampling technique, establishment of steady state conditions.

To assess the minimum level of casamino acids required in the medium, the yeast strain was grown under conditions at which the strain metabolizes glucose by

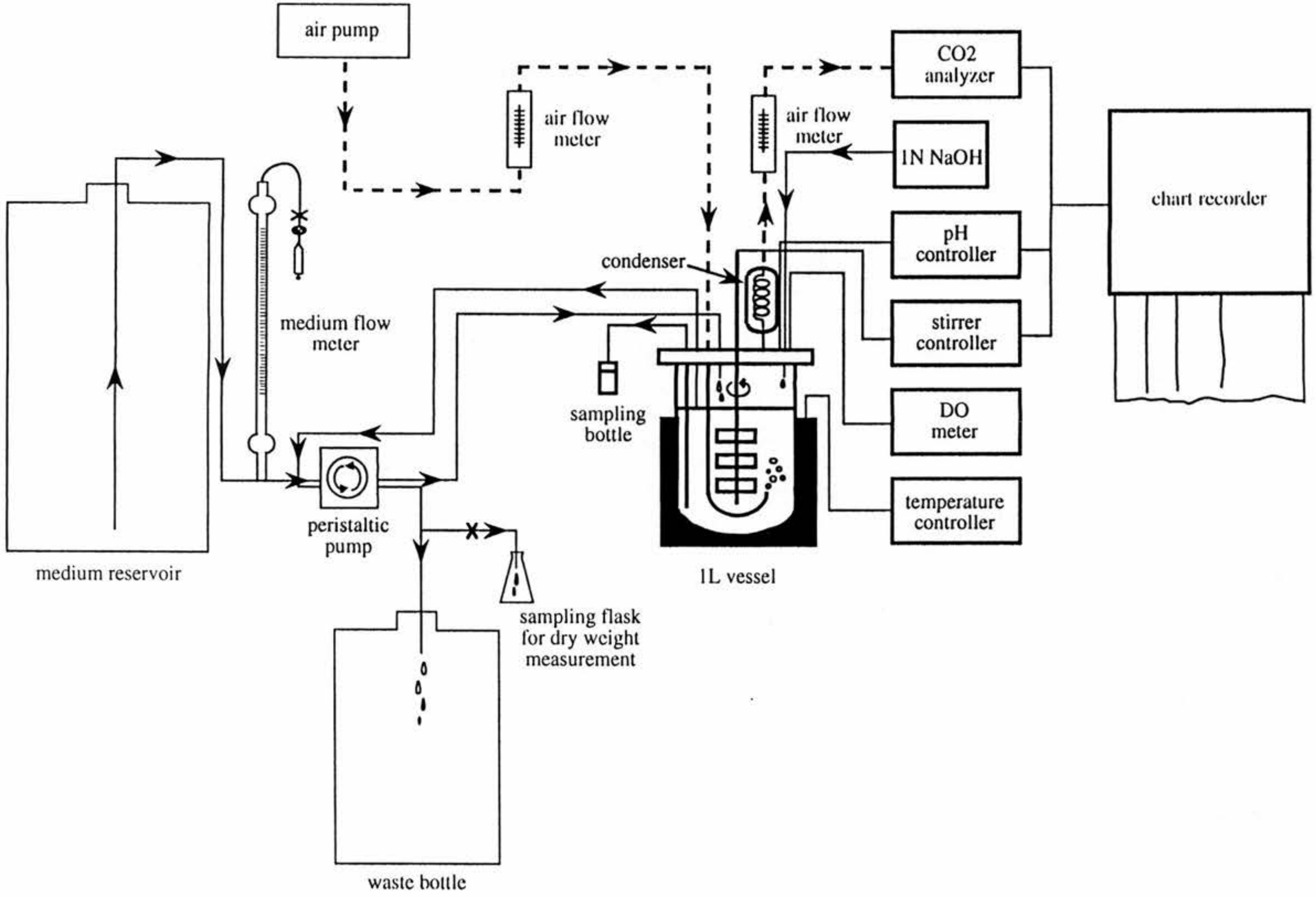


Figure 5.1 The culture system used in yeast chemostat culture.

respiration only and varying concentrations of casamino acids in the medium until the biomass yield is 0.55 g biomass.g glucose^{-1}without ethanol production.

The dilution rate was set at 0.11 h^{-1} throughout this study because ethanol was not detected at this dilution rate in the preliminary experiment (data shown in Appendix C, Table C.4) although casamino acids were present in "excess". Casamino acids and glucose concentrations in the feed medium were varied and studied individually. Several samples were taken after the steady state was reached (constant OD_{600} and $\%CO_2$ reading were observed). At least three steady state samples were taken and analyzed for biomass and metabolite concentrations.

5.1.1 Optimization of Casamino Acids Concentration

Glucose concentration was set at 0.5% (w/v) throughout this experiment and only the concentration of casamino acids was varied. The culture was grown in the medium containing 0.1, 0.2 and 0.3% (w/v) casamino acids respectively. The results of biomass yield and qCO_2 (Figure 5.2) as well as qEthanol (specific rate of ethanol production, millimolar of ethanol produced per gram of biomass per hour) and qGlycerol (specific rate of glycerol production; millimolar of glycerol produced per gram of biomass per hour) are presented as a function of casamino acids concentration (Figure 5.3). The results shown in these figures also include the results obtained from a preliminary experiment at the same culture conditions except 1% (w/v) casamino acids was used.

At 0.1% (w/v) casamino acids, a very low biomass yield was observed (0.22 g biomass.g glucose^{-1}). This result may be a consequence of amino acid limitation because higher biomass yields, 0.50 and 0.63 g biomass.g glucose^{-1}, were observed when the concentration of casamino acids was increased to 0.2 and 0.3% (w/v) respectively. However, it should be noticed that the biomass yield obtained at 0.3% was similar to that obtained at 1% (w/v) casamino acids. No ethanol formation was found at 0.2 and 0.3% (w/v) casamino acids but glycerol was present at 0.1 and 0.2%

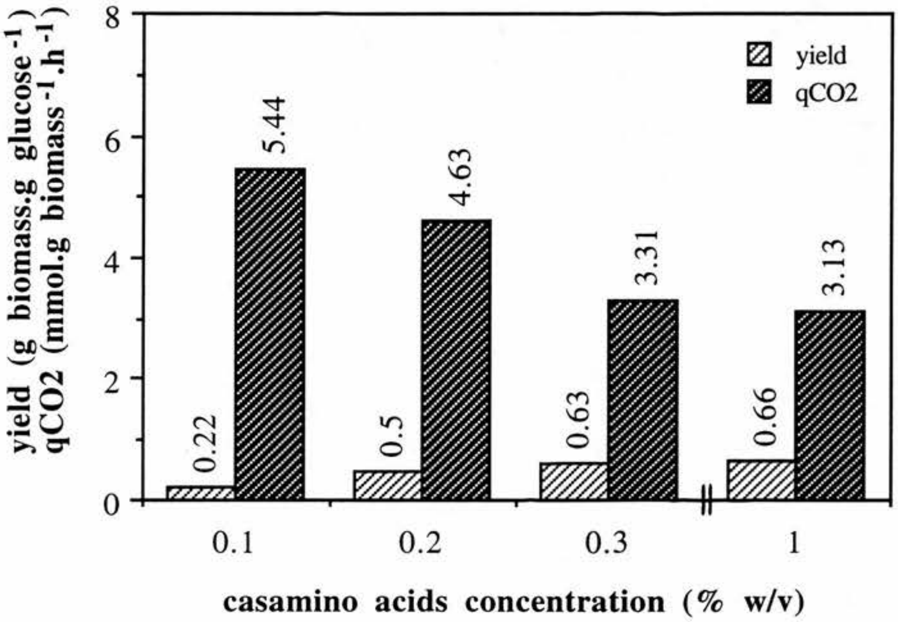


Figure 5.2 Biomass yield and specific rate of carbon dioxide production (qCO_2) at four different casamino acids concentrations (% w/v) in aerobic chemostat culture of strain REF1 with 0.5% (w/v) glucose supplement.

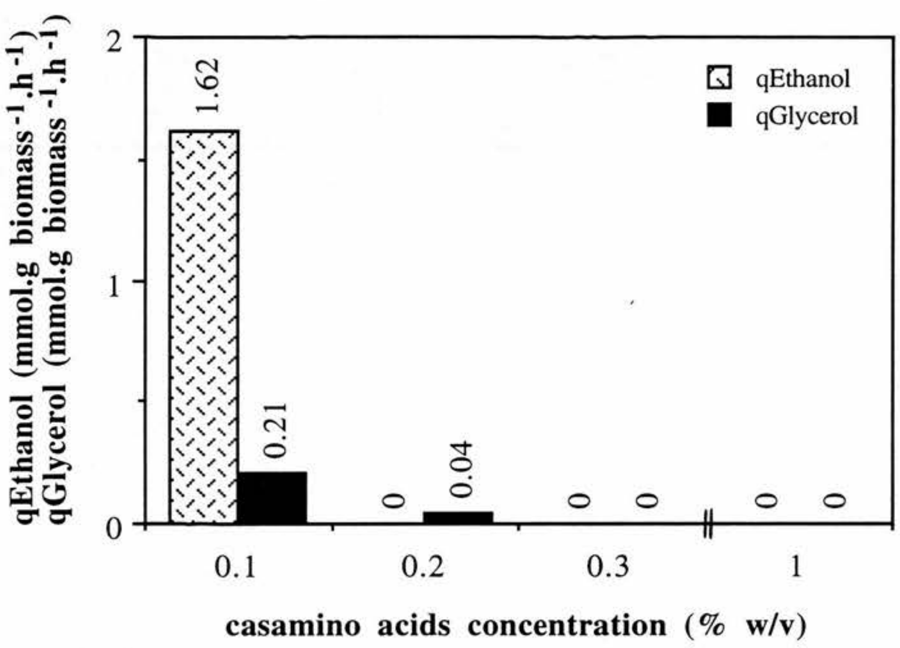


Figure 5.3 Fluxes of ethanol (qEthanol) and glycerol (qGlycerol) at four different casamino acids concentrations (% w/v) in aerobic chemostat culture of strain REF1 with 0.5% (w/v) glucose supplement.

(w/v) casamino acids.

As similar results were observed at 0.3% and 1% (w/v) casamino acids, it could be assumed that 0.3% (w/v) casamino acids concentration was the saturated level that the yeast strain could utilize this compound. Further increases in casamino acids concentration would no longer promote any metabolic change in the yeast strain.

Surprisingly, at 0.1% (w/v) casamino acids where a very low biomass yield was found, ethanol was produced. The ethanol production observed here has raised another possible explanation for a very low biomass yield obtained under these conditions, as it is known that when glucose is fermented to ethanol, less biomass yield is observed. However, the reason why ethanol formation was triggered under these conditions was unknown and this problem was not considered further.

It was decided that 0.2% (w/v) casamino acids would be used in further experiments because amino acid limitation would be shown at lower concentration. In addition, the biomass yield of 0.50 g biomass.g glucose^{-1} (which is below the theoretical maximum) was obtained at 0.2% (w/v) casamino acids concentration.

5.1.2 Optimization of Glucose Concentration at 0.2% (w/v) Casamino Acids

After the desired casamino acids concentration was obtained, glucose content in the medium was varied to study and, eventually, to ensure that a glucose-limited condition was established. The culture was grown at different glucose concentrations but the casamino acids concentration was kept constant at 0.2% (w/v) throughout the study. Cultures were grown at four different glucose concentrations (0.25, 0.5, 0.75 and 1.0% w/v), and, for each of these, at least three samples were taken at steady state for the assessment of growth and metabolite production. The results shown in Figure 5.4 are the biomass yield and qCO_2 of steady state samples recorded as a function of glucose concentration. Figure 5.5 shows ethanol and glycerol fluxes at the corresponding glucose concentrations.

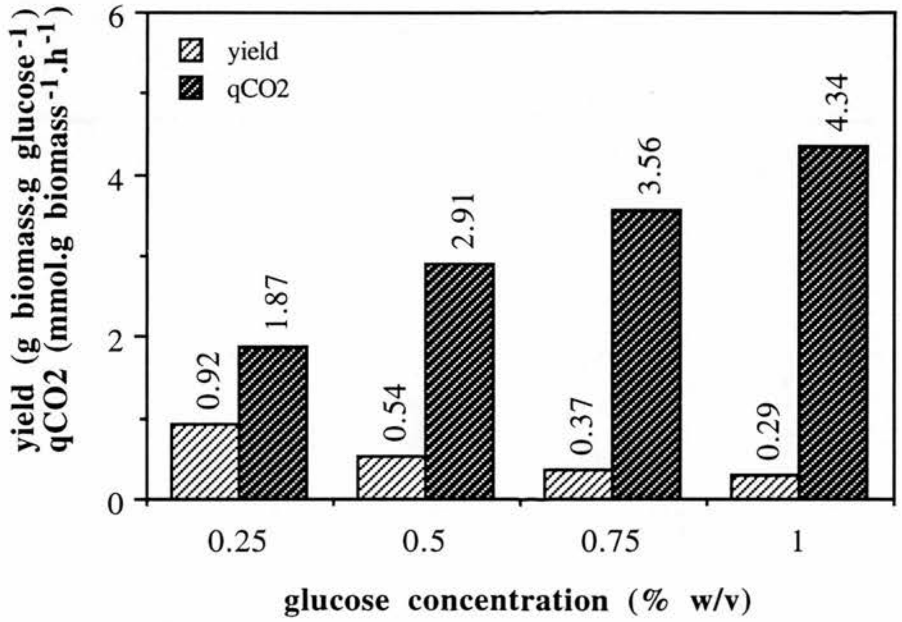


Figure 5.4 Biomass yield and specific rate of carbon dioxide production (qCO_2) at four different glucose concentrations (% w/v) in aerobic chemostat culture of strain REF1 with 0.2% (w/v) casamino acids supplement.

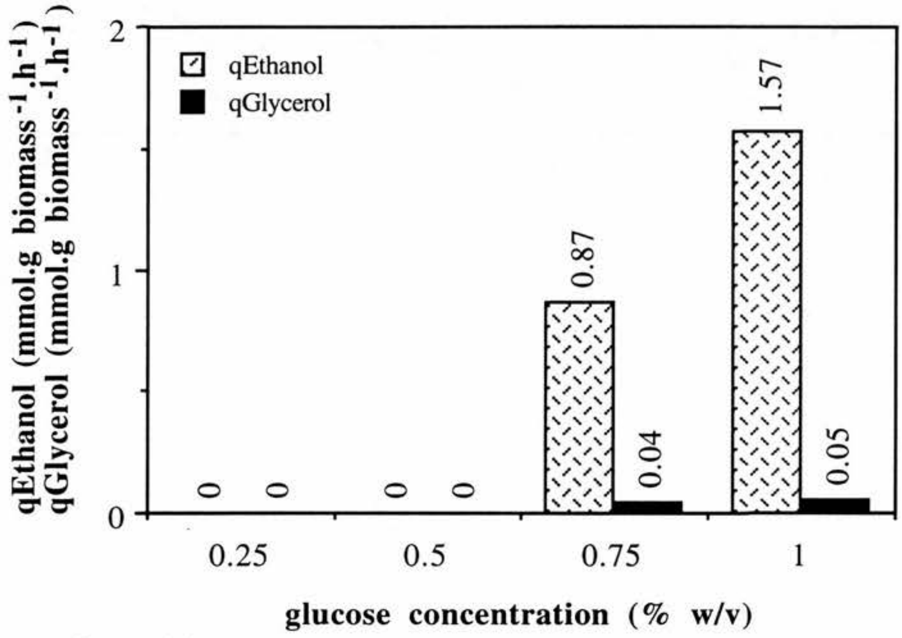


Figure 5.5 Fluxes of ethanol (qEthanol) and glycerol (qGlycerol) at four different glucose concentrations (% w/v) in aerobic chemostat culture of strain REF1 with 0.2% (w/v) casamino acids supplement.

At 0.25% (w/v) glucose, the highest biomass yield (0.92 g biomass. g glucose^{-1}) was observed, compared to those obtained from higher concentrations, and was accompanied by qCO_2 of 1.87 mmol.g biomass^{-1}h^{-1}. This result was somewhat puzzling. The very high biomass yield observed here could be the result of an overestimation of cell dry weight under these conditions because cell density was found to be low (OD_{600}=3 unit.ml^{-1}) when compared to the values observed at other concentrations. Ethanol and glycerol were not detected at 0.25 and 0.50% (w/v) glucose. The biomass yield of 0.54 g biomass.g glucose^{-1} was observed at 0.50% (w/v) glucose. At glucose concentrations above 0.50 % (w/v), biomass yield dropped to 0.37 and 0.29 g biomass.g glucose^{-1} at 0.75 and 1.0% (w/v) glucose respectively as a result of ethanol formation found at these concentrations. Glycerol was also detected at these glucose concentrations but it was present at very low level.

Changes in the concentration of a limiting substrate should result in a proportional change in biomass concentration. However, in this study, a proportional increase in biomass concentration was not observed at high concentration of glucose because alcoholic fermentation occurred at glucose concentrations above 0.5% (w/v). In addition, a proportional decrease in biomass concentration was not observed after the reduction of glucose concentration to 0.25% (w/v). This was believed to be due to an experimental error in dry weight measurement of a low cell density culture.

Although none of the proportional changes were observed from the variation of glucose content, an assay for the residual glucose in the cultures taken at D=0.11 to 0.32 h^{-1} (in subsequent experiments) showed negligible amount of glucose (less than 0.28 mM in all three strains studied) in comparison with the glucose-input concentration (28 mM). This ensured that the glucose-limited condition has been established at 0.5% (w/v) glucose supplement.

5.2 The Dilution Rate Profile Study

The dilution rate profiles of the yeast strains REF1, OP2 and OP7 were studied individually in the defined medium (Appendix A, Table A.7) supplemented with 0.5% (w/v) glucose and 0.2% (w/v) casamino acids in a chemostat culture. The chemostat was prepared and experiments were carried out using the techniques described in section 2.6. Steady state conditions were established and several samples were taken from each steady state. Biomass and metabolite production were analyzed and recorded as a function of dilution rate. It should be noted that the dilution rate profiles of the three yeast strains described below would not be studied at a dilution rate higher than 0.36 h^{-1}. This was because in the preliminary dilution rate profile experiment carried out in strain REF1 the *wash out phenomenon* (see introduction of this chapter) was observed after a further increase in dilution rate to 0.42 h^{-1}. It could therefore be stated that the $D_{\text{wash out}}$ of the REF1 is somewhere in between 0.36 and 0.42 h^{-1}. This outcome was consistent with the μ_{max} of this strain (0.376 h^{-1}) observed in batch culture (μ assessed by OD_{600} measurement in Chapter 4, section 4.2).

5.2.1 Dilution Rate Profile of the Strain REF1

The dilution rate profile of the yeast strain REF1 was analyzed at different dilution rates varying from 0.11 to 0.36 h^{-1}.

At D=0.11 h^{-1}, a biomass yield of 0.60 g biomass.g glucose^{-1} and a qCO_2 of 2.61 mmol.g biomass$^{-1}h^{-1}$ was observed. Ethanol and glycerol were not detected at this dilution rate. At D=0.20 h^{-1}, the formation of ethanol was detected and this resulted in a significant drop in biomass yield to 0.48 g biomass. g glucose^{-1}. The decrease in biomass yield continued with increasing dilution rate above 0.20 h^{-1}. It was accompanied by an enhanced qCO_2 (Figure 5.6) as well as increases in qEthanol and qGlycerol (Figure 5.7). Pyruvate and acetate were present at all dilution rates and the fluxes of these two metabolites increased with increasing dilution rates

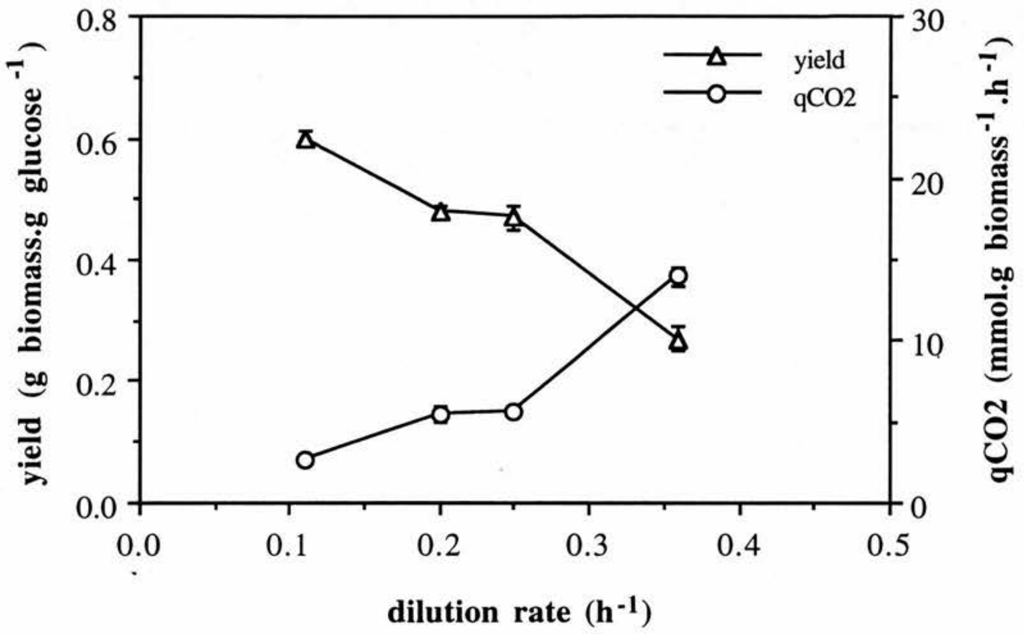


Figure 5.6 Biomass yield and specific rate of carbon dioxide production (qCO_2) as a function of dilution rate in aerobic glucose-limited chemostat culture of strain REF1 with 0.2% (w/v) casamino acids supplement.

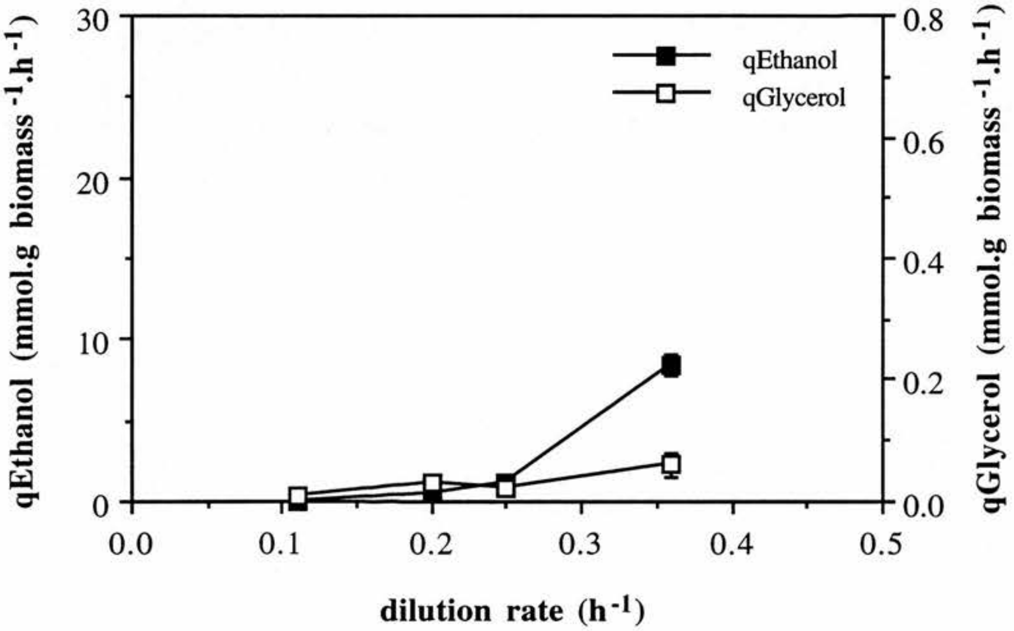


Figure 5.7 Fluxes of ethanol (qEthanol) and glycerol (qGlycerol) as a function of dilution rate in aerobic glucose-limited chemostat culture of strain REF1 with 0.2% (w/v) casamino acids supplement.

