AN ANALYSIS OF GLYCEROL SYNTHESIS BY *SACCHAROMYCES CEREVISIAE*

BY

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DECLARATION

I, the undersigned hereby declare that the work contained in this thesis is my original work and has not previously in its entirety or in part been submitted to any university for a degree

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SUMMARY

Glycerol metabolism is paramount to the physiological adaptation by *Saccharomyces cerevisiae* to hyper-osmotic stress conditions. Glycerol metabolism also plays a fundamental role in maintaining a redox state favourable for growth under fermentative conditions. All aspects of the relationship between redox balancing and glycerol metabolism are not yet fully defined and attempts to manipulate this relationship, i.e., to increase or decrease glycerol yields from fermentation, result in a redox disturbance that is often detrimental to other aspects of metabolism. Another aspect of glycerol metabolism that is not thoroughly understood, is how the various parameters of the glycerol synthesis pathway, each independently and in conjunction with each other, control the rate at which glycerol is synthesized. Addressing these questions has been the topic of this thesis. In this regard, the theory of metabolic control analysis (MCA) was adopted and calculations were performed with the aid of an experimentally validated kinetic model.

To ascertain the *in vivo* substrate, product, coenzyme and known modifier concentrations of the glycerol synthesis pathway, reliable techniques to halt metabolism, extract and measure these metabolites had to be established. The metabolite concentrations constitute a portion of the parameters of the pathway and are necessary to construct a detailed kinetic model. Measuring the concentration of an intracellular metabolite enzymatically requires the cell extract to have an adequate quantity of the metabolite in question. This may be achieved by concentrating the cells, before extracting the metabolite, by means of rapid filtration. Then by freezing the cells with liquid nitrogen, metabolism can be halted instantly. It was found that when metabolites were measured, yields were largely dependent on the method of extraction, since different metabolites are sensitive to different pH and temperature conditions. Methods of extraction found to be reliable for the metabolites of interest in this study are presented in Chapter 3.

Metabolic control coefficients calculated by the model helped identify the parameters that control flux through the glycerol synthesis pathway most rigidly. The first reaction of the pathway, catalyzed by NAD^+-dependent glycerol 3-

phosphate dehydrogenase, had a flux control coefficient (C^J_{v1}) of 0.83 to 0.87 and exercises the majority of control of flux through the pathway, while the subsequent reaction, catalyzed by glycerol 3-phosphatase, had far less control ($C^J_{v2} = 0.13$ to 0.17).

The response coefficients ($R^J_{[x]}$) of various parameter metabolites indicate that flux through the pathway is most responsive to the concentration of the substrate, DHAP ($R^J_{[DHAP]} = 0.48$ to 0.69), followed by the concentration of the inhibitor, ATP ($R^J_{[ATP]} =$ -0.21 to -0.5). Interestingly, the model also predicts that the pathway responds far more severely to the ATP/ADP ratio than to the NADH/NAD ratio, because of the weak response coefficient attributed to NADH ($R^J_{[NADH]} = 0.03$ to 0.08). Thus, the model suggests that the targets most strategic for altering glycerol synthesis would be the V_{max} of the glycerol 3-phosphate dehydrogenase reaction and the concentrations of DHAP and ATP. Ideally, the approach would entail manipulating each of these parameters to their optimal levels in conjunction with each other, with the least detrimental physiological effect possible.

OPSOMMING

Gliserolmetabolisme is noodsaaklik tydens die fisiologiese aanpassing van gis onder hiperosmotiese strestoestande. Dit speel ook 'n fundamentele rol in die handhawing van 'n voordelige redokstoestand, tydens groei onder fermentatiewe kondisies. Alle aspekte rondom die verwantskap tussen redoksbalansering en gliserolmetabolisme is nog nie ten volle gedefinieer nie en pogings om hierdie verwantskap te manipuleer, m.a.w. om gliserolproduksie tydens fermentasie te verhoog of verlaag, het 'n redoksversteuring tot gevolg, wat dikwels nadelig teenoor ander aspekte van metabolisme is. Verder is die meganisme van hoe verskeie parameters in die gliserolsintese pad, beide afsonderlik en gesamentlik, die tempo van gliserolsintese beheer, nie ten volle duidelik nie. Die doel van hierdie studie was dus om die bogenoemde onduidelikhede te probeer verklaar. In hierdie verband is die teorie van metaboliese kontrole analise (MCA) gebruik, en berekeninge is uitgevoer met behulp van 'n eksperimenteel gevalideerde kinetiese model.

Ten einde, die *in vivo* substraat-, produk-, koensiem-, asook bekende aktiveerder en inhibeerder-konsentrasies van die gliserolsintese pad te bepaal, moes betroubare tegnieke ontwikkel word om die metabolisme vinnig te stop en sodoende metaboliete te ekstraheer en te meet. Dié metabolietkonsentrasies vorm 'n deel van die parameters in die pad, en word dus benodig om 'n gedetailleerde kinetiese model saam te stel. Die ensiematiese bepaling van intrasellulêre metabolietkonsentrasies vereis dat die selekstrak genoegsame hoeveelhede van die metaboliete bevat. Dit kan verkry word deur die selle te konsentreer deur middel van 'n vinnige filtrasiestap voordat ekstraksie van die metaboliete plaasvind. Metabolisme word onmiddellik gestop deur die selle te vries met vloeibare stikstof. Die metaboliet-hoeveelhede het grootliks afgehang van die ekstraksie-metode gebruik, aangesien verskillende metaboliete gevoelig is vir verskeie pH en temperatuurkondisies. Betroubare ekstraksiemetodes vir die metaboliete van belang vir hierdie studie word aangedui in Hoofstuk 3 van die tesis.

Metaboliese kontrole koëeffisiënte wat met die model bereken is, het daardie parameters geïdentifiseer wat die fluksie deur die gliserolsintese pad die meeste beïnvloed. Die eerste reaksie in die pad, wat deur NAD^+-afhanklike gliserol 3-fosfaat dehidrogenase gekataliseer word, besit 'n fluksie kontrole-koëeffisiënt (C_{v1}^J) van 0.83 tot 0.87, en oefen die grootste beheer uit oor fluksie deur die pad. Die daaropvolgende reaksie, gekataliseer deur gliserol 3-fosfatase, handhaaf minder kontrole oor fluksie deur die pad (C_{v2}^J = 0.13 tot 0.17).

Responskoëeffisiënte ($R_{[x]}^J$) van verskeie parametermetaboliete dui daarop dat die fluksie deur die pad die sterkste deur substraat (DHAP) konsentrasie beïnvloed word ($R_{[DHAP]}^J$ = 0.48 tot 0.69), gevolg deur inhibeerder (ATP) konsentrasie ($R_{[ATP]}^J$ = -0.21 tot -0.5). Daarbenewens dui die model ook aan dat die pad meer sensitief is teenoor die ATP/ADP verhouding, relatief tot die NADH/NAD^+ verhouding, wat die gevolg is van die klein responskoëeffisiënt teenoor NADH ($R_{[NADH]}^J$ = 0.03 tot 0.08). Die model suggereer dus dat die V_{maks} van die gliserol 3-fosfaat dehidrogenase reaksie, asook die DHAP en ATP konsentrasies, die mees strategiese teikens vir manipulering van gliserolsintese behoort te wees. Die ideale benadering sal dus wees om al hierdie parameters in samehang met mekaar te kan manipuleer tot hul optimale vlakke, met die minste ontwrigting van die sel se fisiologie.

BIOGRAPHICAL SKETCH

Garth Rupert Cronwright was born in Johannesburg, South Africa, on July the 29th, 1974. He grew up in the Lowveld and completed his secondary education in 1992 at Lowveld High School in Nelspruit. He then completed his national service in 1993 at 44 Parachute Battalion in Bloemfontein. In 1995, Garth enrolled at Stellenbosch University and completed a B.Sc. in Human Physiology and Genetics. Thereafter, he completed a B.Sc. Hons. (Genetics) in 1998, at the same university.

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COMMONLY USED ABBREVIATIONS

METABOLITES

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
DHAP	Dihydroxyacetone phosphate
F1,6BP	Fructose 1,6-bisphosphate
G3P	Glycerol 3-phosphate
Gla3P	Glyceraldehyde 3-phosphate
Glu6P	Glucose 6-phosphate
NAD^+	Nicotinamide adenine dinucleotide (oxidized form)
NADH	Nicotinamide adenine dinucleotide (reduced form)

GENES

GPD	NAD^+-dependent glycerol 3-phosphate dehydrogenase
GPP	DL-glycerol 3-phosphatase

OTHER

V_{max}	Maximum enzyme activity (mM/min)
K_{eq}	Equilibrium constant
K_m	Michaelis constant (mM)
K_i	Inhibition constant (mM)
MCA	Metabolic Control Analysis
MFA	Metabolic Flux Analysis
J	Flux (mM/min)
C_{vi}^{y}	Control coefficient (see Sec. 2.2.4.1)
ε_{p}^{vi}	Elasticity coefficient (see Sec. 2.2.4.1)
R_{p}^{y}	Response coefficient (see Sec. 2.2.4.1)

CONTENTS

1. INTRODUCTION

An essential function of life for all organisms is the capacity to adapt to environmental changes. In the case of microorganisms, the ability to adapt to a sudden decrease in environmental water activity (a_w) is vital for survival in nature. The ability to react and cope with these environmental changes rests in the so-called osmotic stress response. This physiological response entails the production and accumulation of "physiologically friendly" compounds, often referred to as compatible solutes since, they help maintain turgor without interfering with cellular functions (Yancey *et al.,* 1982). In *Saccharomyces cerevisiae*, glycerol metabolism plays a very significant role in this respect, since adaptation to and survival of hyper-osmotic conditions is dependent on glycerol production, uptake and retention [reviewed by Prior & Hohmann (1997)].

Glycerol metabolism plays another important role, which under certain growth conditions may be indispensable in catabolic metabolism. In this case however, the significance of glycerol is not linked to its amphipatic properties, but is linked specifically to the redox reaction leading to the formation of glycerol. Glycerol is produced by the reduction of dihydroxyacetone phosphate to glycerol 3-phosphate. This reaction is catalyzed by a cytosolic glycerol 3-phosphate dehydrogenase, and in the process, NADH is oxidized to NAD^+. Glycerol 3-phosphate is subsequently dephosphorylated to glycerol by glycerol 3-phosphatase (Gancedo *et al.*, 1968). Under aerobic growth conditions *S. cerevisiae* produces NADH in the mitochondria as well as in the cytosol from catabolic and biosynthetic reactions (van Dijken *et al.*, 1986). Since it is necessary to re-oxidize NADH to NAD^+ to maintain sustained metabolism, *S. cerevisiae* has evolved several different mechanisms to achieve this (Nissen *et al.*, 1997; Larsson *et al.*, 1998; Luttik *et al.*, 1998; Pahlman *et al.*, 2001). However, when *S. cerevisiae* cells are cultured in the absence of oxygen or in the presence of high concentrations of glucose, these mechanisms are strongly repressed. Under these conditions, NADH formed in glycolysis is primarily oxidized through the formation of ethanol, resulting in a redox-neutral process (van Dijken *et al.*, 1986). However, since a portion of glycolytic intermediates are consumed in anabolic reactions and converted to oxidized by-products such as acetate, an excess of reducing equivalents is generated. Consequently, NAD^+ is regenerated through the production of glycerol (van Dijken *et al.*, 1986), which may end up consuming up to 4-10% of the carbon source (Radler & Schutz, 1982).

The property of redox balancing and glycerol synthesis by yeast is of major importance to various fermentation industries. On one side, where high levels of glycerol and low yields of ethanol are desired from sugar fermentation, i.e. in the low-alcoholic beverage and wine industry, yeast strains capable of directing a large portion of carbon flux toward glycerol are of commercial value. This is illustrated by the effort spent on various strategies to achieve this objective. Strategies

that have been attempted included; the manipulation of environmental parameters (Rankine & Bridson, 1971; Ough *et al.*, 1972; Radler & Schutz, 1982; Watanabe *et al.,* 1982; Gardner *et al.,* 1993; Albers *et al.,* 1996), breeding of glycerol hyperproducing strains (Eustace & Thornton, 1987; Prior *et al.*, 1999) and through genetic manipulation (Compagno *et al.,* 1996; De Barros *et al.,* 1996; Michnick *et al.,* 1997; Remize *et al.*, 1999; Compagno *et al.,* 2001; Remize *et al.*, 2001). While success has been achieved in raising glycerol production in all three cases, a number of drawbacks to other aspects of metabolism and growth have been encountered.

In contrast, as ethanol produced by *S. cerevisiae* is expected to become an important transportation fuel in the future, much effort is being devoted to reduce carbon flux towards the formation of biomass and major by-products, such as glycerol (von Sivers & Zacchi, 1995). With regard to decreasing glycerol formation during the fermentation process, a number of molecular approaches have been undertaken (Valadi *et al.*, 1998; Anderlund *et al.*, 1999; Nissen *et al.*, 2000). In a number of cases, glycerol yields have been reduced and ethanol yields have been increased; nevertheless, these manipulations also have an impact on the growth properties of the strain.

It is evident that attempts to manipulate this relationship, whether to increase or decrease the amount of carbon directed toward or from glycerol formation, invariably results in unforeseen metabolic occurrences. No doubt this is because of the limited of understanding of the complex relationship between glycerol metabolism and the maintenance of a yeast cell's redox state during fermentation. To overcome this shortfall, numerous studies on the metabolism of glycerol and its role in redox balancing have been carried out. A number of these have shown that under fermentative conditions yeast cells possess mechanisms to regulate the amount of glycerol synthesizing enzymes present to maintain a favourable redox state (Ansell *et al.*, 1997; Costenoble *et al.*, 2000; Pahlman *et al.*, 2001). However, how the various parameters of the glycerol synthesis pathway interact and independently affect the rate at which glycerol is synthesized has not been explored at all. Thus, the purpose of this thesis has been to address this gap in our present understanding of glycerol metabolism. More specifically it has been to:

1) Identify all relevant parameters of the glycerol synthesis pathway in *S. cerevisiae* that would help define the control of glycerol synthesis.
2) Once identified, collect data on kinetic parameters, and calculate parameter values relating to enzyme activity, substrate, coenzyme, product, and known modifier concentrations.
3) Use these parameters to construct a detailed mathematical model of the glycerol synthesis pathway.
4) Compare variables calculated by the model with those determined experimentally to validate the model.

5) If valid, use the model to ascertain the parameters that exercise strong control on glycerol synthesis and ultimately help identify more strategic targets for the manipulation of glycerol production.

2. LITERATURE REVIEW

2.1 GLYCEROL METABOLISM IN *SACCHAROMYCES CEREVISIAE*

2.1.1 GLYCEROL SYNTHESIS

2.1.1.1 Synthesis pathway

Glycerol is produced in a two-step process, beginning with the reduction of dihydroxyacetone phosphate to glycerol 3-phosphate coupled with NADH oxidation, followed by dephosphorylation of glycerol 3-phosphate. Dihydroxyacetone phosphate originates as an intermediate in the glycolytic pathway as one of the two cleavage products from the fructose 1,6-bisphosphate aldolase reaction. Most of the dihydroxyacetone phosphate, however, is converted to glyceraldehyde 3-phosphate by the interconverting enzyme triose phosphate isomerase (Figure 2.1).

The first reaction is catalyzed by two isoforms of the NAD^+-dependent glycerol 3-phosphate dehydrogenase, encoded by the *GPD1* and *GPD2* genes (Larsson *et al.*, 1993; Albertyn *et al.*, 1994a; Eriksson *et al.*, 1995), and the subsequent reaction is carried out by the action of a glycerol 3-phosphate specific phosphatase, encoded by two isogenes *GPP1* and *GPP2* (Norbeck *et al.*, 1996).

2.1.1.2 Biochemistry and transcriptional regulation of *GPD1*

GPD1 encodes the cytosolic NAD^+-dependent glycerol 3-phosphate dehydrogenase and is located on chromosome IV of *S. cerevisiae*. The predicted size of the enzyme is 391 amino acid residues with a calculated mass of 42,8 kDa, in agreement with that of the 42 kDa subunit of the purified protein from *S. cerevisiae* (Nader *et al.*, 1979; Albertyn *et al.*, 1992). The deduced amino acid sequence indicates a 43-50% identity with glycerol 3-phosphate dehydrogenase of other species and reveals a two-domain protein structure for the binding of a coenzyme and substrate (Larsson *et al.*, 1993; Albertyn *et al.*, 1994a). A significant difference between Gpd1p and its counterparts from other eukaryotes is the presence of an amino-terminal extension of about 30 amino acids. The extension contains characteristic sequences of mitochondrial signal peptides, the function of which is still unclear. (Larsson *et al.*, 1993; Albertyn *et al.*, 1994a). The pI of Gpd1p is 5,3. Kinetic data have shown NADH and dihydroxyacetone phosphate to be the primary substrates of glycerol 3-phosphate dehydrogenase in *S. cerevisiae* since the reverse reaction is 30-fold slower than the forward reaction (Nader *et al.*, 1979; Chen *et al.*, 1987; Nilsson & Adler, 1990; Albertyn *et al.*, 1992). Using Lineweaver-Burk plots, the K_m values of Gpd1p were shown to be 0.018 - 0.023 mM for NADH and 0.37 - 0.54 mM for dihydroxyacetone phosphate (Albertyn *et al.*, 1992; Chen *et al.*,

1987). The Gpd1p shows no activity against dihydroxyacetone or NADPH at physiological levels. Gpd1p is specific for NADH and not NADPH, which indicates that the pentose phosphate pathway does not play a role in supplying reducing equivalents for the production of glycerol (Nader *et al.*, 1979).

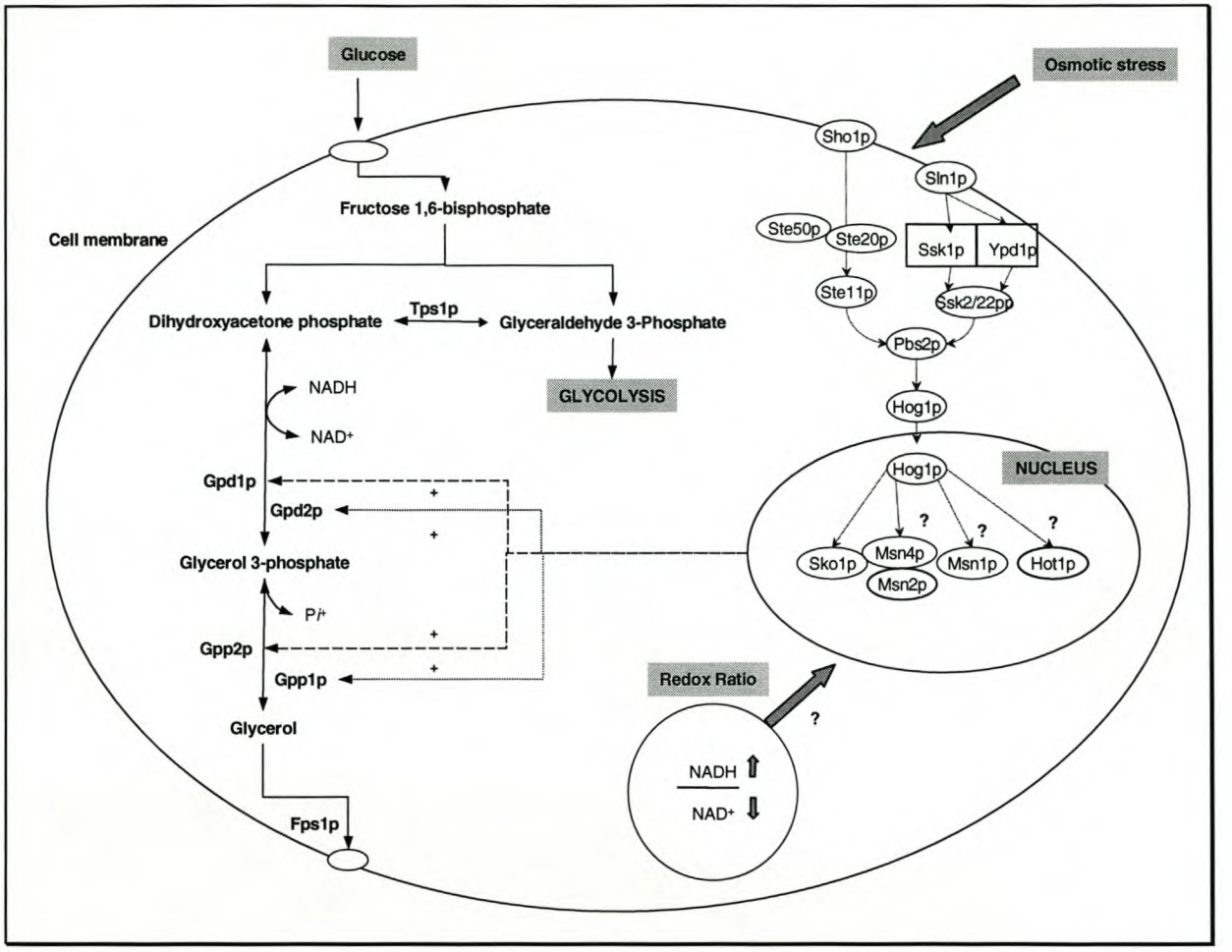


Figure 2.1 The glycerol synthesis pathway and the response to osmotic stress via the HOG pathway and the redox balance during anaerobic fermentation. Abbreviations of enzymes: (Tpi1p) triosephosphate isomerase; (Gpd1p/Gpd2p) NAD^+-dependent glycerol 3-phosphate dehydrogenase; (Gpp1p/Gpp2p) glycerol 3-phosphatase; (Fps1p) glycerol facilitator.

