Engineering of *Saccharomyces cerevisiae* for the production of L-glycerol 3-phosphate

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Abbreviations

Amp	Ampicillin
ATP	Adenosine triphosphate
bp	base pair(s)
DHAP	Dihydroxyacetone phosphate
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EDTA	Ethylendiaminetetraacetic acid
EtBr	Ethidium Bromide
Fig.	Figure
G418	Geneticin
GPD	Glycerol 3-phophate dehydrogenase
h	hour
kb	kilobase
Km^R	Kanamycin resistance
LB	Luria-Bertani
L-G3P	L-Glycerol 3-phosphate
M, mM	Molar, Millimolar
min	minute
NAD	Nicotinamide adenine dinucleotide (oxidised)
NADH	Nicotinamide adenine dinucleotide (reduced)
NADP	Nicotinamide adenine dinucleotide phosphate (oxidised)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
nm	nanometer
nt	nucleotide
OD	optical density
ori	origin of replication
PCR	polymerase chain reaction
PDC	Pyruvate decarboxylase
PMSF	phenylmethylsulfonyl flourid
rpm	Rotations per minute
RT	room temperature
S. cerevisiae	*Saccharomyces cerevisiae*
SDS	Sodium dodecyl sulphate
sec	second
Tab.	Table
Tris	Tris (hydroxymethyl) aminomethane
U	Unit
Vol	Volume
w/v	weight/volume
wt	wild type
YPD	Yeast Peptone Glucose medium

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I. Literature Review

Redox balance and its important role in metabolic engineering of *Saccharomyces cerevisiae*

Saccharomyces cerevisiae has been widely used for the production of fermented food and alcoholic beverages for a long time. Nowadays, thanks to the development of modern biology, physiological and genetic characters of *S. cerevisiae* have been well studied. *S. cerevisiae* is the first eukaryotic organism whose genome is completely sequenced (Goffeau *et al*., 1997) and the data can be easily accessed from Internet. This useful knowledge has been applied in metabolic engineering to improve *S. cerevisiae* to become a more useful microorganism with new properties. The concept "metabolic engineering" was newly given by Stephanopoulos *et al*. (1998) even though the process has been established since a long time ago. It was defined as "*the directed improvement of product formation or cellular properties through the modification of specific biochemical reaction(s) or the introduction of new one(s) with the use of recombinant DNA technology*".

In fact, metabolic engineering of *S. cerevisiae* has been established for several decades in order to obtain either cells constituents, e.g. dry whole cells, proteins, enzymes, lipids, coenzymes, vitamins, amino acids, or excretion products, e.g. ethanol, glycerol, carbon dioxide. Engineering the relevant pathways has been done in all these processes to create strains having desired properties. During these processes, one common limiting factor must be carefully considered: *redox status*, represented by two pyridine nucleotide pairs NAD^+/ NADH and $NADP^+$/ NADPH and their ratio. Redox balance must be always maintained to keep the cells functioning. In biotechnological processes, redox balance plays an essential role in determining the maximal yield of the desired products.

However, regulation of redox balance can be used as one strategy of metabolic engineering in *S. cerevisiae*. In general, the principle of this approach is to change the redox balance of the cells by using different techniques to amplify, inhibit, delete, transfer or deregulate the corresponding genes or enzymes having essential roles in redox metabolism. These oriented changes can either directly balance the redox metabolism and thereby improve the production of desired products or break the redox balance of the cells. In the later case, the cells have to regulate the relevant biochemical reactions to regain the redox balance in a manner that leads to an overproduction of the desired metabolite.

In this thesis, a short overview of the redox metabolism on glucose of *S. cerevisiae* is provided in the first part. In the second part, studies concerning the improvement of *S. cerevisiae* to become a pentose-fermenting yeast are presented as an example for the important role of redox balancing in metabolic engineering.

1. Redox metabolism in *S. cerevisiae*

Metabolism can be divided into two major categories: catabolism (dissimilation) and anabolism (assimilation). Dissimilatory pathways are processes in which nutrients and cell constituents are oxidised, resulting in the removal of electrons from intermediates and generation of energy. Assimilatory pathways, in contrast, are reductive processes, consuming energy and synthesizing complex biomolecules from simpler components.