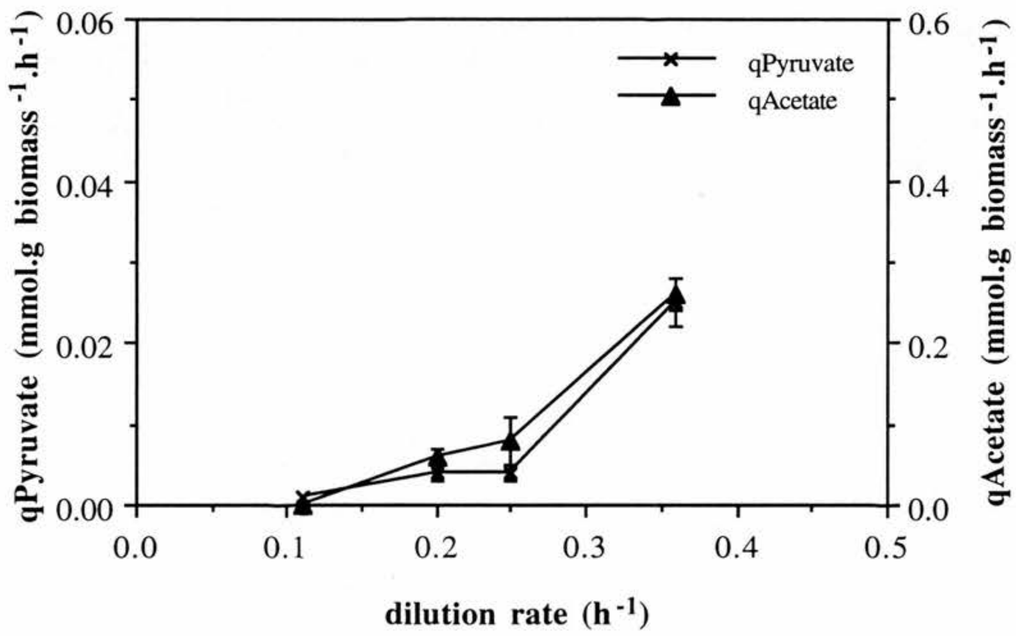


Figure 5.8 Fluxes of pyruvate (qPyruvate) and acetate (qAcetate) as a function of dilution rate in aerobic glucose-limited chemostat culture of strain REF1 with 0.2% (w/v) casamino acids supplement.

(Figure 5.8).

The biomass yield obtained at D=0.11 h^{-1}(0.60 g biomass.g glucose^{-1}) appeared to be higher than the theoretical level although the casamino acids content in the medium had been reduced by a factor of five i.e. from 1 to 0.2% (w/v). However, the casamino acids content could not be further reduced because the results from section 5.1.1 suggested that a further decrease in casamino acids concentration to 0.1% (w/v) may cause a limitation of amino acids.

5.2.2 Dilution Rate Profile of the Strain OP2

The yeast strain OP2 was grown and studied in glucose-limited chemostat culture at three steady state conditions (D=0.11, 0.32 and 0.36 h^{-1}). Plots of biomass yield and qCO_2 against dilution rate are shown in Figure 5.9. Changes in biomass yield and qCO_2 were found to be quantitatively similar to those observed in the strain REF1 (Figure 5.6). At D=0.11 h^{-1}, biomass yield was 0.58 g biomass.g glucose^{-1} and qCO_2 was 3.48 mmol.g biomass^{-1}h^{-1}. HPLC analysis showed that pyruvate which was produced at a rate of 0.002 mmol.g biomass^{-1}h^{-1} was the only metabolite that could be detected at this dilution rate. All other metabolites (ethanol, glycerol and acetate) were found at D=0.32 and 0.36 h^{-1}. Enhanced rate of ethanol, glycerol and acetate production were found with increasing dilution rate from 0.32 to 0.36 h^{-1} (Figure 5.10 and 5.11).

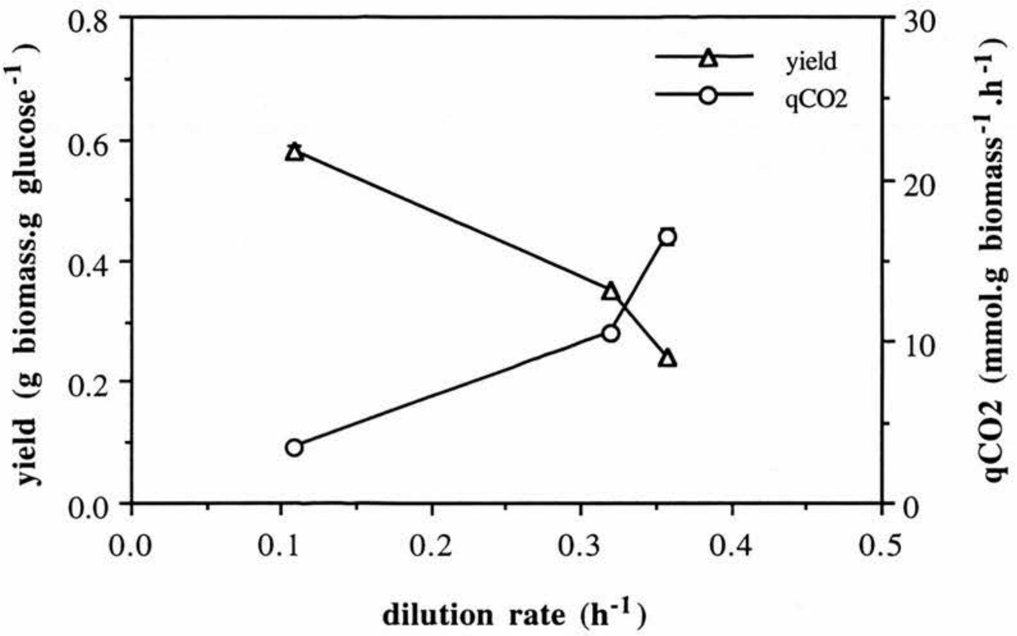


Figure 5.9 Biomass yield and specific rate of carbon dioxide production (qCO_2) as a function of dilution rate in aerobic glucose-limited chemostat culture of strain OP2 with 0.2% (w/v) casamino acids supplement.

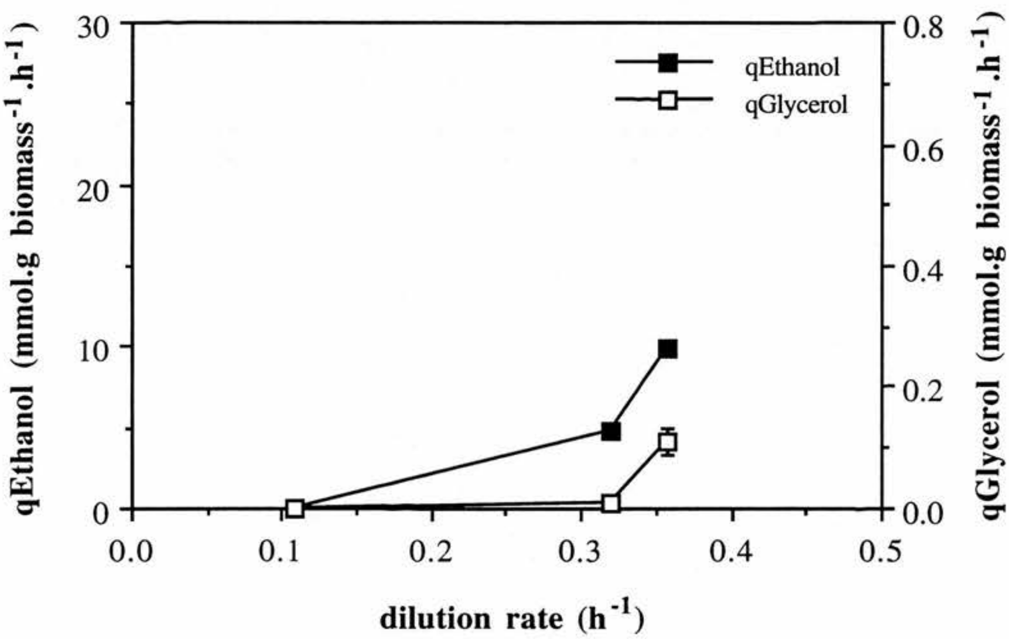


Figure 5.10 Fluxes of ethanol (qEthanol) and glycerol (qGlycerol) as a function of dilution rate in aerobic glucose-limited chemostat culture of strain OP2 with 0.2% (w/v) casamino acids supplement.

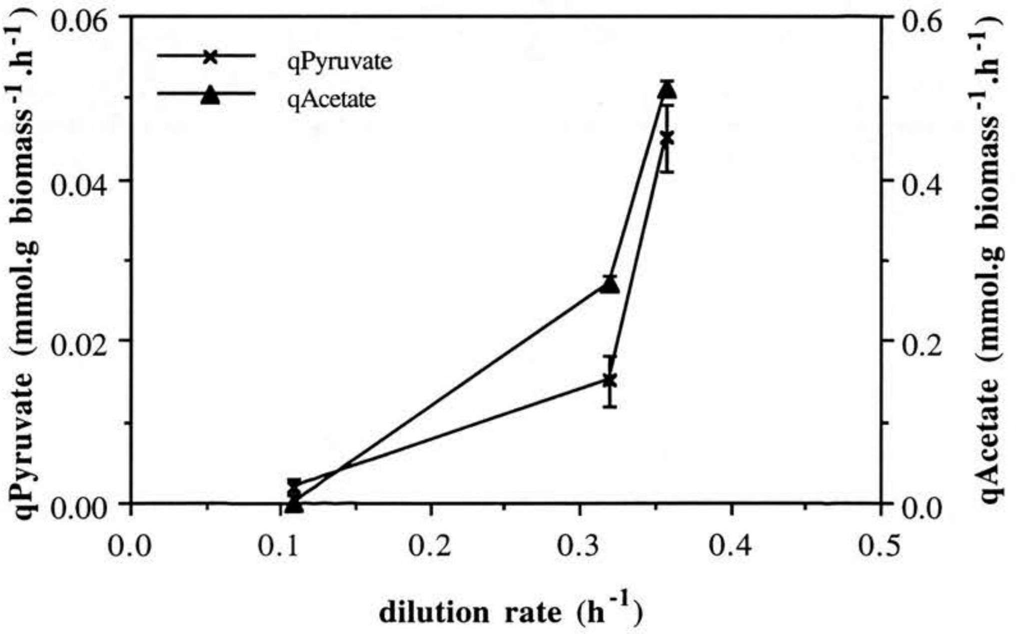


Figure 5.11 Fluxes of pyruvate (qPyruvate) and acetate (qAcetate) as a function of dilution rate in aerobic glucose-limited chemostat culture of strain OP2 with 0.2% (w/v) casamino acids supplement.

5.2.3 Dilution Rate Profile of The Strain OP7

The dilution rate profile of the strain OP7 was obtained at four steady state conditions (D=0.11, 0.20, 0.25 and 0.32 h^{-1}). The steady state values of biomass yield and qCO_2 were plotted as a function of the dilution rate as shown in Figure 5.12. The change in qCO_2 of this strain was found to be quantitatively similar to those observed in the strains REF1 and OP2 shown in Figure 5.6 and 5.9 respectively. However, when the pattern of biomass change of the strain OP7 was compared to the pattern of the strains REF1 and OP2, a slight difference was found. At D=0.11 h^{-1}, this strain showed a biomass yield of 0.44 g biomass.g glucose^{-1}. This value appeared to be lower than those observed in the other strains (0.58 g biomass.g glucose^{-1} in both REF1 and OP2). This reduction in biomass yield was believed to be due to a switch to respiro-fermentative catabolism as ethanol production was found to occur (0.04 mmol.g biomass^{-1} h^{-1}, Figure 5.13). In addition, strain OP7 also produced pyruvate and acetate at higher rates than strains REF1 and OP2. This could also be associated with ethanol formation. Glycerol was detected at D=0.11 h^{-1} (0.02 mmol.g biomass^{-1} h^{-1}) but disappeared at dilution rates above this level (Figure 5.13). Pyruvate and acetate were present at all the dilution rates studied (Figure 5.14).

Above D=0.11 h^{-1}, increases in the rates of ethanol, pyruvate and acetate production were observed (Figure 5.13 and 5.14). Changes in the biomass yield were found to be quantitatively similar to those observed in strains REF1 and OP2.

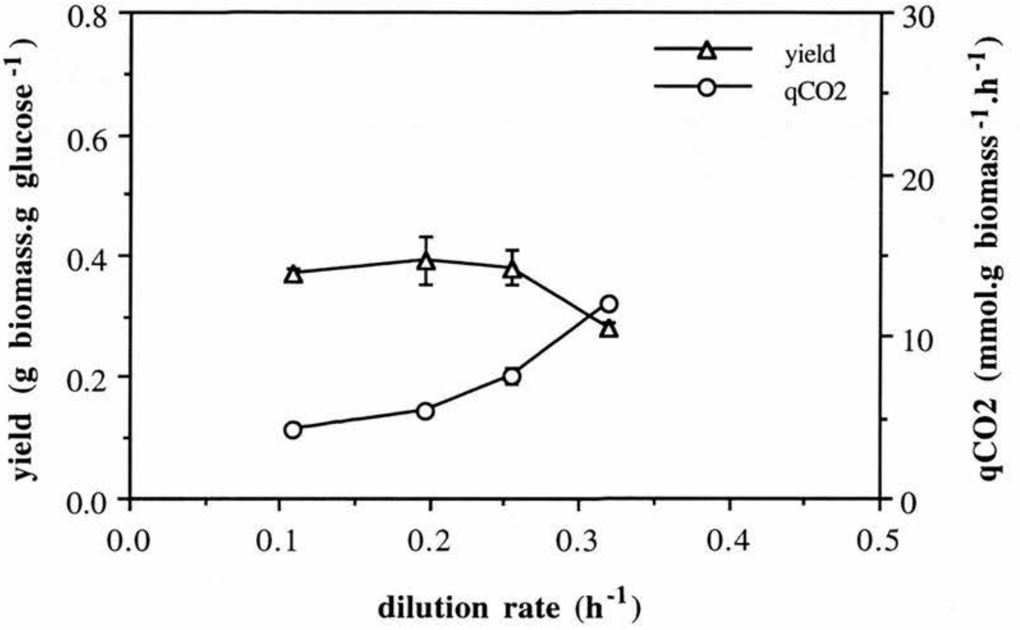


Figure 5.12 Biomass yield and specific rate of carbon dioxide production (qCO_2) as a function of dilution rate in aerobic glucose-limited chemostat culture of strain OP7 with 0.2% (w/v) casamino acids supplement.

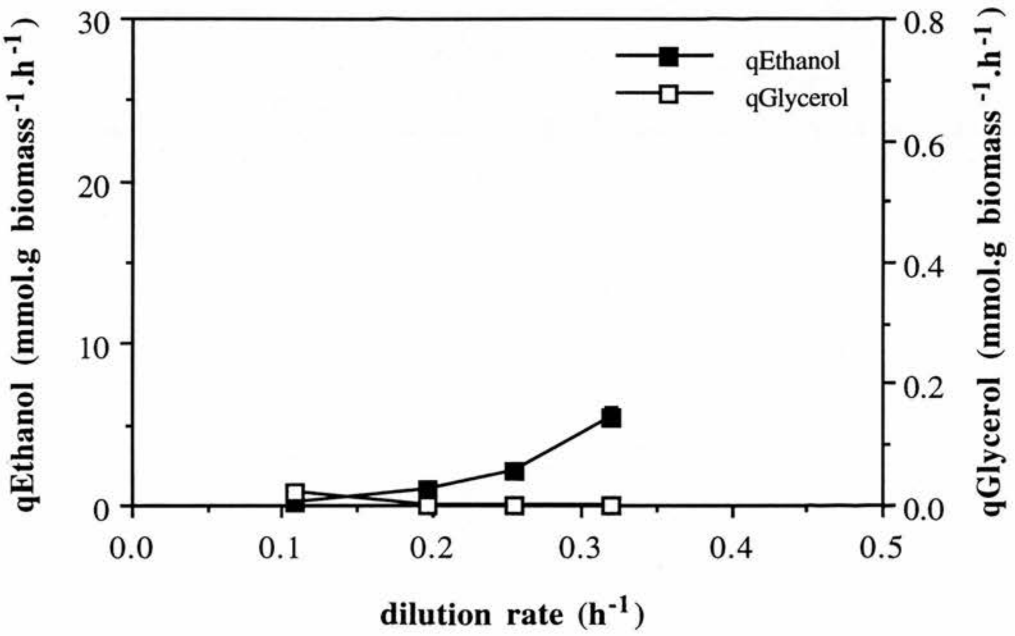


Figure 5.13 Fluxes of ethanol (qEthanol) and glycerol (qGlycerol) as a function of dilution rate in aerobic glucose-limited chemostat culture of strain OP7 with 0.2% (w/v) casamino acids supplement.

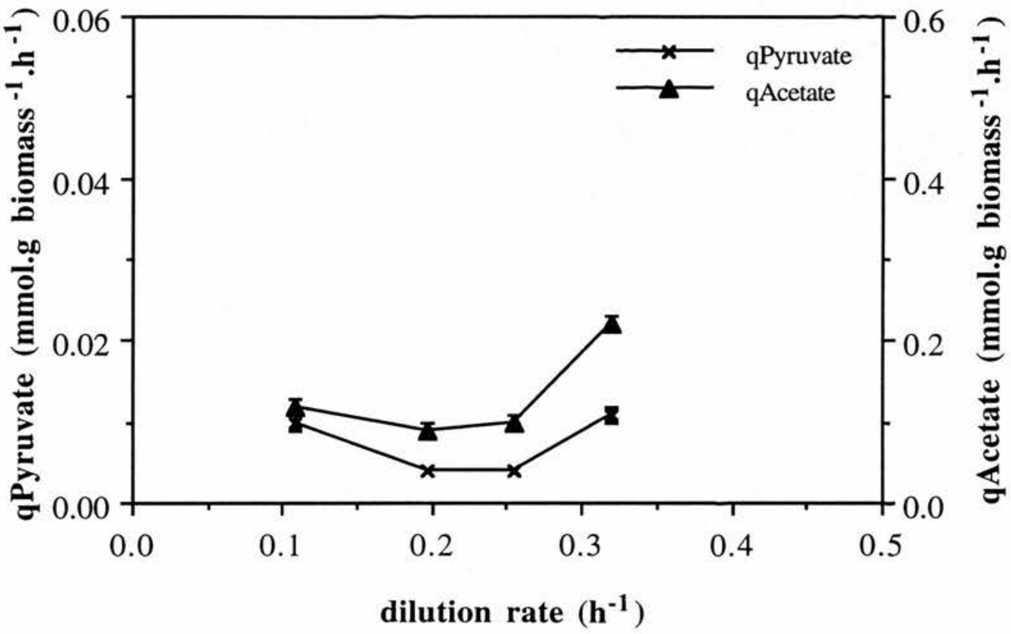


Figure 5.14 Fluxes of pyruvate (qPyruvate) and acetate (qAcetate) as a function of dilution rate in aerobic glucose-limited chemostat culture of strain OP7 with 0.2% (w/v) casamino acids supplement.

5.3 Comparison of The Three Constructed Yeast Strains at D=0.11 and 0.32 h^{-1}

Dilution rate profile studies of the yeast strains REF1 and OP2 suggested that these two yeast strains metabolized glucose mainly by respiration at D = 0.11 h^{-1} because no ethanol formation was detected in either of them. However, strain OP7 appeared to be using respiro-fermentative glucose catabolism at this dilution rate. At D=0.32 h^{-1}, all strains showed ethanol production. These two dilution rates were chosen to be the conditions of study to compare metabolic activity between TPI overproducing strains and the reference. In addition to the data obtained in section 5.2, the strains REF1 and OP7 were re-cultivated in an aerobic glucose-limited chemostat at D=0.11 and 0.32 h^{-1} using the same culture system and cultivation techniques described above. Steady state samples were collected and analyzed for growth, metabolite production and glycolytic enzyme production. Results were analyzed together with those were obtained in section 5.2 under the same growth conditions i.e. D=0.11 and 0.32 h^{-1}.

5.3.1 Steady State Growth and Metabolite Production

Physiological parameters obtained from strains REF1, OP2 and OP7 are summarized and compared in Table 5.1. Statistic significance was determined within 95% confidence and shown in Appendix B section 4.2.

At D=0.11 h^{-1}, no significant difference between strains REF1 and OP2 was observed with respect to biomass yield, ethanol production and pyruvate production. The OP7 showed significant decrease in biomass yield (from 0.58 to 0.44 g biomass.g glucose^{-1}) accompanied by a detectable level of ethanol production (0.04 mmol.g biomass^{-1}h^{-1}). qCO2 of strain REF1 was found to be significantly lower than those of the overproducers. The three strains appeared to produce acetate at three different rates. Glycerol was found at small amount (0.01 mmol.g biomass^{-1}h^{-1}) in strains REF1 and OP7 whilst it was not detected in the OP2.

Table 5.1 Summary of physiological parameters at steady state of the strain REF1, OP2 and OP7 at D = 0.11 and 0.32 h^{-1}.

strain	D^a	biomass yield^b	$qCO_2{}^c$	qEthanol^c	qGlycerol^c	qPyruvate^c	qAcetate^c
REF1	0.11**	0.58±0.02	2.70±0.22	0	0.01±0.00	0.002±0.001	0.02±0.00
	0.32 *	0.31±0.01	10.71±0.65	6.06±0.67	0	0.010±0.000	0.20±0.01
OP2	0.11 *	0.58±0.01	3.48±0.06	0	0	0.002±0.001	0
	0.32 *	0.35±0.00	10.55±0.09	4.71±0.46	0.01±0.00	0.015±0.003	0.27±0.01
OP7	0.11**	0.44±0.08	3.58±0.73	0.04±0.06	0.01±0.01	0.007±0.003	0.06±0.06
	0.32**	0.28±0.01	11.69±0.71	5.62±0.72	0	0.016±0.004	0.23±0.02

* data recorded at this condition were obtained from single chemostat

** data recorded at this condition were averages obtained from two repeated chemostats

[a] h^{-1}

[b] g biomass.g $glucose^{-1}$

[c] mmol.g $biomass^{-1}h^{-1}$

At D=0.32 h^{-1}, the rate of CO_2, ethanol and pyruvate production of the three strains studied were essentially the same but the biomass yields were found significantly different in all strains. Acetate production of the OP2 was found significantly higher than those observed in the others. Glycerol was found at small amount (0.01 mmol.g biomass^{-1}h^{-1}) in strain OP2 only. In all strains, higher rates of ethanol, pyruvate and acetate production and lower biomass yields were observed when compared to the results obtained at D=0.11 h^{-1}.