Glycerol may accumulate to molar concentrations within cells under certain stress conditions, and at these concentrations (up to 1 M), glycerol fails to inhibit Gpd1p activity (Albertyn *et al.*, 1992). NAD^+ inhibits glycerol 3-phosphate dehydrogenase competitively and at physiological concentrations ATP, ADP and fructose 1,6-bisphophate were found to inhibit Gpd1p noncompetitively (inhibition constants are presented in Table 4.1), indicating *in vivo* regulation by these metabolites (Albertyn *et al.*, 1992).

The production and intracellular accumulation of glycerol plays a crucial role in the ability of yeasts to adapt to hyperosmotic conditions (Brown, 1978; Brown *et al.*, 1986). Consequently, when yeast cells are exposed to increased external osmolarity, expression of *GPD1* is elevated and glycerol production increases (Andre *et al.*, 1991; Albertyn *et al.*, 1994; Eriksson *et al.*, 1995; Ansell *et al.*, 1997; Rep *et al.*, 1999). This response to hyperosmotic stress forms part of a broader osmoregulatory response and contributes significantly to the physiological process by which yeast cells regulate their water content. The physiological effect of accumulating a compound such as glycerol is that the cells' cytosolic osmotic potential decreases, thus remaining at a similar level to the growth medium (Yancey *et al.*, 1982). Because of its amphipatic properties, glycerol belongs to a group of compounds referred to as "compatible solutes" (Brown *et al.*, 1972) or "osmolytes" (Yancey *et al.*, 1982). These compounds have minimal deleterious effect on protein structure when accumulated to high concentrations, and are known to alleviate some of the inhibitory effects of high concentrations of ionic compounds (Yancey *et al.*, 1982). Most osmolytes are derivatives of amino acids or higher alcohols, and the exact nature by which they are accumulated and retained is a point of divergence between species. Nevertheless, most often they are accumulated by endogenous production or by uptake from the medium.

Numerous studies have revealed that mRNA transcript levels of *GPD1* increase drastically when *S. cerevisiae* cells are exposed to hyperosmotic conditions, whether the stress be induced by elevated NaCl or sorbitol concentrations (Eriksson *et al.*, 1995; Ansell *et al.*, 1997; Rep *et al.*, 1999; Rep *et al.*, 2000). The osmotic induction of *GPD1* expression is executed via an osmosensing signal transduction cascade, called the high osmolarity glycerol (HOG) pathway (Brewster *et al.*, 1993; Albertyn *et al.*, 1994b). The HOG pathway is a mitogen-activated pathway (MAP) kinase cascade which is related to three other MAPK pathways, known as the pheromone response pathway (Herskowitz *et al.*, 1995), the pseudohyphal/invasive growth pathway (Liu *et al.*, 1993; Roberts & Fink *et al.*, 1994) and the PKC-regulated pathway. The latter responds to heat and hypo-osmotic stress (Davenport *et al.*, 1995; Kamada *et al.*, 1995). Two transmembrane proteins, Sho1p and Sln1p, appear to serve as independent osmosensors and mediate activation of the HOG pathway (Figure 2.1) (Maeda *et al.*, 1994; Maeda *et al.*, 1995). The Sln1p histidine kinase receptor feeds into a three-component system consisting of Ypd1p and Ssk1p as response regulators (Posas *et al.*, 1996). Stimulation of the Sln1p kinase results in the activation of a pair of MAP kinase kinase kinases (MAPKKK's), Ssk2p and Ssk22p, which in turn activate a MAPKK, Pbs2p (Maeda *et al.*, 1995). Sho1p transmits its signal via Ste50p and Ste20p to the MAPKKK Ste11p, which in turn phosphorylates Pbs2p, thus converging with the signal from Sln1p. The MAPKKK, Ste11p, is also an integral component of the mating pheromone response pathway. There has been speculation whether "cross-talk" occurs between the different MAP-kinase pathways (Davenport *et al.*, 1995);

however, it appears that the uncontrolled interference between pathways is eliminated by the formation of enzyme complexes. In this complex, Pbs2p acts as a scaffold protein in the HOG pathway, thereby preventing "cross-talk" with the pheromone-mating pathway in which Ste5p acts as the scaffold protein (Posas *et al.*, 1997). Once Pbs2p is activated by the two upstream branches it stimulates tyrosine phosphorylation of Hog1p, a MAPK, at T174 and T176, which leads to translocation of the phosphorylated Hog1p to the nucleus (Brewster *et al.*, 1993; Gustin *et al.*, 1998; Reiser *et al.*, 1999). Transfer to the nucleus results in a three-fold induction in the mRNA level of some 186 genes. Many of the induced genes encode proteins that presumably contribute to protection against different types of damage or encode enzymes in glycerol, trehalose and glycogen metabolism (Rep *et al.*, 2000). There is also new evidence that a novel mechanism is involved in activation of the HOG pathway. This mechanism operates in severe osmostress conditions (1.4 M NaCl) and is independent of the Sln1p and Sho1p osmosensors. The alternative input activates Pbs2p by phosphorylation, however Hog1p phosphorylation and nuclear accumulation is delayed, which results in delayed stress-responsive gene expression (van Wuytswinkel *et al.*, 2000).

One of the major unresolved problems concerning this transcriptional response is the identity of the molecular targets recognized by the signaling pathway. Recently, two transcription factors, Msn2p and Msn4p, have been found to respond to a large variety of stress situations, including osmostress. These factors are translocated to the nucleus where they target and bind to a common promoter element called the stress response element (STRE). STRE elements promote the transcription of stress responsive genes such as the *CTT1* (encodes catalase) or *HSP12* (encodes the small heat shock protein) genes (Gorner *et al.*, 1998); however, HOG pathway mediated transcriptional activation can still be found in the absence of Msn2p and Msn4p function (Martinez-Pastor *et al.*, 1996; Rep *et al.*, 1999). In a recent study, the previously undescribed nuclear protein Hot1p has been shown to be important in the HOG pathway-dependent osmotic induction of *GPD1* and *GPP2*. A protein with a similar putative DNA-binding domain, Msn1p, contributes to both heat and osmotic stress induction of *GPD1, CTT1* and *HSP12*. Although the relationship with Hog1p is not well understood, together, Hot1p and Msn1p seem to mediate the bulk of Msn2p- and Msn4p-independant osmostress activation of the genes *GPD1, GPP2, CTT1* and *HSP12* (Rep *et al.*, 1999). Furthermore, stimulated expression of some HOG pathway-dependent genes after osmotic stress appears to be the result of transcriptional derepression (Marquez *et al.*, 1998). It appears as if derepression is brought about by Hog1p dependent phosphorylation of Sko1p, a bZIP transcriptional repressor. Phosphorylation of Sko1p disrupts the Sko1p-Ssn6p-Tup1p repressor complex. A strain harbouring a mutant allele of Sko1p, cannot be phosphorylated by Hog1p and exhibits less derepression than wild-type strains, and is consequently more osmosensitive (Proft *et al.*, 2001).

2.1.1.3 Biochemistry and transcriptional regulation of *GPD2*

GPD2 located on chromosome VII, encodes a second NAD^+-dependent glycerol 3-phosphate dehydrogenase (Eriksson *et al.*, 1995), which consists of 385 amino acids, has an estimated molecular mass of 42,3 kDa and a theoretical pI of 6,8. Sequence comparison exhibits a high degree of homology to amino acid sequences from various sources. The amino acid sequence shows 69% homology to *GPD1* and between 44 and 47% to other GPD enzymes. Juxtaposition of the *GPD2* and *GPD1* sequences revealed that the central and major parts of the proteins were strikingly homologous, exhibiting only 68 amino acid substitutions, the majority of which were interchangeable with regard to their chemical properties. The Gpd2p polypeptide, like the Gpd1p, contains an amino-terminal extension; however, it differs remarkably from that of Gpd1p, and its function still remains unknown. The promoter regions of *GPD1* and *GPD2* have a very low degree of homology, which points to different regulatory mechanisms (Eriksson *et al.*, 1995); however, there is no evidence that the two gene products are functionally different since there is a high degree of homology at the amino acid level and the isoforms have a similar affinity for their substrates (Ansell *et al.*, 1997).

GPD2 is subject to redox control and is strongly induced under anoxic conditions or when yeast cells are grown in the presence of glucose (Crabtree affect) (Ansell *et al.*, 1997; Bjorkvist *et al.*, 1997; Costenoble *et al.,* 2000). In *S. cerevisiae,* the role of *GPD2* in redox regulation can be illustrated by growing *gpd2*Δ mutants in the absence of oxygen. Under these conditions, *gpd2*Δ mutants are unable to respond to the unfavourable redox ratio ($NADH/NAD^+$) and consequently grow at a far slower rate, produce 40% less glycerol and 8% more ethanol (Valadi *et al.*, 1998), whilst *gpd1*Δ mutants exhibit no significant deviation from the growth pattern of wild-type cells (Bjorkvist *et al.*, 1997; Valadi *et al.*, 1998). These findings have been confirmed by other reports, and they indicate that, of the two isogenes known to encode a cytosolic NAD^+-dependent glycerol 3-phosphate dehydrogenase, *GPD2* is primarily responsible for redox maintenance (Ansell *et al.*, 1997; Valadi *et al.*, 1998; Costenoble *et al.*, 2000).

Little is known about the regulation of functions required for anaerobic growth of *S. cerevisiae*. A set of hypoxic genes encoding selected enzymes in heme, sterol and fatty acid biosynthesis are repressed under aerobic conditions and stimulated under oxygen limiting conditions (Zitomer & Lowry, 1992). It is assumed that the increased expression of these genes is required to circumvent the effects of hypoxic conditions. The availability of oxygen seems to be sensed through the heme level, the synthesis of which is heme dependent. Heme activates the transcription of *ROX1*, a hypoxic repressor (Balasubramanian *et al.*, 1993; Deckert *et al.*, 1995a). The *ROX1* controlled genes encode products that are required only when oxygen is limiting.

Another gene, *ROX3*, encodes a nuclear protein that appears to be involved in the control of anaerobic expression of heme regulated genes (Rosenblum-Vos *et al.*, 1991) and participates in mediating diverse stress responses in yeast (Evangelista *et al.*, 1996). Nevertheless, a specific regulatory mechanism independent of *ROX1* and *ROX3* should exist, since induction of *GPD2* occurs in *ROX1* and *ROX3* deficient strains under anoxic conditions. The mechanism/s that regulate the expression of *GPD2* and other more general metabolic functions under anaerobic conditions are still unknown. Preliminary evidence suggests that oxygen itself is not directly involved as a regulatory signal. Instead the redox-state of the cytosol appears to be sensed, possibly by the $NADH/NAD^+$ ratio. This is supported by the observation that under anaerobic conditions, *GPD2* mRNA levels respond to artificial manipulation of the cytosolic $NADH/NAD^+$ ratio (Figure 2.1) (Ansell *et al.*, 1997).

2.1.1.4 Biochemistry and transcriptional regulation of *GPP1* and *GPP2*

GPP1 and *GPP2* encode two isoforms of DL-glycerol 3-phosphatase in *S. cerevisiae*. Both isoforms are found in the cytosol. The predicted amino acid sequences of the two isozymes are 95% identical, have molecular masses of 30,4 and 27,8 kDa respectively, are catalyticaly active as monomers with pH optima at 6,5 and have respective K_m values of 3,1 and 3,9 mM for glycerol 3-phosphate (Norbeck *et al.*, 1996).

A contribution from Gpp2p to the osmo-stress response is indicated by the finding that this isoform, which is the minor form under non-stressed conditions, increases as cells are cultured at elevated osmolarity. The *GPP1* encoded isoform is abundantly present under normal growth conditions, and is regulated independently of external osmolarity (Norbeck *et al.*, 1996). Induction of the *GPP1* gene appears to play a decisive role at elevated growth rates or hypoxic conditions, when there is a high demand for NADH re-oxidation (Costenoble *et al.*, 2000).

2.1.2 Glycerol Assimilation

Glycerol can be used as a carbon source by *S. cerevisiae* in the absence of glucose (Sprague *et al.*, 1977). The glycerol catabolic pathway involves phosphorylation of glycerol by a glycerol kinase, followed by the reduction of glycerol 3-phosphate to dihydroxyacetone phosphate by the flavin-dependent mitochondrial glycerol 3-phosphate dehydrogenase (mtGpd), located on the outer surface of the mitochondrial inner membrane (Gancedo *et al.*, 1968; Klingenberg *et al.*, 1970). The dihydroxyacetone phosphate formed enters the glycolytic pathway (Figure 2.2).

Synthesis of glycerol kinase and mtGpd is predominantly controlled by glucose repression and there is no marked induction of these enzymes by glycerol (Sprague *et al.*, 1977). The structural

genes for glycerol kinase (*GUT1*) and for mtGpd (*GUT2*) have been cloned by functional complementation of the corresponding *gut1*Δ and *gut2*Δ mutants (Pavlik *et al.*, 1993; Ronnow *et al.*, 1993). *GUT1*, located on chromosome VIII, encodes a protein of 709 amino acids with 40,8% homology with the *Escherichia coli* glycerol kinase and 42,1% homology with the *Bacillus subtilis* enzyme. *GUT2* encodes a protein with a predicted mass of 68,8 kDa, is 615 amino acids in length and has been mapped to chromosome IX. The *GUT2* gene shares a low degree of homology with the anaerobic (27%) and the aerobic (32%) glycerol 3-phosphate dehydrogenase of *E. coli* (Ronnow *et al.*, 1993).

The mitochondrial Gpd, in conjunction with cytoplasmic NAD^+-dependent Gpd, participates in the aerobic glycerol 3-phosphate shuttle system (G3P) (Haddock *et al.*, 1977). In the G3P shuttle, cytosolic NADH reduces dihydroxyacetone phosphate (DHAP) and the resulting glycerol 3-phosphate (G3P) passes through the permeable outer mitochondrial membrane where it is re-oxidized to DHAP by the mtGpd. The DHAP produced then returns to the cytoplasm by an unknown mechanism. Hence, the overall process provides a means for mitochondrial oxidation of NADH that is produced in the cytoplasm (Larsson *et al.*, 1998). Various yeasts have an alternative means of assimilating glycerol, which involves a putative $NADP^+$-dependent glycerol dehydrogenase (encoded by the isogenes *GCY1* and *YPR1*), which oxidizes glycerol to dihydroxyacetone and a dihydroxyacetone kinase (encoded by isogenes *DAK1* and *DAK2*) that phosphorylates dihydroxyacetone (Hofmann & Babel, 1982; Forage & Lin, 1982). These genes are also found in *S. cerevisiae* and *GCY1, YPR1* and *DAK1* are known to be induced by elevated salt concentrations (Norbeck & Blomberg, 1997; Rep *et al.*, 2000). However, glycerol utilization by this pathway seems to be less important in this species, since mutants defective in glycerol kinase and mtGpd are unable to grow when glycerol is used as the sole carbon source (Sprague *et al.*, 1977; Pavlik *et al.,* 1993). Fine-tuning of the intracellular glycerol concentration has been suggested as a function of the pathway (Norbeck & Blomberg, 1997).

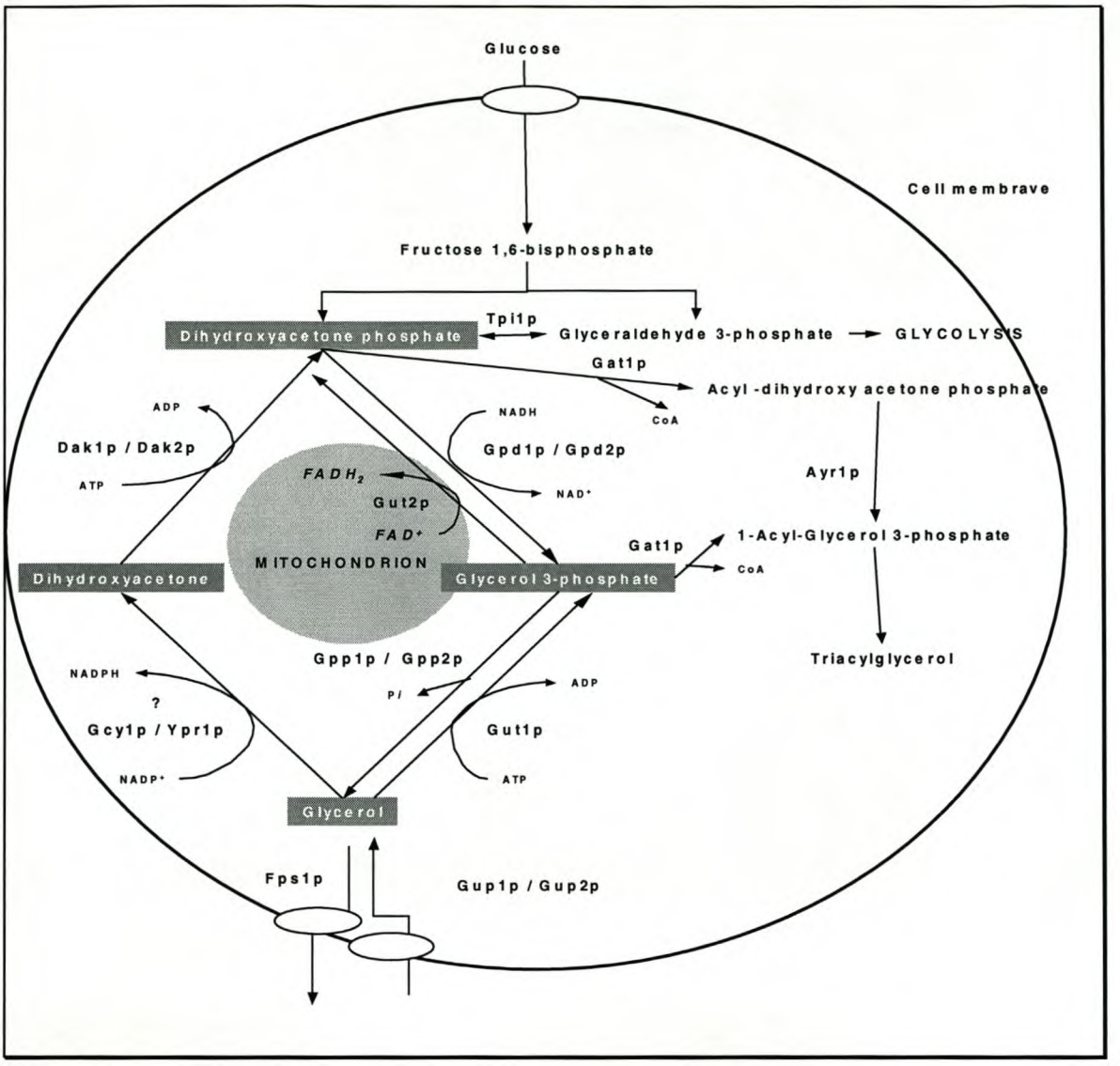


Figure 2.2 Pathways involved in glycerol assimilation in *S. cerevisiae.* Abbreviations of enzymes are: (Ayr1p) 1-acylhydroxyacetone-phosphate reductase; (Dak1p/Dak2p) putative hydroxyacetone kinase; (Fps1p) glycerol facilitator; (Gup1p/Gup2p) glycerol uptake protein; (Gat1p) glycerol 3-phosphate acyltransferase and dihydroxyacetone phosphate acyltransferase; (Gcy1p/Ypr1p) putative $NADP^+$-dependent glycerol dehydrogenase; (Gpd1p/Gpd2p) glycerol 3-phosphate dehydrogenase; (Gpp1p/Gpp2p) glycerol 3-phosphatase; (Gut1p) glycerol kinase; (Gut2p) mitochondrial glycerol 3-phosphate dehydrogenase ($FADH_2$). Adapted form Costenoble *et al.* (2000).