The oxidative and reductive processes of anabolism and catabolism are mediated by dehydrogenases, which predominantly use NAD^+ or NADH and $NADP^+$ or NADPH, respectively, as redox cofactors (Walker 1998). In fact, *cozymase,* which later known the mixture of cofactors as NAD^+, ATP, ADP, as well as metal ions, was first discovered in yeast extract by Harden and Young (1906). After the discovery of the related coenzyme $NADP^+$ (Warburg, 1935), the crucial role of NAD(H)/NADP(H)-dependent redox reactions in the metabolism of *S. cerevisiae* has been asserted and studied more deeply (for reviews see van Dijken and Scheffers, 1996; Bakker *et al.*, 2001).

In general, the two coenzyme systems NAD(H) and NADP(H) have different functions in the metabolism of *S. cerevisiae*. NAD(H) mainly participates in oxidative processes, particularly in glycolysis (cytosol), the citric acid cycle (mitochondria), and the respiratory chain (mitochondria). NADP(H), in contrast, is predominantly used in reductive syntheses, especially of fatty acids (mitochondria) and amino acids (cytosol and mitochiondria). Indeed, cells normally maintain their NAD^+/NADH ratio near 1000, which favours metabolite oxidation, while keeping their $NADP^+$/NADPH ratio near 0.01, which favours metabolite reduction (Voet and Voet, 1995). However, since the catabolic and anabolic processes are two sides of the metabolism that tightly involved with each other, i.e some central carbon metabolism pathways as glycolysis and TCA cycle are amphibolic, metabolism of NAD(H) and NADP(H) cannot be simply considered as separated processes. This will be explained more cautiously in the sections 1.4 and 1.5.

1.1 A brief overview of metabolism of *S. cerevisiae* during growth on glucose

S. cerevisiae exhibits different modes of metabolism to gain energy when glucose is used as a carbon source (Table 1). In general, the route of metabolic flux starting from pyruvate, the end product of glycolysis, can be alcoholic fermentation (fermentative metabolism) or respiration (respiratory metabolism) through tricarboxylic acid (TCA) cycle and respiratory chain. However, both types of metabolism can also occur at the same time (respirofermentative metabolism) in *S. cerevisiae*.

There are two important regulatory phenomena depending on the availability of oxygen and the concentration of glucose in the medium, referred to as Pasteur Effect and Crabtree Effect (for details see Walker, 1998). Pasteur Effect can be defined as the suppression of fermentation by oxygen. However, this phenomenon is only observable when glucose concentrations are low (e.g. below around 5 mM in *S. cerevisiae*) or under certain nutrient-limited conditions (Lagunas, 1979). Crabtree Effect, also referred to as the "glucose effect" or "contre-effect Pasteur" was defined as the suppression of respiration by high glucose. As other Crabtree-positive yeasts, *S. cerevisiae* has a strong tendency towards alcoholic fermentation. It shows a respirofermentative metabolism when the sugar concentration in the

cultivation medium exceeds a threshold value of about 5 mM even under fully aerobic conditions (Verduyn *et al*., 1984) or when the specific growth rate is higher than two third of the maximal growth rate (Postma *et al*., 1989). Respiration is the sole route of glucose catabolism when the concentration of glucose is very low. Fermentative metabolism alone occurs only under completely anaerobic conditions. Glycerol is formed as the quantitatively most important by-product of fermentative metabolism and is therefore also mentioned in Table 1.

By using glucose-limited chemostat, the mode of metabolism can be adjusted. In this case, the specific growth rate and the rate of glycolysis can be controlled by changing the dilution rates (Petrik *et al*., 1983).

Table 1. Type of metabolism of *S. cerevisiae* during growth on glucose depending on glucose and oxygen availability

Glucose concentrations	**Oxygen supply**	
	Aerobic conditions	Anaerobic conditions
2% - Batch culture	Respirofermentative	Fermentative
Low dilution rate - chemostat	Respiratory	
High dilution rate - chemostat	Respirofermentative	

Respirofermentative: $Glucose + O_2 = biomass + ethanol + CO_2 + glycerol + H_2O$

Respiratory: $Glucose + O_2 = biomass + CO_2 + H_2O$

Fermentative: $Glucose = biomass + ethanol + CO_2 + glycerol$

1.2 Interconnection between NAD(H) and NADP(H)

In several organisms, e.g. animals and bacteria, hydrogen can be transferred between NAD(H) and NADP(H) through transhydrogenase activity. However, there are some contradictory findings about the presence of transhydrogenase in *S. cerevisiae.* Griffiths and