To determine the balance of the input and output carbon content between strains, all the results summarized in Table 5.1 are transformed to a unit of carbon production per hour. For the glycolytic metabolites such as ethanol, pyruvate etc. the calculations are obvious. For CO_2, the gas flow rate had to be taken into account since CO_2 was recorded in terms of %CO_2 in the gas above the culture fluid. The biomass has been reported as $C_{3.75}H_{6.6}O_{2.18}N_{0.54}$ (Verduyn *et al.*, 1991). From this information, the input and output carbon content can be calculated and compared. Results are summarized in Table 5.1a.

It can be seen that one can account for all the "carbon in" (in the form of glucose) and the "carbon out" in the form of sum of all the major products (and the residual glucose at D=0.32 h^{-1}). There is in all the strains, at both dilution rates studied, a slightly higher sum of carbon than the apparent input. This can be assumed to be due to the possible contribution of additional carbon source (i.e. casamino acids) in the growth medium.

Table 5.1a Summary of carbon production at steady state of the strain REF1, OP2 and OP7 at D=0.11 and 0.32 h^{-1} where the input carbon (in form of glucose) are 0.154 and 0.448 g carbon. h^{-1} respectively.

strain	D[a]	residual glucose[b]	biomass production[b]	CO_2 production[b]	ethanol production[b]	glycerol production[b]	pyruvate production[b]	acetate production[b]	total carbon production[b]
REF1	0.11	0	0.107±0.006	0.065±0.007	0	0.0008±0.0001	0.0002±0.0001	0.002±0.00001	0.175±0.013
	0.32	0.008±0.003	0.160±0.004	0.136±0.007	0.156±0.014	0	0.0005±0.00005	0.005±0.0001	0.466±0.028
OP2	0.11	0	0.105±0.002	0.086±0	0	0	0.0001±0	0	0.191±0.002
	0.32	0.004±0.004	0.184±0.002	0.154±0	0.140±0.014	0.0006±0	0.0007±0.0001	0.008±0.0004	0.491±0.020
OP7	0.11	0	0.080±0.017	0.065±0.004	0.004±0.001	0.001±0.0003	0.0004±0.0001	0.004±0.0004	0.154±0.023
	0.32	0.009±0.005	0.148±0.008	0.140±0.004	0.133±0.018	0	0.0006±0.0002	0.006±0.0006	0.437±0.036

[a] h^{-1}

[b] g carbon. h^{-1}

5.3.2 Steady State Glycolytic Enzyme Production

Steady state samples of the TPI overproducers and the strain REF1 were collected at the dilution rate of 0.11 and 0.32 h^{-1} and assayed for TPI, PGK, PYK and HK activities. The specific activities of these glycolytic enzymes for each strain are summarized in Table 5.2. Statistical significance was determined within 95% confidence and shown in Appendix B section 4.3.

The assay of TPI activity indicated that, at both steady state conditions studied, the strains OP2 and OP7 showed relative increases in specific activity to the reference level by approximately factors of two and seven respectively. This was consistent with the results obtained in batch experiments (Chapter 4, section 4.6). The samples were assayed for activities of the three other glycolytic enzymes i.e. PGK, PYK and HK. At D=0.11 h^{-1}, the level of PGK activity of the REF1 was appeared to be significantly higher than those observed in the overproducers whilst it was found to be different in all strains at D=0.32 h^{-1}. No significant difference in the level of PYK and HK activities could be observed between the strains REF1 and OP2 at both steady states. The OP7 showed a statistically significant lower level of these enzymes than those observed in the other two strains at both dilution rates.

Table 5.2 Summary of glycolytic enzyme production at steady state of the strain REF1, OP2 and OP7 at D = 0.11 and 0.32 h^{-1}.

strain	D^a	specific TPI activity^b	specific PGK activity^b	specific PYK activity^b	specific HK activity^b
REF1	0.11**	78.9±16.5	11.7±1.0	5.7±0.5	2.2±0.3
	0.32 *	76.9±7.9	9.6±0.7	5.1±1.4	2.0±0.5
OP2	0.11 *	150.0±5.9	8.9±0.4	6.2±0.2	2.1±0.1
	0.32 *	186.8±15.8	7.6±0.2	6.1±0.1	2.3±0.1
OP7	0.11**	510.0±66.7	8.7±1.6	3.6±1.0	1.5±0.3
	0.32**	566.5±97.6	6.5±0.2	1.8±0.5	1.1±0.3

* data recorded at this condition were obtained from single chemostat

** data recorded at this condition were averages obtained from two repeated chemostats

a h^{-1}

b unit.mg protein^{-1}

Discussion

The individual chemostat experiments studied here showed that the reference strain REF1, and probably the two-fold TPI overproducer, produced ethanol at $D=0.20\ h^{-1}$but not at $D=0.11\ h^{-1}$. The critical dilution rate, the dilution rate at which alcoholic fermentation was triggered in aerobic glucose-chemostat culture, of these two strains may be between D=0.11 and $0.20\ h^{-1}$. The critical dilution rate is strain specific. Postma *et al.* (1989) showed that the yeast *S. cerevisiae*CBS 8066 exhibits oxidative respiration up to dilution rate of $0.38\ h^{-1}$ in aerobic glucose-limited chemostat culture. The value they found is high when compared to the results described in this thesis and elsewhere (van der Aar *et al.*, 1990a,b). A PGK overproducing strain (van der Aar *et al.*, 1990b) carrying 80 extra copies of the *PGK1* gene integrated in the genome and a wild type strain, showed purely oxidative glucose metabolism at $D<0.11\ h^{-1}$. Respiration was accompanied by ethanol formation at dilution rates above this critical value.

The study of the seven-fold overproducer showed different metabolism when compared to the reference and the two-fold overproducer. This strain exhibited ethanol production at $D=0.11\ h^{-1}$whereas the others did not. Ethanol formation was accompanied by a drop in biomass yield and also an increase in pyruvate and acetate production rates. In addition, a seven-fold increase in TPI production has lowered the activities of PYK and HK (63.5% and 71.6% with respect to reference levels), the two enzymes of which the steady state level was also found to be lowered in the OP7 in batch experiments (Chapter 4). Taken together these results indicate that some metabolic changes have taken place as a result of the overproduction of TPI. Whether the pool movements of ethanol, pyruvate and acetate (and others not monitored) could result in changes in repression/induction of other glycolytic enzymes must remain speculative. However, these outcomes are unexpected because if, as is usually claimed, TPI is an "equilibrium enzyme" increase in its activity should not affect metabolism at all. The results were found even more unexpected when compared to the results of Davies & Brindle (1992) who showed no changes in PYK and HK activities in a five-fold overproduction of phosphofructokinase1, an enzyme which is often described as a regulatory enzyme to the glycolytic pathway.

Although some differences were found in the OP7, the apparent lack of differences in ethanol production between the OP2 and the REF1 may be due to the limits of detection. It may be possible that the two-fold overproducer also showed ethanol production at $D=0.11\ h^{-1}$ but at such a low rate that the difference could not be detected in the HPLC analysis.

As the results obtained in this chapter are still, however, ambiguous in some areas, the differences between the TPI overproducers and the reference strain were examined in competitive chemostat cultures which provides a much more sensitive procedure than an individual chemostat study. The study is described and discussed in the following chapter.

Chapter 6

Study of the TPI-overproducing Strains in Competitive Chemostat Culture

Introduction

The physiological study of micro-organisms can be achieved by a number of cultivation techniques, among which the chemostat has been found to be an effective tool (see Chapter 5). However, a comparison of the results obtained from the individual study of two different strains in chemostat experiment may suffer from experimental errors especially when there are only small differences between the strains. If this is the case, a much more sensitive technique is required.

To demonstrate the relative sensitivity of different cultivation techniques, the behaviour of two strains grown under four different growth conditions i.e. individual batch, competitive batch, individual chemostat and competitive chemostat, was simulated by Dr. J. R. Small. The model was simulated assuming the rate of substrate consumption and the rate of biomass production by each strain are as follows:

$$V_{\text{uptake}} = K_{\text{uptake}} \cdot X . \frac{\mu_{\max} . S}{K_s + S}$$

$$V_{\text{biomass}} = X . \frac{\mu_{\max} . S}{K_s + S}$$

where V_{uptake} is the rate of substrate uptake

K_{uptake} is the substrate affinity uptake

V_{biomass} is the rate of biomass production

$\mu_{\max}$ is the maximal specific growth rate

K_s is the half maximal saturation constant

S is substrate concentration

X is biomass concentration

Under all conditions the parameter values for the strain X_1 and X_2 were shown in Table 6.1.

Table 6.1 Parameters used in the simulation for growth of the strains X_1 and X_2.

parameter	strain X_1	strain X_2
μ_{max}	0.36	0.355
K_s	5	10
K_{uptake}	10	10

For the batch growth simulations, the levels of initial substrate and biomass concentration (for both strains) were set at 120 and 0.01 units respectively. For chemostat simulations, the levels of the feed-in substrate concentration, the initial biomass concentration and dilution rate were set at 12, 1 and 0.1 units respectively. The results of these simulations are shown in Appendix C, Table C.10 and the plots of biomass against time for all four cases are shown in Figures 6.1-6.4.

Figure 6.1 shows the biomass change with time of the strains X_1 and X_2 when grown individually under batch condition. During an exponential phase of growth, the strain X_1 grew faster than the strain X_2. However, the growth rates of these strains are very close and, in addition, the difference could be observed within only a short period of time as a result of substrate depletion. The depletion of substrate would be observed earlier when the two strains are grown competitively under batch conditions (Figure 6.2). The biomass changes observed with these growth conditions appear to be smaller than those observed in individual studies due to an early depletion of substrate. As a result, the biomass changes of each strain are stabilized at lower levels than those shown in Figure 6.1. Under chemostat conditions, the growth rate of the two strains grown individually is obtained at steady state i.e. an exponential phase growth. Although the steady state can be maintained

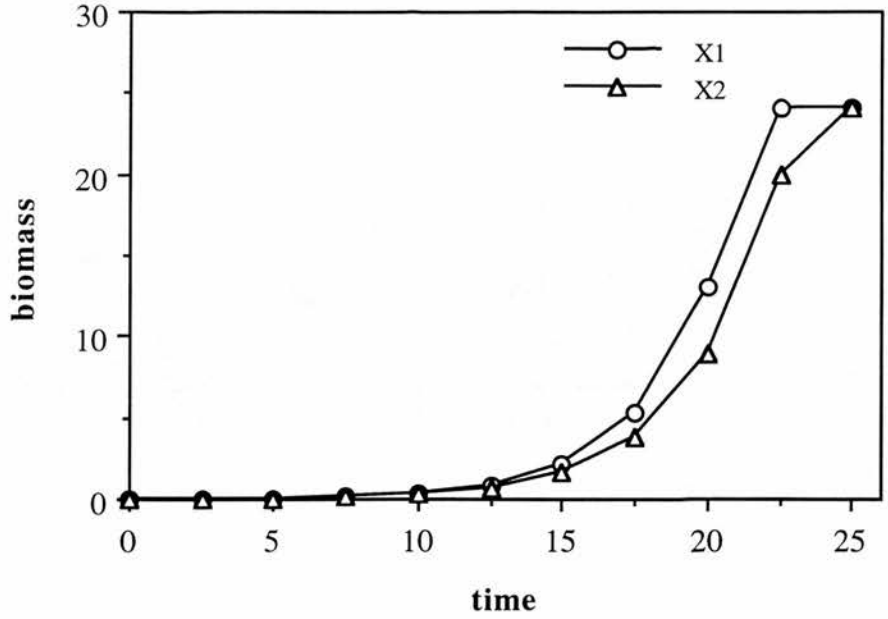


Figure 6.1 Changes in biomass with time of strains X_1 and X_2 obtained from a simulation model when the two strains were grown individually under batch conditions.

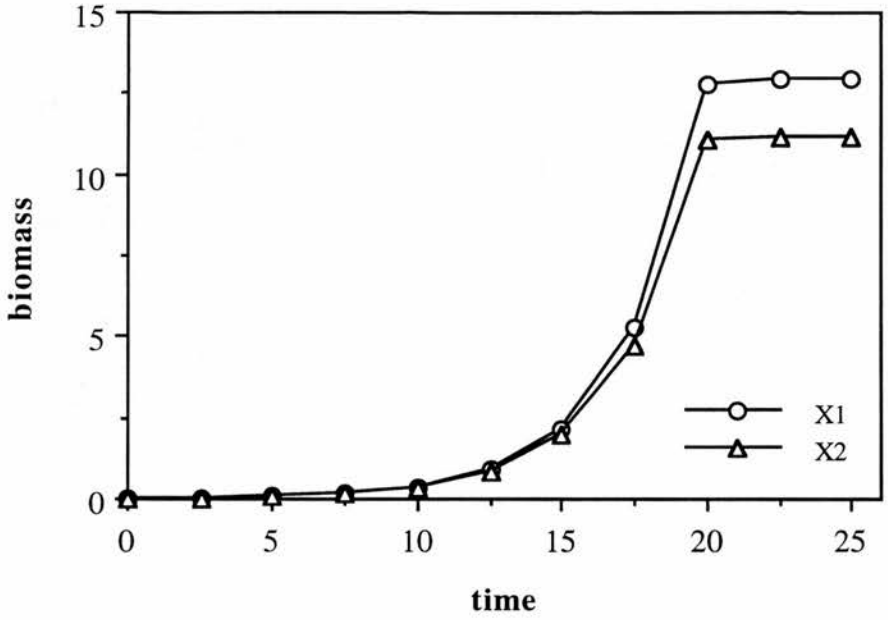


Figure 6.2 Changes in biomass with time of strains X_1 and X_2 obtained from a simulation model when the two strains were grown competitively under batch conditions.

indefinitely the difference in the biomass is very small (Figure 6.3) and may not be identified if the experimental error is high. The change in the biomass concentration with time when the two strains are grown competitively in the chemostat is shown in Figure 6.4. This figure shows the outgrowth of strain X_1 which has a higher maximal specific growth rate than strain X_2. The culture will ultimately appear to be solely strain X_1 as shown by the plot of the proportion of the two strains against time (Figure 6.5).

Competitive chemostat experiments have been successfully employed to study the behaviour of micro-organisms in a number of applications e.g. the estimation of bacterial growth rates in natural water (Jannasch, 1969), the study of wild yeast contamination in bakers' yeast production (Postma *et al.*, 1989a) and the study of selection and relative fitness in bacteria (Dean *et al.*, 1986; Dean, 1989; Dykhuizen & Davies, 1980). The success of such experiments is due to the sensitivity of this technique to very small differences in the specific growth rates of two strains, for example 0.005 h^{-1} between strains X_1 and X_2, and the result could be precisely examined (Figure 6.5).

The constructed yeast strains have already been studied individually in batch and chemostat culture (Chapters 4 and 5, respectively), and no obvious differences were found. These strains were now studied and compared under competitive chemostat conditions to assess the possible effect of TPI overproduction with a more sensitive technique.

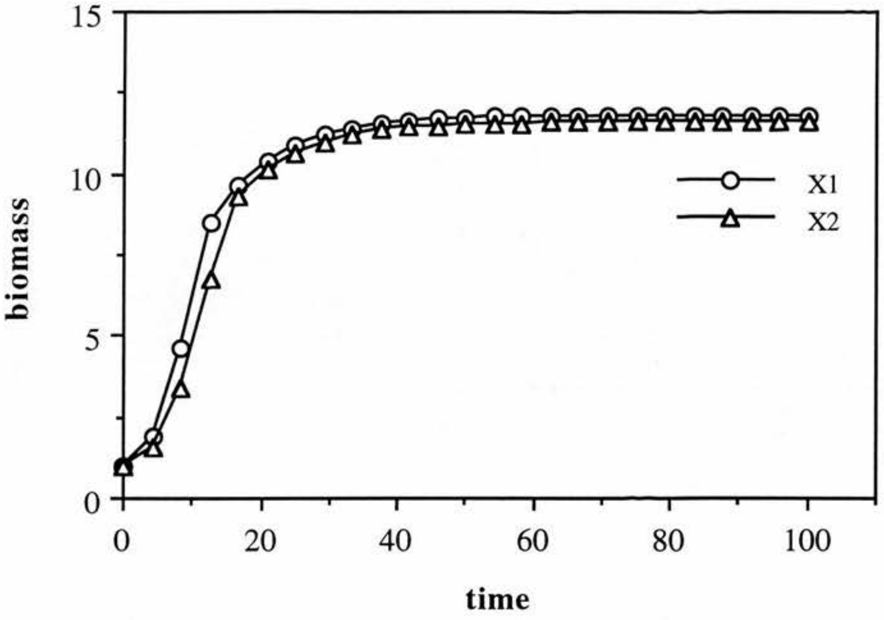


Figure 6.3 Changes in biomass with time of strains X_1 and X_2 obtained from a simulation model when the two strains were grown individually under chemostat conditions.

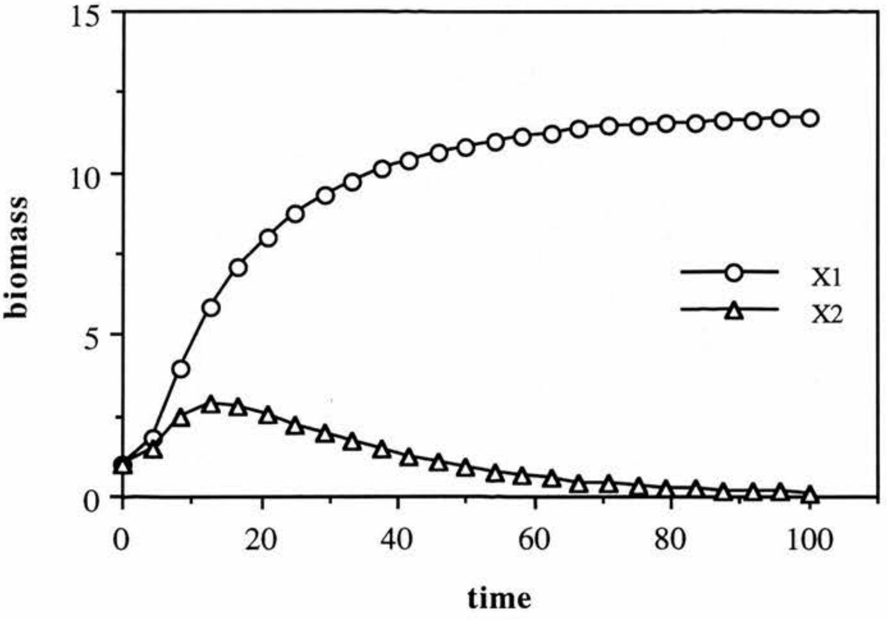


Figure 6.4 Changes in biomass with time of strains X_1 and X_2 obtained from a simulation model when the two strains were grown competitively under chemostat conditions.

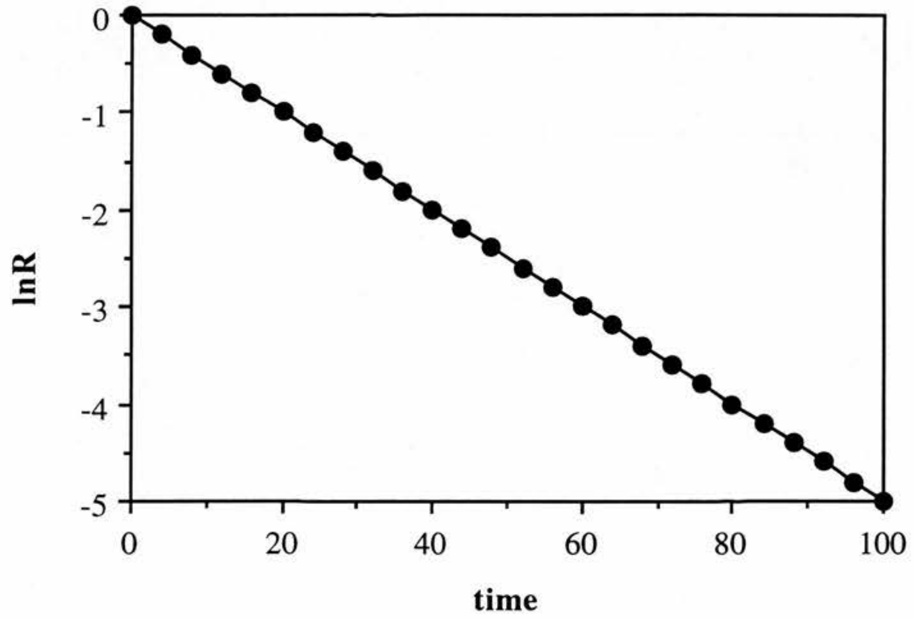


Figure 6.5 Change in the proportion of the two strains with time obtained from a simulation model when the two strains were grown competitively under chemostat conditions. R is the ratio between the two strains.

Results

In a competitive chemostat experiment, a detection marker which is neutral under the conditions of study is required to assess the ratio of the two yeast strains with time. The yeast *S. cerevisiae* DBY747, the host strain used in the construction of TPI overproducers, requires uracil, leucine, histidine and tryptophan for growth. The uracil auxotrophic phenotype had been removed after the integration of the plasmid vector carrying the *URA3* gene as well as the*TPI1* extra copies into the yeast genome. As a result, the constructed yeast strains are auxotrophic for leucine, histidine and tryptophan. The reference strain REF1, constructed by transforming the strain DBY747 with the plasmid vector alone, also showed the identical phenotype. Any of these three amino acid auxotrophies could be used as a marker. The leucine auxotrophy was chosen to be the genetic marker to distinguish the strains in the competition experiments.

YEp13 (Broach *et al.*, 1979), a plasmid vector which is widely used in many laboratories, carries the *LEU2* gene as a selective marker for cloning purposes. This could therefore be used to construct the Leu$^+$ strain by introducing the *LEU2* gene fragment isolated from YEp13 into the Leu$^-$ strain. As a result, the leucine auxotrophy would be eliminated. The Leu$^+$ versions of the yeast strains REF1 and OP2 were constructed by Dr. F. Stuart. The *LEU2* gene fragment was isolated from the yeast episomal plasmid vector, YEp13, at the *Pst*I sites. The fragment was introduced into chromosomal DNA using lithium acetate yeast transformation (section 2.7). The Leu$^+$ transformants were selected on minimal medium without leucine supplement. The transformants which obtained a single copy of *LEU2* gene (assessed by Southern analysis, data not shown) were kept for future competition experiments. The transformants derived from the strains REF1 and OP2 are designated as REF2 and OP2L respectively.