Glycerol metabolic pathways also play an important role as a source of precursors necessary for the synthesis of triacyl-glycerol and glycerophospholipids. Glycerol 3-phosphate acyltransferase, encoded by the *GAT1* gene (Racenis *et al.*, 1992; Athenstaedt *et al.*, 1999), can use glycerol 3-phosphate or dihydroxyacetone phosphate as a substrate. In the latter case, the formed acyl-dihydroxyacetone phosphate can be reduced in a NADPH-coupled reaction by acyl-dihydroxyacetone phosphate reductase, encoded by the *AYR1* gene (Athenstaedt & Daum, 2000)

2.1.3 Glycerol Transport

The accumulation of compatible solutes, such as glycerol in yeast *Sacchromyces cerevisiae*, is a ubiquitous mechanism in cellular osmoregulation (Brown, 1976). In yeast, this accumulation is controlled at different levels. In *S. cerevisiae* the regulation of glycerol production plays a central role (Larsson *et al.*, 1993; Albertyn *et al.*, 1994b; Hohmann *et al.*, 1997). Glycerol crosses all biological membranes to some extent by passive diffusion due to its lipophilic nature (Hohmann *et al.*, 1997). Microorganisms also produce specific transport proteins that aid in the rapid transport of glycerol across the membrane; for example, in *Escherichia coli* glycerol crosses the cytoplasmic membrane via a proteinaceous pore mechanism (Heller *et al.*, 1980). Active transport mechanisms requiring the expenditure of energy are also found, and have been identified in *Zygosacchromyces rouxii, Debaryomyces hansenii, Pichia sorbitophila* and *S. cerevisiae* (Van Zyl *et al.*, 1990; Lages *et al.*, 1995; Holst *et al.*, 2000).

2.1.3.1 The glycerol facilitator, Fps1p

Until recently, it was assumed that glycerol was only taken up by passive diffusion in *S. cerevisiae*. *FPS1*, which encodes a protein belonging to the yeast "major intrinsic protein" (MIP) (Reizer et al., 1993) family of channel proteins, was shown to play a role in facilitating the movement of glycerol across the membrane in this species (Luyten *et al.*, 1995). The MIP family is a group of channel proteins present in organisms ranging from bacteria to humans (Reizer, 1993; Hohmann *et al.*, 2000). Relatively little is known about the molecular mechanisms of MIP channel regulation. However, it has been suggested that the transport activity of at least some of these proteins might be directly regulated by phosphorylation (Maurel *et al.*, 1995).

It has been shown that *fps1*Δ mutants accumulate large amounts of glycerol, and grow far slower than the wild-types cells under anaerobic growth conditions. These growth defects are probably caused by osmotic problems, due to the high intracellular levels of glycerol (Tamas *et al.*, 1999). In addition, during adaptation to hypo-osmotic shock, yeast cells reduce their intracellular glycerol content by 50-75% within 3 min of the shock, whereas mutants lacking the Fps1p require more than 1 hour to achieve the same glycerol loss (Luyten *et al.*, 1995). *Fps1*Δ mutants survive hypo-osmotic shock 50- to 100-fold less than wild type cells.

The rate of glycerol export is greatly reduced during growth at high osmolarity, indicating that cells have mechanisms to control the transport rate of glycerol across the cell wall (Luyten *et al.*, 1995; Sutherland *et al.*, 1997). It has been found that *fps1*Δ mutants have a low glycerol uptake rate in the presence and absence of salt. When wild-type cells were shifted to 5% NaCl, within one minute the glycerol transport rate had been reduced to levels similar to those observed for the *fps1*Δ

mutant. A shift to a higher sucrose rather than NaCl concentration at the same water activity caused the same reduction in the glycerol transport rate, demonstrating that the effect is osmotic and not salt dependent (Tamas *et al.,* 1999).

To determine whether Fps1p-faciltated transport of glycerol depended on metabolic energy, metabolic inhibitors were used to determine the effects on glycerol uptake (Sutherland *et al.,* 1997). Measurements of the pH changes during uptake showed no evidence of simultaneous uptake of glycerol and protons in glucose-grown cells, with either wild type or *fps1*Δ mutants, and the intracellular concentration did not exceed the diffusion equilibrium. This indicates that Fps1p-dependent transport occurs by facilitated diffusion and not active transport. Interestingly, deletion of the *FPS1* gene also affects cellular lipid composition and results in lower apparent membrane permeability. The phospholipid and glycolipid fractions of total lipid composition in *fps1*Δ mutants were 25% lower and 62% higher, respectively, than in the wild type cells (Sutherland *et al.*, 1997). The HOG MAP kinase pathway is induced by an increase in external osmolarity (Brewster *et al.,* 1993), and the PKC MAP kinase pathway is stimulated by a drop in external osmolarity (Davenport *et al.,* 1995), yet none of these pathways have been found to be involved in the rapid regulation of Fps1p-mediated glycerol transport (Tamas *et al.,* 1999).

2.1.3.2 Glycerol uptake proteins, Gup1p and Gup2p

In contrast to glucose grown cells, glycerol uptake in either glycerol- or ethanol-grown cells of both wild-type and *fps1*Δ mutants was accompanied by the simultaneous uptake of protons. Glycerol was accumulated with inside-to-outside ratios of approximately 12 and 15 in the wild type and *fps1*Δ respectively. This indicates the production of an Fps1p-independent proton symport system (Sutherland *et al.*, 1997) in which other transport proteins are involved in the uptake of glycerol (Lages & Lucas, 1997) in *S. cerevisiae.* Two such glycerol uptake proteins, namely *GUP1* and *GUP2,* have subsequently been identified and cloned (Holst *et al.*, 2000). Their ORFs were identified by functional complementation of mutants defective in the transport of glycerol into cells. The amino acid sequence of *GUP1* indicates that the protein consists of 560 residues, with a predicted 8-10 transmembrane domains (Holst, *et al.,* 2000), and has been suggested to belong to the major facilitator superfamily (Nelissen *et al.*, 1997).

Deletion of *GUP1* was found to result in slow growth on glycerol as a carbon source. In addition, the *gup1*Δ mutant also had a moderate growth defect on rich media with glucose as a carbon source when supplemented with 1 M NaCl. This may be interpreted to mean that even when glycerol production is not affected, salt stress requires uptake of glycerol as a result of glycerol diffusing across plasma membrane (Holst *et al.*, 2000). These proteins are essential for proton symport of glycerol, and are active in cells grown on ethanol or glycerol, but are repressed by cells

grown on glucose (Sutherland *et al*, 1997). The facilitated diffusion system, involving Fps1p, seems to function in osmotic regulation (Luyten *et al.*, 1995), whereas the active system, involving Gup1p and Gup2p, seems to have a metabolic role since it is induced by a shift from glycolysis to gluconeogenesis (Lages & Lucas, 1997).

2.2 Manipulation of Yeast Metabolism

2.2.1 Introduction

Genetically modified industrial microorganisms are used for the synthesis of a vast array of products, such as hormones, enzymes, precursors, intermediates, chiral compounds, steroids, vitamins, unnatural amino acids, etc. In such cases the maximal production of the product is essential, and invariably, other physiological properties are unimportant unless they affect maximal production. When yeast is used in food or beverage fermentation, the situation is different because the product of interest is not a single component but a balanced mixture of hundreds of compounds. Glycerol is a major by-product of fermentation by *S. cerevisiae,* and is therefore of major importance to various fermentative industries. When production of ethanol is of primary concern, the less glycerol produced during fermentation, the better. Yet, inhibiting glycerol production for the sake of increased ethanol formation hampers the cell's ability to respond to varying osmotic and anaerobic conditions (Blomberg & Adler, 1992; Albertyn *et al.*, 1994b; Ansell *et al.*, 1997). In contrast, optimal levels of glycerol in wine are high. When glycerol is overproduced in engineered strains, other by-product yields are altered and growth is sluggish (Remize *et al.*, 1999). In light of this, the ensuing section will introduce the concept of metabolic engineering (Bailey, 1991) and address some of the strategies and methodologies employed in this field to optimize engineered metabolic systems. Thereafter, examples of the different metabolic engineering strategies used to alter glycerol metabolism in various *S. cerevisiae* strains will be described.

2.2.2 Metabolic Engineering

The term metabolic engineering was coined by Bailey (1991) who defined it as "Improvement of cellular activities by the manipulation of enzymatic transport and regulatory functions of the cell with use of recombinant DNA technology". An essential part of improving biotechnological production processes is to improve the strains productivity. This may entail enhancing a strains' ability to metabolize cheaper raw materials, to increase product and by-product yield, or to improve a strain's tolerance to environmental conditions, which may include high substrate and/or product

concentrations. The concept of improving or manipulating metabolism originated some time before the term metabolic engineering was coined. Before the advent of recombinant DNA technology, a less directed approach of metabolic engineering was achieved by classical breeding techniques. These classical approaches are effective and still have an important part to play. For example, in the wine industry where there is stringent regulation against the use of genetically engineered yeast strains, classical techniques have been very successful in improving glycerol yields (Eustace *et al.*, 1987; Prior *et al.*, 1999). Another successful approach, which has led to many highly productive industrial strains, entails repeated rounds of mutagenesis and selection. For example, production of penicillin by *Penicillium chrysogenum* has been increased over 500-fold (Nielsen, 1998). It is important to note that with either of the approaches described above, the precise genetic and metabolic alterations that lead to increased metabolite yields in many of these organisms remain unknown. Therefore, increasing productivity in these already highly productive strains depends on the availability of detailed information on the regulation of flux through the relevant metabolic pathways. In contrast to classical techniques, recombinant DNA technology has made it possible to alter and improve the inherent and novel capabilities of various organisms in a rational manner.

There are numerous fields of biotechnology in which metabolic engineering is applied and they all require various strategies depending on the novel function required of the cell. A review by Chartrain *et al.* (2000) provides insight on how engineered microorganisms with a diverse set of modified or non-native enzyme activities are being used to generate improved processes and novel products, such as in the production of precursors, intermediates, chiral compounds and compounds of importance to the pharmaceutical industry, including polyketides, nonribosomal peptides, steroids, vitamins and unnatural amino acids. An area of metabolic engineering that has received much attention over the last decade, has been the effort to extend the range of utilizable substrates, such as lignocelluloses, hemicelluloses and cellulose, by various fermentative microorganisms (Hahn-Hagerdal *et al.*, 1993; Ingram *et al.*, 1998; Parekh *et al.*, 2000) with the intent to replace the use fossil fuels with a more environmentally friendly and renewable fuel such as ethanol (Wyman *et al.*, 1995). Another is the production of products that are entirely novel to the host cell, such as human serum albumin, polyketides, and biopolymers (Fleer *et al.*, 1991; Hutchinson, 1994, McDaniel *et al.*, 1993). Also included is the improvement of general cellular properties, such as the ability to withstand hypoxic fermentation conditions, increased tolerance toward inhibitory substances, the elimination of inhibitory by products, as well as various environmental applications (Stephanopoulos, 1998).

The sequencing of several entire microbial genomes over the last few years has been of significant value to field of metabolic engineering. In the past, progress had been slowed by the lack of gene sequence information (Cameron, 1997). Now that genomic sequence information is more

readily available, it has become possible to apply transcriptome analysis with the aid of microarray technology. This will lead to a better understanding of cellular physiology and will help develop more rational approaches to metabolic engineering, which in turn may be exploited to advance human health, agricultural production, and industrial fermentation processes (Cornish-Bowden & Cardenas, 2000).

2.2.3 Strategies Employed and Problems Encountered

The overproduction of metabolites is one of the goals of metabolic engineering, and the goal may be achieved by the amplification of the biosynthetic pathway (productivity improvement) and alteration of flux distributions of key metabolic branch points (yield improvement) (Simpson *et al.*, 1998). To amplify the flux of a particular pathway requires the identification of the reaction(s) with the greatest impact on the overall pathway flux, followed by the appropriate modification of the corresponding enzymatic activity. Often the outcome of the strategy described above delivers disappointing or negative results. Most often the gene encoding the enzyme in a pathway that is thought to be the "rate limiting" step (flux controlling step) is cloned into a high copy number plasmid and/or placed under control of strong promoter, which results in a 10-100 fold increase in expression of the cloned gene (Jensen & Hammer, 1998). Metabolic control analysis (Heinrich & Rapoport, 1974; Kacser & Burns, 1973) has shown that control can be distributed over many enzymes in a pathway, and experimental determination of control by individual steps in a pathway have shown that this is often the case (Niederberger *et al.*, 1992; Snoep *et al.*, 1995). It has been reported that reaction steps that were thought to be "rate limiting" with respect to a particular flux, may turn out to have no, or very little control over the flux. For example, the negligible effects on glycolytic flux to ethanol observed in yeast with individual glycolytic enzymes overexpressed up to 14-fold (Schaaff *et al.*, 1989). Small & Kacser (1993) and Fell & Thomas (1995) have subsequently shown mathematically that even a near-infinite increase in enzyme activity causes a limited increase in metabolic flux if the flux control coefficient (definition provided in section 2.2.4) is less than 0.6. This phenomenon may also be attributed to the homeostatic control mechanisms of the cell (either at the metabolic or genetic level) working to maintain constant concentrations of the intracellular metabolites (Jensen & Hammer, 1998). For this reason an alternative approach suggested by Kacser & Acerenza (1993) was the"Universal Method", which aimed to produce unbound flux increases to the desired product while maintaining all fluxes to other products, and metabolite concentrations at constant levels. This is achieved by increasing all the enzymes in the product branch by the same amount, so that none of the metabolites in the branch change. The aim is to avoid the intrinsic regulatory mechanisms by generating any concentration changes in metabolites to act as regulatory

signals. For this reason, the Universal Method has been termed an "evasion" strategy (Cornish-Bowden, 1995). The accurate control of the overexpression of a large number of genes is beyond the current capability of molecular genetics (Fell, 1998). However, a partial demonstration exists, in which five genes were simultaneously overexpressed to approximately 23-fold their usual levels, which led to a 9-fold increase in the tryptophan synthesis flux in yeast (Niederberger *et al.*, 1992).

Another strategy considered to increase flux, is to change the control structure of a pathway. Abolishing the feed back inhibition on an enzyme at the start of a pathway should increase its flux control coefficient, thereby allowing its raised activity to increase flux (Hofmeyr & Cornish-Bowden, 1991). However, further simulation studies suggests that the major effect of abolishing feedback inhibition will be to increase intermediate metabolite concentrations much more than fluxes. This has been illustrated by increased accumulation and excretion of intermediates in feedback resistant mutants of amino acid synthetic pathways (Katsumata & Ikeda, 1993). Thus, according to Hofmeyr and Cornish-Bowden (2000) synthetic fluxes towards most metabolites are regulated by metabolic demand and that increasing the supply has little or no effect because in the absence of demand, the feedback mechanisms simply switch off the excess capacity. In light of this, the best strategy might then be to subvert feedback inhibition of the cell (Cornish-Bowden, 1995) by retaining it and transferring control to the demand for the feedback metabolite. If this demand is a single step (e.g. excretion), it is possible that it will have a relatively high flux control coefficient, and therefore activating it could lead to increased flux in a system with feedback control (Fell, 1998).

Yet another strategy used to increase flux is to delete or mutate genes encoding enzymes used in the production of undesired products. For example, Lomovskaya *et al.* (1999) have been able to show dramatic improvements in doxorubicin production by *Streptomyces peucetius* by disrupting genes whose products catalyze conversion to unstable or unwanted side products. However, such a disruption could also negatively affect homeostasis, not because the product of the reaction is indispensable to the cell, but because a redox imbalance might be created by the loss of a cofactor-dependent enzymatic reaction, or because of a metabolite in the branch that regulates another part of metabolism. The elimination of such fluxes may then slow down the general metabolic performance of the cell with respect to the desired product (Jensen & Hammer, 1998).

In general, there is a tendency to overlook the subtlety of control and regulation in the metabolic network that constitutes the living cell, and therefore understanding metabolic fluxes and their control becomes an integral component of the field of metabolic engineering. The combination of analytical techniques to quantify fluxes and their control using molecular biological techniques to implement suggested genetic modification is the essence of metabolic engineering (Stephanopoulos,

1998). The following section will describe some of the techniques developed to quantify flux distribution and the control thereof through a pathway.

2.2.4 MEANS OF ANALYZING AND QUANTIFYING THE DISTRIBUTION OF FLUX

This section provides an overview of the different methodologies and concepts developed to analyze and quantify flux in metabolic systems. The quantitative methodology that is used in this thesis, to analyze the control of glycerol synthesis in *S. cerevisiae,* is metabolic control analysis aided by a computer simulated kinetic model. Hence, metabolic control analysis will receive a more detailed description than the other methodologies. Nielsen (1998), Gombert & Nielsen (2000) and Eggeling *et al.* (1996) provide reviews on the topic.

2.2.4.1 Metabolic Control Analysis

Several control theories have been developed with an aim to quantify the control that each constitutive reaction exerts on flux through a metabolic pathway. They are the biochemical systems theory (Savageau, 1971), the flux-oriented theory (Crabtree & Newsholme, 1987) and metabolic control analysis (Kacser & Burns, 1973; Heinrich & Rapoport, 1974). Metabolic control analysis (MCA) is the most widely recognized and applied theory, and will therefore be outlined in greater detail below. MCA is a theoretical framework designed to analyze quantitatively the steady state behavior of metabolic pathways. It enables one to determine the extent to which a perturbation of a parameter a system (e.g. kinetic properties of an enzyme, enzyme activity, substrate, product, cofactor, and modifier concentration) will affect the variable of a system (e.g. flux or intermediate concentration). The principles of MCA are defined by various coefficients. There are two types of control coefficients, namely the flux and the concentration control coefficient, which quantify the effects of a change in a system parameter on a system variable at steady state. Control coefficients (C) can be viewed as systemic properties, as they depict the behavior of the system as a whole, and can be defined as follows:

$$C_{v_i}^{y} = \frac{\partial \ln y}{\partial \ln v_i}$$

where y is the pathway variable (flux or intermediary metabolite concentration), i the step (enzyme) and v the steady-state activity of the perturbed step (Kacser and Burns, 1973; Heinrich and Rapoport, 1974). The elasticity coefficients (elasticities; ε) however, are a property of a particular

step. Elasticities are indicative of the sensitivity of a reaction to the concentration of its substrate, product, cofactor or modifier, and is given by:

$$\varepsilon_{p}^{v_i} = \frac{\partial \ln v_i}{\partial \ln [p]}$$

where, v is the rate of the enzyme in question and p is the parameter of the perturbation.

It is possible to relate the two to each other through the response coefficient. For example if one wanted to determine how a change in enzyme activity would affect a change in flux, one could change a parameter that specifically affects the enzyme, and the resultant change in flux would in effect be the response of the system to the change in the parameter. The response coefficient (R) is defined as follows (Kacser & Burns, 1973):

$$R_{p}^{y} = \frac{\partial \ln y}{\partial \ln v_i} \cdot \frac{\partial \ln v_i}{\partial \ln [p]} = C_{v_i}^{y} \cdot \varepsilon_{[p]}^{v_i}$$

where, p is the parameter of the pathway that is perturbed, v_i is the rate of reaction i, and y the pathway variable.

The ability to relate control coefficients to elasticies is made possible by the summation and connectivity theorems (Kacser & Burns, 1973; Heinrich & Rapoport, 1974; Westerhoff & Chen, 1984). This relationship makes it possible to express a control coefficient in terms of elasticities of the enzymes towards internal metabolite concentrations. In principle this means that one can determine the amount of control each reaction of a pathway has, by knowing how each enzyme responds in isolation to substrate, product, and modifier concentrations.

2.2.4.2 Kinetic models

To simulate metabolic systems, several programs have been written. These programs include Gepasi (Mendes, 1997), SCAMP (Sauro, 1993), MetaModel (Cornish-Bowden & Hofmeyr, 1991), Dbsolve (Goryanin, 1993), MetaCon (Thomas & Fell, 1993), CONTROL (Letellier *et al.*, 1991), and are freely available to the public. These programs are capable of calculating various control and elasticity coefficients by simulating the time-dependent and steady state behavior of a metabolic pathway.

To build a complete kinetics-based model one requires knowledge of the kinetic parameters of the individual enzymes of the pathway. Depending on the types of enzyme-catalyzed reactions that constitute the pathway, various, but not necessarily all, kinetic parameters will be required.

Kinetic parameters include Michaelis constants for substrates, cofactors and products, inhibition and activation constants of modifiers for allosterically controlled enzymes, equilibrium constants, and V_{max} values for each enzyme. The kinetic model also requires details on the intracellular concentration of substrates, products, cofactors, and known modifiers. These values will constitute the parameters of the pathway, which in order for the model simulation to calculate a steady state, need to be clamped. The intermediate metabolites of the pathway are variables and are therefore not clamped. The reason for such an approach is that metabolic control analysis is based on an assumption of steady state or pseudo-steady state. The availability of relevant information depends on the current knowledge of the metabolic pathway's structure and on kinetic data of the constitutive enzymes, all of which may vary between different organisms. However, owing to evolutionary constraints, kinetic data on the corresponding enzymes of other organisms may serve as a good approximation. Once the model has been constructed, to ensure with certainty that the parameters that define the pathway offer a reliable quantitative framework, it is necessary to validate the model's predictions. This may be achieved by comparing pathway variables (e.g. flux or intracellular intermediate concentrations) calculated by the model with pathway variables determined experimentally. One of the disadvantages of such an approach is that for it to be complete and accurate all detailed information required needs to be available. Another problem is that not all reaction properties and regulatory properties that occur *in vivo* may have been identified, hence, there is no guarantee that all expressions are comprehensive (Eggeling *et al.*, 1996). In part, this may be because the *in vivo* electrolytic conditions and protein concentrations are very different from the *in vitro* situations from which the enzyme constants are derived.