Bernofsky (1970) have detected the activity of a mitochondrial NADH kinase, which can convert NADH to NADPH. This transhydrogenase system requires one ATP for each NADPH (NADH + ATP → NADPH + ADP). Nevertheless, the activity of this enzyme is very low and the reaction is only limited in the mitochondria. A cytosolic transhydrogenase activity in *S. cerevisiae* has also been detected by Evans *et al*. (1985). However, in contrast to these findings, from the theoretical calculations and studies on enzymes of *S. cerevisiae* and *C. utilis* cells which were grown on mixtures of glucose and formate, Bruinenberg *et al.* (1985) suggested that transhydrogenase is absent in yeast. This conclusion was generally accepted in all the later papers.

1.3 Compartmentation of redox reactions

Balancing the redox equivalents plays a crucial role in keeping homeostasis of the cells. Since there is no transhydrogenase in yeast (as generally accepted), the balance must be separately maintained for each cofactor pair. The rates of formation and consumption of NAD^+/NADH or $NADP^+$/NADPH have to be balanced in order to avoid depletion of either the reduced or the oxidised state.

Redox reactions in the metabolism of *S. cerevisiae* happen mainly in the cytosol, the place where most of the cellular metabolism occurs, and the mitochondria. Nevertheless, other organelles as glyoxisomes and peroxisomes also play an important role when cells are grown on oleic acid or some other non-fermentable carbon sources (Veenhuis *et al.*, 1987). The membranes of these organelles as well as the inner membrane of mitochondria are not permeable to NAD(H) and NADP(H) (von Jagow *et al.*, 1970). Therefore, redox balance is regulated in each compartment. The exchange of the reducing equivalents between cytosol and compartments only occurs with the help of metabolite shuttles through the membranes. Fig. 1 shows an overview of the flows of reduced nicotinamide nucleotides.

NAD(H) turnover occurs both in cytosol and in mitochondria. In contrast, generation and consumption of NADP(H) take place mainly in the cytosol (Albers *et al.*, 1998). The detailed metabolism of NAD(H) and NADP(H) will be explained in the next two sections.