Competition experiments were conducted between pairs of the Leu$^+$ and Leu$^-$ strains in a glucose-limited chemostat using the culture system and culture conditions described in chapter 5. Each inoculum was prepared separately in shake flask culture as for the individual chemostat cultures (section 2.6.4). The proportion of the two inoculums was varied, as desired, by mixing the two cell suspensions to a total volume of 100 ml in an inoculation bottle. The dilution rate was set at 0.11 h^{-1} throughout the study. Samples were taken directly from the culture vessel and serially diluted in phosphate buffer pH 5 to prevent cell lysis caused by osmotic pressure and plated onto minimal medium (YO-leucine, Appendix B, Table B.3) in the presence and absence of leucine. The number of colonies formed in the presence of leucine yields an estimate of the total number of yeast cells per sample, and the number of colonies formed in the absence of leucine yields an estimate of the number of Leu^+ cells. The number of the Leu^- cells was therefore estimated by taking the difference. An initial ratio of the two inoculums could also be estimated by sampling immediately after inoculation. The selection coefficient (s) was calculated as the slope of the regression line ln(R) against time where R is the ratio of Leu^+ cells to the Leu^- cells in the sample. Relative fitness (w) was determined as follows (Dean, 1989):

$$w = 1 - sH$$

where s is the selection coefficient

H is the holding time of the chemostat which is 9.17 h throughout the study

6.1 Competitive Chemostat Culture Between the Strains REF1 and REF2 (Experiment 1)

A competition experiment between the original reference strain REF1 and the reference strain carrying the *LEU2* gene (REF2) was carried out to investigate the consequence of having the *LEU2* gene in the chromosome. A zero-slope straight line of the relationship between the lnR and time i.e. no change in the ratio between the two yeast strains with time was expected if the *LEU2* gene is neutral. Two experiments were carried out to obtain the result of the competition between the reference strains, REF1 and REF2. One is discussed here and the other will be discussed in section 6.4.

The first competition experiment was inoculated with equal proportions of the REF2 (Leu$^+$) and the REF1 (Leu$^-$). The first sample was not taken until 30 h of incubation as a small preliminary experiment suggested that sampling earlier resulted in scattered values for the ratio. Changes in the ratio were followed from 30-148 h and a straight line with approximately zero slope was observed (Figure 6.6). From the results, it was concluded that the *LEU2* gene marker on the strain REF2 was neutral. This result led to the study between TPI overproducers and the REF2 strain described in the following sections. However, it should be noted that the results obtained here were not extended beyond 150 h.

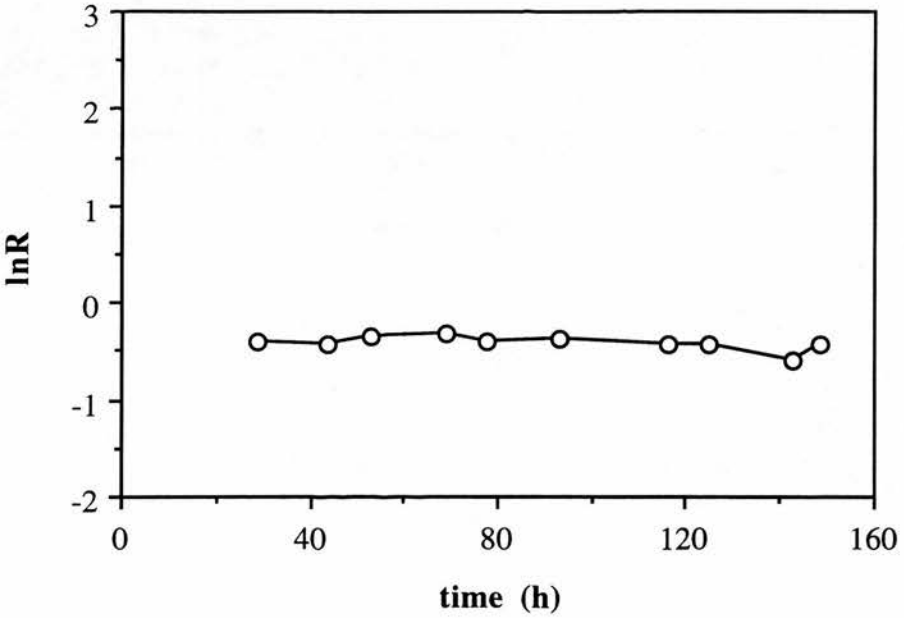


Figure 6.6 Change in ratio of strains REF2 and REF1 as a function of time during competition experiment at $D=0.11h^{-1}$ in an aerobic glucose-limited chemostat culture. R is the ratio of REF2 to REF1.

6.2 Competitive Chemostat Culture Between the Strains OP7 and REF2

The consequence of seven-fold TPI overproduction in strain OP7 compared to normal level in REF2 was investigated in this section. Two identical chemostats were set up and inoculated with two different inoculums, one was 70% REF2 and 30% OP7 and the other was 30% REF2 and 70% OP7. By doing this, the influence of the initial ratio of the two yeast strains on changes in the ratio during the course of the competition could be studied. The two chemostats were fed with fresh medium taken from the same medium reservoir in order to eliminate all possible variations that may occur during the experiments. This would allow only the proportion between the two strains in the inoculum to be different. Changes in the ratio of the two strains were followed for 45-280 h of incubation. Figure 6.7 shows the plot of ln(R) against incubation time, where R, in these experiments, is the ratio of REF2 to OP7.

The results indicated that the changes in ratio of the two yeast strains were not dependent on the initial ratio because both curves were qualitatively similar throughout the period of study. In both experiments, REF2 which is the Leu$^+$ strain appeared to be strongly inferior during 45-150 h. From 150-200 h, the converse changes in the ratio were observed i.e. the REF2 became superior to the OP7. After 200 h, the ratio appeared to be approaching a plateau and no more changes could be observed in both experiments.

Assays for the activities of TPI and HK as well as protein concentration were carried out. Total specific TPI and HK activities of the mixed culture are shown in Figure 6.8 and 6.9 respectively. Total specific TPI activity of the mixed culture in both vessels (Figure 6.8) reflected the changes in the ratio indicated in Figure 6.7. Between 45-150 h, as the ratio of REF2 to OP7 decreased, an increase in specific TPI activity was observed in both experiments. A converse change in the total specific TPI activity was shown between 150 h and 200 h. After 200 h, the specific TPI activity appeared to stabilize whilst the change in the ratio was approaching a plateau. The total specific HK activity in the mixed culture shown in Figure 6.9 indicates that,

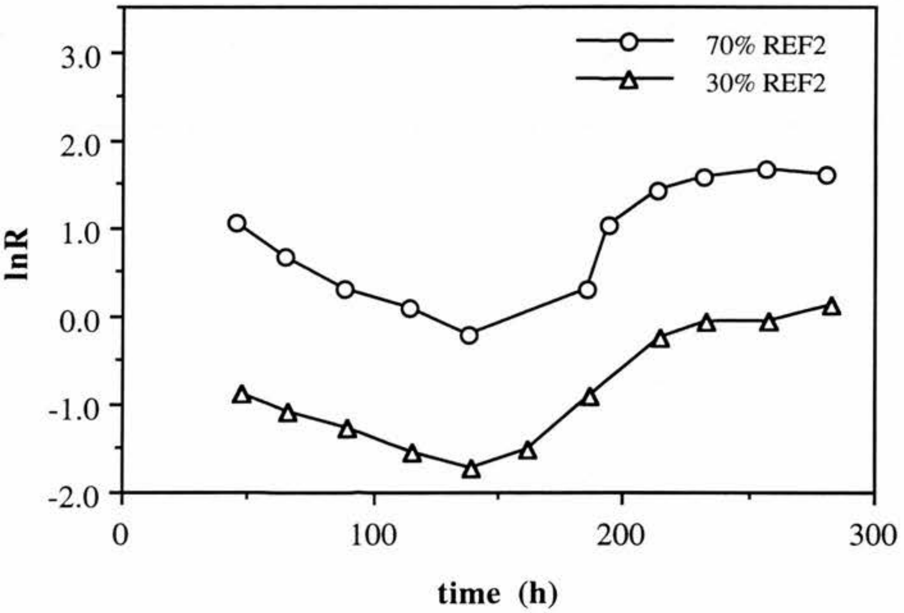


Figure 6.7 Changes in ratio of strains REF2 and OP7 as a function of time during competition experiments, inoculated with 70% REF2 and 30% OP7 and with 30% REF2 and 70% OP7, at $D=0.11h^{-1}$ in an aerobic glucose-limited chemostat culture. R is the ratio of REF2 to OP7.

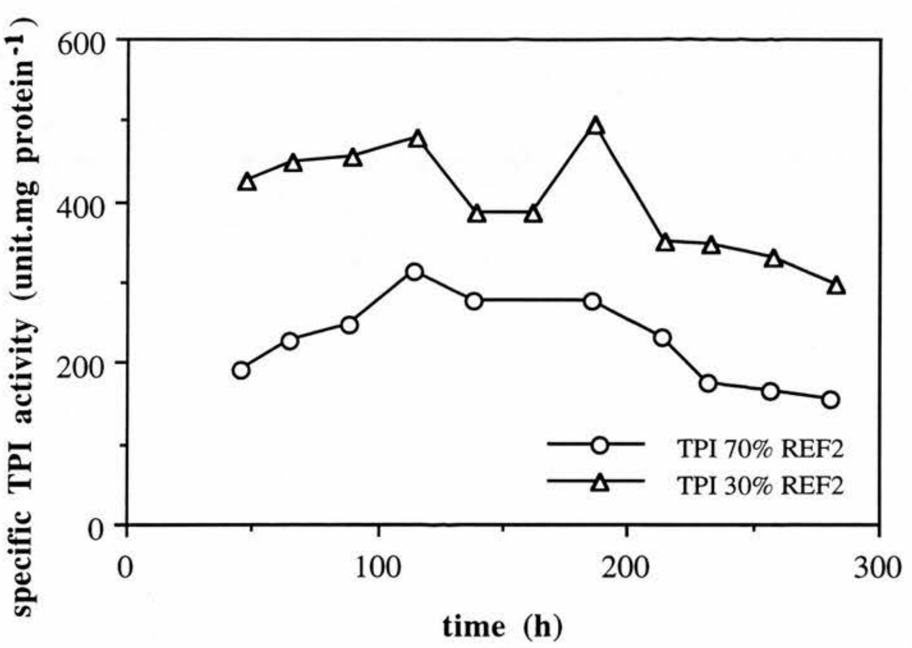


Figure 6.8 Changes in total specific TPI activity of a mixed culture of strains REF2 and OP7 as a function of time during competition experiments at $D=0.11h^{-1}$in an aerobic glucose-limited chemostat culture. The patterns shown by circle and triangle symbols correspond to two competitions inoculated with 70% REF2 and 30% OP7 and with 30% REF2 and 70% OP7, respectively.

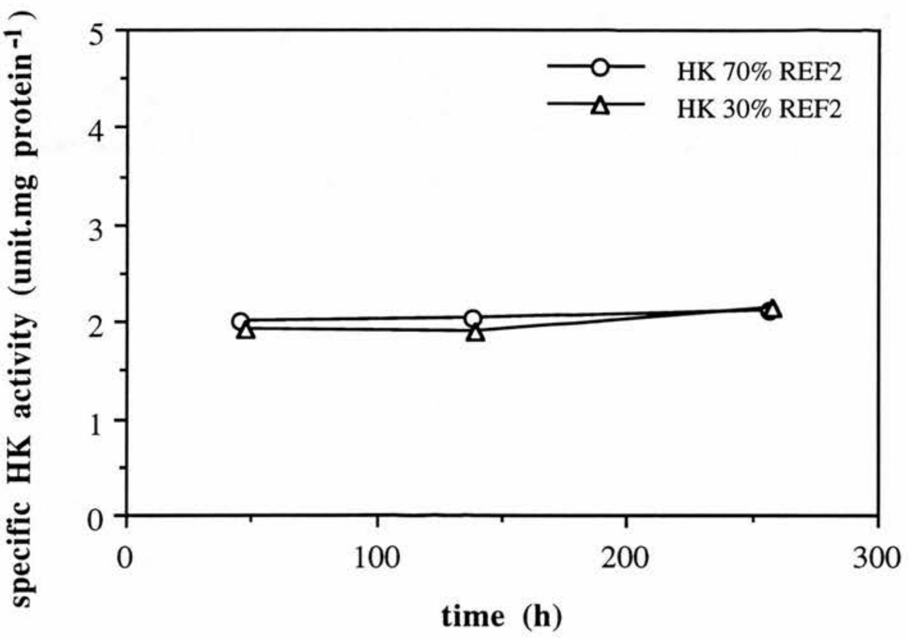


Figure 6.9 Changes in total specific HK activity of a mixed culture of strains REF2 and OP7 as a function of time during competition experiments at D=0.11h^{-1}in an aerobic glucose-limited chemostat culture. The patterns shown by circle and triangle symbols correspond to two competitions inoculated with 70% REF2 and 30% OP7 and with 30% REF2 and 70% OP7, respectively.

although the ratio between the two strains changed as well as total specific TPI activity, the level of HK production remained constant in both experiments.

An initial conclusion which could be drawn from these results is the difference in a starting ratio of the two strains did not affect the changes in ratio during the experiment. However, an explanation is needed for the biphasic curves of the ratio and, consequently, of the total specific TPI activity. The loss of the *LEU2* gene from the chromosome is unlikely to be the cause of this outcome because the reversion of the Leu$^+$ to the Leu$^-$ cells is known to occur at very low frequency i.e. 10^{-12} (Broach *et al.*, 1979).

A possible explanation was sought for the decline in population ratio of REF2 to OP7 during 0-150 h in terms of a hypothetical 2μm plasmid loss. Cells containing the 2μm plasmid (cir$^+$) were found to have a 1.5-3% longer generation time than plasmid free cells (cir^o) (Walmsley *et al.*, 1983; Mead *et al.*, 1986). The original population of both strains is assumed to contain only cir$^+$ cells. If the OP7 cells were to lose their plasmids, for some unknown reason, at a faster rate than the REF2, the former could grow faster. OP7 therefore would overtake REF2 over the period of 150 h from the beginning of the competition. After 150 h (Figure 6.2), the yeast population at this stage would have contained a majority of cir^o cells. This then implies that some mechanism other than the 2μm plasmid loss, possibly by actual selection for TPI overproduction, may have to be taken into account for any further changes. According to an increase in the proportion of the REF2 in the mixed culture from 150-200 h (seen as positive slopes in Figure 6.7), it would follow that the overproduction of TPI is disadvantageous to the yeast growth. No more change could be observed after 200 h. However, at this stage of cultivation both vessels were still expected to contain cir^o cell type population. To clarify this hypothetical 2μm plasmid loss, the presence of the 2μm plasmid in the yeast population at various stages of the competition was investigated.

Southern analysis of the samples taken at 45 h, 150 h and 200 h was carried out by Dr. C. S. Carmichael. Samples of yeast total DNA were prepared, blotted and probed with the *EcoR*I fragment of the 2µm plasmid isolated from pJDB36 (the plasmid was kindly provided by Dr. J. D. Beggs, Institute of Cell and Molecular Biology, University of Edinburgh).

Results indicated that no cir$^+$ cell could be observed at any stage of the competition. The hypothesis on the loss of the 2µm plasmid was therefore abandoned. This outcome had raised a difficulty in explaining the results obtained because some other complicated mechanism may be involved in the occurrence of the biphasic curve. The competition between the strains OP2, the two-fold TPI overproducer, and REF2 was studied to investigate whether or not the biphasic curve would be observed.

6.3 Competitive Chemostat Culture Between the Strains OP2 and REF2

The chemostat was inoculated with 61% REF2 and 39% OP2. From 45-189 h, a biphasic pattern (Figure 6.10), similar to those observed in Figure 6.7, was observed. After 189 h, the ratio appeared to be approaching a plateau the level of which was lower than that observed in Figure 6.7. The total specific TPI activity of the mixed culture showed a slight change over a period of 212 h (Figure 6.11, triangles). The correlation between the changes in ratio and TPI activity could be seen although not as clearly as that seen in the competition between the REF2 and the OP7 because the difference in TPI production between these two strains is only a factor of two. Figure 6.11 (circles) shows only slight increase in total specific HK activity of the mixed culture during 45-287 h.

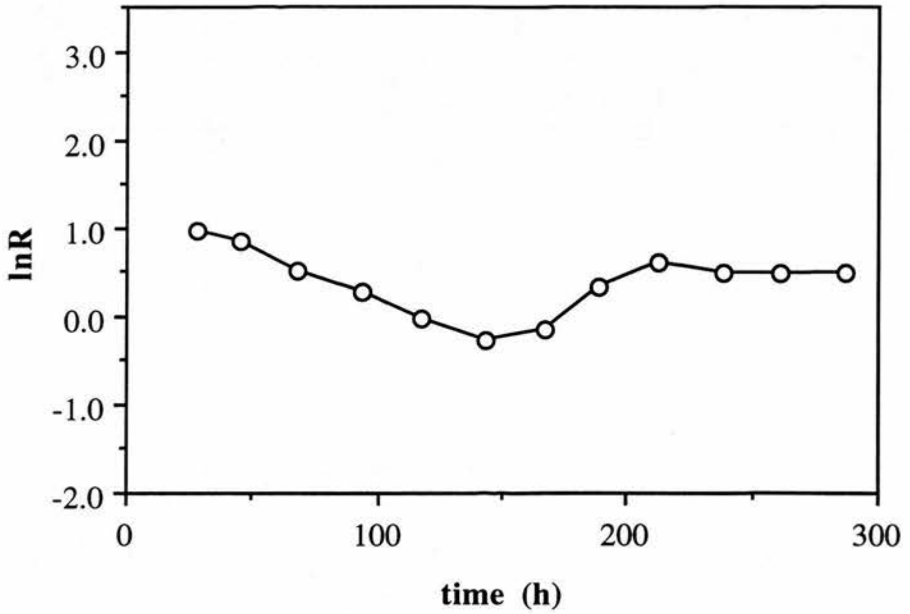


Figure 6.10 Change in ratio of the strains REF2 and OP2 as a function of time during the competition experiment at $D=0.11h^{-1}$in aerobic glucose-limited chemostat culture. R is the ratio of REF2 to OP2.

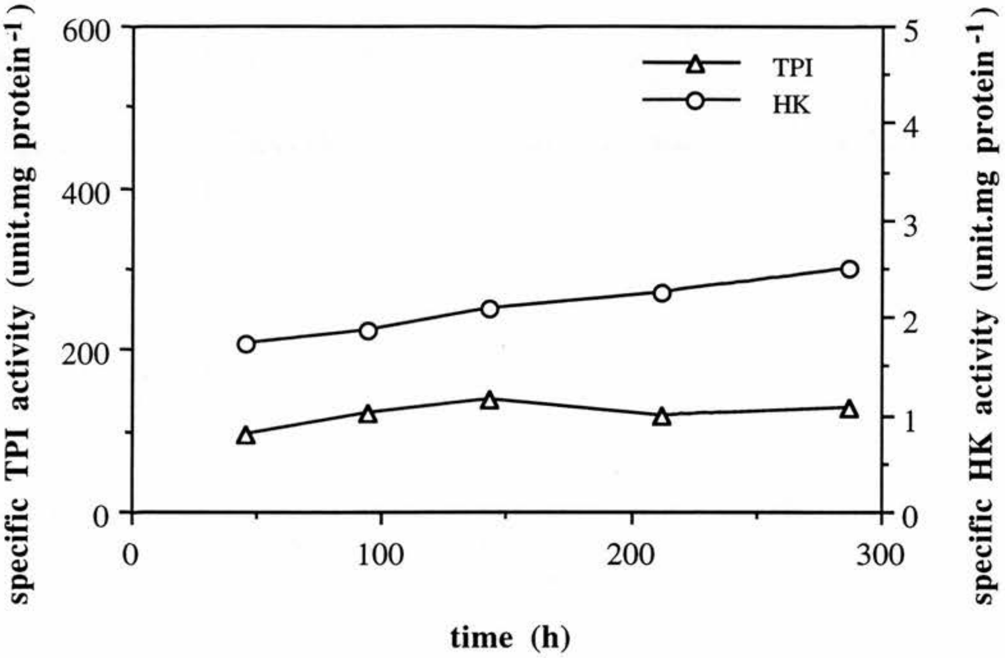


Figure 6.11 Change in total specific TPI activity and total specific HK activity of a mixed culture of strains REF2 and OP2 as a function of time during competition experiment at $D=0.11h^{-1}$in an aerobic glucose-limited chemostat culture.

6.4 Competitive Chemostat Culture Between the Strains REF1 and REF2 (Experiment 2)

In view of the ratio changes observed after 150 h in the previous experiments (section 6.2 and 6.3), it was decided to repeat the competition experiment between the REF1 and the REF2 which had previously shown "neutral marker" behaviour over a 150 h period (Figure 6.6). The chemostat was inoculated with 77% REF2 and 23% REF1. The experiment was carried out in the same manner as that described in section 6.1 except that the cultivation time was extended beyond 150 h and the specific enzyme activities were assayed.

Change in the ratio of REF2 to REF1 were followed from 48-262 h (Figure 6.12). The pattern of the ratio change was found to be quantitatively similar to the result obtained in the competition between the REF2 and the OP2 shown in Figure 6.10. An average of the total specific TPI activity of the mixed culture (Figure 6.13, triangles) was found to be approximately half the level of that obtained in the competition between the REF2 and the OP2 (Figure 6.11, triangles). The level of total specific HK activity of the mixed culture (Figure 6.13, circles) appeared to be approximately the same as that shown in Figure 6.11 (circles).

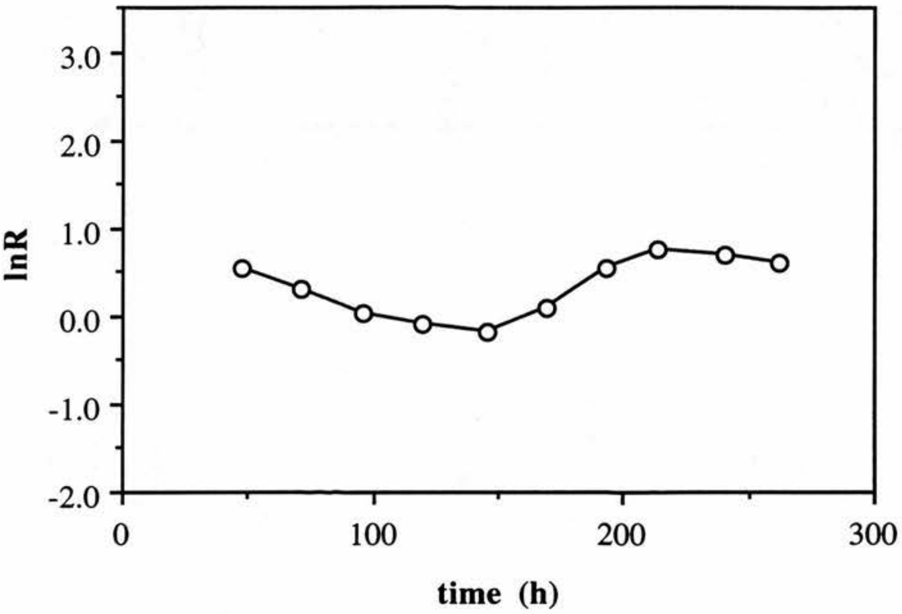


Figure 6.12 Change in ratio of strains REF2 and REF1 as a function of time during competition experiment at $D=0.11h^{-1}$ in an aerobic glucose-limited chemostat culture. R is the ratio of REF2 to REF1.