It is evident that the requirements of this analytical technique make it less suitable for the quantification of large, complex metabolic networks. Nevertheless, Rizzi *et al.* (1997) have applied this methodology to model glycolysis in *S. cerevisiae* and were able to predict the levels of intracellular and extracellular metabolites after a glucose pulse in continuous culture. In a similar study, it was examined whether the *in vivo* behaviour of yeast could be understood in terms of the *in vitro* kinetic properties of the constituent enzymes. In the first model, branch reactions were ignored. This model failed to reach the stable steady state that was observed in the experimental flux measurements. Introduction of branches towards trehalose, glycogen, glycerol and succinate did allow such a steady state, and half of the enzymes matched their predicted flux *in vivo* within a factor of 2. Results from this model also illustrated the implications of a Tps1-mediated feedback on hexokinase (Teusink *et al.*, 2000). A model of the pentose phosphate pathway of the same organism was capable of accurately predicting the intracellular concentration of various metabolites (Vaseghi *et al.*, 1999). The glucose phosphotransferase system in *E. coli* has been modeled in detail and showed quantitative agreement between model and experimental results. The predicted control

coefficients agreed well with the measured ones, and the model offered an explanation for the discrepancies between the protein-dependency of the *in vivo* and *in vitro* uptake rates (Rohwer, 1997; Rohwer *et al.,* 2000). Glycolysis of *Trypanosoma brucei* has been modeled, with the aim to increase drug selectivity, by identifying those steps in glycolysis whose inhibition would affect the glycolytic rate most (Bakker *et al.*, 1997). In another instance, the predictions of a kinetic model on the optimal xylose reductase/xylitol dehydrogenase/xylulokinase ratio, for minimizing xylitol formation in xylose utilizing yeast, was found to correspond with the enzyme ratio found in the most efficient of three xylose utilizing strains (Eliasson *et al.*, 2000). In Chapter 4 it will be demonstrated how a model, based on a relatively simple and well-characterized pathway, can provide insight into the extent to which various parameters affect and control flux through a metabolic pathway.

2.2.4.3 Metabolic Flux Analysis

The primary objective of metabolic engineering is to manipulate metabolism in such a way as to maximize the flux from substrate to product. Quantification of metabolic fluxes is therefore an integral component of metabolic engineering, and metabolic flux analysis provides us with an effective methodology to achieve this. Intracellular fluxes of a metabolic network are calculated using a stoichiometric model of the reactions that constitute a network of interest, by applying mass balances of metabolites that link the network. The benefit of metabolic flux analysis is that no kinetic information is required. Estimations of flux distribution are most useful when they have been determined at different operating conditions or with different mutants. In such cases for example, one can identify a correlation between a flux split ratio and productivity (Nielsen, 1998). Various strategies for measuring fluxes will be described under ensuing headings. Based on metabolic flux analysis, the concept of 'elementary flux modes' was introduced (Schuster & Hilgetag, 1994). An elementary mode is a minimal set of enzymes that could operate at steady state. 'Minimal' means that if only the enzymes belonging to this group were operating, complete inhibition of one of these would lead to a complete pause of steady state flux in the system (Schuster *et al.*, 2000). Developed more recently, metabolic network analysis combines metabolic flux analysis with isotopic labeling experiments for a more reliable estimation of intracellular fluxes, as well as analysis of the metabolic pathway structure and compartmentation of enzymes and metabolites (Christensen & Nielsen, 1999). Nielsen (1998) provides a review of various useful applications of MFA, in understanding metabolic systems.

2.2.4.4 Stoichiometric models

Stoichiometric models are concerned with flux analysis, and offer another approach to model metabolism. These models are based on steady state mass balances and stoichiometric constraints, which are used to calculate the fluxes through the different branches of a metabolic system. These models do not make use of kinetic information regarding the constituent enzymes. This type of modeling has been used abundantly to quantify intracellular fluxes in microorganisms, filamentous fungi and plant and animal cells. For example, a model of sucrose metabolism in sugar cane (*Saccharum officinarum)* has been employed to analyse futile cycling and provide a strategy to maximize culm sucrose accumulation. This model made use of stoichiometric analysis and elementary modes to delineate futile cycles in the network. A combination with kinetic modelling then yielded rational enhancement strategies (Rohwer & Botha, 2001). A more elaborate model has incorporated all known reactions in *E. coli*, and has been used to analyze the phenotypic effects of 66 different deletion mutants, of which 60 agreed with experimental results (Schilling *et al.,* 1999). These models are very useful; however, due to the lack of regulatory information in the model formulation, their predictive abilities are stunted (Gombert *et al.*, 2000).

2.2.4.5 Tracer experiments and nuclear magnetic resonance spectroscopy

A useful way to quantify flux distribution is by using tracers that are either radio-active or stable isotopes. Tracer experiments are also used to analyze the structure of pathways (Eggeling *et al.*, 1994). Detailed analysis of ^{13}C label incorporated into glutamate, combined with a series of mass conservation equations, enabled the flux through the tricarboxylic acid and dicarboxylic acid cycle to be quantified (Walsh *et al.*, 1984; Walsh *et al.*, 1985). Using nuclear magnetic resonance (NMR) spectroscopy, the incorporation of ^{13}C label in metabolites can be followed *in vivo*, assuming that a high intracellular concentration of metabolites exists (Bhaumik *et al.*, 1994). ^{13}C isotopes can also be used to decipher between isotopomers. This kind of analysis gives a very detailed view of cellular fluxes, enabling the quantification of back fluxes and futile cycles (Eggeling *et al.*, 1996). When combined with metabolic flux analysis, isotopic labeling also provides a means for reliable estimation of intracellular fluxes, as well as the analysis of the metabolic pathway structure and compartmentation of enzymes and metabolites (Christensen & Nielsen, 1999). The only direct method to quantify *in vivo* fluxes is by NMR magnetization transfer (Eggeling *et al.*, 1996). Nevertheless, its application is limited because of the inherent insensitivity of NMR spectroscopy and because it requires the spin-lattice relaxation rates of the reactants and products to be of the same order as the reaction rate constants (Ross *et al.*, 1994). The complicated data analysis allows only relatively simple unbranched reaction sequences to be studied *in vivo* (Eggeling *et al.*, 1996).

2.2.4.6 Metabolite balancing

This type of analysis is used for specific purposes, such as when only specific pathways are to be considered, or for the calculation of maximal yields of a metabolite of interest (Stephanopoulos *et al.*, 1991). Flux rates are determined by measuring the change in quantity of substrate, product, side products, carbon dioxide, ammonium, cell material, and yields data on the respective *in vivo* transport reactions. This, combined with known demands of central metabolites for cellular growth (oxalacetate, pyruvate, α-ketoglutarate), allows for estimation of flux through the basic metabolic pathways. However, it results in a number of assumptions and simplifications of cellular activities. Flux analysis of a metabolic system is then brought about by perturbations introduced by mutations or by the addition of specific inhibitors, precursors, or a sudden substrate increase, which could cause various alterations in flux (Weuster-Botz *et al.*, 1996).

2.2.5 Metabolic Engineering of Glycerol Metabolism in *Saccharomyces cerevisiae*

2.2.5.1 The role of glycerol metabolism in redox balancing

Under respiratory conditions glucose and fructose are oxidized by a sequence of glycolytic reactions to pyruvate; concomitantly, NAD^+ is reduced to NADH. *S. cerevisiae* utilizes at least two different systems to re-oxidize cytosolic NADH to NAD^+, and both entail mitochondrial electron transport. The one system involves an external NADH dehydrogenase situated on the inner mitochondrial membrane which presents its catalytic side to the cytosol (Luttik *et al.,* 1998; Small & McAlister-Henn, 1998; Pahlman *et al.*, 2001). The other system is known as the glycerol 3-phosphate shuttle (G3P shuttle) (Larsson *et al.*, 1998; Pahlman *et al.*, 2001). The action of the G3P shuttle entails the transfer of electrons to FAD via oxidation of G3P by a mitochondrial glycerol 3-phosphate dehydrogenase (Gut2p). The dihydroxyacetone phosphate (DHAP) formed, is then reduced by a cytosolic glycerol 3-phosphate dehydrogenase (Gpd p) at the expense of NADH. But, when yeast cells are cultured in the presence of glucose (Crabtree effect), or with a lack of oxygen, mitochondrial activities are strongly repressed. Under these growth conditions, NADH produced during the glyceraldehyde 3-phosphate dehydrogenase reaction in glycolysis is re-oxidized in a process whereby pyruvate is decarboxylated to acetaldehyde, which is subsequently reduced to ethanol by alcohol dehydrogenase. In principle, the formation of ethanol and CO_2 from a fermentable carbon sources is a redox neutral process. However, since a certain amount of glycolytic intermediates are converted to oxidized by-products, or siphoned off to the pentose phosphate pathway (PPP), the glyoxylate pathway and the tricarboxylic acid (TCA) cycle for biosynthetic processes, an excess of reducing equivalents is generated. Under these conditions,

where there is an absence of mitochondrial electron transport, excess reducing equivalents are re-oxidized via the cytosolic glycerol 3-phosphate dehydrogenase reaction, resulting in increased glycerol formation. This process plays a central role in the ability of *S. cerevisiae* to maintain a favourable redox state during fermentative metabolism (Nordstom *et al.*, 1966; Lagunas *et al.*, 1973; Oura *et al.*, 1977; Van Dijken *et al.*, 1986; Ansell *et al.*, 1997).

Since the formation of both glycerol and ethanol play a role in balancing the redox state of the cell, it might be assumed that amplification or limitation of the steps involved in their production would result in a change of the glycerol/ethanol ratio. Alterations in the rate of either of these processes do indeed affect this ratio. For example, when acetaldehyde is trapped with sulphite, there is an increase in the amount of glycerol formed. This phenomenon is also observed in strains with low Adh p activity or *ADH1* deficient mutants (Ciriacy *et al.*, 1975). Alternatively, in the case of Gpd p-deficient strains, increased levels of ethanol are observed as a result of reduced glycerol production (Valadi *et al.*, 1998).

Alteration of the glycerol/ethanol ratio during fermentation is of major concern to various ethanol bioprocesses, such as beer, wine and spirits production. Accordingly, over the last century numerous investigations have been carried out to determine the optimal process conditions to maximize the production of either glycerol or ethanol. Significant improvements in glycerol (reviewed by Wang *et al.*, 2001) and ethanol (reviewed Zaldivar *et al.*, 2001) production from industrial-scale fermentations have been achieved by manipulating various process parameters and by the breeding and genetic engineering of industrial microorganisms.

The extent to which the metabolic engineering of redox and glycerol metabolism in *S. cerevisiae* has contributed to increasing the amount of glycerol or ethanol produced during fermentation will be summarised below.

2.2.5.2 Increasing glycerol production

As mentioned above, substantial advances in increasing the glycerol yield and productivity of industrial-scale fermentations have been achieved. One of the most successful strategies entails the use of a "steering agent" such as bisulphite. In this case the steering agent prevents the reduction of acetaldehyde to ethanol, resulting in increased glycerol formation as a mechanism to re-oxidize excess NADH (Freeman & Donald, 1957a). This method has resulted in final glycerol concentrations reaching 30-40 g/l (Harris & Hajny, 1960). Similarly, by conducting fermentations at an alkaline pH, elevated acetaldehyde dehydrogenase activity occurs, which results in increased oxidation of acetaldehyde to acetate. This reaction generates NADH, which is subsequently re-oxidized by the formation of glycerol (Freeman & Donald, 1957b). This process also yields final glycerol concentrations of 30-40 g/l (Underkofler *et al.*, 1954). Another, more recent and very

successful fermentation process yields glycerol concentrations as high as 110-130 g/l by employing osmotolerant yeast strains in the fermentation process (Zhuge & Fang, 1994).

Metabolic engineering approaches to increase the glycerol/ethanol ratio have primarily arisen from the wine industry's interest in glycerol metabolism (Scanes *et al.*, 1998). Glycerol, next to ethanol, is the most abundant by-product in wine (4-9 g/l) (Oura, 1977) and plays an important role in wine quality, as it is assumed to affect the sweetness and viscosity of wine (Hinreiner *et al.*, 1955). It is also believed that a yeast strain that harbours the ability to produce larger than normal amounts of glycerol would be beneficial for the production of wines that ordinarily lack body (Noble *et al.*, 1984). Similarly, in the light-alcoholic beverage industry, a yeast strain that produces more glycerol at the expense of ethanol would provide an alternative to the organoleptic diminishing, physical techniques used to remove alcohol (Michnick *et al.*, 1997). In light of this, various strategies to engineer or breed high glycerol producing strains have been attempted. Classical genetic techniques have been reasonably successful, in this regard. This procedure entails spore-cell hybridization of selected strains, followed by backcrossing of the resultant spores with the original parent to maintain genetic stability. For example, a breeding programme led to an increase in glycerol production from 3-6.6 g/l in the original breeding stock, to 10-11 g/l in the hybridized yeast strain. The hybridized strains also produced less ethanol, which is indicative of a higher glycerol/ethanol ratio compared to the original strains (Eustace *et al.*, 1987). Prior *et al.* (1999) applied a similar technique and obtained glycerol levels as high as 18 g/l. Concomitantly, there was an increase in acetaldehyde and acetic acid, indicating that the redox imbalance created by the overproduction of glycerol was compensated for by a reduction in the amount of acetaldehyde reduced to ethanol. It was also reported that 2,3-butanediol and acetoin concentrations were elevated in the hybridized strains.

The glycerol 3-phosphate dehydrogenase reaction is considered to be rate limiting in the production of glycerol and therefore overexpression of this enzyme should result in increased levels of glycerol synthesis (Radler *et al.*, 1982). This hypothesis has been confirmed in numerous studies, which have involved overexpression of both of the *GPD* genes (Nevoigt *et al.*, 1996; Michnick *et al.*, 1997; Remize *et al.*, 1999; Remize *et al.*, 2001). Incidentally, overexpression of *GPD1* and *GPD2* are equally effective at increasing glycerol production and both have similar effects on yeast metabolism (Remize *et al.*, 2001). In contrast, the glycerol 3-phosphatase reaction is believed to exercise very little control of flux through the glycerol synthesis pathway. Recently, this has been confirmed by overexpressing the *GPP1* gene alone and in conjunction with the *GPD1* gene. Overexpression of *GPP1* alone had no effect on glycerol production. Similarly, strains overexpressing *GPP1* and *GPD1* simultaneously did not produce higher amounts of glycerol than *GPD1* overexpressing strains (Remize *et al.*, 2001).

Nevoigt & Stahl (1996) have investigated the metabolic prerequisites for glycerol overproduction. The impact of reduced pyruvate-decarboxylase activity and increased levels of glycerol 3-phosphate dehydrogenase was analysed. The glycerol yield was fivefold higher for the *pdc•* mutant and sevenfold higher for the *GPD1* overexpressed strain, than that of the wild-type strain. In a strain carrying both enzyme activity alterations, the glycerol yield was 8 times higher than that of the wild-type. In all cases, the substantial increase in glycerol yield was associated with a reduction in ethanol yield and an increase in oxidized by-product formation. Michnick *et al.* (1997) studied the consequences of glycerol overproduction on by-product formation, growth and fermentation kinetics in wine and champagne yeast strains. A similar investigation by Remize *et al.* (1999) compared the effects of glycerol overproduction on wine and laboratory yeast strains. In both cases *GPD1* was overexpressed in order to increase glycerol yield. Both studies revealed an increase in by-product formation, namely acetaldehyde, acetate, acetoin, 2,3-butanediol, pyruvate and succinate, with a marked variation in by-product formation between industrial and laboratory strains. The laboratory strains produced exceptionally high acetaldehyde and acetate levels and their final cell counts were less than half of those obtained by industrial strains. There was also a decrease in ethanol yield and biomass formation in all strains overproducing glycerol. It has been proposed (Michnick *et al.*, 1997; Remize *et al.*, 1999) that the observed alterations in by-product formation that occur when glycerol is overproduced, are the result of the cell's attempt to maintain a suitable redox balance. The reduced activity of alcohol dehydrogenase could be responsible for the observed increases in acetaldehyde, acetate and pyruvate. The increased levels of acetaldehyde may be responsible for the higher levels of acetoin, which when reduced, lead to the observed increase in 2,3-butanediol. A benefit of the consumption of acetaldehyde leading to the formation of acetoin, lies in the toxic effects of high acetaldehyde concentrations on yeast cells (Jones, 1989; Otsuka *et al.*, 1996; Remize *et al.*, 1999). Increased levels of succinate could possibly be the result of elevated flux through the glyoxylate cycle, which may have been initiated by the increased levels of acetate. Incidentally, operation of the glyoxylate cycle results in a net gain of NADH, which may compensate for the increased NADH consumption during glycerol formation.

It is also proposed that the increase of carbon flux towards glycerol may lead to a shortage of ATP. This might explain why glycerol-overproducing strains also exhibit a higher rate of glucose consumption and rate of fermentation at stationary phase (Michnick *et al.*, 1997; Remize *et al.*, 1999).

2.2.5.3 Decreasing glycerol production

In the brewing industry the opposite scenario is favoured, since one of the many attributes preferred from a brewing strain is the ability to produce low levels of glycerol. When glycerol levels in beer exceed a certain threshold, sweetness increases and bubble formation is hindered, which are both undesirable characteristics. Hitherto, decreasing the amount of glycerol produced from fermentation has probably been of more importance to the ethanol production industry. The production of ethanol as an alternative, less environmentally destructive fuel source has received a great deal of attention since the 1970s. Of the 31.2 billion litres of ethanol produced in 1998, over 90% was produced by fermentation (Zaldivar *et al.*, 2001). Bioethanol (ethanol from biomass) production is dependent on the fermentation of carbohydrate monomers (hexoses), which are primarily derived from sugar cane and corn crops, and as such, raw material costs can constitute approximately 40% of the eventual product price (Wyman, 1999). To increase the competitiveness of bioethanol as an alternative energy source, utilization of a cheaper, abundant carbon source such as lignocellulose may help significantly in this regard. Over the last decade, substantial effort has been devoted to engineering various microorganisms (i.e. *Zymomonas mobilis, Escherichia coli* and *S. cerevisiae*), capable of fermenting a variety of sugars (hexoses and pentoses), as well as the ability to withstand various stress conditions (recently reviewed by Zaldivar *et al.*, 2001).

There are however, various aspects to improving the efficiency of ethanol production. One of these entails the redirection of carbon flux from by-product formation toward ethanol formation. As mentioned above, after ethanol, glycerol is the second most abundant metabolite produced during anaerobic fermentation, and therefore various metabolic engineering approaches to decrease the glycerol/ethanol ratio have been taken. In one of the first studies to characterize the anaerobic performance of *gpd1*Δ and *gpd2*Δ mutants of *S. cerevisiae*, it was found that *gpd2*Δ strains produced 40% less glycerol and 13% more ethanol than did wild-type strains. In terms of growth, the *gpd1*Δ and the wild-type strain were indistinguishable. In contrast, the *gpd2*Δ strain displayed an extended lag phase as well as a reduced growth rate at exponential phase (Valadi *et al.*, 1998). An alternative strategy to reduce glycerol formation involved transgenic expression of the *E. coli pntA* and *pntB* genes in *S. cerevisiae*. The heterologous membrane-bound transhydrogenase was intended to reoxidize NADH to NAD^+ thereby reducing glycerol formation during anaerobic fermentation. Unfortunately, the expressed transhydrogenase favoured the reverse reaction, whereby reducing equivalents were transferred from NADPH to NAD^+, leading to increased formation of glycerol (Anderlund *et al.*, 1999). A similar approach entailed the expression of a cytoplasmic transhydrogenase from *Azotobacter vinelandii*, encoded by *CTH*, in *S. cerevisiae* *gpd1*Δ, *gpd2*Δ or *gpd1*Δ *gpd2*Δ mutants. The *CTH* expressing *gpd1*Δ strain exhibited a similar

growth rate and product yield to the wild-type strain, whereas the *CTH* expressing *gpd2*Δ strain produced far less glycerol, but suffered from retarded growth, exhibiting a maximum specific growth rate of 0.08/h, 5-fold lower than that of the parent strain (0.41/h). The *gpd1*Δ *gpd2*Δ mutant expressing *CTH* was unable to grow under anaerobic conditions due to its inability to reoxidize NAD^+ to NADH. As in the previous example, the transhydrogenase reaction led to the formation of NADH and $NADP^+$ from NAD^+ and NADPH (Nissen *et al.*, 2000). A study by the same group was undertaken with an objective to evaluate whether a reduction in the formation of NADH and an increase in the consumption of ATP from biomass synthesis would result in a decreased glycerol yield and an increased ethanol yield. The study entailed metabolic engineering of ammonium assimilation in *S. cerevisiae*. This was achieved by substituting the NADPH-dependent glutamate dehydrogenase reaction with an NADH-dependent glutamate synthase (*GLT1*) reaction and an ATP-consuming glutamine synthetase (*GLN1*) reaction. The resulting strain yielded 10% more ethanol and 38% less glycerol compared to the wild-type strain in anaerobic batch fermentations. It is noteworthy that this approach was successful at achieving its objective with minimal deleterious effect, since the engineered strain managed to maintain a maximum specific growth rate only slightly lower (10%) than the wild-type (Nissen *et al.,* 2000). Results from this study also agree with those presented by Albers *et al.* (1996), in which it was reported that surplus NADH derived from the *de novo* biosynthesis of amino acids as from ammonia, can be circumvented by cultivating *S. cerevisiae* in the presence of alternative nitrogen sources, i.e., glutamic acid or amino acids. It was shown that the reduction in NADH generated under these growth conditions leads to lower glycerol yields [glutamic acid (19%), mixture of amino acids (52%)] and higher ethanol yields [glutamic acid (9%), mixture of amino acids (14%)]. Cultures grown on amino acids also have a higher specific growth rate (0.52/h) than both ammonium-grown (0.45/h) and glutamic acid-grown (0.33/h) cells.