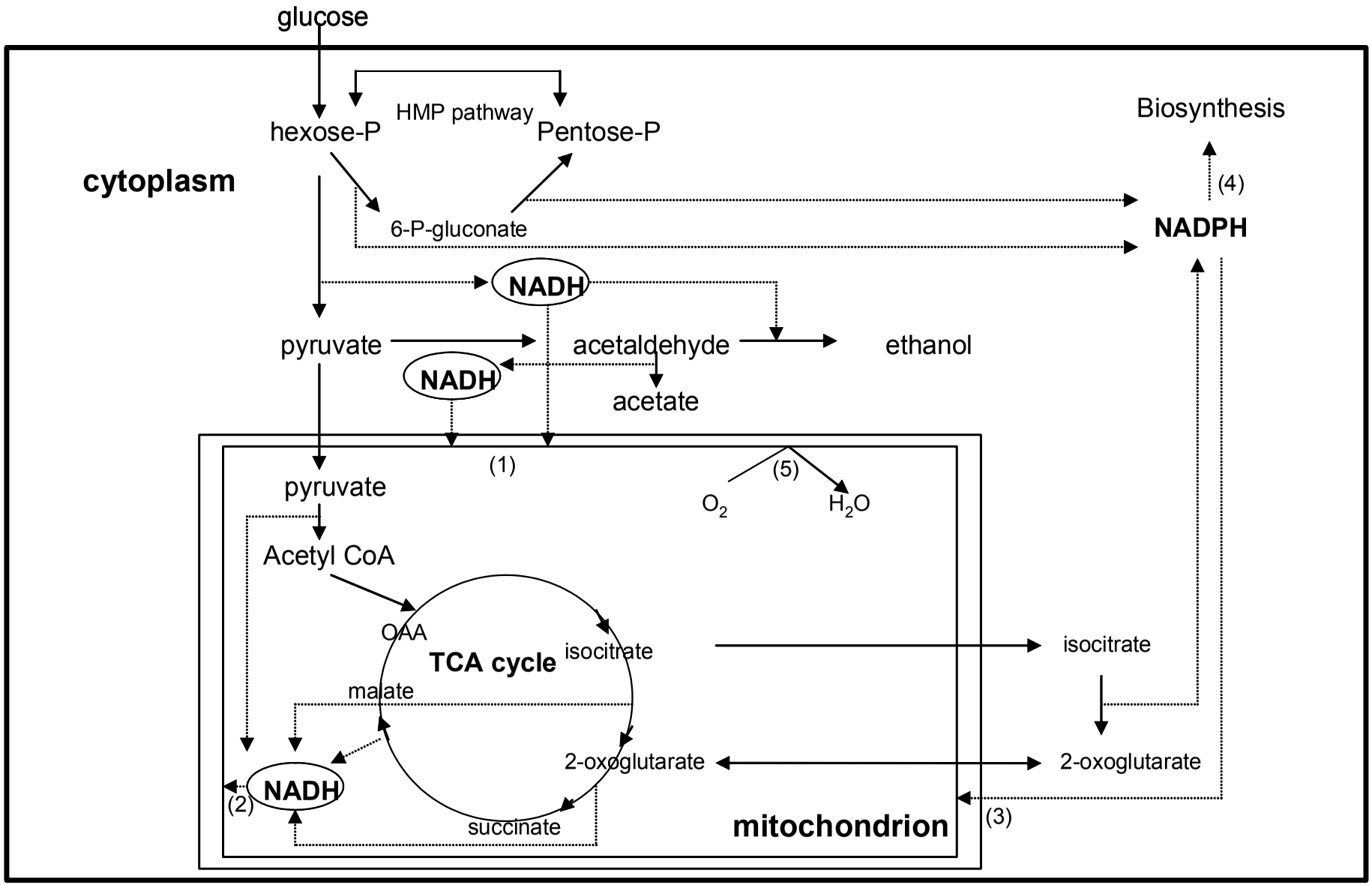


Fig. 1 Schematic representation of the flows of reduced nicotinamide nucleotides in glucose metabolism and their subcellular location (van Dijken and Scheffers, 1986). (1) Oxidation of cytoplasmic NADH by NADH dehydrogenase. (2) Oxidation of mitochondrial NADH by internal NADH dehydrogenase. (3) Oxidation of NADPH by dehydrogenase at the outer surface of of the inner membrane. (4) Ultilisation of NADPH in assimlatory processes. (5) Reduction of oxygen by cytochrom oxidase.

1.4 NAD(H) metabolism

NADH is formed from both catabolic and anabolic pathways since these two processes share the initial reactions of sugar metabolism.

The main source of NADH production in cytosol is the glycolytic pathway. It is formed by glyceraldehyde 3-phosphate dehydrogenase. Since yeast biomass ($C_{3.75}H_{6.6}N_{0.63}O_{2.1}$) is slightly more reduced than glucose, it was expected that the conversion of glucose and other nutrients to biomass would cause a net input of reducing equivalents during assimilatory sugar metabolism. In fact, formation of 1 g yeast dry biomass from glucose and ammonia accompanied by the net production of ca. 10 mmol NAD^+ to NADH (Verduyn *et al*., 1990). The assimilatory NADH production reactions occur both in cytosol and mitochondria. It has been estimated that 60-80% of the NADH generated in biosynthetic reactions come from the synthesis of amino acids and 30-50% of that NADH is generated in the mitochondrial matrix, mainly from synthesis of 2-oxoglutarate, a precursor of glutamate (Albers *et al*., 1998). Another source of NADH production is biomass synthesis. In addition to the formation of biomass, the excretion of oxidised low-molecular mass metabolites, e.g. pyruvate, acetaldehyde or acetate during growth on glucose may also lead to the production of NADH.

The generated NADH must be reoxidised in order to maintain the redox balance. There are different mechanisms of the reoxidation of NADH. The major mechanisms suggested are: i) by mitochondrial internal NADH dehydrogenase; ii) by external NADH dehydrogenase; iii) by alcohol dehydrogenase Adh1p; iv) by cytosolic glycerol-3-phosphate dehydrogenase and v) by the mitochondrial redox shuttles.