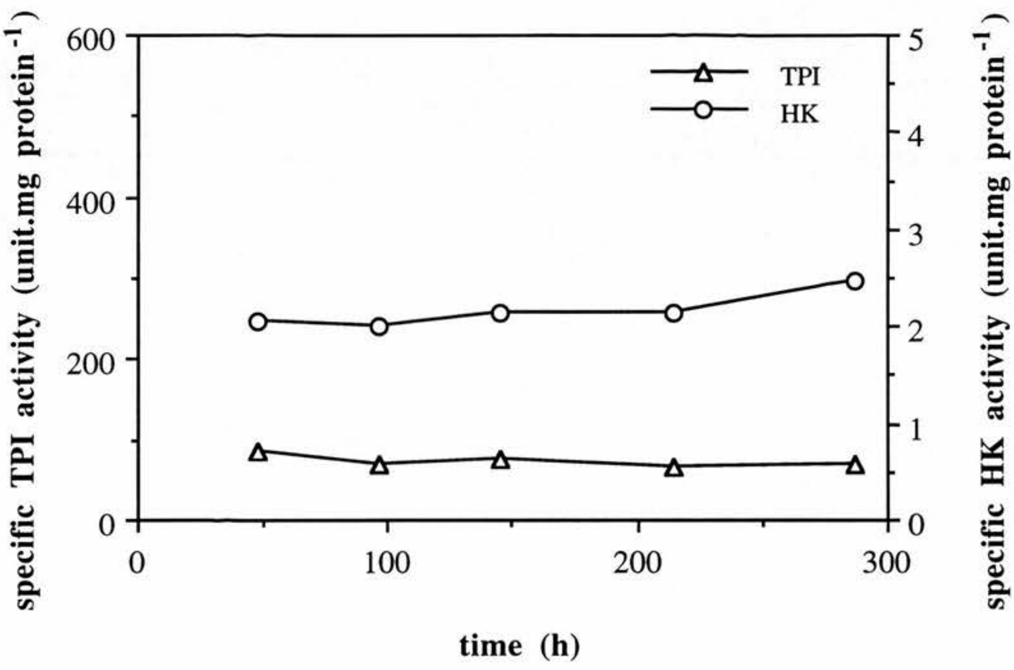


Figure 6.13 Change in total specific TPI activity and total specific HK activity of a mixed culture of strains REF2 and REF1 as a function of time during competition experiment at $D=0.11h^{-1}$ in an aerobic glucose-limited chemostat culture.

The results shown above (section 6.1-6.4) describe the competition between the reference and the TPI overproducers. These results were obtained when the *LEU2* marker was placed on the reference strain only. It was therefore of interest to know what the result would be if the overproducer carried the *LEU2* marker. The OP2L which is a Leu$^+$ derivative of OP2, simultaneously constructed with the REF2, was used to clarify this query. The experiment described below was the reciprocal experiment of that studied in section 6.3.

6.5 Competitive Chemostat Culture Between the Strains OP2L and REF1

OP2L was placed in competition with REF1 which is a leucine auxotroph in the chemostat. Two identical chemostats were set up and inoculated with two different inoculums, one was 74% OP2L and 26% REF1 and the other was 34% OP2L and 66% REF1.

Figure 6.14 shows the plot of lnR against incubation time, where R in these experiments is the ratio of OP2L to REF1. Unexpectedly, biphasic curves were obtained in both experiments. These curves are qualitatively similar to those observed in the competition between the REF2 and the OP2 (section 6.3) except that the turning point appeared at 100 h instead of 150 h as shown before (Figures 6.7, 6.10 and 6.12). In addition, both experiments showed steeper positive slopes in the second part of the curves than those observed in Figures 6.7, 6.10 and 6.12. As a result of these steeper slopes, the saturated levels of the ratio after 200 h were higher than those observed in all previous experiments when compared to the previous results studied at the same proportion between the Leu$^+$ and the Leu$^-$ strains (Figures 6.7-upper curve, 6.10 and 6.12). Change in the total specific TPI activity of the mixed culture followed the same pattern as the change in the ratio of the two strains (Figure 6.15). The level of total specific HK activity throughout the cultivation (Figure 6.16) was similar to the levels observed in all previous experiments (circles in Figures 6.9, 6.11 and 6.13).

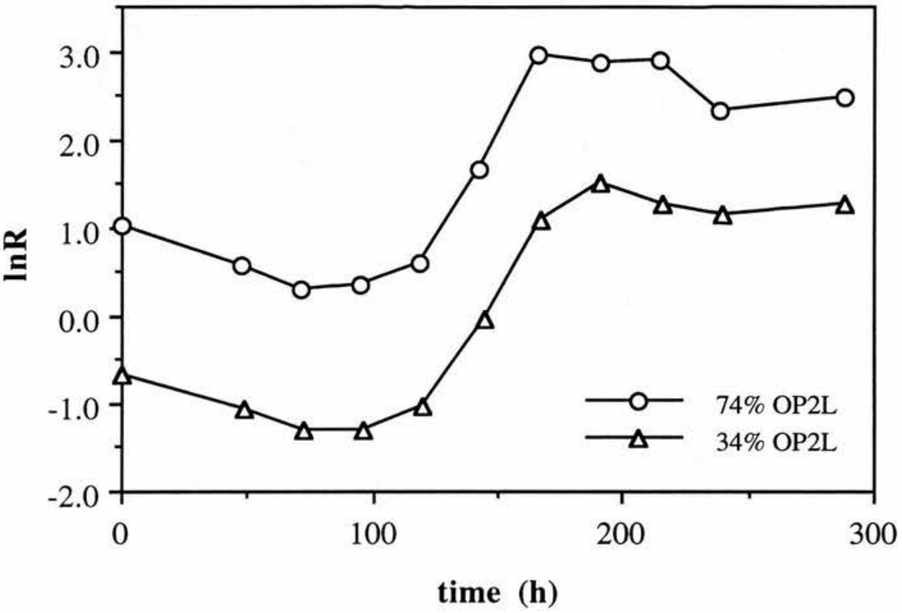


Figure 6.14 Changes in ratio of strains OP2L and REF1 as a function of time during competition experiments, inoculated with 74% OP2L and 26% REF1 and with 34% OP2L and 66% REF1, at $D=0.11h^{-1}$ in an aerobic glucose-limited chemostat culture. R is the ratio of OP2L to REF1.

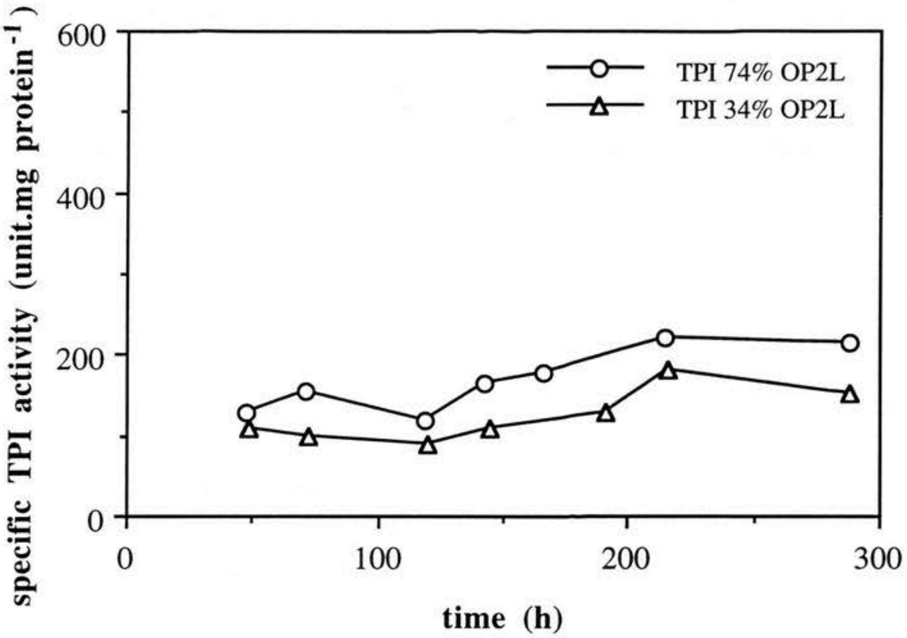


Figure 6.15 Changes in total specific TPI activity of a mixed culture of strains OP2L and REF1 as a function of time during competition experiments at $D=0.11h^{-1}$ in an aerobic glucose-limited chemostat culture. The patterns shown in circle and triangle symbols correspond to two competitions inoculated with 74% OP2L and 26% REF1 and with 34% OP2L and 66% REF1, respectively.

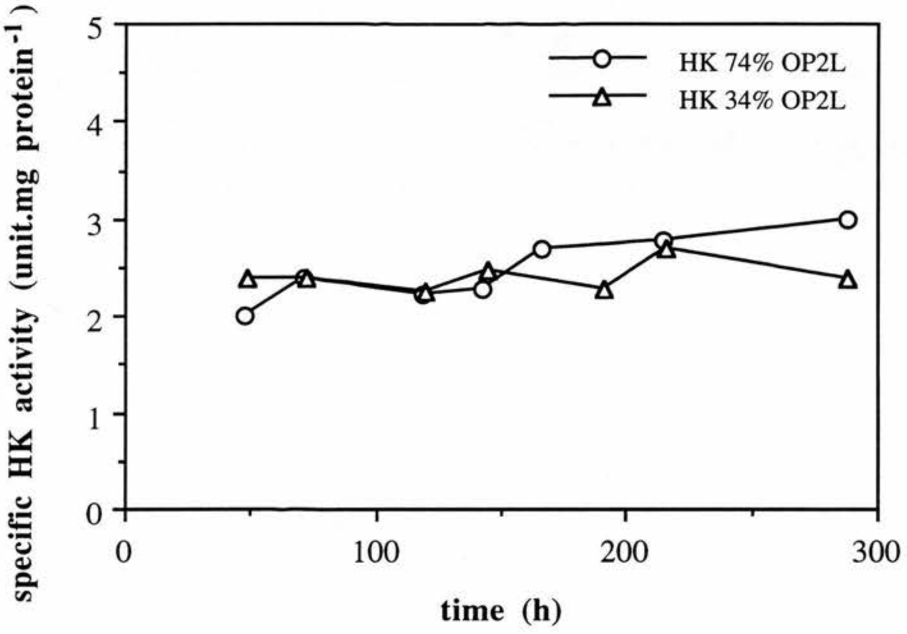


Figure 6.16 Changes in total specific HK activity of a mixed culture of strains OP2L and REF1 as a function of time during competition experiment at $D=0.11h^{-1}$ in an aerobic glucose-limited chemostat culture. The patterns shown in circle and triangle symbols correspond to two competitions inoculated with 74% OP2L and 26% REF1 and with 34% OP2L and 66% REF1, respectively.

All results shown above (sections 6.1-6.5), particularly the pattern of the ratio change, have indicated that the biphasic pattern was the result of the *LEU2* marker placed in the yeast strain REF2. This suggests that the *LEU2* marker had, somehow, altered the metabolism of the original reference strain (REF1). It is therefore concluded that the *LEU2* marker is not neutral under the present conditions of study. The mechanism of this marker effect was not comprehensively investigated. The amino acid contents of the samples taken at 47, 71, 119, 143 and 200 h of the competition between the strains OP2L and REF1 (starting ratio was 74% : 26%) was kindly analyzed by the Roslin Institute, Edinburgh. The results indicated that after 47 h of cultivation most amino acids analyzed including leucine were present at very low concentrations, when compared to the concentration in the feed medium, (see data in Appendix C, Table C.19). The leucine concentration in the feed medium (1.98 mg.100 ml^{-1}) rapidly declined to 45.5 μg. 100 ml^{-1} from 47 h to the end of the experiment. This therefore suggested that leucine limitation (or the combination of other amino acid limitations also) may well have had an effect on the selection of the Leu^+ and Leu^- cells.

The consistency of the correlations between the change in ratio and the change in total specific TPI activity of the mixed culture has provided the possibility of using specific TPI activity as a self-detecting marker for the competition experiment. The following section describes the competition of the reference strain REF1 and the TPI overproducers using the differences in the level of TPI production as an estimate of the proportions of the strains in the mixed culture.

6.6 The Study of Competitive Chemostat Culture Using Specific TPI Activity As a Self-Detector

Two competition experiments were carried out simultaneously, between OP7 and REF1 and between OP7 and OP2. Any possible effects of the *LEU2* gene were excluded because all the strains used are leucine auxotrophs. Two identical chemostat vessels were prepared as described previously. Each vessel was inoculated with 50% of each strain. After inoculation, cell density was very low (OD_{600} was approximately 1.1 unit in both vessels), and a large sample would thus have been needed for an accurate assay for enzyme activity and protein concentration. However, it was undesirable to take too large a sample from the vessel because it would result in longer time needed to resume the steady state. In these experiments, an initial ratio was therefore not assessed. Samples were taken from 45-218 h to assay for TPI activity and protein concentration.

The different proportions of the strains was calculated using the relationship between the specific TPI activity of each strain obtained from an individual chemostat (Chapter 5) and the total specific TPI activity of the mixed culture. This relationship is shown below.

$$\mathrm{spAB} = \mathrm{spA}\left(\frac{\mathrm{A}}{\mathrm{T}}\right) + \mathrm{spB}\left(1 - \frac{\mathrm{A}}{\mathrm{T}}\right)$$

therefore:

$$\frac{\mathrm{A}}{\mathrm{T}} = \frac{\mathrm{spAB} - \mathrm{spB}}{\mathrm{spA} - \mathrm{spB}}$$

where spA is the specific TPI activity of strain A obtained from an individual chemostat culture

spB is the specific TPI activity of strain B obtained from an individual chemostat culture

spAB is the total specific TPI activity of the mixed culture obtained from a competitive chemostat and $\frac{A}{T}$ is the proportion of the strain A in a mixed population

Specific TPI activity of strains REF1, OP2 and OP7 obtained from individual chemostats used in this experiment were 78.9±16.5, 150.0±5.9 and 510.0±66.8 unit.mg protein^{-1} respectively. To assess the selection coefficient, a plot of the natural logarithm of the ratio of the two populations with time was carried out. It appeared from the experimental data (Appendix C, Table C.20-21) that some values of the total specific TPI activity of the mixed culture would generate negative values of the ratio between the two strains and, as a result, the transformation to the logarithmic value would be impossible. This could be due to experimental errors. To overcome this problem, the value of $\frac{A}{T}$ at the first sampling time was calculated assuming that the mixed culture contained an equal proportion of each strain. Having done this, the spB was obtained and it was used to calculate the values of $\frac{A}{T}$ in all the rest of the samples in that vessel. The natural logarithm of the adjusted data, shown in Appendix C, Table C.20-21, were plotted against time as shown in Figure 6.17 and 6.18 for the competition between the strains OP7 and OP2 and between the OP7 and REF1, respectively. The linear regression lines of these plots were obtained by the least squares fit method using the program Minitab for the Macintosh computer.

Test for evidence of a zero slope was carried out using the program Minitab for the Macintosh computer (Appendix B, section 3.2). Results indicated that the regression line computed for the competition between strains OP7 and OP2 was not significantly different from a zero slope line (p=0.238). This implies that strain OP7 appeared to be neutral to strain OP2 and, therefore, the relative fitness of strain OP7 against OP2 could be assumed as 1. An evidence of a non-zero slope was observed for the regression line computed for the competition between the OP7 and the REF1 (p=0.058) which indicated that strain OP7 was selectively inferior to strain REF1 with the selection coefficient and relative fitness of -0.0132 and 0.8789, respectively.

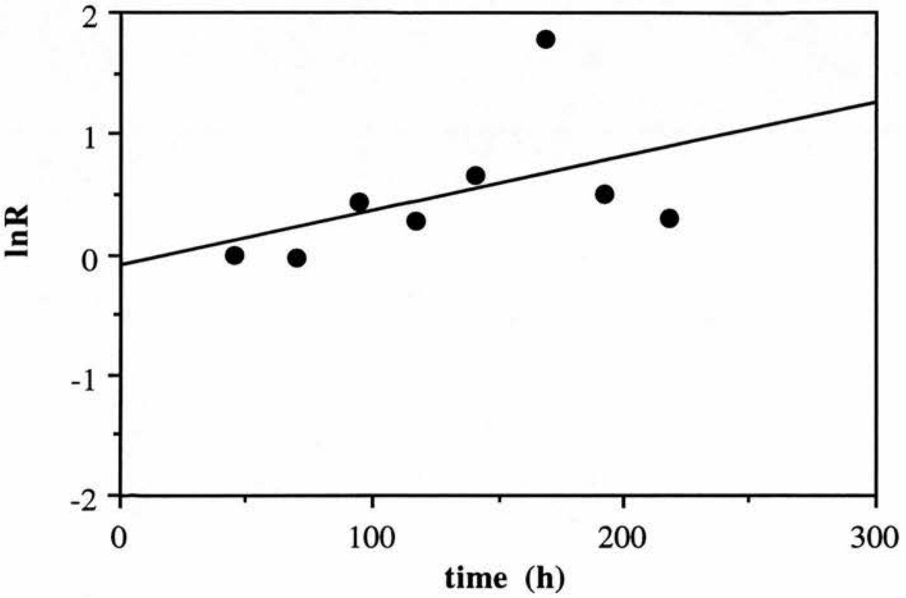


Figure 6.17 Changes in ratio of strains OP7 and OP2 as a function of time during a competition experiment at $D=0.11h^{-1}$ in an aerobic glucose-limited chemostat culture. R is the ratio of OP7 to OP2.

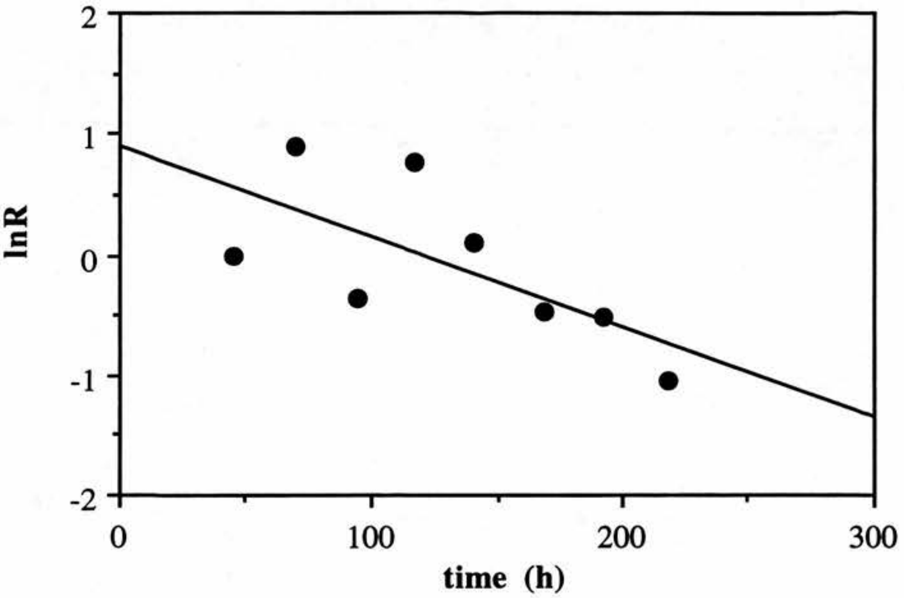


Figure 6.18 Changes in ratio of strains OP7 and REF1 as a function of time during a competition experiment at $D=0.11h^{-1}$ in an aerobic glucose-limited chemostat culture. R is the ratio of OP7 to REF1.

The relative fitness of strains OP2 and OP7 with respect to strain REF1 are shown in Table 6.2.

Table 6.2 The relative fitness of strains OP2 and OP7 with respect to strain REF1.

strain	relative fitness
REF1	1
OP2	0.8789*
OP7	0.8789

* this figure was obtained as described below

From Figure 6.17, the relative fitness of strain OP2 to strain OP7 (or strain OP7 to strain OP2) = 1

From Figure 6.18, the relative fitness of strain OP7 to strain REF1 = 0.8789

Therefore, the relative fitness of strain OP2 to strain REF1 = 1x0.8789

= 0.8789

Plot of the relative fitness of the three yeast strains studied with respect to the normal level TPI producing strain (REF1) against enzyme concentration is shown in Figure 6.19. The plot suggested that an increase in the *in vivo* TPI activity by a factor of two has been disadvantageous to the overproducer, as shown by the reduction in relative fitness when compared to the normal strain. However, further increase in the *in vivo* TPI activity by a factor of seven showed no effect on the relative fitness of the yeast strain when compared to that of the two-fold TPI overproducer. This is not entirely surprising in view of known non-linearities of enzyme activities and flux consequences (Kacser & Burns, 1973). The relationship can well reach a plateau of effects after doubling of TPI activity.

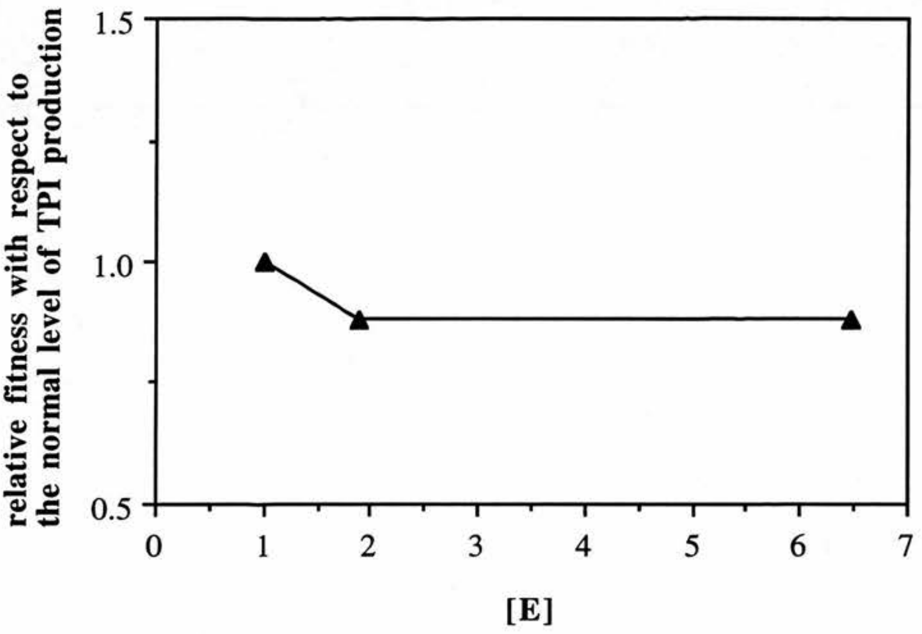


Figure 6.19 Relative fitness of strains REF1, OP2 and OP7 with respect to the normal level of TPI production. [E] represents TPI concentration (or activity) observed in an individual chemostat at D=0.11 h^{-1}.

Discussion

The *LEU2* gene placed in the yeast genome in the present study has been found to produce an unexpected effect on the yeast strains. The effect was shown as a biphasic pattern of the change in ratio of Leu^+ and Leu^- strains. This outcome was obtained repeatedly irrespective of which strain the marker was placed on. The cause of this outcome has not been comprehensively investigated. Because the culture was grown under non-selective conditions i.e. leucine supplemented medium, the auxotrophic Leu^- strain could have a selective advantage over the prototrophic strain due to energetic economy (Harder *et al.*, 1977). During the first 150 h, the leucine auxotroph used leucine in the medium straight away while the leucine prototroph synthesized its own leucine. Cell energy must be required for this purpose. The selective advantage of the prototrophic Leu^+ strain during the second part of the biphasic pattern could be a consequence of the depletion of leucine (and other amino acids in the medium observed in section 6.5 after 45 h).

Moreover, an adaptive mutation was unlikely to be the cause of the converse change found after 150 h, which corresponds to approximately 24 yeast generations (number of generation = $\frac{\text{time} \times \text{D}}{\ln 2}$). This period of time appeared to be too soon to take into account an adaptive mutation in the yeast *S. cerevisiae* which was previously shown to appear only after 100 generations in a glucose-limited chemostat culture (Paquin & Adams, 1983a,b).