As is evident, ethanol and glycerol yields from anaerobic fermentations are inversely related and this relationship is linked to, and regulated by the redox state of the cell. As described, attempts to manipulate this relationship often result in unwanted and occasionally satisfactory perturbations to metabolism. It is likely that these metabolic changes arise from attempts by the cell to compensate for the imbalanced redox state induced by the metabolic manipulations. From this, it is apparent that a complex network of reactions in *S. cerevisiae* helps to regulate and maintain a redox state that supports catabolism during fermentation. To manipulate the flux of carbon through these pathways successfully, specifically with the intent of increasing or decreasing glycerol production, it will be necessary to perform the following:

1) Metabolic flux analysis with the aid of stoichiometric modelling (described in Sec. 2.2.4.3; 2.2.4.4), which will provide a more thorough understanding of how flux is distributed amongst this network of reactions, and where their key flux-regulatory sites lie.

2) Metabolic control analysis with the aid of kinetic modelling (described in Sec 2.2.4.1; 2.2.4.2), which will assist by defining the parameters that exercise strong control on flux through the glycerol synthesis pathway.

This thesis deals with the latter aspect. Thus, an attempt is made to identify the parameters that exercise a strong control of flux through the glycerol synthesis pathway and this knowledge will assist in future efforts to manipulate the glycerol/ethanol ratio with the least deleterious physiological side effects possible.

3. EVALUATION OF EXTRACTION TECHNIQUES FOR INTRACELLULAR METABOLITE QUANTIFICATION

3.1 SUMMARY

Metabolite extraction methods employing various extraction solutions (6% and 35% perchloric acid, boiling buffered ethanol and hot alkalised ethanol) were examined for their efficacy to recover adenine nucleotides, pyridine nucleotides and glycolytic intermediate metabolites from *Saccharomyces cerevisiae* cells. Yeast cells grown to early stationary phase (OD_{600} = 1) were concentrated and simultaneously separated from the growth media by rapid filtration. Immediately thereafter, metabolism was inactivated by instantly freezing the filtered yeast pellicle with liquid nitrogen. The frozen yeast cells were then immersed in various extraction solutions to test the efficacy of the solution to extract different metabolites. It was found that the success of recovery of each metabolite was largely dependent on the sensitivity of the metabolite to pH and temperature. ATP and ADP were most efficiently extracted in a 35% perchloric acid solution, while NAD^+, F1,6BP and DHAP were most efficiently extracted in a 6% perchloric acid solution. The recovery of NADH occurs best when a hot (70°C)-alkalised ethanol solution is used in the extraction process. It was found that the concentrations of the metabolites tested fell within the range of values previously reported. Thus, the procedures employed in this study apparently offer a simple and reliable method to inactivate metabolism rapidly and to recover intracellular metabolites successfully.

Abbreviations can be found on page v.

3.2 INTRODUCTION

To analyse the control of specific components of a metabolic pathway in an organism, information on the various pathway parameters is required. The pathway parameters include the kinetic properties and the maximum activities of the enzymes that constitute the pathway, as well as the concentrations of intracellular metabolites that affect the pathway in one way or another. Thus, to complete an analysis of this type, the concentration of the pathway substrate, product, coenzyme, and known modifier metabolites need to be determined. To ensure that the values obtained deviate minimally from the prevailing *in vivo* metabolite concentrations, the following three steps in the metabolite determination procedure are critical (Lohmeier-Vogel *et al.*, 1983):

1. Sampling and arresting metabolism instantly.
2. Gathering a sufficient quantity of biomass, to ensure that adequate amounts of each metabolite are extracted.
3. Extracting metabolites from the cells as efficiently as possible.

Since metabolite concentrations are more prone to rapid changes induced by environmental alterations than are enzyme activities (de Koning and van Dam, 1992), various methods have been developed to sample cells suspended in culture and to halt metabolism in the fastest manner possible. The approach of the different methods varies; however, most proceed according to one of the following three routines:

1. The first routine entails quenching a volume of cell culture in a solution prepared to halt metabolism instantly and to extract metabolites simultaneously, as in the boiling buffered ethanol method described by Gonzalez *et al.* (1997).
2. The second routine involves quenching a cell culture in one chilled solution and thereafter, the chilled cells are collected, removed and immersed in an extraction solution, as described in the method by de Koning & van Dam, (1992).
3. With the third routine, cells are first concentrated and removed from the growth medium before metabolism is halted and extraction takes place. The method of Saez & Lagunas (1976) is an example of such a routine.

Independent of the routine followed, most methods have proven to be effective at providing reproducible concentration values for a wide range of glycolytic intermediates. Therefore the method selected will depend on certain factors. One determinant is the availability of specialized equipment as employed in certain techniques, such as a specialized sampling device (Theobald *et al.,* 1993), a regulated liquid nitrogen cooled incubator (de Koning & van Dam, 1992) or rotavapor apparatus (Gonzalez *et al.,* 1997). Another determinant will be whether the metabolite of interest is located intracellularly only, or intracellularly and extracellularly. Latter case circumstances will require the cells to be removed from the growth media before extraction takes place. In addition, another determinant will be the technique employed to detect and quantify the metabolites, since different detection techniques have varying degrees of sensitivity. For example, when measuring metabolites enzymatically, metabolites that only occur at low concentrations suffer the possibility of being diluted during the extraction process to levels that will not enable accurate determination. Therefore, when measuring metabolites enzymatically, it is important to select a method where an adequate amount of biomass will be harvested. In this case, a method that follows routine 2 or 3 should be selected. Because of simplicity, reliability and no requirement for specialized equipment,

the rapid filtration technique described by Saez & Lagunas (1976) was used in this study, to sample and quench cells suspended in culture.

The third critical step in the metabolite determination procedure is the method of metabolite extraction. Since various metabolites are only stable at acidic or alkaline conditions and sensitive to extreme temperatures, the method of extraction is critical (Bergmeyer, 1974). The conflicting values of intracellular metabolites found in literature might be explained in part by the use of different extracting agents (Gancedo & Gancedo, 1973; Weuster-Botz & de Graaf, 1996). In this chapter, extraction methods of Klingenberg (1974a) (6% perchloric acid); Theobald *et al.* (1993) (35% perchloric acid); Klingenberg (1974b) (hot alkalised ethanol) and Gonzalez *et al.* (1997) (boiling buffered ethanol) have been evaluated to determine the simplest and most reliable metabolite extraction method. This was done more specifically for metabolites that affect flux through the glycerol synthesis pathway.

3.3 Materials and Methods

3.3.1 Organism and growth conditions

The *Saccharomyces cerevisiae* haploid laboratory strain W303-1A (Thomas and Rothstein, 1989) was used in this study. In all experiments, yeast cells were cultured in glucose (2 g/l), yeast nitrogen base (6.7 g/l) medium, with amino acids (United States Biological, Swampscott, Ma, USA). All yeast cells were maintained on agar plates at 4°C for short-term storage and at –80°C in (15% w/v) glycerol for extended periods of storage. Ten-millilitre cultures were used to inoculate 100 ml of media in 250 ml Erlenmeyer Flasks. Cultures were incubated at 30°C and 140 rpm on an orbital shaker (New Brunswick Scientific Co., Edison. N.J., USA) until an OD_{600} of one was reached.

3.3.2 Yeast dry weight determination

Depending on the stage of growth, volumes of 20 ml (from early to mid exponential phase) or 10ml (late exponential to early stationary phase) of culture were filtered under vacuum through Whatman Glass Microfibre Filters (25 mm diameter; Cat no. 1822 025). The filters were then dried at 80°C until a constant weight was achieved (usually within 24 hrs).

3.3.3 Filtration of cultures

Cultures grown to an OD_{600} of one were filtered under vacuum through Whatman Glass Microfibre Filters (25 mm diameter; Cat no. 1822 025) as described by Saez & Lagunas (1976). Filtration took approximately 5 s and immediately thereafter, cells were frozen with liquid nitrogen and stored at -80°C until the extraction procedure was performed.

3.3.4 Metabolite extraction with a 6% or 35% perchloric acid solution

The frozen cells on the filter were transferred and immersed in 6 ml of a 6% (Klingenberg, 1974a) or 35% (Theobald *et al.*, 1993) $HClO_4$ solution held at –10°C. The suspension was held at –5°C for 10 min, during which 3 sessions of vigorous shaking occurred (1 min per session). During shaking, samples thawed to approximately 0°C. Thereafter, samples were centrifuged for 7 min at 24 000 g at 0°C to remove cell debris. For determination of NAD^+, DHAP, F1,6BP and G3P, the supernatant from the 6% $HClO_4$ extraction solution was adjusted to pH 4 with 5 M K_2CO_3. For determination of ATP and ADP, the supernatants from 6% and 35% $HClO_4$ extraction solutions were adjusted to pH 9 with 5 M K_2CO_3. The $KClO_4$ precipitate was removed by centrifugation as described above, and the supernatant was removed and stored at –80°C until further analysis.

3.3.5 Metabolite extraction with hot alkalized ethanol

The frozen cells on the filter were immersed in 6 ml 0.5 M KOH-50% (v/v) ethanol solution. The suspension was incubated at 70°C for 1 min, chilled on ice, vortexed for 30 s, and then 3 ml of TAP buffer (0.5M triethanolamine-HCl; 0.4 M KH_2PO_4; 0.1 M K_2HPO_4) was added, resulting in a suspension of pH 7 (Klingenberg, 1974b). The suspension was centrifuged at 24 000 g for 7 min at 0°C and the supernatant was stored at –80°C.

3.3.6 Metabolite extraction with boiling buffered ethanol

The frozen cells on the filter were immersed in 5 ml 80% (v/v) ethanol solution -0.25 M Hepes buffer (pH 7.5) held at –10°C. The suspension was incubated at 80°C for 4 min and thereafter cooled for 3 min on ice. The volume was then reduced by rotary vacuum evaporation (Bruchi Rotavapor R-134, Bruchi Waterbath-480, Julabo F-12 cooling system, France) for 10 min at 45°C. The residue was suspended to a final volume of 2 ml with distilled water and then centrifuged at 24 000 g for 7 min at 0°C. The supernatant was removed and stored at –80°C until further analysis.

3.3.7 Assay of metabolites

Metabolites were determined enzymatically by measuring the oxidation or reduction of NADH or NAD^+ respectively at 340nm in a Beckman Coulter DU640 spectrophotometer as described in Bergmeyer (1974). To calculate the intracellular concentrations, a yeast cytosolic volume of 1.67 µl per milligram of dry yeast biomass was assumed (de Koning & van Dam, 1992).

3.4 RESULTS AND DISCUSSION

A comparison of different extraction techniques for various metabolites is presented in Table 3.1. Since certain metabolites are sensitive to harsh pH and/or temperature conditions not all methods were tested on each metabolite (Bergmeyer, 1974). Instead, a comparison was made between methods previously prescribed for a specific metabolite. For ATP and ADP however, all four techniques were attempted since both acid extraction methods have been employed before, as has the boiling buffered ethanol method, whereas the boiling alkalized ethanol method has not. This method was included in the evaluation procedure, since ATP and ADP are stable under alkaline conditions.

3.4.1 Extraction of NAD^+

To determine NAD^+ concentration, extraction with 6% perchloric acid was compared to extraction with boiling buffered ethanol. Neither the hot alkalised ethanol nor the 35% perchloric acid extraction methods were evaluated, as NAD^+ is sensitive to alkaline conditions (Klingenberg, 1974a) and is unstable in perchloric acid at concentrations higher than 14% (Gonzalez *et al.,* 1997). Extraction with 6% perchloric acid yielded quantities very similar to those observed with extraction by boiling ethanol, with the intracellular concentration being 0.49 mM and 0.5 mM respectively (Table 3.1). Reported intracellular concentrations of NAD^+ in *S. cerevisiae* range from 5 mM (Gonzalez *et al.,* 1997) to 1.2 mM (Saez & Lagunas, 1976) and 0.8 mM (de Koning & van Dam, 1992). Albeit under different growth conditions, this illustrates the variation in values reported in literature. The NAD^+ concentration found in this study falls below those values previously reported, but when measured at earlier phases of growth (results presented in the next chapter) the NAD^+ concentration falls within the reported ranges. This variation in metabolite concentrations at different phases of growth might shed light on the variation in values reported by Saez & Lagunas (1976) and de Koning & van Dam (1992), but does not offer any explanation for the substantially higher value reported by Gonzalez *et al.* (1997). The extent of degradation of commercial metabolites exposed to each of the respective extraction procedures yielded similar values of 12% and 9% (Table 3.1). Although the NAD^+ concentrations obtained in this study were below those previously reported, it was found that both extraction methods gave similar concentrations and degrees of degradation. Therefore it is believed that the values are accurate for the specific growth conditions.

Table 3.1 Comparison of various techniques used to extract metabolites from *Saccharomyces cerevisiae*[a]

METABOLITE[b]	INTRACELLULAR METABOLITE EXTRACTION METHOD							
	Perchloric acid (6%)[c]		Perchloric acid (35%)[d]		Boiling buffered ethanol[e]		Hot alkalised ethanol[f]	
	[mM][g]	% Recovered[h]	[mM]	% Recovered	[mM]	% Recovered	[mM]	% Recovered
NAD^+	0.49 ± 0.04	88	na	na	0.5 ± 0.04	91	na	na
NADH	na[i]	na	na	na	0.18 ± 0.07	54	0.53 ± 0.08	91
ATP	1.22 ± 0.1	90	1.53 ± 0.1	88	0.72 ± 0.09	58	0.49 ± 0.06	49
ADP	0.74 ± 0.09	91	0.93 ± 0.17	86	0.36 ± 0.03	52	0.33 ± 0.18	54
F1,6BP	2.47 ± 0.12	92	na	na	1.70 ± 0.35	64	na	na
DHAP	0.45 ± 0.04	84	na	na	0.12 ± 0.05	32	na	na
G3P	nd[j]	87	na	na	nd	48	na	na

[a]Cells were cultured in 2% glucose, YNB media at 30°C and harvested at OD_{600} = 1.0 (early stationary phase).
[b]Metabolite abbreviations are as follows: ATP (adenosine triphosphate), ADP (adenosine diphosphate), NADH (nicotinamide adenine dinucleotide, reduced form), NAD^+ (nicotinamide adenine dinucleotide, oxidised form), F1,6BP (fructose-1,6-bisphosphate), DHAP (dihydroxyacetone phosphate), G3P (glycerol 3-phosphate).
[c]Metabolites extracted in 6% perchloric acid (Klingenberg, 1974a).
[d]Metabolites extracted in 35% perchloric acid (Theobald *et al.*, 1993).
[e]Metabolites extracted in 85% buffered ethanol solution (Entian *et al.*, 1977, revised by Gonzalez *et al.*, 1997).
[f]Metabolites extracted in 50% basic ethanol solution (Klingenberg, 1974b).
[g]Average of triplicate determinations ± the standard deviation.
[h]Commercial preparations of metabolites were exposed to the extraction procedure and the percentage of metabolite recovered was recorded.
[i]na: Metabolite was not analysed by this procedure.
[j]nd: No detection of the metabolite when measured.

3.4.2 Extraction of NADH

To determine the optimum extraction procedure for NADH determination, two methods of extraction were compared. Since NADH is acid labile (Klingenberg, 1974b), the boiling buffered ethanol method (Gonzalez *et al.,* 1997) was compared to the hot alkalised ethanol method described by Klingenberg (1974b). The latter method was more efficient at extracting NADH, with a concentration over twofold higher (0.53 mM) than that obtained by the boiling buffered ethanol method (0.18 mM). Also, the recovery of NADH from the hot alkalised ethanol method was 91%, whereas the amount recovered from the boiling buffered ethanol technique was substantially lower at 54%. NADH is sensitive to high-temperatures (Klingenberg, 1974b), and possibly the extended period of heat incubation in boiling buffered ethanol (3 min at 80°C) led to increased NADH degradation, whereas in the hot alkalised ethanol method, heat incubation continues for a shorter period and at a lower temperature (1 min at 70°C), and therefore less NADH degradation is observed. The reported concentration values for NADH range from 1.2 mM (Saez & Lagunas, 1976), 0.8 mM (Gonzalez *et al.,* 1997) and 0.23 mM (de Koning & van Dam, 1992). The results reported in Table 3.1 fall approximately in the middle of this range, and when cells were harvested at an earlier and later phase of growth, as reported in the following chapter, concentrations fell within the upper and lower limits of the reported ranges.

3.4.3 Extraction of ATP and ADP

In the case of adenine metabolite determination, four methods of extraction were evaluated (Table 3.1). Extraction by means of perchloric acid is possibly the most widely used. In agreement with results recorded by Theobald *et al.* (1993), extraction with a high concentration of perchloric acid (35%) resulted in a 25% increase in the quantity of ATP liberated from the cell compared to the method where a low concentration (6%) perchloric acid solution was used. This was possibly be due to increased cytoplasmic and mitochondrial membrane disruption as a result of the high acid concentration (Theobald *et al.*, 1993). A similar result was recorded for the amount of ADP liberated. The quantity of metabolite recovered in the boiling buffered ethanol method was approximately half of that obtained by extraction in a 6% perchloric acid solution and even less when extracted in a 35% perchloric acidic solution. It is unlikely that this is due to insufficient membrane disruption and decreased metabolite liberation, but probably due to increased metabolite degradation from prolonged exposure to a high temperature, since approximately only half of both commercial metabolites was recovered when exposed to the extraction process. ATP is stable under alkaline conditions and therefore the hot alkalised ethanol extraction method by Klingenberg (1974b) was used in an attempt to extract ATP and ADP. The concentration values obtained were substantially lower than with any of the other techniques tested. This technique includes a short

incubation period at a high temperature; this combined with the effects of a high pH, might in part, be responsible for the low recovery levels observed. Intracellular ATP concentrations reported in literature range from 4.8 mM (Theobald *et al.,* 1993) and 4.2 mM (Gonzalez *et al.,* 1997) to 1.2 mM (Saez & Lagunas, 1976) and 0.04 mM (de Koning & van Dam, 1992). Those for ADP range from 4.2 mM (Gonzalez *et al.,* 1997) to 0.6 mM (Theobald *et al.,* 1993) and 0.4 mM (Saez & Lagunas, 1976; de Koning & van Dam, 1992). Although the growth conditions and strains used in the various reports were different, there is significant variation in ADP and ATP concentrations. The values presented in Table 3.1, for ATP (1.53 mM – 0.49 mM) and ADP (0.93 mM – 0.33 mM) extraction by each of the methods tested, all fall within the range of previously reported values. The ATP/ADP ratios determined by each of the methods ranged from 1.5 to 2.0, whereas reported ratios vary vastly (between 8 and 0.1). This may be an indication that without a rapid halt in metabolism followed by efficient metabolite extraction, inaccurate estimations of a cell's energetic state may occur. When commercial ATP and ADP were added to the 6% and 35% perchloric acid extraction procedures, there was slightly less recovery of metabolites exposed to the harsher of the two acidic conditions. Nevertheless, cellular recovery of ATP and ADP using the 35% perchloric acid method was more successful, and since the amount of degradation that occurred during testing was less than 14%, values obtained using this method appear to be the most representative of *in vivo* values.

3.4.4 Extraction of DHAP, G3P and F1,6BP

For the extraction of DHAP, G3P and F1,6BP, the 6% perchloric acid method was compared to the boiling buffered ethanol method. There was a marked decrease in DHAP and F1,6BP concentration when extracted with the boiling buffered ethanol method (Table 3.1). In addition, the level of degradation of both metabolites was substantially higher when extraction was carried out with boiling buffered ethanol. Exposure of DHAP to the 6% perchloric acid extraction procedure resulted in 15% degradation, whereas only 8% of F1,6BP was degraded. Reported concentrations for DHAP and F16BP by de Koning & van Dam (1992) are 0.4 mM and 0.5 mM respectively. The value recorded for DHAP in Table 3.1 falls within this range, yet the F1,6BP concentration obtained was approximately five-fold higher than previously reported values. This may be result of differences in growth conditions and strains used. As the 6% perchloric acid extraction procedure did not drastically affect the recovery of either metabolite, it is believed the results obtained using this extraction method provide an accurate representation of the intracellular concentration of both metabolites.