The internal mitochondrial NADH dehydrogenase (NDI) reoxidises the intramitochondrial NADH formed during TCA cycle and the pyruvate-dehydrogenase complex by transferring two electrons to ubiquinone. This enzyme is encoded by the *NDI1* gene and located in the inner membrane of mitochondria with the active site facing the mitochondrial matrix (de Vries and Grivell, 1988; Marres *et al.*, 1991). NDI was supposed to replace the respiratory complex I, which is lacked in mitochondria of *S. cerevisiae* (Grandier-Vazeille *et al.*, 2001). However, in contrast to complex I, NDI is neither inhibited by rotenone nor coupled to the

generation of a proton-motive force (de Vries and Marres, 1997). This partly explains the lower phosphorylation efficiency of yeast mitochondria in comparison to mammalian mitochondria.

The external NADH dehydrogenase (NDE) is also located in the inner mitochondrial membrane. However, in contrast to NDI, the active site of NDE faces to the space between inner and outer mitochondrial membranes (von Jagow and Klingenberg, 1970). In fact, the enzyme is encoded by two isogenes *NDE1, NDE2* (Luttik 1998; Small and McAlister-Henn, 1998). Similar to NDI, NDE isoenzymes do not pump protons (von Jagow and Klingenberg, 1970; Ohnishi, 1973; de Vries and Marres, 1987).

The alcohol dehydrogenase 1 (ADH1) catalyses the conversion of acetaldehyde, the product of the decarboxylation of pyruvate, to ethanol, thereby oxidising cytosolic NADH.

The cytosolic glycerol-3-phosphate dehydrogenase (GPD) is the major enzyme of glycerol biosynthesis. GPD catalyses the reduction of dihydroxyacetone phosphate (DHAP) to L-glycerol 3-phosphate, the precursor of glycerol. GPD is encoded by two isoenzymes *GPD1* and *GPD2* (Albertyn *et al.*, 1994; Eriksson *et al.*, 1995). This enzyme plays a crucial role in reoxidising the cytosolic NADH, maintaining the redox balance under anaerobic conditions.

In general, cytosolic NADH can also be partially reoxidised by the L-G3P shuttle. This shuttle comprises a pair of metabolites, DHAP/L-G3P, which can pass the outer mitochondrial membrane. Hence, the shuttle acts in carrying electrons from cytosolic NADH to the respiratory chain (Fig. 2) with the help of the cytosolic and mitochondrial G3P-dehydrogenases. The cytosolic glycerol-3-phosphate dehydrogenase (GPD), which uses NADH as substrate, is encoded by two isoenzymes *GPD1* and *GPD2* (Albertyn *et al.*, 1992). The second is mitochondrial FAD^+- dependent glycerol-3-phosphate dehydrogenase encoded by *GUT2* (Rønnow and Kielland-Brandt, 1993) gene. The cytosolic DHAP generated from glycolysis is reduced in a reaction catalysed by cytosolic GPD. The resulting G3P will diffuse through the permeable outer membrane of mitochondria. In the space between inner and outer mitochondria, G3P is reoxidised back to DHAP by mitochondrial GPD with the concomitant

transfer of the electrons from reduced cytosolic NADH to mitochondrial oxidised FAD^+. The produced DHAP then returns back to the cytosol.

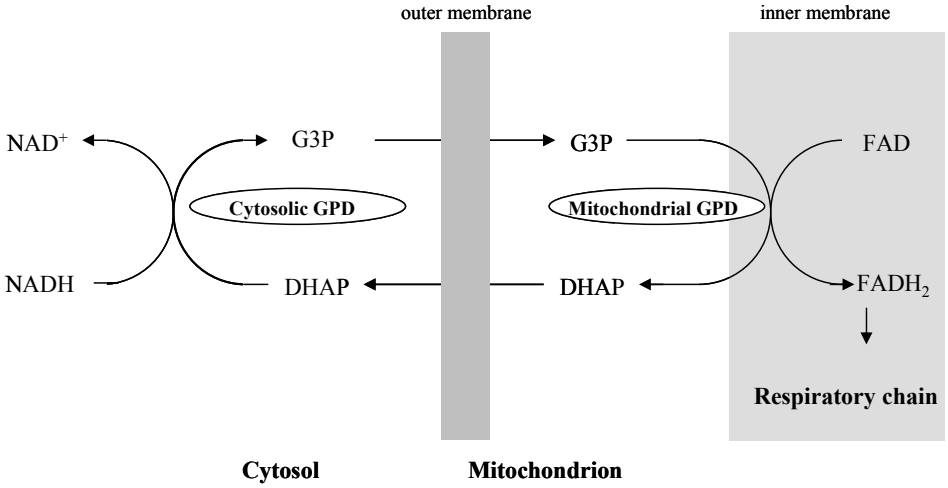


Fig. 2 Glycerol-3-phosphate shuttle (Murray *et al.* 1996)