It should be noted that all the results obtained after 300 h of the competition between Leu^+ and Leu^- strains described in section 6.2-6.5 were excluded. This was due to the small colonies, appearing in plates during the colony counting, which were found to be Leu^- and subsequently overtook the yeast population in the culture vessel. According to a low leucine content in the growth medium observed in section 6.5, these small colonies were considered as leucine 'scavengers' which adapted from the phenotypically and genotypically (*leu2-3,112*) Leu^- cells of *S. cerevisiae* when grown in a leucine-limiting, but not deprived, environment (Heinemann *et al.*, 1994).

The use of the *LEU2* gene as a marker during the competition experiment between the PGK overproducer and the normal level PGK producing strains has been reported (van der Aar, 1990a). The decline in the proportion of the PGK overproducer which carried the *LEU2* gene in the chromosome was found during 0-150 h cultivation in a similar manner to the decline of the Leu$^+$ population repeatedly observed in the competition carried out in this work. The authors discussed their results as a consequence of the high level of the PGK overproduction. However, the experiment was not conducted further than 150 h where the converse change in the ratio was consistently observed in all the competition experiments carried out in this work.

Competition experiments in which the *LEU2* marker had been replaced by a self-indicative marker were carried out, and the reduction of relative fitness of the yeast strain was found in the two-fold TPI overproducer. The disadvantage observed in this case could not be due to the extra energetic cost of the extra amount of TPI synthesis in yeast cell because, if so, a larger reduction in the relative fitness would be seen in the case of seven-fold TPI overproduction. Metabolic regulation within the glycolytic pathway itself or, by some unknown mechanism, in connection with other parts of cell metabolism may have been involved. The possibility of metabolic compensation in the glycolytic pathway was considered because of the lower levels of PYK and HK activities observed in individual chemostat study (Chapter 5) of the OP7 compared with the REF1 and OP2. In addition, the fact that ethanol, acetate and pyruvate production were elevated in the OP7 compared to the REF1 and OP2 when studied in individual chemostats (Chapter 5) may also indicate some such metabolic compensation as a result of TPI overproduction. Moreover, TPI overproduction could affect cell physiology e.g carbohydrate accumulation, responses to environmental challenges and sporulation as found in the overproduction of other glycolytic enzymes (Rosenzweig, 1992).

The non-linear relationship between the relative fitness and the amount of TPI (Figure 6.19) suggested that TPI may be not an "equilibrium enzyme" as is usually claimed. These results indicated that increase in TPI activity up to seven times normal level had less effect to the pathway than that observed in the two-fold increase. This is not surprising, in terms of non-linearity of response in the relative fitness to a large increase in enzyme activity. Based on steady state analysis of linear pathways, metabolic control theory predicts that a large increase in the activity of one enzyme will have negligible effect on the flux through the pathway when compared to a large decrease in the activity of that enzyme (Kacser & Burns, 1973, 1981). This prediction has been discussed as if flux is held to co-vary with fitness and both are maximized, a plot of average fitness versus the activity of a single enzyme will result in a saturating, hyperbolic function (Harlt *et al.*, 1985; Dean *et al.*, 1986; Dykhuizen *et al.*, 1987; Dean, 1989). The results obtained in this thesis, therefore, appear to be consistent with these circumstances.

Chapter 7

General Discussion and Future Study

7.1 General Discussion

The investigations described in this work started by asking the apparently simple question: Does the insertion of several copies of a *TPI* gene (and hence an increase in enzyme activity) alter the flux through the glycolytic pathway? The proposed method (discussed below) was to integrate one to several gene copies of the TPI specifying gene (*TPI1*) into the yeast chromosome. Thereafter, enzyme activity determinations showed that two strains, one containing one extra copy and showing twice the enzyme activity (strain OP2) and one containing several copies and showing approximately seven times the normal level of enzyme activity (strain OP7), were chosen from ten isolates studied. Growth experiments of the TPI overproducers and the reference strain, both in batch and in individual chemostat, showed very small differences between the strains or in the output of the pathway (glycolytic metabolites). Two competitions were carried out using enzyme activity as a self indicative marker to avoid the undesired effect of the *LEU2* marker (see Chapter 6). Results from competition experiments indicated that an increase of TPI activity to two-fold has introduced a disadvantage to yeast growth as observed by a reduction in relative fitness of this strain with respect to the reference strain. A further increase of the TPI activity up to seven-fold did not result in any further reduction in relative fitness of the yeast strain.

Several methods have now become available to use to introduce extra copies of genes into yeast cells in order to get a high level of gene expression. Genetic stability of the recombinant DNA obtained from different means of introducing extra copies of genes into genome has been shown to vary with the method used. The yeast integrative plasmid was favoured and employed in this work to introduce extra copies of glycolytic genes into yeast cells instead of an episomal plasmid. The use of an episomal plasmid was rejected because of the variation in gene copy number which could occur due to unequal segregation during cell division leading to a heterogeneous yeast population. This would therefore make the interpretation of the results difficult as well as creating problems of reproducibility. In addition, it is well known that a spontaneous loss of plasmid and the recombinant DNA could occur during the long-term growth on non-selective medium (Futcher & Cox, 1984; DiBiasio & Sardonni, 1986; Parker & DiBiasio, 1987). The latter disadvantage was seriously considered in particular view of growth in chemostat culture where the culture would be subjected to a weak selective growth medium over a very long-term cultivation. All the problems mentioned above i.e. genetic and metabolic variation as well as the heterogeneity of yeast population and the plasmid loss during long-term cultivation could be avoided using integrative plasmid. By integrating extra copies of genes into chromosome, these extra copies were inherited in a Mendelian fashion. As a result, homogeneity yeast population was obtained. In addition, the integration of the extra copies into chromosome also resulted in a more genetically stable recombinant DNA than that obtained when using episomal plasmid. The merit of integrative plasmids has been shown in this thesis from the stability in enzyme activity levels found during the long-term growth in chemostat culture at different rates of growth (see Chapter 5). van der Aar *et al.* (1990a,b) found that the phosphoglycerate kinase overproducing strain of yeast *S. cerevisiae* carrying 80 copies of *PGK1* gene integrated into the genome was genetically unstable. In a subsequent study, van der Aar *et al.* (1992) preferred to use the multicopy 2μ-based episomal plasmid instead of the integrative plasmid they used previously. The unexpected genetic instability observed by van der Aar *et al.* (1990a,b) appeared to conflict with the widely accepted finding that the recombinant DNA located on the 2μ-based episomal plasmid is genetically unstable. However, the results observed by van der Aar *et al.* (1990a,b) could be due to very high level of *PGK1* overexpression (approximately 40% of soluble cell proteins) i.e. gene dosage effect which might intensify selective pressure to the yeast strains under conditions of study and therefore resulted in the instability of recombinant DNA.

The *in vivo* effects of enzyme activity alterations could be measured in two ways. On the one hand, flux through glycolysis from glucose to ethanol of the yeast strain carrying extra copies of particular genes and the wild-type strain could be quantitated and determined by assay of metabolite concentration. On the other hand, the *in vivo* effects could be measured by determining relative fitness of the modified strain in competition with the wild-type strain.

The study of yeast metabolism in batch culture may have limited accuracy due to the continuous changes in environmental conditions. As discussed in Chapter 4, the characteristic of batch cultivation as a closed-system was still present although a much more controllable culture system than shake flask cultivation was employed. Metabolic products can be well accumulated and utilized by the culture after the actual substrate becomes exhausted. In addition, continuous changes in concentration of glucose supplied to batch growth of yeast (Chapter 4) may also affect the glucose transport system. If the glucose transport is altered as a consequence of continuous change of glucose levels in the medium, the interpretation of results will be very difficult as the results would be consequences of more than one metabolic alteration i.e. both enzyme activity and glucose transport alterations. The use of chemostat therefore diminishes this possibility because glucose concentration in chemostat culture can be kept constant as long as the dilution rate of the culture is unchanged.

In this work the study,in chemostat culture, of TPI-overproducing strains along with the reference strains did not show obvious differences between the strains as might have been expected. This could be due to several factors. Non-significant differences between the two-fold TPI overproducer and the reference could be due to the fact that very small differences exist between these two strains. On the other hand, very small differences when compared to the reference strain may be present in the two-fold overproducer. As a result, within the limits of detection used in this work e.g. HPLC analysis of metabolite concentrations, such a small difference could not be detected. In the case of the seven-fold TPI overproducer, some differences could be seen although these were not very clear. The small, but still detectable, amount of ethanol found in this strain under conditions at which the two-fold TPI overproducer and the reference strain did not show ethanol production i.e. at low dilution rate, could indicate that the seven-fold overproducer may possess a distinct metabolism. The lowered steady state activities of pyruvate kinase and hexokinase in the seven-fold TPI overproducer were consistent with those observed in batch experiments. These results therefore confirm that this strain does differ from the others.

The strains were then subjected to the much more sensitive competition experiments where the two strains were grown together in chemostat culture. Competitive chemostats were successfully used in study of relative fitness of bacteria (Dean *et al.*, 1986; Dean, 1989; Dykhuizen & Davies, 1980). Competitive chemostat culture has also been applied in yeast studies, although not in as many cases as in bacterial studies. Postma *et al.* (1989) employed competitive chemostat to study contamination of wild yeast in bakers' yeast production. It was also used to study the physiological consequences of phosphoglycerate kinase overproduction (van der Aar *et al.*, 1990a).

A genetic marker is required to distinguish one strain from the other in a competitive chemostat study. The consequences of introducing a genetic marker into a strain may requires thorough examination to ensure that the marker is definitely neutral under the conditions of study. The *LEU2* marker used in this work is an obvious example of a non-neutral marker effect which has never been reported for any previous use of this gene. The *LEU2* marker which was originally thought to be a neutral marker ultimately showed an undesirable effect upon the selection of the yeast strain under the conditions of study. van der Aar *et al.* (1990a) also employed the *LEU2* marker to distinguish the PGK overproducer and the wild-type yeast strain in competitive chemostat experiments and did not report any effect of the *LEU2* marker. It should be noted that the time course of this competition experiment was not long enough to ensure the neutrality of the *LEU2* marker under the experimental conditions used in this study (van der Aar *et al.*, 1990a) with respect to the results observed in this thesis (Chapter 6). However, the effect of *LEU2* gene may vary under different circumstances i.e. depending on yeast strain or culture conditions. This is why a thorough primary investigation is required prior to the actual study being undertaken.

7.2 Future Study

Although the original aim of this thesis, which was to study the entire glycolytic pathway was not accomplished due to limitations in time, the details and experimental results obtained here can be, at least, used as preliminary information to help future investigation of other glycolytic enzyme modulations.

As mentioned previously some results observed in both batch and chemostat cultures are still unclear. This could be due to the fact that the magnitude of changes in enzyme activity i.e. two and seven times the normal level was not high enough to illustrate clear differences between the strains. One simple thing which could be done to test this point of view is to screen for TPI overproducing strains which possess very high levels, e.g. 20-fold, of enzyme production using similar experimental approaches to those carried out here. However, one should bear in mind that a very high level of the expression of one gene may stimulate some metabolic regulation of the cell leading to mis-interpretation of the results.

Alternatively, instead of using the TPI overproducer, a TPI underproducing strain can also be employed as a model of study. Several methods may be used to construct mutant strains which possess less than the normal level of *TPI* gene expression and hence less *in vivo* enzyme concentration. This could be achieved, for example, by replacing the chromosomal TPI gene with one in which the promoter had been partially deleted (Rothstein, 1983). Site-directed mutagenesis to alter enzyme activity may also be employed although, in some cases, site-directed mutagenesis of the TPI subunit interface has been shown to alter the physical characteristics of the enzyme such as to make it less resistant to environmental change than the wild-type strain (Casal *et al.*, 1987). In addition, the use of antisense mRNAs may be used to decrease enzyme activity (Haeuptle *et al.*, 1986; Izant & Weintraub, 1985; van der Krol *et al.*, 1988; Lichtenstein, 1988; Stitt *et al.*, 1991).

To achieve the original aim of the project, namely to study the changes in metabolic flux of all the modified yeast strains which have been altered in their glycolytic enzyme production, other glycolytic genes will therefore be the next targets of the study. All the other glycolytic genes can be studied in the same manner as TPI, except that the method to achieve the alteration of enzyme activity may be required to be different because different genes possess different structures and may differ in their expression.

Once all the glycolytic enzymes have been modulated, the yeast strains possessing single enzyme activity changes in their metabolism can be characterized in the same manner as with the TPI overproducers i.e. study in individual chemostat culture and competitive chemostat culture. In addition, it would be of interest to study and characterize the metabolism of strains which possess two or more enzymes modulated in their activity.

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Appendix A
Composition of Media

Table A.1 Composition of LB medium.*

component	composition $(g.L^{-1})$
bacto-tryptone	10
bacto-yeast extract	5
NaCl	10

* To obtain LB agar, 15 g of bacto-agar is added per 1 L of medium.

Table A.2 Composition of YEPDmedium*.

component	composition $(g.L^{-1})$
yeast extract	10
bacto-peptone	20
glucose	20

* To obtain YEPD agar, 15 g of bacto-agar is added per 1 L of medium.

Table A.3 Composition of Y0-uracil or leucine medium*.

component	composition $(g.L^{-1})$
yeast nitrogen base (without amino acids)	6.7
glucose	10

- 10 ml 100x amino acids mixture without uracil (Table A.4) was added per 1 L of medium for YO-Ura. Same amino acids mixture, except that 0.5 $g.100ml^{-1}$ leucine was replaced by 0.1 $g.100ml^{-1}$ uracil, was added (10 ml) to obtain YO-Leu.

* To obtain YO-Ura or Leu agar, 15 g of bacto-agar is added per 1 L of medium.

Table A.4 Composition of 100x amino acids mixture (uracil omission stock).

amino acid	composition (g 100 ml^{-1})
aspartic acid	0.5
glutamic acid	0.5
histidine	0.1
isoleucine	1.5
leucine	0.5*
lysine	0.15
methionine	0.1
phenylalanine	0.25
serine	1.75
threonine	1.0
tryptophan	0.2*
tyrosine	0.15
valine	0.75

* prepared separately and added before used

note 1) Arginine (0.1 g) was also added per 100 ml stock solution

2) This stock solution did not contain alanine, cysteine, glycine and proline.

Table A.5 Composition of 20%(w/v) casamino acids stock solution (modified from Difco Catalogue.

component	concentration (% w/v)
ash	3.40
total nitrogen	11.15
arginine	3.80
aspartic acid	0.49
glutamic acid	5.10
glycine	1.10
histidine	2.30
isoleucine	4.60

Table A.5 (cont.)

component	concentration
leucine	9.90
lysine	6.70
methionine	2.20
phenylalanine	4.00
threonine	3.90
tryptophan	0.80
tyrosine	1.90
valine	7.20
phosphorus	0.35
iron	0.0006
SiO_2	0.053
potassium	0.88
sodium	0.77
magnesium	0.0032
calcium	0.0025
chloride	11.20
manganese	7.60
lead	4.00
arsenic	0.50
copper	10.00
zinc	8.00
pyridoxine	0.073
biotin	0.102
thiamine	0.12
nicotinic acid	2.70
riboflavin	0.03

Table A.6 An amino acid composition in the defined medium supplemented with amino acids stock solution (Wilson, 1985) and commercial casamino acids supplied by Difco.

composition	casamino acids (mg.100 ml medium^{-1})	amino acids mixture (mg.100 ml medium^{-1})
aspartic acid	0.10	0.50
glutamic acid	1.02	0.50
histidine	0.46	0.10
isoleucine	0.92	1.50
leucine	1.98	0.50[a]
lysine	1.34	0.15
methionine	0.44	0.10
phenylalanine	0.80	0.25
serine	nil	1.75
threonine	0.78	1.00
tryptophan	0.36[b]	0.20[a]
tyrosine	0.38	0.15
valine	1.44	0.75

[a] final concentration made up from pure stock solutions only

[b] final concentration made up by adding pure tryptophan stock solution into the original tryptophan available in casamino acid

Table A.7 Composition of defined medium components (modified from Postma *et al.* (1989 and Bruinenberg *et al.* (1983)).

component	composition (mg.L^{-1})
I Salt solution	
$(NH_4)_2SO_4$	7.5×10^3
KH_2PO_4	3.5×10^3
$MgSO_4.7H_2O$	750
EDTA	15

Table A.7 (cont.)

component	composition (mg.L^{-1})
II Trace element solution	
$ZnSO_4.7H_2O$	4.5
$CoCl_2.6H_2O$	0.3
$MnCl_2.4H_2O$	1.0
$CuSO_4.5H_2O$	0.3
$CaCl_2.2H_2O$	4.5
$FeSO_4.7H_2O$	3.0
$NaMoO_4.2H_2O$	0.4
H_3BO_3	1.0
KI	0.1
III Vitamin solution	
Biotin	0.05
Calcium Pantothenate	1
Nicotinic acid	1
Inositol	25
Thiamine HCl	1
Pyridoxine HCl	1
para-Aminobenzoic acid	0.2
IV Glucose stock solution	
V amino acids / casamino acids	
- amino acids	diluted from 100x amino acids mixture stock solution prepared as describe in Table A.4.
- casamino acids	diluted from 20% (w/v) stock solution

Appendix B
Statistical Procedures

1 Linear Regression

The linear regression equation was computed using the program Cricket Graph for the Macintosh computer. The program is based on the Least square fit method.

1.1 Batch Data

The slopes of growth, carbon dioxide production rate (CPR) and metabolite concentration curves described in section 4.1-4.3 (Chapter 4) were obtained from the linear regression equations shown in Table B.1.

1.2 Competitive Chemostat Data

Linear regression equations for the competition experiments carried out in section 6.6 (Chapter 6) are shown in Table B.2.

2 Homogeneity of The Regression Coefficients

2.1 Batch Data

The rate of growth and metabolite production between the three yeast strains studied in section 4.1-4.3 (Chapter 4) were tested for a homogeneity of the regression coefficients. The procedure was carried out using the null hypothesis and tested by an appropriate *F*- test in an analysis of covariance (Steel & Torrie, 1980). Results are shown in Table B.3-B.7.

Table B.1 Linear regression equations for experimental data of three strains studied in batch culture.

measurement	strain	regression equation	coefficient of determination (r^2)
OD_{600}	REF1	lnOD600 = -1.9639+0.37624(time)	0.996
	OP2	lnOD600 = -2.5447+0.40003(time)	0.998
	OP7	lnOD600 = -2.2808+0.39018(time)	0.995
dry weight	REF1	lndry weight = -2.2321+0.32992(time)	0.992
	OP2	lndry weight = -1.7810+0.26769(time)	0.990
	OP7	lndry weight = -3.2949+0.41365(time)	0.993
CPR	REF1	lnCPR = -3.3448+0.34657(time)	0.945
	OP2	lnCPR = -4.0402+0.40584(time)	0.995
	OP7	lnCPR = -3.9359+0.40727(time)	0.997
[ethanol]	REF1	ln[ethanol] = 1.25780+0.38925(time)	0.998
	OP2	ln[ethanol] = 0.84711+0.39378(time)	1.000
	OP7	ln[ethanol] = 0.87027+0.41318(time)	1.000
[glycerol]	REF1	ln[glycerol] = -1.1572+0.30345(time)	0.994
	OP2	ln[glycerol] = -1.4197+0.30334(time)	1.000
	OP7	ln[glycerol] = -1.6227+0.34544(time)	0.993

Table B.2 Linear regression equations for experimental data of two competitions studied in competitive chemostat culture.

competition	regression equation	coefficient of determination (r^2)
OP7 vs OP2	lnR = -0.093267+0.0044598(time)	0.224
OP7 vs REF1	lnR = 0.90468-0.0075246(time)	0.469

Table B.3 Test for homogeneity of the regression coefficients obtained from OD600 lines (Figure 4.4) of the three yeast strains

treatment	df	$E_{xx}=\Sigma(x-x)^2$	$E_{xy}=\Sigma(x-x)(x-y)$	$E_{yy}=\Sigma(y-y)^2$	df	Residual SS
REF1 strain	5	17.5	6.5820	2.4930	4	0.01742
OP2 strain	5	17.5	7.0010	2.8154	4	0.01460
OP7 strain	5	17.5	6.8255	2.6881	4	0.02596
Residuals from individual regressions					12	0.05798
Total for single regression	15	52.5	20.4085	7.9965	14	0.06304
Difference for homogeneity of regressions					2	0.06304-0.05798 = 0.00506

$$F = \frac{\frac{0.00506}{2}}{\frac{0.05798}{12}} = 0.52\text{, with 2 and 12 df}$$

Table B.4 Test for homogeneity of the regression coefficients obtained from dry weight lines (Figure 4.4) of the three yeast strains

treatment	df	$E_{xx}=\Sigma(x-x)^2$	$E_{xy}=\Sigma(x-x)(x-y)$	$E_{yy}=\Sigma(y-y)^2$	df	Residual SS
REF1 strain	5	17.5	5.7730	1.9349	4	0.03047
OP2 strain	5	17.5	4.6850	1.2748	4	0.02056
OP7 strain	5	17.5	7.2370	3.0380	4	0.04519
Residuals from individual regressions					12	0.09622
Total for single regression	15	52.5	17.6950	6.2477	14	0.28364
Difference for homogeneity of regressions					2	0.28364-0.09622 = 0.18742

$$F = \frac{\frac{0.18742}{2}}{\frac{0.28364}{12}} = 3.96^{*}, \text{ with 2 and 12 df}$$

Table B.5 Test for homogeneity of the regression coefficients obtained from CPR lines (Figure 4.5) of the three yeast strains

treatment	df	$E_{xx}=\Sigma(x-x)^2$	$E_{xy}=\Sigma(x-x)(x-y)$	$E_{yy}=\Sigma(y-y)^2$	df	Residual SS
REF1 strain	5	17.5	6.0630	2.3033	4	0.20273
OP2 strain	5	17.5	7.1000	2.8993	4	0.01873
OP7 strain	5	17.5	7.1265	2.9126	4	0.01048
Residuals from individual regressions					12	0.23194
Total for single regression	15	52.5	20.2895	8.1152	14	0.27398
Difference for homogeneity of regressions					2	0.27398-0.23194 = 0.04204

$$F = \frac{\frac{0.04204}{2}}{\frac{0.23194}{12}} = 1.09, \text{ with 2 and 12 df}$$

Table B.6 Test for homogeneity of the regression coefficients obtained from ethanol production lines (Figure 4.6) of the three yeast strains

treatment	df	$E_{xx}=\Sigma(x-x)^2$	$E_{xy}=\Sigma(x-x)(x-y)$	$E_{yy}=\Sigma(y-y)^2$	df	Residual SS
REF1 strain	5	17.5	6.8115	2.6610	4	0.00977
OP2 strain	5	17.5	6.8920	2.7151	4	0.00083
OP7 strain	5	17.5	7.2290	2.9877	4	0.00150
Residuals from individual regressions					12	0.01210
Total for single regression	15	52.5	20.9325	8.3638	14	0.01771
Difference for homogeneity of regressions					2	0.01771-0.01210 = 0.00561

$$F = \frac{\frac{0.00561}{2}}{\frac{0.01210}{12}} = 2.78, \text{ with 2 and 12 df}$$

Table B.7 Test for homogeneity of the regression coefficients obtained from glycerol production lines (Figure 4.7) of the three yeast strains

treatment	df	$E_{xx}=\Sigma(x-x)^2$	$E_{xy}=\Sigma(x-x)(x-y)$	$E_{yy}=\Sigma(y-y)^2$	df	Residual SS
REF1 strain	5	17.5	5.3110	1.6294	4	0.01759
OP2 strain	5	17.5	5.3085	1.6110	4	0.00070
OP7 strain	5	17.5	6.0460	2.1088	4	0.01999
Residuals from individual regressions					12	0.03828
Total for single regression	15	52.5	16.6655	5.3492	14	0.05894
Difference for homogeneity of regressions					2	0.05894-0.03828 = 0.02066

$$F = \frac{\frac{0.02066}{2}}{\frac{0.03828}{12}} = 3.24\text{, with 2 and 12 df}$$

3 Test for Evidence of Zero Slope

Evidence of zero slope was tested using the program Minitab for the Macintosh computer. The result would be interpreted as significant evidence of non-zero slope when a p-value of zero was obtained from the analysis of variance.