G3P was not detected with either of the extraction methods used. The reason for this is unknown, since 87% of G3P added to the acid extraction method was recovered. It is possible that the intracellular concentration of G3P is too low to detect using the current assay method.

Nevertheless, this is doubtful, since the model of the glycerol synthesis pathway, presented in Chapter 4, indicates that the G3P concentration fluctuates between 0.2 and 0.4 mM at the different growth phases measured. Thus, it seems likely that a large percentage of G3P degradation might occur during the filtration process. Although the filtration process is brief (5 to 10 s), and most metabolites are stable for a few minutes before having been frozen on the filter, it has been shown that the levels of certain metabolites (e.g., pyruvate and glucose 6-phosphate) may drop a few hundred μM within seconds after completion of filtration (Saez & Lagunas, 1976). A drop in G3P concentration of this magnitude would make it impractical to measure the remaining G3P enzymatically. Thus, should G3P be prone to such drastic fluctuations, a rapid quenching technique (de Koning & van Dam, 1992) might provide the best results when attempting to determine the *in vivo* concentration of G3P.

3.5 CONCLUSION

Being able to accurately determine the concentration of intracellular metabolites is an essential part of metabolic control and flux analysis. The disparity in reported concentrations of various metabolites in yeasts could be due to a range of experimental factors. These may include the strain used, the phase of growth at which cells are harvested, and the availability of nutrients and carbon source. Nevertheless, a number of other factors will impact on the accuracy of intracellular metabolite determination, and these include the time taken to collect cells, the speed at which metabolism is halted and, as found in this study, the extraction procedure employed.

On the basis of the results reported in this chapter, a simple and reliable sampling and extraction procedure for the estimation of metabolites in yeast is recommended. A volume of culture corresponding to 200 mg (dry weight) should be filtered in a vacuum through Millipore (glassfibre) filters (0.45 μm pore size, 47 mm in diameter). Immediately thereafter, liquid nitrogen should be poured onto the filtered cells to halt metabolism. The filter and cells should then to be immersed in the extraction solution. The extraction procedure used will depend on the sensitivity of the individual metabolite to pH and temperature. The recommended methods for the extraction of NAD+, NADH, ATP, ADP, F1,6BP and DHAP are summarized in Table 3.2:

Table 3.2 **Optimal extraction methods for metabolites from *Saccharomyces cerevisiae***

METABOLITE	EXTRACTION METHOD	REFERENCE
NAD^+	Boiling buffered ethanol /	Gonzalez *et al.* (1997)
	Perchloric acid (6%)	Klingenberg (1974a)
NADH	Boiling alkalised ethanol	Klingenberg (1974b)
ATP	Perchloric acid (35%)	Theobald *et al.* (1993)
ADP	Perchloric acid (35%)	Theobald *et al.* (1993)
F1,6BP	Perchloric acid (6%)	Klingenberg (1974a)
DHAP	Perchloric acid (6%)	Klingenberg (1974a)
G3P	ur[a]	

[a]ur: Unable to make a recommendation.

4. A MODEL FOR GLYCEROL SYNTHESIS BY *S. CEREVISIAE*, BASED ON KINETIC AND EXPERIMENTAL DATA

4.1 SUMMARY

Glycerol is a major by-product of aerobic and anaerobic fermentation by *Saccharomyces cerevisiae*, and is of significant importance to the wine, beer, and ethanol production industry. To gain a clearer understanding of, and to quantify the parameters that affect the rate of glycerol synthesis in *Saccharomyces cerevisiae,* a kinetic model of the glycerol synthesis pathway has been constructed. The kinetic parameters of the pathway enzymes were collected from published values. Other parameters, such as, maximal enzyme activities and intracellular substrate, coenzyme, known modifier and product concentrations, were determined experimentally. The model was validated by comparing experimental results on the rate of glycerol synthesis obtained at various phases of growth, to the rates calculated by the model at the corresponding phases of growth. Values calculated by the model agree well with measured glycerol synthesis rate. The model also mimics the trend of change in the rate of glycerol synthesis at the various phases of growth. Thus, the model has been used to provide insight on the extent to which various parameters affect the rate of glycerol synthesis under standard growth conditions. Metabolic Control Analysis of the model indicates that the NAD^+-dependent glycerol 3-phosphate dehydrogenase (Gpd p) catalyzed reaction has a flux control coefficient (C^J_{v1}) of between 0.83 and 0.87, depending on the growth phase and exercises the majority of control of flux through the pathway. The response coefficients of various parameter metabolites indicate that flux through the pathway is most responsive to the concentration of the substrate DHAP (R^J_{DHAP} = 0.48 to 0.69), followed by the concentration of the inhibitor ATP (R^J_{ATP} = -0.21 to -0.50). Interestingly, the model predicts that the pathway responds weakly to NADH concentration (R^J_{NADH} = 0.03 to 0.08). The model indicates that the best strategy to increase flux through the pathway is not to increase enzyme activity, substrate or coenzyme concentration alone, but to increase all these parameters in conjunction with each other.

Abbreviations can be found on page v.

4.2 INTRODUCTION

Glycerol is a major by-product of anaerobic and aerobic fermentation by *S. cerevisiae*. Glycerol is formed by the reduction of dihydroxyacetone phosphate (DHAP) to glycerol 3-phosphate concomitant with NADH oxidation by NAD-dependent glycerol 3-phosphate dehydrogenase (Gpd p) (Gancedo *et al.*, 1968; Merkel, *et al.*, 1982; Albertyn *et al.*, 1992). Glycerol-3-phosphate (G3P) is then dephosphorylated to glycerol by glycerol-3-phosphatase (Gpp p) (Gancedo *et al.*, 1968; Norbeck *et al.*, 1996) (summarized in Fig. 4.1). The physiological role of NADH-consuming glycerol formation is thought to be the maintenance of the cytosolic redox balance under anaerobic conditions, thereby compensating for cellular reactions that produce NADH (Nordstrom, 1966; van Dijken *et al.*, 1986; Albers *et al.*, 1996). The inability of a mutant defective in glycerol production to grow under anaerobic conditions bears out this observation (Ansell *et al.*, 1997; Bjorkvist *et al.*, 1997; Costenoble *et al.*, 2000; Nissen *et al.*, 2000). Glycerol has another important physiological function, in that it is the primary compatible solute in *S. cerevisiae*, and is accumulated intracellularly when cells are exposed to decreased extracellular water activity (Reed *et al.*, 1987; Meikle *et al.*, 1991; Blomberg & Adler, 1992; Albertyn *et al.*, 1994b).

The value of creating an industrial strain that produces larger than normal amounts of glycerol is illustrated by the number of recent studies to increase glycerol production in industrial and laboratory strains (Radler & Schulz, 1982; Prior *et* al., 1999; Remize *et al.*, 1999). So far, three strategies have been employed to attain this objective: One approach has been to alter growth conditions (Gardner *et al.*, 1993); a second has been to improve wine producing strains by classical genetic techniques involving spore-cell hybridisation followed by repeated back-crossing (Eustace &Thornton, 1987; Prior *et al.*, 1999); and a third has been to improve strains through genetic engineering, by overexpressing either the *GPD1* or the *GPD2* genes, which encode isoenzymes of NAD-dependent glycerol-3-phosphate dehydrogenase, or by deleting either the acetaldehyde dehydrogenase or the pyruvate decarboxylase genes (Remize *et al.*, 1999; Remize *et al.*, 2000).

These strategies have proven to be successful as a result of the current understanding of the physiological conditions under which increased glycerol formation occurs. Concomitant to increased glycerol synthesis, decreased levels of ethanol occur, which is considered to be a positive attribute in the production of alcoholic beverages (Prior *et al.*, 2000). However, increased quantities of acetaldehyde and acetate have also been observed, which are unfavourable in wine. These alterations to the metabolism of the cells seem to be related to a redox imbalance created by the increased flux of carbon towards the formation of glycerol. In light of an incomplete understanding of glycerol synthesis, a detailed kinetic model of the glycerol synthesis pathway has been constructed to evaluate and to quantify the parameters that control the rate of glycerol synthesis. Attention has been placed on glycerol

synthesis, and not on glycerol assimilation, since the enzymes involved in glycerol assimilation, [glycerol kinase (Gut1 p), and mitchondrial FAD-dependant glycerol-3-phosphate dehydrogenase (Gut2 p)], are repressed by glucose at the transcriptional level during fermentative growth (Pavlik *et al.*, 1993; Ronnow & Kielland-Brandt, 1993). The model provides insight to the role and extent to which the redox balance, substrate availability, modifier concentrations and intrinsic enzyme capacity control the flux of carbon through the glycerol synthesis pathway. The data generated by the model may shed some light on the intrinsic capacities of the pathway, and may provide a more insightful approach to controlled glycerol synthesis in industrial strains of *S. cerevisiae.*

4.3 MATERIALS AND METHODS

4.3.1 Organism and growth conditions

A *S. cerevisiae* haploid laboratory strain W303-1A (*MATa leu2-3/112 ura3-1 trp1-1 his3-11/15 ade2-1 can1-100 GAL SUC2 mal0*) (Thomas & Rothstein, 1989) was used in this study. In all experiments, yeast cells were cultured in a medium consisting of glucose (2 g/l) and yeast nitrogen base (6.7 g/l) (United States Biological, Swampscott, Ma, USA), with amino acids [Adenine (10 mg/l); Arg, His, Lys, Met, Trp and Ura (20 mg/l); Ile, Leu, Ser, Thr, Tyr and Val (30 mg/l); Phe (50 mg/l)] (Sigma-Aldrich, St. Loius MO, USA). All yeast cells were maintained on agar plates at 4°C for short-term storage and at –80°C in (15% w/v) glycerol for extended periods of storage. Ten-milliliter starter cultures were used to inoculate 100 ml and 250 ml batch cultures in 250 ml and 500 ml Erlenmeyer Flasks respectively. Cultures were incubated at 30°C and 140 rpm on an orbital shaker (New Brunswick Scientific Co., Edison. N.J., USA).

4.3.2 Sampling of the yeast

Volumes of culture containing at least 100 mg of dry weight biomass were harvested. The culture was rapidly filtered under vacuum through Millipore® glassfiber prefilters (0.45 µm pore size, 47 mm diameter). While still on the filter the cells were instantly frozen with liquid nitrogen. For intracellular glycerol determination, cells were first rinsed with 5 ml of 60 % methanol solution kept at –40°C (rinse step; complete within 5 s) before being frozen with liquid nitrogen and stored as described previously (Saez & Lagunas, 1976).

4.3.3 Determination of yeast dry weight

Volumes of 20 ml [from early to mid exponential phase (OD_{600} 0 to 0.7; approximately 0 to 450 min)] and 10ml [late exponential to early stationary phase (OD_{600} 0.7 to 1.2; approximately 450 to 1100 min)] (Fig. 4.2) of culture were filtered under vacuum through Whatman Glass Microfibre

Filters (25mm diameter; Cat no. 1822 025). The filters were dried at 80°C until a constant weight was obtained (usually 24 h).

4.3.4 Extraction of metabolites

Intracellular metabolites were extracted at three different phases of growth; early exponential phase (approximately OD_{600} 0.4; 400-430 min), mid exponential phase (approximately OD_{600} 0.7; 600-630 min) and early stationary phase (approximately OD_{600} 1.1; 970-1000 min). ATP and ADP were extracted in a 35% perchloric acid solution, whereas DHAP, F1,6BP and NAD^+ were extracted in a 6% perchloric acid solution as described in Chapter 3. (Sec. 3.3.3). NADH was extracted with boiling alkalized ethanol as described in Sec. 3.3.4. Glycerol was extracted in boiling 0.1 M Tris/HCL buffer (pH 7.7) as previously described (van Eck *et al.,* 1989).

4.3.5 Assay of metabolites

Intracellular metabolite concentrations were determined enzymatically by measuring the oxidation or reduction of NADH or NAD^+ respectively at 340nm in a Beckman Coulter DU640 Spectrophotometer as described in Bergmeyer (1974). Extracellular glycerol was determined by high-performance liquid chromatography (Dionex). Anion exchange chromatography followed by pulsed amperometric detection (gold electrode detector) was used with a CarboPac™ MA1 analytical column plus a CarboPac™ MA1 guard column, run at a flow rate of 0.25 ml/min, eluted with a 125 mM NaOH solution. Samples were diluted with double distilled milli-Q water and filtered through 0.22 µm filters (Millex®). To calculate the intracellular concentrations, a yeast cytosolic volume of 1.67 µl per milligram of dry yeast biomass was assumed (van Dam & de Koning, 1992).

4.3.6 Preparation of cell-free extracts for enzyme activity assays.

Yeast cells were harvested at growth phases as described above. Cells were washed twice in ice-cold TRED buffer (10 mM triethanolamine, 1 mM EDTA, 2 mM 2-mercaptoethanol, 1 mM dithiotreitol, pH 7.5) and resuspended in the same buffer with a 0.5 µl/ml protease inhibitor mix (70 mg/ml phenylmethylsulfonyl fluoride, 1 mg/ml pepstatin, 12 mg/ml antipain) (Sigma-Aldrich).
Extracts were prepared by disrupting cells with acid-washed glass beads [400-625 microns (Sigma-Aldrich)] twice for 2 min, with a 1 min interval of cooling on ice. Cell debris was removed by centrifugation at 24, 000 × g for 30 min, and the supernatant was removed and kept on ice until assayed for enzyme activity.

4.3.7 Enzyme activity assay

Cell free extracts were assayed using a Beckman Coulter DU640 spectrophotometer. One unit (U) of enzyme activity is defined as the rate of conversion of 1 µmol of substrate or product per min, and specific activities are given as U/mg of protein. For modeling purposes the specific activities were converted to mM/min, assuming a yeast cytosolic volume of 3.75 µl per milligram of protein (de Koning & van Dam, 1992).

Gpd p activity was assayed by measuring the maximum rate of DL-glycerol 3-phosphate oxidation and NAD^+ reduction, as previously described (Nilsson & Adler, 1990). This assay measures the reverse rate of the GPD reaction and was subsequently converted to forward reaction rate by a conversion factor of 33 (Albertyn *et al.*, 1992). Determinations were started by the addition of 20-320 µl of a 10-fold diluted cell free extract to the cuvette, resulting in a total reaction mixture volume of 1.0 ml. This yielded linear reactions for 1 min under standard conditions with an absorption change of less than 0.1/min.

Gpp p activity was assayed as described Norbeck *et al.,* (1996b) with minor modifications. Determinations were started by the addition of 20-320µl of a 10-fold diluted cell free extract to a test tube, resulting in a total reaction mixture volume of 1.1 ml. After starting the reaction 5 samples of 200 µl were removed from the reaction mixture at 15 s intervals, and instantly quenched in a chilled 600 µl solution of 1% (v/v) sodium dodecyl sulphate, 100 mM zinc acetate, and 15 mM ammonium molybdate, pH 5.0 (Bencini *et al.,* 1983b). Released inorganic phosphate was analyzed (Bencini *et al.*, 1983a), and the reaction rate was calculated from the slope of the linear plot of released phosphate versus time.

4.3.8 Protein determination

Protein concentrations were estimated by determining the absorbance of dilute samples at 280 nm in a 100 µl quartz microcuvette (Ausubel *et al.*, 1999). A_{280} values were compared with those of a bovine serum albumin standard (Sigma-Aldrich). These values were verified by comparing them to values obtained from the Bradford method (Bradford, 1971)

4.3.9 Calculation of the glycerol synthesis rate

The change in extracellular glycerol concentration and biomass versus time from three experiments were averaged and plotted. Because extracellular glycerol and biomass readings were taken approximately only every sixty minutes, a five parameter sigmoidal function was fitted to each curve (Fig. 4.2) [Correlation coefficients (R^2) were above 0.989 for both curves], which enabled us to determine, with more accuracy, the rate at which glycerol was produced at very small time intervals. The glycerol production rate was calculated as the change in the amount of extracellular

glycerol per gram of dry weight biomass per minute [mol/g /min]. The change in intracellular glycerol was not taken into account, since intracellular glycerol never exceeded 0.2% of the total glycerol content of the flask. The model calculates the rate of glycerol production as flux (J) through the pathway, and expresses it as the rate of change in intracellular glycerol concentration (mM/min). To compare values calculated by the model with those determined experimentally, the measured glycerol synthesis rate [mol/g /min] was converted to the rate of change in intracellular glycerol concentration (mM/min) by converting biomass to intracellular volume [1 g dry weight biomass ≈ 1.67 ml cytosolic volume (van Dam & de Koning, 1992)].

4.3.10 Kinetic model

A kinetic model of glycerol synthesis via glycerol 3-phosphate (Fig. 4.1) was constructed using the metabolic simulation program Gepasi (Mendes, 1997). Three phases of growth, as described above, were modeled. The parameters for each phase of growth were derived from *in vivo* values measured at each of the growth phases. Steady-state calculations of the kinetic model were performed on an IBM compatible personal computer.

Details on the kinetics of the enzyme-catalyzed reactions that form the core reaction sequence of the model, and the aspects of Metabolic Control Analysis (MCA) used in the presentation of results, are provided in the appendix.

4.4 RESULTS

4.4.1 Construction of the kinetic model

In *S. cerevisiae,* oxidation of NADH and reduction of DHAP by NAD^+-dependent Gpd p results in the formation of G3P, which is then dephosphorylated to glycerol by Gpp p. To assess the importance and to quantify the control various pathway parameters have on flux, a kinetic model of the glycerol synthesis pathway was constructed (Fig. 4.1). The kinetic parameters of the pathway enzymes, (Gpd p and Gpp p), were collected from reported values and are presented in Table 4.1 (Bergmeyer *et al.*, 1974; Nader *et al.*, 1979; Merkel *et al.,* 1982; Nilsson & Adler, 1990; Albertyn *et al.*, 1992; Norbeck *et al.*, 1996). Maximal enzyme activities were determined at three phases of growth (Table 4.1). The intracellular concentrations of substrates, cofactors, products, and known effector metabolites were also determined at the above-mentioned phases of growth (Table 4.2). Besides the variable metabolite, glycerol 3-phosphate, all metabolites were fixed and therefore not modeled as system variables.

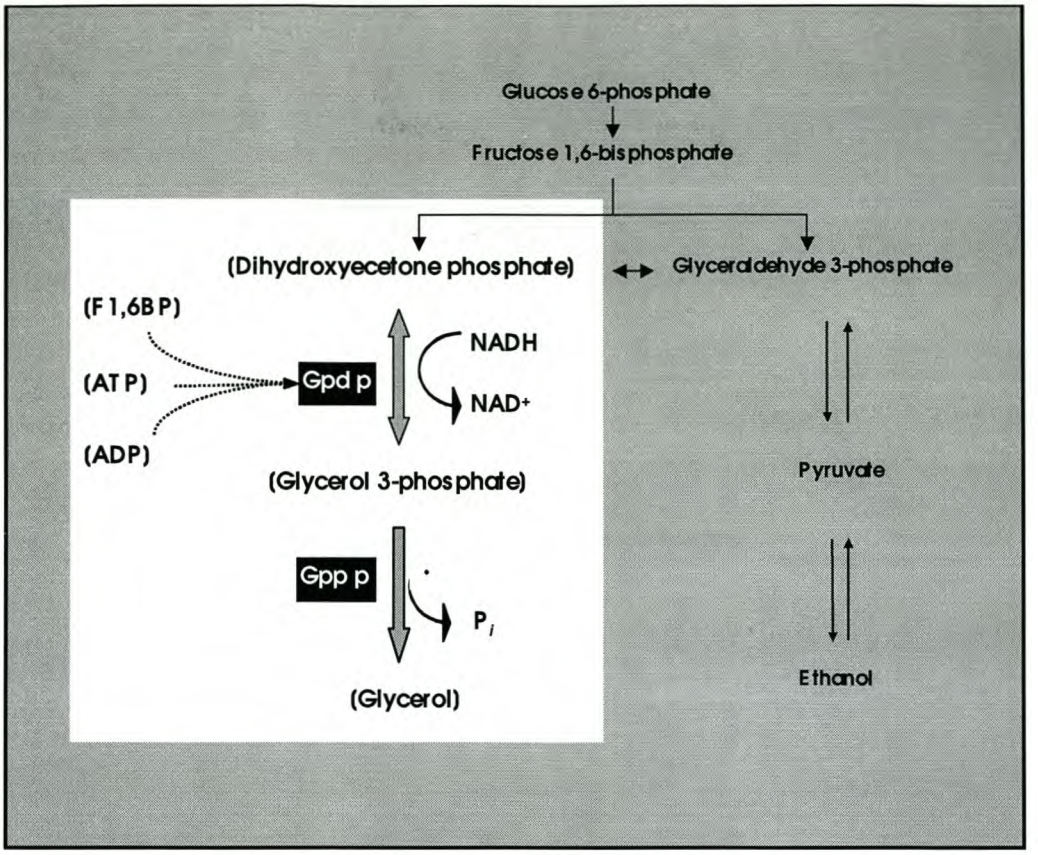


Figure 4.1. The glycerol synthesis pathway in *S. cerevisiae*. The highlighted area indicates the metabolites and enzymes considered in the kinetic model. Abbreviations: Gpd p (NAD+-dependent glycerol 3-phosphate dehydrogenase), Gpp p (Glycerol 3-phosphatase), F1,6BP (fructose 1,6-bisphosphate).