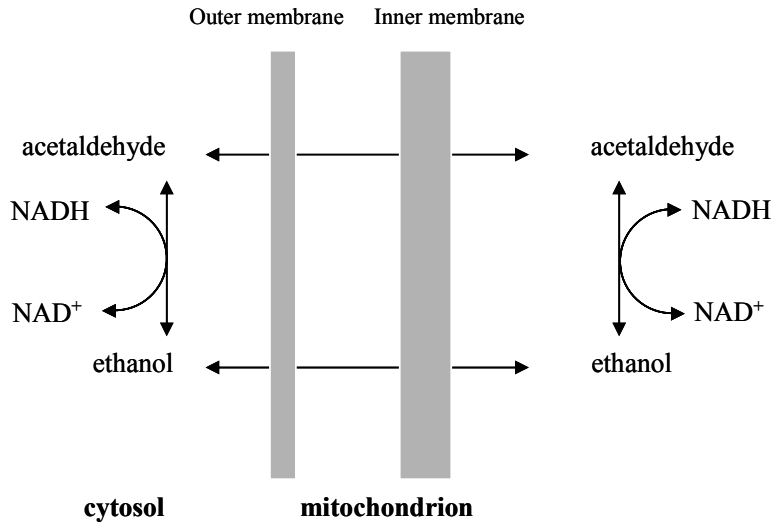


Fig. 3 A scheme presentation of ethanol-acetaldehyde shuttles slightly modified from Bakker *et al.*, 2001.

Beside L-G3P/ DHAP shuttle, yeast cells can also indirectly exchange the NADH between mitochondria and cytosol by some other metabolite shuttles, e.g. the ethanol-acetaldehyde shuttle (von Jagow and Klingenberg, 1970), the malate-oxaloacetate shuttle (Borst, 1963), the malate-aspartate shuttle (Borst, 1963). Like L-G3P shuttle, these shuttles contain pairs of reduced-oxidised substrates which are symmetrical in most cases. However, unlike L-G3P and DHAP, the reversible metabolite pairs can pass through the inner mitochondrial membrane and serve as substrates of the dehydrogenases with NADH as cofactor.

While it is still unclear whether the malate-oxaloacetate shuttle and the malate-aspartate shuttle work *in vivo* in *S. cerevisiae* even though the required enzymes are present (Overkamp *et al*., 2000), *in vivo* activity of the ethanol-acetaldehyde shuttle in *S. cerevisiae* has recently been confirmed (Bakker *et al*., 2000, 2001). This shuttle contains three alcohol dehydrogenases (ADH). *ADH1* and *ADH2* are two cytosolic isogenes encoding the cytosolic dehydrogenase (Lupiañez, 1974, Ciriacy, 1997) (Fig. 3). Adh1p reduces acetaldehyde to ethanol and consumes NADH as cofactor under anaerobic conditions. Adh2p oxidises the ethanol to acetaldehyde. *ADH3* (Young and Pilgrim, 1985) encodes a mitochondrial alcohol dehydrogenase which converts acetaldehyde to ethanol.

The extent at which the different mechanisms for reoxidation of NADH in *S. cerevisiae* are used is dependent on the mode of carbon metabolism.

When the metabolism is completely respiratory, mitochondrial and cytosolic NADH is reoxidised by internal and external NADH dehydrogenases, respectively. G3P shuttle is also an option of *S. cerevisiae* to maintain the cytosolic NAD/NADH balance by transfer of the equivalents into the mitochondrial electron transport chain (Larsson *et al*., 1998; Overkamp *et al*., 2000, Påhlman *et al*., 2001). However, the investigation of growth of the *gut2*Δ mutant under well-aerated conditions showed that the absence of G3P shuttle does not affect the growth rate of the cells. It is assumed that the shuttle only consumes a small fraction of the total cytoplasmic NADH and the lack of its activity would be complemented by external NADH dehydrogenase activity. The ethanol-acetaldehyde shuttle does not play an essential role in these conditions because NADH is oxidised sufficiently by NDE and NDI. The fact is

that deletion of *ADH3* did not affect to neither the maximum specific growth rate nor biomass yield (Bakker *et al.*, 2000).