3. 1 Enzyme Production in Batch Culture

The steady state glycolytic enzyme production experiments observed in section 4.4 (Chapter 4) were tested for evidence of zero slope and the results are shown as follow:

3.1.1 TPI

REF1

```
The regression equation is    specific TPI activity = 78.2 + 1.02 time
Prediction Table
Predictor      Coef     Stdev    t-ratio      p
Constant      78.16     15.20      5.14   0.007
time          1.019     1.976      0.52   0.633
s = 8.264     R-sq = 6.2%     R-sq(adj) = 0.0%
Analysis of Variance
SOURCE        DF        SS        MS         F       p
Regression     1     18.17     18.17      0.27   0.633
Error          4    273.20     68.30
Total          5    291.36
```

OP2

```
The regression equation is    specific TPI activity = 122 + 5.39 time
Prediction Table
Predictor      Coef     Stdev    t-ratio      p
Constant     122.21     16.97      7.20   0.002
time          5.393     2.207      2.44   0.071
s = 9.232     R-sq = 59.9%    R-sq(adj) = 49.9%
Analysis of Variance
SOURCE        DF        SS        MS         F       p
Regression     1    509.00    509.00      5.97   0.071
Error          4    340.88     85.22
Total          5    849.89
```

OP7

```
The regression equation is    specific TPI activity = 576 + 2.0 time
Prediction Table
Predictor      Coef     Stdev    t-ratio      p
Constant      576.2     147.6      3.90   0.018
time           1.97     19.19      0.10   0.923
s = 80.29     R-sq = 0.3%     R-sq(adj) = 0.0%
Analysis of Variance
SOURCE        DF        SS        MS         F       p
Regression     1        68        68      0.01   0.923
Error          4     25788      6447
Total          5     25856
```

3.1.2 HK

REF1

```
The regression equation is      specific HK activity= 1.75 - 0.0074 time
Prediction Table
Predictor        Coef      Stdev   t-ratio       p
Constant     1.7457     0.2665      6.55   0.003
time       -0.00743   0.03465     -0.21   0.841
s = 0.1449     R-sq = 1.1%     R-sq(adj) = 0.0%
Analysis of Variance
SOURCE       DF          SS            MS          F        p
Regression     1    0.00097    0.00097     0.05   0.841
Error             4    0.08403    0.02101
Total             5    0.08500
```

OP2

```
The regression equation is      specific HK activity = 1.26 + 0.0540 time
Prediction Table
Predictor        Coef      Stdev   t-ratio       p
Constant     1.2633     0.2061      6.13   0.004
time        0.05400   0.02679      2.02   0.114
s = 0.1121     R-sq = 50.4%     R-sq(adj) = 38.0%
Analysis of Variance
SOURCE       DF          SS            MS          F        p
Regression     1    0.05103    0.05103     4.06   0.114
Error             4    0.05025    0.01256
Total             5    0.10128
```

OP7

```
The regression equation is      specific HK activity = 0.398 + 0.0614 time
Prediction Table
Predictor        Coef      Stdev   t-ratio       p
Constant     0.3976     0.1964      2.02   0.113
time        0.06143   0.02554      2.41   0.074
s = 0.1068     R-sq = 59.1%     R-sq(adj) = 48.9%
Analysis of Variance
SOURCE       DF          SS            MS          F        p
Regression     1    0.06604    0.06604     5.79   0.074
Error             4    0.04565    0.01141
Total             5    0.11168
```

3.1.3 PGK

REF1

```
The regression equation is      specific PGK activity = 15.6 - 0.322 time
Prediction Table
Predictor        Coef      Stdev   t-ratio       p
Constant     15.590      2.140      7.29   0.002
time         -0.3217    0.2782     -1.16   0.312
s = 1.164     R-sq = 25.1%     R-sq(adj) = 6.3%
Analysis of Variance
SOURCE       DF          SS          MS          F        p
Regression     1      1.811      1.811     1.34   0.312
Error             4      5.416      1.354
Total             5      7.227
```

OP2

```
The regression equation is     specific PGK activity = 13.9 - 0.033 time
Prediction Table
Predictor       Coef      Stdev    t-ratio      p
Constant      13.919      1.042      13.36  0.000
time         -0.0334     0.1354      -0.25  0.817
s = 0.5666    R-sq = 1.5%    R-sq(adj) = 0.0%
Analysis of Variance
SOURCE       DF         SS          MS         F        p
Regression    1     0.0196      0.0196      0.06    0.817
Error         4     1.2839      0.3210
Total         5     1.3035
```

OP7

```
The regression equation is     specific PGK activity = 8.83 + 0.133 time
Prediction Table
Predictor       Coef      Stdev    t-ratio      p
Constant       8.832      1.328       6.65  0.003
time          0.1326     0.1727       0.77  0.485
s = 0.7223    R-sq = 12.8%    R-sq(adj) = 0.0%


Analysis of Variance
SOURCE       DF         SS          MS         F        p
Regression    1     0.3076      0.3076      0.59    0.485
Error         4     2.0868      0.5217
Total         5     2.3943
```

3.1.4 PYK

REF1

```
The regression equation is     specific PYK activity = 10.6 - 0.298 time
Prediction Table
Predictor       Coef      Stdev    t-ratio      p
Constant      10.647      1.816       5.86  0.004
time         -0.2983     0.2361      -1.26  0.275
s = 0.9876    R-sq = 28.5%    R-sq(adj) = 10.7%
Analysis of Variance
SOURCE       DF         SS          MS         F        p
Regression    1     1.5571      1.5571      1.60    0.275
Error         4     3.9017      0.9754
Total         5     5.4588
```

OP2

```
The regression equation is     specific PYK activity = 10.4 - 0.197 time
Prediction Table
Predictor       Coef      Stdev    t-ratio      p
Constant      10.448      1.351       7.73  0.002
time         -0.1966     0.1757      -1.12  0.326
s = 0.7349    R-sq = 23.8%    R-sq(adj) = 4.8%
Analysis of Variance
SOURCE       DF         SS          MS         F        p
Regression    1     0.6762      0.6762      1.25    0.326
Error         4     2.1603      0.5401
Total         5     2.8365
```

OP7

```
The regression equation is    specific PYK activity = 7.92 - 0.165 time
Prediction Table
Predictor      Coef     Stdev   t-ratio       p
Constant      7.925     1.357      5.84   0.004
time        -0.1649    0.1764     -0.93   0.403
s = 0.7381    R-sq = 17.9%    R-sq(adj) = 0.0%
Analysis of Variance
SOURCE        DF        SS        MS       F       p
Regression     1    0.4756    0.4756    0.87   0.403
Error          4    2.1791    0.5448
Total          5    2.6547
```

3.2 Competitive Chemostat Data

The regression lines obtained for the competitions studied in section 6.6 (Chapter 6) were tested for evidence of zero slope and the results are shown below.

3.2.1 Competition Between Strains OP7 and OP2

```
The regression equation is    ln(R) = - 0.099 + 0.00449 time


Prediction Table
Predictor      Coef           Stdev         t-ratio         p
Constant      -0.0991         0.4883        -0.20           0.846
time           0.004492       0.003425       1.31           0.238
s = 0.5412    R-sq = 22.3%    R-sq(adj) = 9.3%
Analysis of Variance
SOURCE        DF     SS        MS        F       p
Regression    1      0.5039    0.5039    1.72    0.238
Error         6      1.7576    0.2929
Total         7      2.2615
Unusual Observations
Obs.   time   OP7/OP2     Fit Stdev.Fit  Residual   St.Resid
 6      169    1.774       0.660   0.231     1.114      2.28R
R denotes an obs. with a large st. resid.
```

3.2.2 Competition Between Strains OP7 and REF1

```
The regression equation is    OP7/REF1 = 0.926 - 0.00767 time
Prediction Table
Predictor      Coef           Stdev         t-ratio         p
Constant       0.9263         0.4666         1.99           0.094
time          -0.007668       0.003273      -2.34           0.058
s = 0.5172    R-sq = 47.8%    R-sq(adj) = 39.1%
Analysis of Variance
SOURCE        DF     SS            MS            F      p
Regression    1      1.4687        1.4687        5.49   0.058
Error         6      1.6049        0.2675
Total         7      3.0736
```

4 Multiple Comparison Test

Tukey's multiple comparison (Tukey, 1953) was used to determine the differences between the three strains studied in this thesis (REF1, OP2 and OP7).

Three null hypotheses (as shown below) were established and tested using the program Minitab for the Macintosh computer. The test forms confidence intervals for the differences between the strains within each individual parameter measurement.

$$H_0 : \mu_{REF1} = \mu_{OP2}$$
$$H_1 : \mu_{REF1} \neq \mu_{OP2}$$

$$H_0 : \mu_{REF1} = \mu_{OP7}$$
$$H_1 : \mu_{REF1} \neq \mu_{OP7}$$

$$H_0 : \mu_{OP2} = \mu_{OP7}$$
$$H_1 : \mu_{OP2} \neq \mu_{OP7}$$

where μ is an average mean of the experimental data obtained from each individual parameter measurement

These hypotheses would be rejected i.e. the two means are equal, whenever the confidence interval for the difference in the means does not contain zero.

4.1 Enzyme Production in Batch Culture

Steady state enzyme production experiments of strains REF1, OP2 and OP7 studied in section 4.4 (Chapter 4) were compared and the results are shown below.

TPI

Difference between	Confidence Interval 95% Family Confidence	Decision
REF1 and OP2	-140.4 to -13.3	Reject $H_0 : \mu_{REF1} = \mu_{OP2}$
REF1 and OP7	-568.7 to -441.6	Reject $H_0 : \mu_{REF1} = \mu_{OP7}$
OP2 and OP7	-491.8 to -364.7	Reject $H_0 : \mu_{OP2} = \mu_{OP7}$

HK

Difference between	Confidence Interval 95% Family Confidence	Decision
REF1 and OP2	-0.1895 to 0.2328	Accept $H_0 : \mu_{REF1} = \mu_{OP2}$
REF1 and OP7	0.6205 to 1.0428	Reject $H_0 : \mu_{REF1} = \mu_{OP7}$
OP2 and OP7	0.5988 to 1.0212	Reject $H_0 : \mu_{OP2} = \mu_{OP7}$

PGK

Difference between	Confidence Interval 95% Family Confidence	Decision
REF1 and OP2	-1.7703 to 0.7870	Accept $H_0 : \mu_{REF1} = \mu_{OP2}$
REF1 and OP7	2.0713 to 4.6287	Reject $H_0 : \mu_{REF1} = \mu_{OP7}$
OP2 and OP7	2.5630 to 5.1203	Reject $H_0 : \mu_{OP2} = \mu_{OP7}$

PYK

Difference between	Confidence Interval 95% Family Confidence	Decision
REF1 and OP2	-1.8435 to 0.7168	Accept $H_0 : \mu_{REF1}=\mu_{OP2}$
REF1 and OP7	0.4415 to 3.0018	Reject $H_0 : \mu_{REF1}= \mu_{OP7}$
OP2 and OP7	1.0049 to 3.5651	Reject $H_0 : \mu_{OP2}= \mu_{OP7}$

4.2 Steady State Growth and Metabolite Production at D=0.11 and 0.32 h^{-1} in Chemostat Culture

Growth and metabolite production of strains REF1, OP2 and OP7 studied in section 5.3.1 (Chapter 5) were compared and the results are shown below. Glycerol production between strains was not determined due to very small amounts of glycerol detected.

4.2.1 D=0.11 h^{-1}

Biomass Yield

Difference between	Confidence Interval 95% Family Confidence	Decision
REF1 and OP2	-0.07726 to 0.08083	Accept $H_0 : \mu_{REF1}=\mu_{OP2}$
REF1 and OP7	0.07579 to 0.21612	Reject $H_0 : \mu_{REF1}= \mu_{OP7}$
OP2 and OP7	0.06276 to 0.22557	Reject $H_0 : \mu_{OP2}= \mu_{OP7}$

CO_2

Difference between	Confidence Interval 95% Family Confidence	Decision
REF1 and OP2	-1.5324 to -0.0276	Reject $H_0 : \mu_{REF1}= \mu_{OP2}$
REF1 and OP7	-1.5462 to -0.2105	Reject $H_0 : \mu_{REF1}= \mu_{OP7}$
OP2 and OP7	-0.8732 to 0.6765	Accept $H_0 : \mu_{OP2}= \mu_{OP7}$

Ethanol

Difference between	Confidence Interval 95% Family Confidence	Decision
REF1 and OP2	-0.04914 to 0.04914	Accept $H_0 : \mu_{REF1}=\mu_{OP2}$
REF1 and OP7	-0.13077 to -0.02257	Reject $H_0 : \mu_{REF1}= \mu_{OP7}$
OP2 and OP7	-0.13655 to -0.01679	Reject $H_0 : \mu_{OP2}= \mu_{OP7}$

Pyruvate

Difference between	Confidence Interval 95% Family Confidence	Decision
REF1 and OP2	-0.003935 to 0.004602	Accept $H_0 : \mu_{REF1}=\mu_{OP2}$
REF1 and OP7	-0.008608 to -0.001725	Reject $H_0 : \mu_{REF1}= \mu_{OP7}$
OP2 and OP7	-0.009874 to -0.001126	Reject $H_0 : \mu_{OP2}= \mu_{OP7}$

Acetate

Difference between	Confidence Interval 95% Family Confidence	Decision
REF1 and OP2	0.00344 to 0.05656	Reject $H_0 : \mu_{REF1}=\mu_{OP2}$
REF1 and OP7	-0.11410 to -0.05924	Reject $H_0 : \mu_{REF1}= \mu_{OP7}$
OP2 and OP7	-0.13481 to -0.09852	Reject $H_0 : \mu_{OP2}= \mu_{OP7}$

4.2.2 D=0.32 h^{-1}

Biomass Yield

Difference between	Confidence Interval 95% Family Confidence	Decision
REF1 and OP2	-0.056641 to -0.016692	Reject $H_0 : \mu_{REF1}=\mu_{OP2}$
REF1 and OP7	0.019468 to 0.055199	Reject $H_0 : \mu_{REF1}= \mu_{OP7}$
OP2 and OP7	0.056134 to 0.091866	Reject $H_0 : \mu_{OP2}= \mu_{OP7}$

CO_2

Difference between	Confidence Interval 95% Family Confidence	Decision
REF1 and OP2	-1.2414 to 1.5547	Accept $H_0 : \mu_{REF1}=\mu_{OP2}$
REF1 and OP7	-2.2298 to 0.2711	Accept $H_0 : \mu_{REF1}=\mu_{OP7}$
OP2 and OP7	-2.3864 to 0.1144	Accept $H_0 : \mu_{OP2}= \mu_{OP7}$

Ethanol

Difference between	Confidence Interval 95% Family Confidence	Decision
REF1 and OP2	-0.1685 to 2.8685	Accept $H_0 : \mu_{REF1}=\mu_{OP2}$
REF1 and OP7	-0.9162 to 1.8002	Accept $H_0 : \mu_{REF1}=\mu_{OP7}$
OP2 and OP7	-2.2662 to 0.4502	Accept $H_0 : \mu_{OP2}= \mu_{OP7}$

Pyruvate

Difference between	Confidence Interval 95% Family Confidence	Decision
REF1 and OP2	-0.014716 to 0.004716	Accept $H_0 : \mu_{REF1}=\mu_{OP2}$
REF1 and OP7	-0.014505 to 0.003305	Accept $H_0 : \mu_{REF1}=\mu_{OP7}$
OP2 and OP7	-0.008373 to 0.007173	Accept $H_0 : \mu_{OP2}= \mu_{OP7}$

Acetate

Difference between	Confidence Interval 95% Family Confidence	Decision
REF1 and OP2	-0.11287 to -0.02379	Reject $H_0 : \mu_{REF1}=\mu_{OP2}$
REF1 and OP7	-0.06582 to 0.01582	Accept $H_0 : \mu_{REF1}=\mu_{OP7}$
OP2 and OP7	0.00770 to 0.07897	Reject $H_0 : \mu_{OP2}= \mu_{OP7}$

4.3 Enzyme Production at D=0.11 and 0.32 h^{-1} in Chemostat Culture

Steady state glycolytic enzyme production experiments at two different dilution rates of the three yeast strains studied in section 5.3.2 (Chapter 5) were compared and the results are shown below.

4.3.1 D=0.11 h^{-1}

TPI

Difference between	Confidence Interval 95% Family Confidence	Decision
REF1 and OP2	-139.1 to -3.1	Reject $H_0 : \mu_{REF1}=\mu_{OP2}$
REF1 and OP7	-491.4 to -370.8	Reject $H_0 : \mu_{REF1}= \mu_{OP7}$
OP2 and OP7	-430.0 to -290.0	Reject $H_0 : \mu_{OP2}= \mu_{OP7}$

HK

Difference between	Confidence Interval 95% Family Confidence	Decision
REF1 and OP2	-0.4931 to 0.5903	Accept $H_0 : \mu_{REF1}=\mu_{OP2}$
REF1 and OP7	0.1461 to 1.1077	Reject $H_0 : \mu_{REF1}= \mu_{OP7}$
OP2 and OP7	0.0204 to 1.1362	Reject $H_0 : \mu_{OP2}= \mu_{OP7}$

PGK

Difference between	Confidence Interval 95% Family Confidence	Decision
REF1 and OP2	0.767 to 4.785	Reject $H_0 : \mu_{REF1}=\mu_{OP2}$
REF1 and OP7	1.218 to 4.785	Reject $H_0 : \mu_{REF1}= \mu_{OP7}$
OP2 and OP7	-1.844 to 2.295	Accept $H_0 : \mu_{OP2}= \mu_{OP7}$

PYK

Difference between	Confidence Interval 95% Family Confidence	Decision
REF1 and OP2	-1.7247 to 0.7290	Accept $H_0 : \mu_{REF1}=\mu_{OP2}$
REF1 and OP7	0.9899 to 3.1678	Reject $H_0 : \mu_{REF1}= \mu_{OP7}$
OP2 and OP7	1.3132 to 3.8401	Reject $H_0 : \mu_{OP2}= \mu_{OP7}$

4.3.2 D=0.32 h^{-1}

TPI

Difference between	Confidence Interval 95% Family Confidence	Decision
REF1 and OP2	-265.5 to 45.6	Accept $H_0 : \mu_{REF1}=\mu_{OP2}$
REF1 and OP7	-635.2 to -344.2	Reject $H_0 : \mu_{REF1}= \mu_{OP7}$
OP2 and OP7	-525.2 to -234.2	Reject $H_0 : \mu_{OP2}= \mu_{OP7}$

HK

Difference between	Confidence Interval 95% Family Confidence	Decision
REF1 and OP2	-1.1854 to 0.6988	Accept $H_0 : \mu_{REF1}=\mu_{OP2}$
REF1 and OP7	0.0654 to 1.8279	Reject $H_0 : \mu_{REF1}= \mu_{OP7}$
OP2 and OP7	0.3087 to 2.0713	Reject $H_0 : \mu_{OP2}= \mu_{OP7}$

PGK

Difference between	Confidence Interval 95% Family Confidence	Decision
REF1 and OP2	0.8804 to 3.0729	Reject $H_0 : \mu_{REF1}=\mu_{OP2}$
REF1 and OP7	2.0246 to 4.0754	Reject $H_0 : \mu_{REF1}= \mu_{OP7}$
OP2 and OP7	0.0479 to 2.0988	Reject $H_0 : \mu_{OP2}= \mu_{OP7}$

PYK

Difference between	Confidence Interval 95% Family Confidence	Decision
REF1 and OP2	-3.0787 to 1.0520	Accept $H_0 : \mu_{REF1}=\mu_{OP2}$
REF1 and OP7	1.4330 to 5.2970	Reject $H_0 : \mu_{REF1}= \mu_{OP7}$
OP2 and OP7	2.4464 to 6.3103	Reject $H_0 : \mu_{OP2}= \mu_{OP7}$

Appendix C
Experimental Data

Table C.1 Data from batch culture of strains REF1, OP2 and OP7.

strain	time (h)	OD$_{600}$	dry weight (mg ml^{-1})	%CO$_2$
REF1	5	0.920±0.014	0.59±0.05	0.34
	6	1.315±0.021	0.77±0.00	0.49
	7	2.015±0.021	1.00±0.00	0.75
	8	2.950±0.042	1.47±0.00	1.13
	9	3.820±0.000	2.25±0.07	1.55
	10	6.260±0.170	2.88±0.11	1.48
OP2	5	0.565±0.001	0.69±0.02	0.29
	6	0.848±0.004	0.80±0.00	0.41
	7	1.378±0.004	1.08±0.02	0.57
	8	1.944±0.008	1.34±0.02	0.87
	9	2.875±0.035	1.90±0.07	1.27
	10	4.170±0.042	2.55±0.07	1.62
OP7	5	0.728±0.000	0.30±0.01	0.29
	6	1.000±0.010	0.40±0.01	0.39
	7	1.657±0.040	0.70±0.01	0.55
	8	2.412±0.043	1.10±0.03	0.84
	9	3.213±0.081	1.55±0.03	1.24
	10	5.147±0.094	2.20±0.01	1.62

Table C.2 Metabolite concentrations of strains REF1, OP2 and OP7 grown in batch culture.

strain	time (h)	ethanol conc. (mM)	glycerol conc. (mM)
REF1	5	23.80±0.40	1.40±0.04
	6	36.60±0.30	1.90±0.12
	7	54.50±0.40	2.69±0.04
	8	81.90±0.30	3.75±0.05
	9	120.50±1.00	4.99±0.04
	10	163.7±1.60	6.14±0.03
OP2	5	16.60±0.30	1.11±0.01
	6	24.70±0.10	1.47±0.06
	7	36.90±0.30	2.03±0.04
	8	55.00±0.20	2.73±0.02
	9	81.60±1.00	3.77±0.02
	10	117.80±1.70	4.97±0.02
OP7	5	18.71±1.0	1.13±0.01
	6	28.29±0.3	1.48±0.01
	7	43.40±0.2	2.18±0.03
	8	66.04±1.0	3.36±0.02
	9	99.41±1.7	4.64±0.01
	10	145.96±1.9	5.86±0.03

Table C.3 Glycolytic enzyme activities and protein concentration of strains REF1, OP2 and OP7 grown in batch culture.

strain	time (h)	protein conc. (mg ml^{-1})	TPI activity (u ml^{-1})	HK activity (u ml^{-1})	PGK activity (u ml^{-1})	PYK activity (u ml^{-1})
REF1	5	2.10±0.04	198.31±3.14	3.42±0.20	28.83±1.57	18.35±0.48
	6	2.41±0.05	197.16±5.51	4.65±0.30	36.22±0.52	22.45±0.74
	7	2.75±0.34	224.46±2.30	4.63±0.09	35.46±0.73	23.03±0.72
	8	3.11±0.05	231.61±9.18	4.93±0.14	37.95±2.04	27.81±0.89
	9	2.86±0.02	266.77±4.81	4.88±0.15	40.02±1.01	25.73±0.62
	10	3.15±0.08	291.85±6.42	5.27±0.07	36.94±1.01	20.19±0.81
OP2	5	1.03±0.03	151.93±15.29	1.52±0.04	14.42±0.54	10.37±0.40
	6	1.60±0.04	266.32±23.85	2.70±0.06	12.99±0.49	8.22±0.21
	7	1.62±0.10	249.98±18.80	2.67±0.05	13.72±0.86	9.07±0.57
	8	1.82±0.04	283.55±4.59	3.07±0.15	13.54±0.55	8.83±0.20
	9	1.78±0.06	298.57±11.03	2.83±0.03	13.31±1.33	8.44±0.34
	10	2.82±0.08	519.40±71.69	5.38±0.35	14.03±0.42	8.91±0.29
OP7	5	2.72±0.08	1448.7±141.05	1.98±0.09	25.07±0.24	17.14±0.64
	6	3.42±0.02	2074.1±34.07	2.70±0.07	33.04±0.81	24.74±1.45
	7	3.04±0.04	2123.5±58.38	2.60±0.21	32.81±1.35	23.79±0.44
	8	3.99±0.06	2284.3±119.81	3.14±0.08	37.05±0.16	26.85±0.18
	9	3.98±0.22	1989.3±79.56	3.29±0.18	37.47±1.53	23.38±1.00
	10	5.20±0.10	3305.4±13.34	5.99±0.16	55.12±0.18	32.15±0.35

Table C.4 Data from dilution rate profile of strain REF1 with 0.5% (w/v) glucose and 1% (w/v) casamino acids supplement.