There are two types of pathway metabolites: the first are "source" and "sink" (i.e. DHAP and glycerol), which must be clamped in order for a steady state to be achieved. The second type, are "cofactors" (ATP, ADP, NADH and NAD^+). These were clamped because the model only addresses a small part of metabolism. If cofactors were set free to vary, it would be necessary to include all reactions that require them to provide a realistic result.

4.4.2 Evaluation of the kinetic model

To evaluate the ability of the model to calculate correctly the flux through the pathway, experimental data on the rate of glycerol production at three phases of growth were compared to the rate of glycerol production calculated by the model with parameters determined at the corresponding phases of growth. The parameter values are presented in Tables 4.1 and 4.2 and Fig. 4.3. Results from experiments conducted to measure the rate of glycerol synthesis (Fig. 4.3) indicate that flux through the pathway steadily increases throughout the initial phase of growth (lag phase; 0-200 min). Thereafter, during the exponential phase of growth (200-700 min), flux fluctuates

between 3.3 mM/min and 3.6 mM/min. Then towards the onset of stationary phase (≥ 700 min), the rate of glycerol production decreases. This decline could possibly be the result of glucose exhaustion leading to glycerol reoxidation as a result of the derepression of *GUT1* and *GUT2*, but this suggestion requires confirmation, as glucose levels were not analyzed.

Flux values calculated by the model estimate a rate of glycerol production very similar to the measured rate. In addition, flux values calculated by the model, closely mimic the trends of change in the rate at which glycerol is synthesised at the corresponding phases of growth (Fig. 4.3). This indicates that:

1. The parameters determined at each phase of growth are an accurate estimation of *in vivo* metabolite levels and enzyme activities.
2. The kinetic parameters obtained from literature are accurate and allow the model to calculate flux in accordance with results obtained experimentally.
3. The metabolic control analysis values calculated by the model will provide a quantitatively accurate estimation of parameters that have strong control of flux through the pathway.

4.4.3 Modelling results

Since flux values calculated by the model agreed well with those determined experimentally, the model has been used to provide insight on how the various known parameters affect the flux of carbon through the glycerol synthesis pathway. The model employs MCA to study the relative control exerted by each reaction on the system's flux. To relate the effect of a change in a parameter to a change in the steady state of a system, the response coefficient was calculated. The response coefficient (Kacser & Burns, 1973) allows one to relate the elasticity coefficient (quantifies the effect of an effector on a reaction) with the flux-control coefficient (quantifies the control a reaction has on flux) of a step. In this study, response coefficients have been used to provide insight on how various effectors (e.g. substrate, coenzyme) affect the rate of a reaction, and in so doing affect flux through the system. Table 4.3 presents the flux control coefficients calculated by the model for the reactions catalyzed by Gpd p (reaction 1) and Gpp p (reaction 2), based on the requirement that the sum of all flux-control coefficients (all reactions) of a pathway is equal to unity (Kacser & Burns, 1973; Heinrich & Rapoport, 1975; Giersch, 1988; Reder 1988):

$$\sum_i C_{vi}^J = 1$$

This implies that all the steps of a pathway exert a certain amount of control on flux through the pathway.

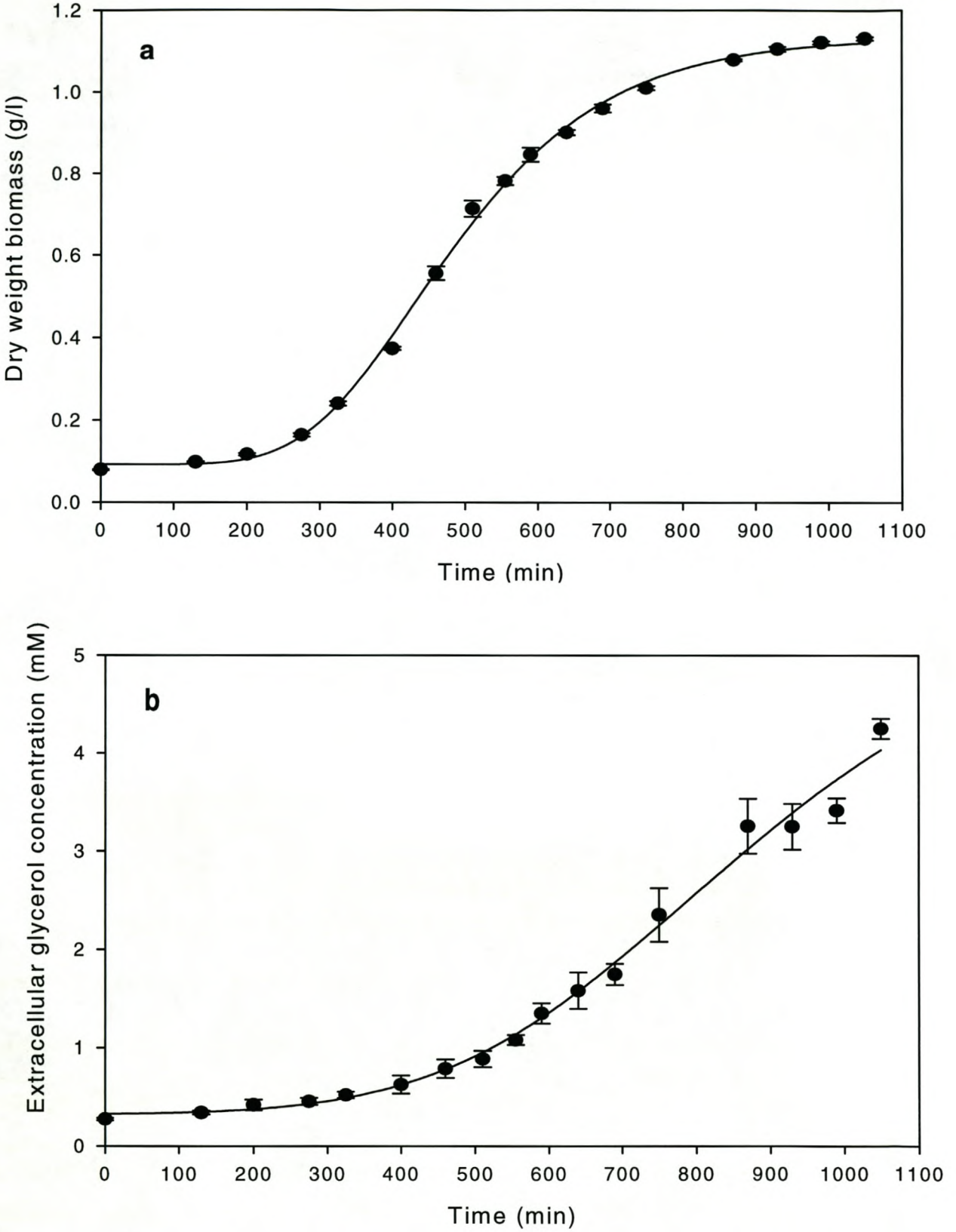


Figure 4.2. Growth of *S. cerevisiae* (a) and extracellular glycerol production (b) during shake flask cultivation in glucose -YNB medium at 30°C. Each data point reflects the mean of triplicate determinations, with error bars indicating the standard error. Values for the growth curve and change in extracellular glycerol concentration were fitted to a five parameter sigmoidal function [Correlation coefficients (R^2) were above 0.989 for both curves].

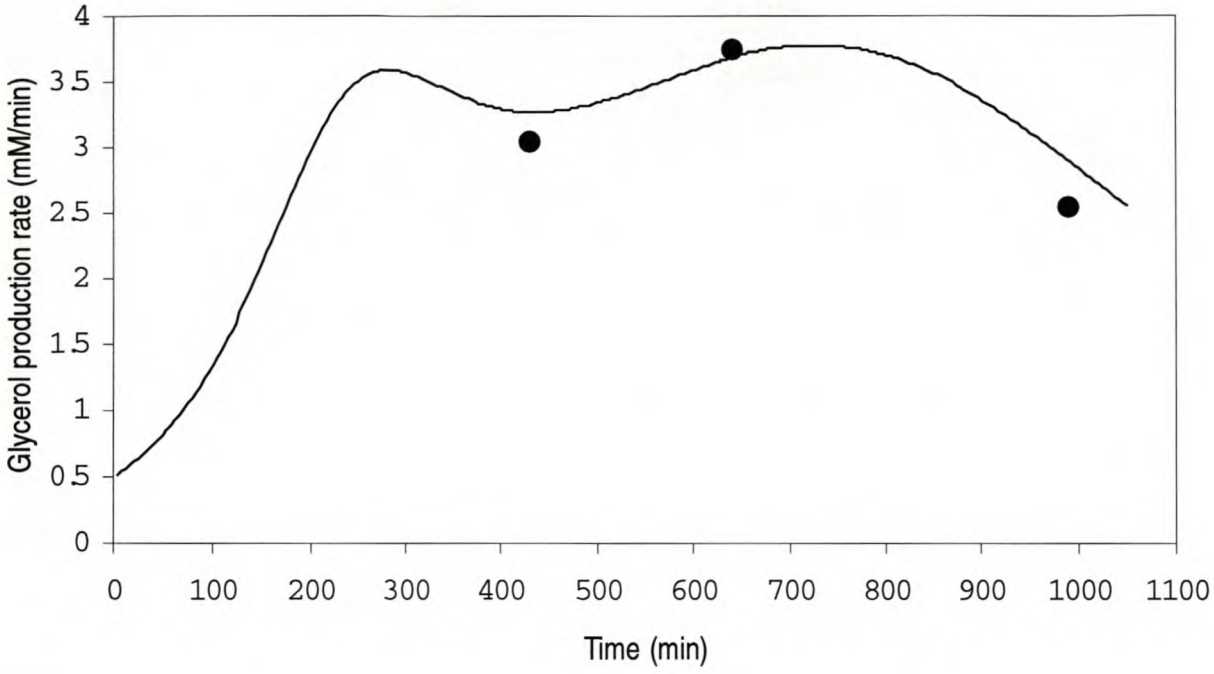


Figure 4.3. The rate of glycerol production in *S. cerevisiae* during shake flask cultivation in glucose-YNB medium (—), compared to the glycerol production rate calculated by the model (•). The glycerol production rate was calculated by plotting the change in extracellular glycerol concentration against time, and the change in dry weight biomass against time. Both of these plots were fitted to a five-parameter sigmoidal function [Correlation coefficients (R^2) were above 0.989 for both curves] from which the glycerol production rate was derived. The three glycerol production rates calculated by the model were based on sets of parameters for each growth phase as defined in Tables 4.1 and 4.2.

The Gpd p reaction flux-control coefficient (C_{v1}^{J}) ranges from 0.83 to 0.88, whilst the Gpp p reaction flux-control coefficient (C_{v2}^{J}) ranges from 0.12 to 0.17. These values were derived from the steady states calculated according to the parameters measured at each of the defined phases of growth. Thus, it is evident that flux through the glycerol synthesis pathway is primarily regulated by the GPD reaction. Fig. 4.4a illustrates how flux through the pathway would be influenced by manipulation of Gpd p and Gpp p activity. Both enzymes activities are increased approximately fivefold in their measured activity at early exponential phase (Table 4.1). It is clear that increased Gpp p activity is futile unless combined with increased Gpd p activity. In contrast, by increasing Gpd p activity fivefold, we see a marked increase in flux (approximately fourfold), this while Gpp p activity remains unaltered from the activity measured.

Table 4.1 Kinetic parameters of enzyme catalyzed reactions

PARAMETER	[a]DETERMINED VALUE	PUBLISHED VALUE	REFERENCE	GROWTH PHASE
Reaction 1: Glycerol 3-phosphate dehydrogenase				
V_{max}		61	(Albertyn *et al.*, 1992)	
V_{max}		36	(Pahlman *et al.*, 2001)	
[b]V_{max}	47 ± 0.14			Early exponential[d]
V_{max}	67 ± 0.09			Mid exponential[e]
V_{max}	46 ± 0.08			Early stationary[f]
K_{eq}		1×10^4	(Bergmeyer *et al.*, 1974)	
[c]K_m^{NADH}		0.023	(Albertyn *et al.*, 1992)	
K_m^{DHAP}		0.54	(Albertyn *et al.*, 1992)	
K_m^{NAD+}		0.93	(Albertyn *et al.*, 1992)	
K_m^{G3P}		1.2	(Nilsson &Adler, 1990)	
$K_i^{F1,6BP}$		4.8	(Albertyn *et al.*, 1992)	
K_i^{ATP}		0.73	(Nader *et al.*, 1979)	
K_i^{ADP}		2.0	(Nader *et al.*, 1979)	
Reaction 2: Glycerol 3-phosphatase				
V_{max}		18	(Norbeck *et al.*, 1996)	
V_{max}	53 ± 2.8			Early exponential[d]
V_{max}	104 ± 6.6			Mid exponential[e]
V_{max}	68 ± 2.3			Early stationary[f]
K_m^{G3P}		3.5	(Norbeck *et al.*, 1996)	
K_i^{Pi}	1.0		Estimated	

[a]Values are presented as the average of three independent experiments, with standard error of the mean. [b] All maximal rates are in mM/min. [c] All K_m and K_i values are in mM. [d] Time (OD_{600} 0.4; 400-430 min). [e] Time (OD_{600} 0.7; 600-630 min). [f] Time (OD_{600} 1.1; 970-1000 min).

Table 4.2 Fixed metabolite concentrations of the kinetic model

METABOLITE	INTRACELLULAR CONCENTRATIONS (mM) AT VARIOUS PHASES OF GROWTH[a]			PUBLISHED VALUES (mM)	REFERENCE
	[b]EARLY EXPONENTIAL	[c]MID EXPONENTIAL	[d]EARLY STATIONARY		
ATP	2.37 ± 0.12	2.25 ± 0.26	0.4 ± 0.03	0.04 – 4.8	(Theobald *et al.*, 1993; (de Koning & van Dam, 1992)
ADP	2.17 ± 0.20	1.31 ± 0.21	0.76 ± 0.07	0.4 – 4.2	(Gonzalez *et al.*, 1997; Saez & Lagunas, 1976)
NADH	1.87 ± 0.24	0.62 ± 0.40	0.33 ± 0.02	0.23 – 1.2	(Gonzalez *et al.*, 1997; Saez & Lagunas, 1976)
NAD^+	1.45 ± 0.17	0.47 ± 0.09	0.48 ± 0.04	0.8 – 5.0	(Gonzalez *et al.*, 1997; Saez & Lagunas, 1976)
F1,6BP	6.01 ± 0.35	3.12 ± 0.58	1.27 ± 0.06	0.5	(de Koning & van Dam, 1992)
DHAP	0.59 ± 0.07	0.31 ± 0.09	0.14 ± 0.02	0.4	(de Koning & van Dam, 1992)
Glycerol	15.10 ± 1.06	21.00 ± 0.99	6.72 ± 0.26	24	(Toh *et al.*, 2001)

[a]Values were derived from experiments performed in triplicate, and are presented as the average with standard error of the mean. [b] Time (OD_{600} 0.4; 400-430 min). [c] Time (OD_{600} 0.7; 600-630 min). [d] Time (OD_{600} 1.1; 970-1000 min).

Table 4.3 **Metabolic Control Analysis on the effects of various parameters on carbon flux (J) through the glycerol synthesis pathway**

METABOLIC CONTROL COEFFICIENTS[a]	PHASE OF GROWTH		
	[b]EARLY EXPONENTIAL	[c]MID EXPONENTIAL	[d]EARLY STATIONARY
Response Coefficient			
R^J_{NADH}	0.03	0.05	0.08
R^J_{DHAP}	0.48	0.60	0.69
R^J_{NAD}	- 0.02	- 0.02	- 0.03
$R^J_{F1,6BP}$	- 0.16	- 0.11	- 0.10
R^J_{ATP}	- 0.42	- 0.50	- 0.21
R^J_{ADP}	- 0.14	- 0.10	- 0.14
Flux Control Coefficient			
C^J_{v1}	0.85	0.88	0.83
C^J_{v2}	0.15	0.12	0.17

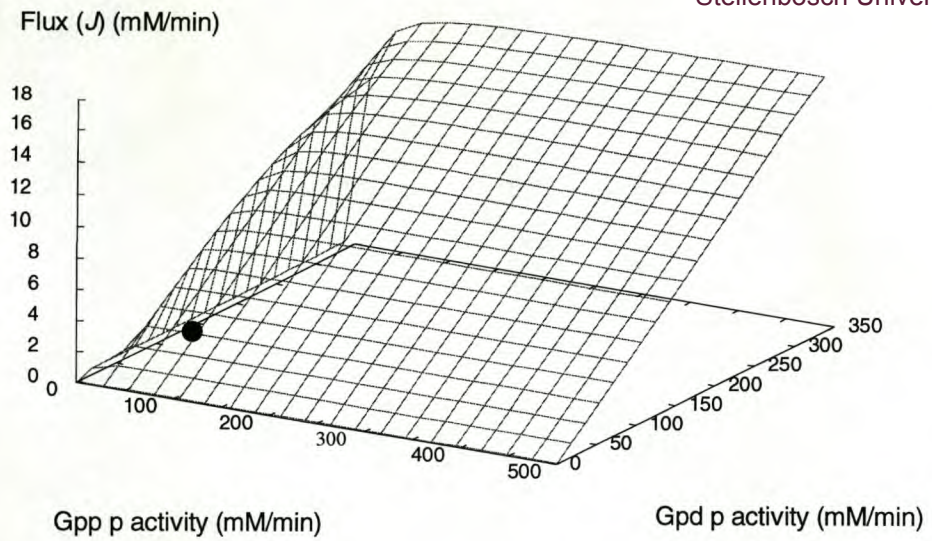
[a] Metabolic Control Coefficients were calculated by the Gepasi simulated model of the glycerol synthesis pathway. v_1 (rate of the Gpd1,2p catalysed reaction), v_2 (rate of the Gpp1,2p catalysed reaction). [b]Time (OD_{600} 0.4; 400-430 min). [c]Time (OD_{600} 0.7; 600-630 min). [d]Time (OD_{600} 1.1; 970-1000 min).

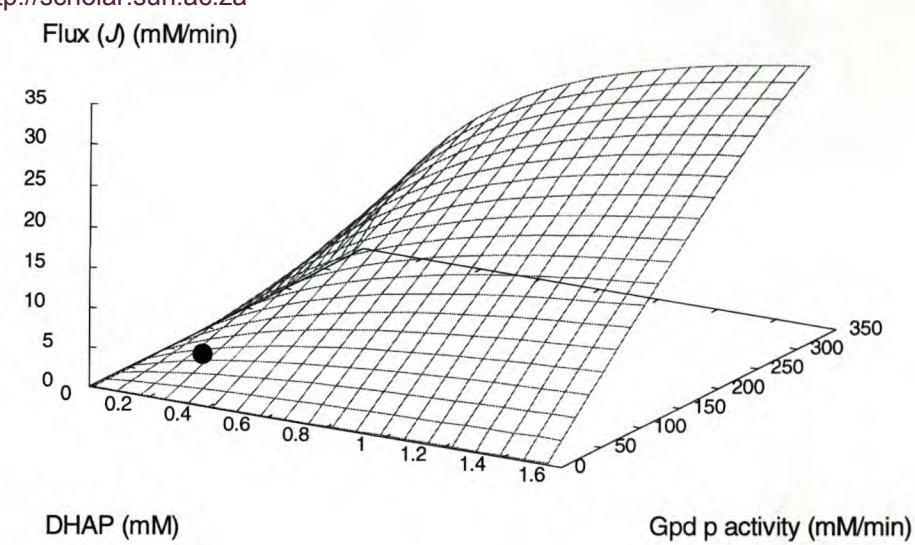
Table 4.3 shows the response coefficients of various metabolites on flux through the pathway. According to MCA, positive flux through the pathway is predominantly controlled by substrate concentration (R^J_{DHAP} = 0.48-0.69). By increasing DHAP concentration fivefold the system realizes a twofold increase in flux (data not shown). This however, can be drastically improved when combined with as little as a twofold increase in Gpd p activity (Fig. 4.4b). Note that by increasing the DHAP concentration in conjunction with Gpd p activity, flux is elevated higher than when Gpd p activity is increased alone.

It is interesting to note that the coenzyme of the reaction, NADH, does not have as much effect on flux as might be expected (R^J_{NADH} = 0.03-0.08). Its effect does however increase slightly as fermentation progresses. The measured NADH/NAD^+ ratio at early exponential phase is approximately 1.5, and by increasing this ratio to 5 the resultant increase in flux is less than 25% (data not shown). This however, may be improved drastically by increasing the NADH/NAD^+ ratio and Gpd p activity simultaneously (Fig. 4.4c). The difference in response DHAP and the NADH/NAD^+ ratio have on the system is clearly illustrated in Fig. 4.4d. This figure also illustrates how a concomitant increase in the DHAP concentration and NADH/NAD^+ ratio can increase the rate of glycerol synthesis to a larger extent than when either of these parameters are increased alone.

Response coefficients with a negative value indicate that these metabolites have a negative effect on the rate that glycerol is synthesized. Three of the four metabolites presented, namely ATP, ADP, and FBP, are known inhibitors with published inhibition constants (Table 4.1) (Nader, *et al.,* 1979; Albertyn, *et al.,* 1992). The other metabolite, NAD^+, is a product of the reversible GPD reaction, and will therefore have a negative effect on the rate of the forward reaction. Of the response coefficients presented, ATP has the strongest negative response on the system (R^J_{ATP} = -0.21 to -0.50). An increase in the ATP/ADP ratio to fivefold its measured ratio results in a 10% decrease in flux through the pathway. When the ATP/ADP ratio is halved (reduced from 1.08 to 0.54), flux through the pathway is increased by approximately 10% (Fig. 4.5a). It is noteworthy that a decrease in the NAD^+/NADH ratio to the same extent only results in a 1% increase in flux through the pathway (Fig. 4.5b).

If the moiety-conserved relationship of ATP, ADP and AMP is ignored, and the ATP concentration is increased fivefold, without affecting the concentration of the other two metabolites, flux through the pathway decreases drastically from 3.1 mM/min to 1.1 mM/min (data not shown). The inhibitory effect of ADP (R^J_{ADP} = -0.10 to -0.14) is less than that of ATP. By increasing the ADP concentration fivefold, flux decreases by 35%. The response coefficient of F1,6BP ($R^J_{F1,6BP}$ = -0.10 to -0.16) is very close to that of ADP, and therefore a fivefold increase in F1,6BP concentration resulted in a 43% decrease in flux.





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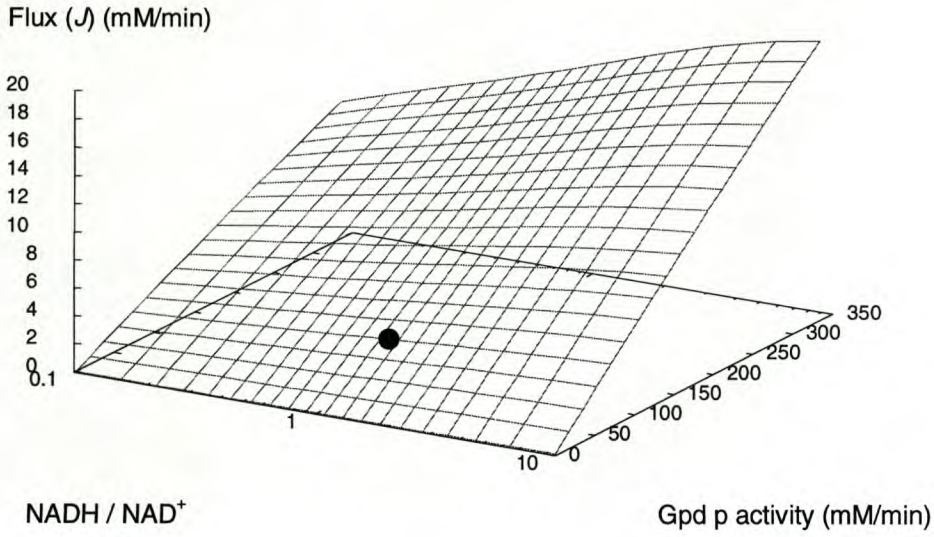

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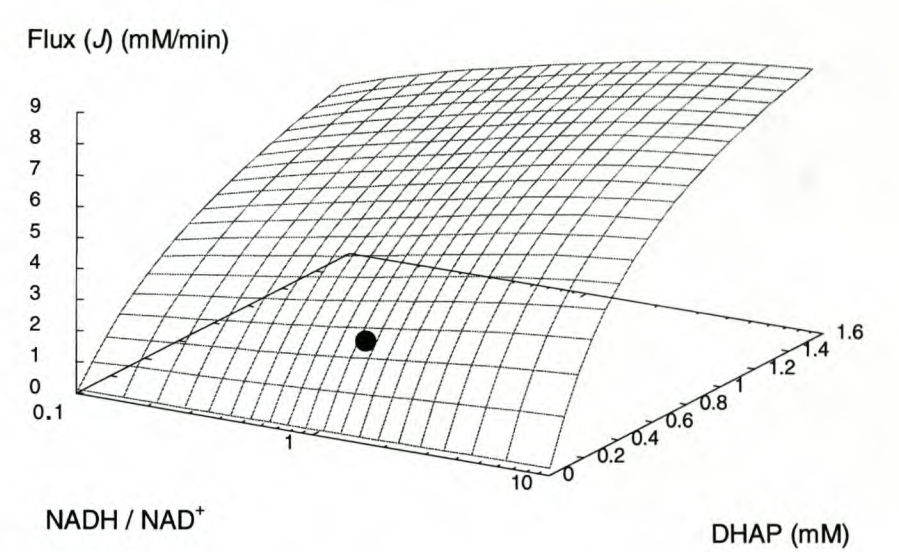

Figure 4.4. Predictions by the model on the effects of manipulating different sets of parameters; (a) Gpd p and Gpp p activity increased fivefold from the measured activity, (b) Gpd p activity and DHAP concentration increased fivefold from the measured activity and concentration, (c) Gpd p activity and the NADH/NAD ratio increased fivefold from the measured activity and approximately a tenfold increase in ratio, (d) DHAP concentration and the NADH/NAD ratio increased fivefold from the measured concentration and approximately a tenfold increase in ratio, on flux through the glycerol synthesis pathway. (•) Reference state, refers to the flux value calculated by the model as defined by parameter values measured at mid-exponential growth phase (Tables 4.1 and 4.2)

4.5 DISCUSSION

4.5.1 Accuracy of the models calculations

The ability of the model to predict flux through the glycerol synthesis pathway was evaluated by selecting three sets of parameters obtained from various stages of growth. In each case the model estimated the rate glycerol synthesis accurately. The intrinsic enzyme activity of both enzymes, at each phase of growth measured, ranged from 46 mM/min to 104 mM/min (Table 4.1). This is well above the maximum steady state flux of 3.75 mM/min (Fig. 4.3), calculated by the model. Therefore, the model was accurately defined by all the parameters employed in its construction. This allowed flux values calculated by the model, not only to be of the same magnitude as determined values, but also to closely mimic the trends of change in the rate at which glycerol is synthesised at different phases of growth (Fig. 4.3). In light of this, the model should be viewed as a quantitative tool with the ability to provide insight to the extent to which known parameters of the pathway affect the quantity of flux that passes through it.

4.5.2 Parameters affecting flux

According to the model developed here, flux through the glycerol synthesis pathway is strongly controlled by the Gpd p reaction ($C_{v1}^{J} = 0.83 - 0.87$). This observation is in accordance with a previously determined flux control coefficient of 0.63 (Blomberg & Adler, 1989). The latter value was derived by plotting a double logarithmic plot of the glycerol production rate versus Gpd p activity, where the slope equaled the estimated flux control coefficient. In their study, increased Gpd p activity was the result of cells being conditioned to media with decreased water activity (0.35-0.7 M NaCl). In effect, their observations of increased glycerol productivity take into account, not only increased Gpd p activity, but also the concomitant alterations to metabolism that would occur under such conditions. The value derived by the model; however, is based on calculations where only perturbations in Gpd p activity occur, since all other parameters are clamped. This may explain the difference in the results.

An increase in Gpd p activity stimulates glycerol flux significantly (Fig. 4.4). Similarly, by overexpressing *GPD1* in industrial and non-industrial *S. cerevisiae* strains, the increase in Gpd p activity results in a two to threefold improvement in glycerol yields from fermentation (Remize *et al.,* 1999; Remize *et al.*, 2001). However, the concomitant enhanced oxidation of NADH affects the redox state of these yeast cells, which results in sluggish growth and increased levels of various by-products, such as acetaldehyde. As mentioned, increased glycerol production occurs naturally when yeast cells are exposed to hyperosmotic or hypoxic conditions. This is achieved by the upregulation of *GPD1*, *GPD2*, *GPP1* and *GPP2* (Blomberg & Adler, 1989; Varela *et al.*, 1992; Albertyn *et al.*,

1994; Ansell *et al.*, 1997; Bjorkvist *et al.*, 1997; Costenoble *et al.*, 2000). The model also indicates, that maximal flux can be achieved with relatively little alteration in Gpp p activity (Fig. 4.4a). Recently this was confirmed; where it was shown that overexpression of *GPP1* has very little effect on flux. Even when overexpressed in a *GPD1* overexpressing strain, there is no enhancement in glycerol production compared to the strain overexpressing *GPD1* alone (Remize *et al.*, 2001).

From the response coefficients generated by the model, it is clear that of the two metabolites consumed in the Gpd p reaction, DHAP has more influence on flux through the pathway than NADH. This is most likely due to the fact that Gpd p has a far greater affinity for NADH than for DHAP (Table 4.1). The physiological NADH concentrations measured at each phase of growth (Table 4.2) are high enough to ensure that Gpd p is saturated, and therefore increasing the NADH concentration would in actual fact have very little effect on the forward reaction rate. However, as fermentation progresses, there is a slight increase in response of the pathway to the NADH concentration, and this may be due to the progressive decrease in concentration of this metabolite (Tables 4.2 & 4.3). In contrast, physiological DHAP concentrations were never high enough to saturate Gpd p, and therefore an increase in DHAP concentration is likely to have more influence on flux through the pathway. Compagno *et al.* (1996) have shown that a *S. cerevisiae* triose phosphate isomerase deficient mutant with elevated levels of DHAP has a glycerol producing ability which is two to threefold that of a wild type strain. This mutant, however, grows at a far slower rate due to a NADH/energy shortage (Compagno *et al.*, 2001).

Surprisingly, MCA has shown that glycerol flux is far more sensitive to the ATP/ADP ratio than it is to $NADH/NAD^+$ ratio (Fig. 4.5). The model provides insight to the coupled effect of ATP and ADP concentration on flux through the pathway. Fig. 4.5a illustrates the extent to which glycerol flux decreases, as the ATP/ADP ratio increases. This is due to ATP having a larger negative response coefficient (Table 4.3). A low ATP/ADP ratio, on the other hand, results in a decrease in the concentration of the metabolite with a stronger response coefficient and an increase in the concentration of the metabolite with a weaker response coefficient, which leads to an increase in flux. However, if the ADP concentration is increased without affecting the ATP concentration, flux decreases. Similarly, if the ATP concentration is increased alone, flux decreases. However, in this case, to a far greater extent. In reality, this would only occur to a limited extent since ATP and ADP variation is constrained by the adenylate kinase equilibrium (Su & Russel, 1968).

The strong response of the Gpd p reaction to ATP concentration has been confirmed experimentally. In cell-free extracts prepared from cells cultivated in carbon limiting chemostats, Gpd p activity was decreased by at least 30% in the presence of 1 mM ATP (Pahlman *et al.*, 2001). Similarly, Albertyn *et al.* (1992) reported an 83% inhibition of purified Gpd p activity by 1 mM ATP.

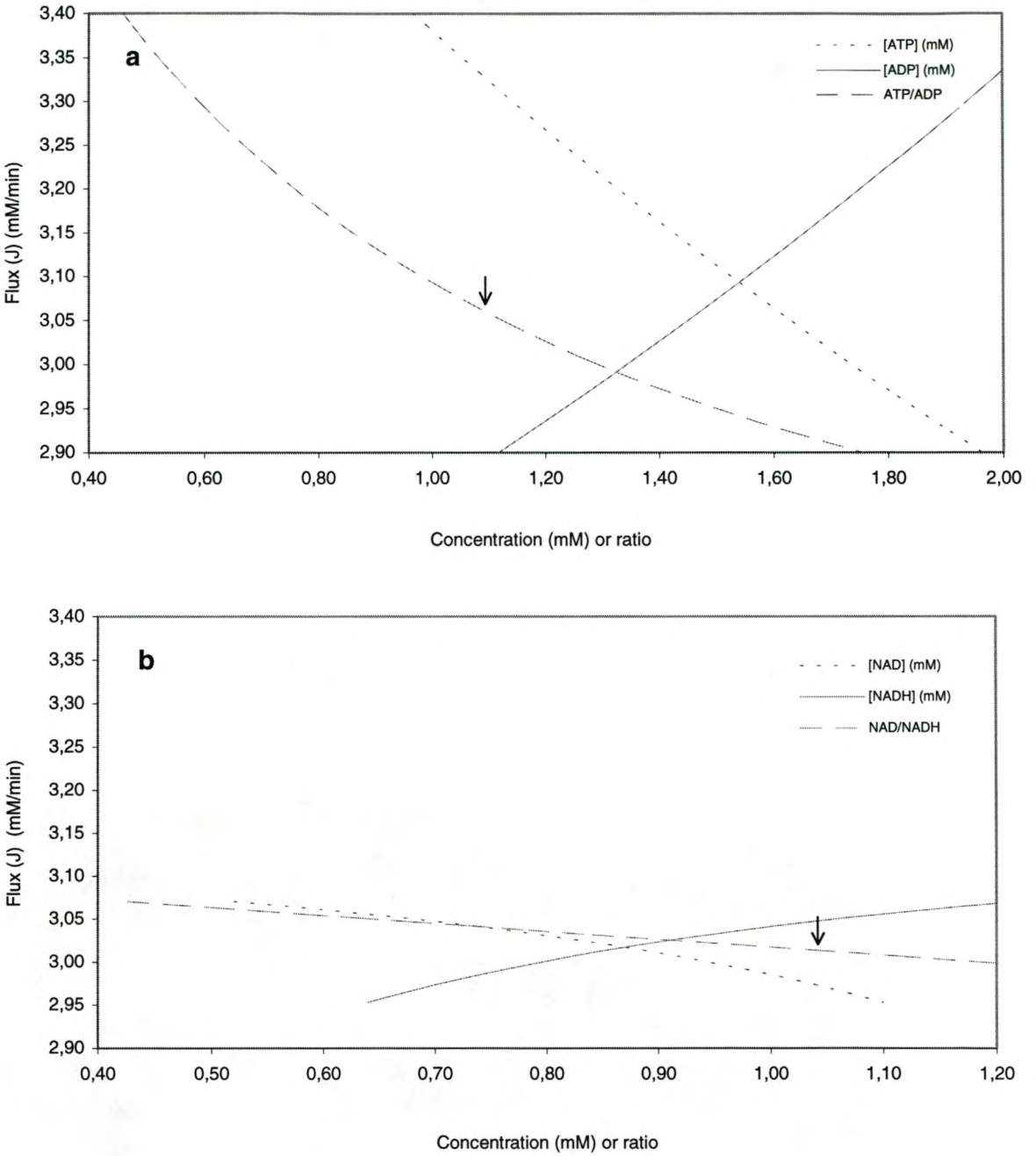

Figure 4.5. The effects of decreasing (a) the ATP/ADP ratio and (b) NAD^+/NADH ratio on flux through the glycerol synthesis pathway. (↓) Refers to the flux value calculated by the model as defined by parameter values measured at mid-exponential growth phase (Tables 4.1 and 4.2).

The effects presented by the model for increased F1,6BP concentration are very similar to those of ADP, in that we could expect a decrease in flux since F1,6BP is a known noncompetitive inhibitor of Gpd p (Albertyn *et al.,* 1992). However, under glycolytic conditions, an increase in F1,6BP concentration might result in an increase in DHAP concentration, since the F1,6BP/DHAP ratio appears to remain relatively constant at different phases of growth (Table 4.2). Therefore, even if F1,6BP concentration increases, flux is also likely to increase, due to the response of the pathway to the increase in DHAP concentration, which holds a stronger response coefficient (Table 4.3).

In conclusion, the model developed here for the glycerol synthesis pathway shows that the rate of glycerol production is primarily controlled by Gpd p activity. There are also a number of metabolites that affect glycerol formation, and at different phases of growth, the concentration of these metabolites vary (Tables 4.1, 4.2 and 4.3). These alterations occur not only in magnitude, but also in relative proportion to each other. Thus, the effects of various parameters on the system do not remain constant. In essence this means, as fermentation progresses, the extent to which the system responds to a parameter alters and therefore, a parameter that affects flux early on in fermentation might not affect flux to the same extent later on. Similarly, in other studies, kinetic models have successfully been used to help researchers understand complex cellular systems. For example, a kinetic model of glycolysis in the bloodstream-form of *Trypanosoma brucei* helped identify factors that affect glycolytic flux (Bakker *et al.,* 1997). The controlling factors of flux through the threonine-synthesis pathway in *Escherichia coli* have also been elucidated (Chassagnole *et al.*, 2001). And in another study, a kinetic model helped identify targets for manipulation in an effort to maximize the conversion efficiency of hexose to sucrose and to minimize futile cycling in sugar cane (Rohwer & Botha, 2001). The quantitative predictions provided by the model in this study have not only agreed with previous findings on the effects of various parameters on glycerol production, but have also allowed us to quantify more accurately the extent to which different parameters affect glycerol flux.

4.6 APPENDIX

4.6.1 Kinetics of enzyme-catalyzed reactions

Glycerol-3-phosphate dehydrogenase: This reaction was simulated using a reversible two-substrate two-product rate equation with non-competitive inhibition. At physiological concentrations ATP, ADP, NAD^+ and F1,6BP inhibit glycerol-3-phosphate dehydrogenase activity (Albertyn *et al.*, 1992), and have been included as modifiers.

The kinetic equation is:

$$v = \frac{\frac{V_f}{K_{NADH} \cdot K_{DHAP}}\left([NADH][DHAP] - \frac{[NAD^+][G3P]}{K_{eq}}\right)}{\left(1+\frac{[F1,6BP]}{K_{F1,6BP}}+\frac{[ATP]}{K_{ATP}}+\frac{[ADP]}{K_{ADP}}\right)\left(1+\frac{[NADH]}{K_{NADH}}+\frac{[NAD^+]}{K_{NAD^+}}\right)\left(1+\frac{[DHAP]}{K_{DHAP}}+\frac{[G3P]}{K_{G3P}}\right)}$$

Glycerol-3-phosphatase: This reaction was simulated using irreversible noncompetitive inhibition kinetics. The reaction has one substrate, two products, one of which is a modifier (inhibitor). The inhibitor is noncompetitive with the substrate, i.e. its effect is only to decrease the apparent limiting rate. The kinetic equation is:

$$v = \frac{V \cdot \frac{[G3P]}{K_{G3P}}}{\left(1+\frac{[G3P]}{K_{G3P}}\right)\cdot\left(1+\frac{[P_i]}{[K_{P_i}]}\right)}$$

4.6.2 Control analysis

In this study, control analysis has been used to quantify the control that the Gpd p and Gpp p catalysed reactions each exert on flux through the glycerol synthesis pathway. Metabolic control analysis (Kacser and Burns, 1973; Heinrich and Rapoport, 1974) provides a means to quantify the link between a system variable (e.g. flux through a pathway or the steady state concentration of a metabolite) and a system parameter (e.g. activity of an enzyme) in terms of a flux or concentration control coefficient. The flux or concentration control coefficient for step *i* of a system, is defined as:

$$C^{y}_{vi} = \frac{\partial \ln y}{\partial \ln v_i}$$

where *y* is the variable, *i* the step (enzyme) and *v* the activity of the perturbed step (Kacser & Burns, 1973, Heinrich & Rapoport, 1974). In metabolic control analysis the properties of an enzyme can be measured relative to change in a parameter. The sensitivity of an enzyme to a metabolite is known as the elasticity coefficient (Kacser & Burns, 1973, Heinrich & Rapoport, 1974). The elasticity coefficients are defined as the ratio of relative change in local rate to relative change in one parameter (normally the concentration of an effector), and is written as:

$$\varepsilon^{v_i}_{p} = \frac{\partial \ln v_i}{\partial \ln [p]}$$

where *v* is the rate of the enzyme in question and *p* is the parameter of the perturbation. Each enzyme has as many elasticity coefficients as the number of parameters that affect it. As parameters

of the reaction, substrates, products and modifiers, will each have an elasticity coefficient. Unlike control coefficients, elasticity coefficients are not systemic properties. Therefore to relate the effect of a change in a parameter to a change in the steady state of a system, we make use of a response coefficient. For example, the response coefficient of a modifier on a system will describe how the modifier affects the rate of a specific reaction, and this change in reaction rate will in turn increase or decrease the flux through a system. Thus, the effect of a parameter (p) on a pathway variable (y) is given by the so-called combined response property (Kacser & Burns 1973):

$$R_p^y = \frac{\partial \ln y}{\partial \ln v_i} \cdot \frac{\partial \ln v_i}{\partial \ln [p]} = C_{v_i}^{y} \cdot \varepsilon_p^{v_i}$$

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