D (h^{-1})	OD_{600}	dry weight mg ml^{-1}	qCO_2 ($mmolg^{-1}h^{-1}$)	residual glucose(gL^{-1})	Y (g biomass g $glucose^{-1}$)	ethanol conc. (mM)	glycerol conc. (mM)
0.11	8.54±0.12	3.30±0.06	3.13±0.12	-	0.66±0.01	-	-
0.19	7.24±0.13	2.93±0.10	5.46±0.30	-	0.58±0.01	5.78	-
0.27	4.74±0.17	1.90±0.13	8.67±0.29	-	0.38±0.01	23.33	-
0.32	3.03±0.10	1.26±0.16	14.13±1.84	6.06	0.25±0.03	33.37	0.82
0.38	2.10±0.22	0.76±0.18	16.83±1.97	13.84	0.19±0.02	27.3	0.79

Table C.5 Data from the dilution rate profile of the REF1 (run no.1) with 0.5% (w/v) glucose and 0.2% (w/v) casamino acids supplement.

D (h^{-1})	time at D (h)	volume changes	OD_{600} (u ml^{-1})	dry weight (mg ml^{-1})	CPR (mmol L^{-1} h^{-1})	residual glucose (g L^{-1})
0.11	20.25	2.2	7.996	3.08	0.12	-
	30	3.3	7.970	3.02	0.13	-
	45	4.9	8.384	3.10	0.15	-
	52	5.7	8.372	3.16	0.13	-
0.20	16	3.1	5.740	2.46	0.24	-
	31.5	6.2	6.112	2.51	0.20	-
	45.75	9.0	6.024	2.57	0.24	-
0.25	13	3.3	5.820	2.50	0.22	-
	17	4.3	6.232	2.48	0.23	-
	22.5	5.7	6.264	2.49	0.23	-
	27	6.8	6.084	2.40	0.23	-
0.36	9	3.2	3.364	1.38	0.31	0.19
	12	4.3	3.136	1.38	0.31	0.20
	15	5.4	3.024	1.33	0.31	0.19
	18	6.4	3.000	1.28	0.31	0.18

Table C.5 (continued)

ethanol conc. (mM)	glycerol conc. (mM)	pyruvate conc. (mM)	acetate conc. (mM)	sp TPI act (u mg prot^{-1})	sp HK act (u mg prot^{-1})	sp PGK act (u mg prot^{-1})	sp PYK act (u mg prot^{-1})
-	0.31	0.03	-	87.50	2.57	12.20	5.11
-	0.31	0.03	-	90.40	2.51	12.08	5.69
-	0.22	0.04	-	103.39	2.71	13.56	6.31
-	-	0.03	-	83.62	2.07	10.19	4.83
7.46	-	0.05	0.91	nd	nd	nd	nd
7.09	0.30	0.04	0.74	nd	nd	nd	nd
5.84	0.38	0.05	0.72	nd	nd	nd	nd
16.12	-	0.03	0.68	nd	nd	nd	nd
11.61	0.14	0.04	1.08	nd	nd	nd	nd
7.17	0.32	0.04	0.97	nd	nd	nd	nd
9.60	0.22	0.04	0.52	nd	nd	nd	nd
29.30	0.19	0.09	1.02	94.78	2.20	7.07	4.15
31.27	0.20	0.09	0.89	88.01	2.22	7.93	5.17
32.10	0.20	0.10	1.01	75.10	1.81	6.99	5.62
32.79	0.27	0.10	0.98	76.65	1.86	6.57	5.00

Table C.6 Data from the dilution rate profile of the REF1 (run no.2) with 0.5% (w/v) glucose and 0.2% (w/v) casamino acids supplement.

D (h^{-1})	time at D (h)	volume changes	OD600 (u ml^{-1})	dry weight (mg ml^{-1})	CPR (mmol L^{-1} h^{-1})	residual glucose (g L^{-1})
0.11	89.5	9.8	7.888	2.93	0.14	0.02
	100.5	10.9	7.972	2.94	0.13	0.02
	113	12.3	7.700	2.64	0.13	0.02
0.32	19.7	6.3	3.744	1.56	0.26	0.07
	28	9.0	3.488	1.49	0.28	0.08
	46.5	14.9	3.512	1.55	0.28	0.08

Table C.6 (continued)

ethanol conc. (mM)	glycerol conc. (mM)	pyruvate conc. (mM)	acetate conc. (mM)	sp TPI act (u mg $prot^{-1}$)	sp HK act (u mg $prot^{-1}$)	sp PGK act (u mg $prot^{-1}$)	sp PYK act (u mg $prot^{-1}$)
-	-	0.09	0.81	62.04	1.89	11.24	6.07
-	-	0.09	-	61.77	1.71	11.13	5.70
-	-	0.09	-	63.62	2.07	11.98	6.17
29.39	-	0.05	0.96	68.36	2.60	10.45	6.32
31.37	-	0.06	0.98	83.95	2.13	9.35	5.65
26.21	-	-	-	78.38	1.44	9.00	3.54

Table C.7 Data from the dilution rate profile of the OP2 with 0.5% (w/v) glucose and 0.2% (w/v) casamino acids supplement.

D (h^{-1})	time at D (h)	volume changes	OD_{600} (u ml^{-1})	dry weight (mg ml^{-1})	CPR (mmol L^{-1} h^{-1})	residual glucose (g L^{-1})
0.11	62	7.1	7.072	2.88	0.17	0.02
	66.5	7.6	7.460	2.90	0.17	0.02
	71.5	8.2	7.185	2.95	0.17	0.03
	87.5	9.9	7.196	3.00	0.17	0.03
0.32	15	4.8	3.632	1.75	0.31	0.08
	41	13.1	3.828	1.78	0.31	0.01
	48	15.4	3.890	1.76	0.31	0.05
0.36	15	5.4	2.644	1.17	0.31	0.22
	18	6.6	2.509	1.18	0.33	0.23
	21	7.5	2.500	1.14	0.32	0.21

Table C.7 (continued)

ethanol conc. (mM)	glycerol conc. (mM)	pyruvate conc. (mM)	acetate conc. (mM)	sp TPI act (u mg $prot^{-1}$)	sp HK act (u mg $prot^{-1}$)	sp PGK act (u mg $prot^{-1}$)	sp PYK act (u mg $prot^{-1}$)
-	-	0.04	-	153.40	2.43	9.68	6.53
-	-	0.04	-	146.60	2.10	8.70	6.10
-	-	0.04	-	156.50	2.17	8.87	6.21
-	-	-	-	143.50	1.98	8.72	5.94
27.92	-	0.10	1.44	201.60	2.38	7.48	6.20
26.86	0.08	0.08	1.59	188.90	2.14	7.96	6.30
23.13	0.08	0.07	1.57	170.10	2.38	7.43	6.05
32.39	0.38	0.16	1.69	198.70	1.92	7.12	7.13
32.22	0.30	0.14	1.68	205.30	1.92	7.36	7.92
32.56	0.34	0.15	1.62	162.20	1.95	6.64	7.34

Table C.8 Data from the dilution rate profile of the OP7 (run no.1) with 0.5% (w/v) glucose and 0.2% (w/v) casamino acids supplement.

D (h^{-1})	time at D (h)	volume changes	OD600 (u ml^{-1})	dry weight (mg ml^{-1})	CPR (mmol L^{-1} h^{-1})	residual glucose (g L^{-1})
0.11	60	6.5	6.944	2.70	0.13	-
	73	8.0	7.013	2.72	0.13	-
	75	8.0	6.980	2.76	0.14	-
0.32	22.5	7.2	3.100	1.41	0.29	0.16
	28.8	9.2	3.228	1.41	0.27	0.15
	40	12.8	3.356	1.53	0.27	0.14

Table C.8 (continued)

ethanol conc. (mM)	glycerol conc. (mM)	pyruvate conc. (mM)	acetate conc. (mM)	sp TPI act (u mg $prot^{-1}$)	sp HK act (u mg $prot^{-1}$)	sp PGK act (u mg $prot^{-1}$)	sp PYK act (u mg $prot^{-1}$)
-	-	0.11	-	518.90	1.67	10.19	4.54
-	-	0.10	-	606.03	1.89	10.46	4.73
-	-	0.10	-	571.01	2.04	10.10	4.43
21.38	-	0.08	1.14	638.24	1.01	6.76	2.40
29.42	-	0.09	1.07	660.48	0.67	6.67	1.07
26.64	-	0.08	1.07				

Table C.9 Data from the dilution rate profile of the OP7 (run no.2) with 0.5% (w/v) glucose and 0.2% (w/v) casamino acids supplement.

D (h^{-1})	time at D (h)	volume changes	OD600 (u ml^{-1})	dry weight (mg ml^{-1})	CPR (mmol L^{-1} h^{-1})	residual glucose (g L^{-1})
0.11	71	7.7	5.307	1.88	0.13	0.03
	89.5	9.8	5.060	1.80	0.12	0.03
	98.7	10.8	5.080	1.86	0.14	0.03
0.20	39	7.7	4.893	1.80	0.16	0.02
	48.6	9.6	4.572	1.76	0.16	0.02
	65	12.8	5.496	2.06	0.19	0.02
	88.3	17.4	5.145	2.08	0.19	0.02
0.25	14.6	3.7	4.816	1.82	0.24	0.02
	38.6	9.9	4.820	2.05	0.24	0.01
	42.8	10.9	4.748	1.86	0.24	0.01
0.32	18.5	5.9	3.652	1.38	0.27	0.03
	22.25	7.1	3.760	1.32	0.27	0.05

Table C.9 (continued)

ethanol conc. (mM)	glycerol conc. (mM)	pyruvate conc. (mM)	acetate conc. (mM)	sp TPI act (u mg $prot^{-1}$)	sp HK act (u mg $prot^{-1}$)	sp PGK act (u mg $prot^{-1}$)	sp PYK act (u mg $prot^{-1}$)
-	0.40	0.17	2.24	450.25	1.43	7.79	3.08
1.59	0.24	0.16	1.85	463.18	1.35	7.04	2.71
2.29	0.50	0.18	1.84	450.75	1.17	7.02	2.22
10.37	-	0.04	0.87	593.71	1.80	9.21	2.26
10.44	-	0.04	0.86	541.87	1.64	9.15	1.88
8.96	-	0.05	0.82	512.93	1.32	8.04	0.96
11	-	0.04	0.77	503.10	1.25	8.12	1.16
17.36	-	0.03	0.81	436.95	1.15	6.22	1.17
15.86	-	0.03	0.78	458.24	1.13	7.06	1.00
15.92	-	0.03	0.75	481.02	1.15	6.96	0.88
21.95	-	0.04	0.92	462.26	1.34	6.28	1.82
24.33	-	0.05	0.92	505.27	1.42	6.49	1.93

Table C.10 Data from the simulation model of growth of two strains grown individually under batch conditions (data were kindly provided by Dr. J. R. Small).

time	S1	X1	X2	S2
0.0	1200.00	0.01	0.01	1200.00
2.5	1199.27	0.02	0.02	1199.33
5.0	1197.50	0.06	0.05	1197.75
7.5	1193.14	0.15	0.13	1194.06
10.0	1182.47	0.36	0.30	1185.40
12.5	1156.33	0.88	0.71	1165.13
15.0	1092.29	2.16	1.66	1117.73
17.5	935.51	5.30	3.86	1007.26
20.0	552.57	12.96	8.96	752.71
22.5	7.21×10^{-10}	24.01	19.93	204.10
25.0	7.99×10^{-25}	24.01	24.01	1.20×10^{-5}

Table C.11 Data from the simulation model of growth of two strains grown competitively under batch conditions (data were kindly provided by Dr. J. R. Small).

time	S	X1	X2
0.0	1200.00	0.01	0.01
2.5	1198.57	0.02	0.02
5.0	1195.09	0.06	0.06
7.5	1186.63	0.15	0.14
10.0	1166.07	0.36	0.34
12.5	1116.10	0.88	0.81
15.0	994.70	2.16	1.96
17.5	700.37	5.29	4.72
20.0	12.42	12.75	11.02
22.5	2.12×10^{-14}	12.90	11.11
25.0	7.69×10^{-15}	12.90	11.11

Table C.12 Data from the simulation model of growth of two strains grown individually under chemostat conditions (data were kindly provided by Dr. J. R. Small).

time	S1	S2	X1	X2
0.00	0.01	0.01	1.00	1.00
4.17	28.27	31.67	1.92	1.58
8.33	26.36	38.75	4.58	3.34
12.50	3.71	21.00	8.48	6.75
16.67	2.58	5.72	9.66	9.35
20.83	2.30	4.76	10.40	10.15
25.00	2.16	4.43	10.88	10.65
29.17	2.07	4.24	11.20	10.98
33.33	2.02	4.12	11.40	11.20
37.50	1.98	4.05	11.54	11.34
41.67	1.96	4.01	11.63	11.43
45..83	1.95	3.98	11.69	11.49
50.00	1.94	3.96	11.73	11.53
54.17	1.93	3.94	11.76	11.56
58.33	1.93	3.94	11.77	11.57
62.50	1.93	3.93	11.78	11.58
66.67	1.93	3.93	11.79	11.59
70.83	1.92	3.93	11.80	11.60
75.00	1.92	3.92	11.80	11.60
79.17	1.92	3.92	11.80	11.60
83.33	1.92	3.92	11.80	11.60
87.50	1.92	3.92	11.80	11.61
91.67	1.92	3.92	11.81	11.61
95.83	1.92	3.92	11.81	11.61
100.00	1.92	3.92	11.81	11.61

Table C.13 Data from the simulation model of growth of two strains grown competitively under chemostat conditions (data were kindly provided by Dr. J. R. Small).

time	S	X1	X2	R
0.00	0.01	1.00	1.00	1.000
4.17	21.02	1.84	1.46	0.795
8.33	12.14	3.93	2.51	0.637
12.50	3.82	5.86	2.89	0.493
16.67	3.02	7.05	2.76	0.392
20.83	2.64	7.97	2.52	0.316
25.00	2.43	8.70	2.24	0.257
29.17	2.29	9.27	1.96	0.211
33.33	2.20	9.73	1.69	0.174
37.50	2.14	10.10	1.45	0.144
41.67	2.09	10.40	1.24	0.119
45..83	2.06	10.64	1.05	0.099
50.00	2.03	10.84	0.89	0.082
54.17	2.01	11.00	0.75	0.068
58.33	1.99	11.13	0.64	0.057
62.50	1.98	11.25	0.54	0.048
66.67	1.97	11.34	0.45	0.040
70.83	1.96	11.42	0.38	0.033
75.00	1.96	11.48	0.32	0.028
79.17	1.95	11.53	0.27	0.023
83.33	1.94	11.58	0.22	0.019
87.50	1.94	11.62	0.19	0.016
91.67	1.94	11.65	0.16	0.014
95.83	1.94	11.67	0.13	0.011
100.00	1.93	11.70	0.11	0.009

Table C.14 Data from the competitive chemostat experiment between strains REF2 and REF1 (experiment 1) when LEU2 gene was used as detective marker.

time (h)	lnR
29.0	-0.416
43.5	-0.436
53.0	-0.348
69.3	-0.324
77.5	-0.396
93.0	-0.383
116.5	-0.442
125.0	-0.438
143.0	-0.610
148.5	-0.460

Table C.15 Data from the competitive chemostat experiment between strains REF2 and OP7 when LEU2 gene was used as detective marker.

inoculum: 70% REF2:30% OP7

time (h)	lnR	sp TPI act	sp HK act
45.75	1.067	189.62±8.04	2.00±0.19
64.50	0.672	226.96±15.63	
88.42	0.294	246.86±29.53	
114.17	0.092	314.58±12.03	
137.75	-0.226	277.41±18.60	2.04±0.19
162.33			
185.33	0.298	276.98±10.84	
213.50	1.424	231.30±7.68	
232.40	1.558	173.76±7.57	
256.75	1.643	165.46±4.20	2.12±0.15

inoculum: 30% REF2:70% OP7

time (h)	lnR	sp TPI act	sp HK act
47.00	-0.882	423.74±30.61	1.94±0.22
65.83	-1.100	448.84±56.18	
89.67	-1.273	454.94±37.16	
115.25	-1.556	478.32±37.03	
138.92	-1.737	387.14±10.31	1.88±0.16
162.33	-1.523	384.81±4.67	
186.50	-0.924	493.98±11.82	
214.50	-0.250	350.86±14.71	
233.50	-0.080	347.37±23.56	
257.83	-0.073	328.88±11.06	2.14±0.12

Table C.16 Data from the competitive chemostat experiment between strains REF2 and OP2 when LEU2 gene was used as detective marker.

time (h)	lnR	sp TPI act	sp HK act
27.75	0.949		
45.08	0.842	94.13±3.88	1.74±0.06
68.25	0.502		
93.75	0.278	123.16±4.58	1.87±0.09
117.50	-0.024		
143.25	-0.274	139.07±10.66	2.08±0.05
166.80	-0.160		
189.25	0.341		
212.25	0.602	119.34±9.32	2.26±0.16
238.00	0.492		
261.42	0.492		
287.00	0.490	128.84±5.97	2.51±0.08

Table C.17 Data from the competitive chemostat experiment between strains REF2 and REF1 (experiment 2) when LEU2 gene was used as detective marker.

time (h)	lnR	sp TPI act	sp HK act
47.85	0.547	84.40±3.27	2.06±0.14
70.75	0.296		
96.50	0.024	69.45±1.31	2.02±0.07
120.08	-0.108		
145.50	-0.177	74.70±4.12	2.15±0.09
169.42	0.090		
192.75	0.552		
213.92	0.754	65.36±2.15	2.14±0.09
240.75	0.686		
262.17	0.597		
286.83	0.566	67.94±10.51	2.46±0.20

Table C.18 Data from the competitive chemostat experiment between strains OP2L and REF1 when LEU2 gene was used as detective marker.

inoculum: 74% OP2L:26% REF1

time (h)	lnR	sp TPI act	sp HK act
47.17	0.580	129.64±9.68	2.01±0.07
71.17	0.300	154.24±26.74	2.40±0.42
95.25	0.344		
118.67	0.593	118.80±16.86	2.23±0.32
147.75	1.669	164.96±8.34	2.29±0.12
166.67	2.944	179.52±10.21	2.70±0.16
190.67	2.873		
214.67	2.893	220.96±4.96	2.77±0.07
238.75	2.320		
287.74	2.463	214.27±7.58	3.00±0.07

inoculum: 34% OP2L:66% REF1

time (h)	lnR	sp TPI act	sp HK act
48.50	-1.075	110.34±1.95	2.39±0.12
72.33	-1.32	100.49±7.60	2.39±0.10
96.04	-1.306		
119.33	-1.030	88.26±4.26	2.25±0.12
144.42	-0.024	108.12±5.24	2.48±0.14
167.33	1.072		
191.33	1.516	130.14±4.18	2.28±0.13
215.33	1.269	179.90±13.58	2.70±0.22
239.66	1.150		
263.66	1.136		
288.66	1.254	153.11±15.61	2.40±0.24

Table C.19 Amino acid contents (µg of amino acid in sample applied to amino acid analyzer) of samples from the competitive chemostat experiment of strains OP2L and REF1with starting ratio of OP2L:REF1=74%:26% (data were kindly provided by Roslin Institute, Edinburgh).

time (h)	Asp	Thr	Ser	Glu	Gly	Ala	Val	Leu	Phe	His	Lys
0.00	2.894	1.976	2.777	7.208	0.863	2.535	3.163	3.394	1.288	1.045	5.431
47.17	0.833	0.076	0.117	1.427	0.071	0.172	0.195	0.078	0.000	0.220	1.848
71.17	0.737	0.000	0.069	1.168	0.074	0.133	0.147	0.050	0.000	0.102	1.413
118.67	0.632	0.127	0.089	1.143	0.078	0.157	0.183	0.000	0.000	0.280	2.345
142.75	0.490	0.059	0.101	0.622	0.052	0.158	0.228	0.078	0.115	0.115	2.136
190.67	0.275	0.000	0.018	0.298	0.014	0.051	0.149	0.053	0.000	0.592	2.161

Table C.20 Data from the competitive chemostat experiment between strains OP7 and OP2 when specific TPI activity was used as a self-detector.

time (h)	spAB	A/T	R	lnR
44.75	453.70	0.500	1.000	0.000
69.75	450.74	0.494	0.976	-0.024
94.75	504.49	0.608	1.551	0.439
117.33	486.04	0.569	1.320	0.278
141.16	526.59	0.656	1.907	0.646
169.00	619.91	0.855	5.896	1.774
192.00	511.79	0.624	1.660	0.507
217.75	488.87	0.575	1.353	0.302

Table C.21 Data from the competitive chemostat experiment between strains OP7 and REF1 when specific TPI activity was used as a self-detector.

time (h)	spAB	A/T	R	lnR
44.75	490.14	0.500	1.000	0.000
69.75	645.48	0.711	2.460	0.900
94.75	424.16	0.410	0.695	-0.364
117.33	624.91	0.683	2.15	0.765
141.16	508.00	0.524	1.101	0.096
169.00	405.27	0.384	0.623	-0.473
192.00	398.08	0.375	0.600	-0.511
217.75	313.02	0.259	0.350	-1.050