When the metabolism is completely fermentative, i.e. in the absence of oxygen, the mechanisms of NADH reoxidation coupled to respiratory chain do not operate. Cytosolic NADH is mainly reoxidised by Adh1p under these conditions, producing ethanol. However, this reaction is only sufficient to neutralise the NADH generated from glycolysis. Cells need an additional mechanism to reoxidise NADH formed during the biosynthesis processes, which is called excess NADH. The reoxidation of the excess NADH is accomplished by GPD in *S. cerevisiae*. Therefore, glycerol production has a crucial role in maintaining the redox balance under anaerobic conditions. The ethanol-acetaldehyde shuttle was proposed to play an important role in exporting the NADH generated during assimilatory reactions from mitochondria into the cytosol where it is reoxidised by ADH1 or GPD (Bakker *et al.*, 2000).

When metabolism is respirofermentative, yeast cells have the possibility to use all the mechanisms listed above to reoxidise NADH in cytolsol and mitochondria. These mechanisms can partly substitute each other. However, the extent of each mechanism depends much on the oxidation state of the cell.

1.5 NADP(H) metabolism

Metabolism of NADP(H) occurs mainly in the cytosol (see Fig. 1). The cytosolic NADPH is generated mainly from the pentose phosphate pathway (PPP) during growth on glucose. NADPH is formed from the first reaction of PPP converting glucose-6-phosphate (G6P) to 6-phosphogluconolactone catalysed by glucose-6-phosphate dehydrogenase. 6-phosphogluconolactone is converted to 6-phosphogluconate which is then oxidised to ribulose-5-phosphate (Ru5P) by phosphogluconate dehydrogenase. This reaction produces another NADPH. Both glucose-6-phosphate dehydrogenase and phosphogluconate dehydrogenase are located in the cytoplasm (Hirai *et al*., 1976*b*). Another part of the cytosolic NADPH can be generated by $NADP^+$-linked isocitrate dehydrogenase catalysing the reaction which converts isocitrate to 2-oxoglutarate. In fact, three isoenzymes of $NADP^+$-linked isocitrate dehydrogenase were identified in *S. cerevisiae.* The cytosolic $NADP^+$-dependent

isocitrate dehydrogenase, encoded by the *IDP2* gene (Loftus *et al*., 1994), contributes directly to cytosolic 2-oxoglutarate and NADPH pool. As suggested by Bruinenberg *et al*. (1983), this route, however, only plays a minor role in comparison to the PPP. Aside from the PPP and $NADP^+$-linked isocitrate dehydrogenase, an $NADP^+$-dependent acetaldehyde dehydrogenase may also contribute to cytoplasmic NADPH synthesis during growth on ethanol (Tamaki and Hama, 1982; Meaden *et al*., 1997; Wang *et al*., 1998).

The primary source of NADPH in yeast mitochondria probably is the mitochondrial $NADP^+$-dependent isocitrate dehydrogenase, which is encoded by the *IDP1* gene (Haselbeck and McAlister-Henn, 1991). Another possible source of mitochondrial NADPH, the $NADP^+$-linked malic enzyme was reported to be only present at a low level in *S. cerevisiae*.

The third $NADP^+$-linked isocitrate dehydrogenase, encoded by the *IDP3* gene, is confined in peroxisomes. It was proposed that cytosolic and peroxisomal $NADP^+$-dependent isocitrate dehydrogenases function in a redox shuttle to refill NADPH consumed in the dienoyl-CoA reductase reaction required for the β-oxidation of polyunsaturated fatty acids in the peroxisome (van Roermund *et al*., 1998).

The generated NADPH is mainly used for biosynthesis, especially lipid synthesis. Synthesis of amino acids and nucleic acids also require NADPH. The percentage of glucose degradation by the PPP pathway in *S. cerevisiae* varies from 2-20% (Walker, 1998) up to 34% (Mian *et al.,* 1974) depending on whether cells are actively growing or not, since NADPH generated from PPP is the main source for biomass production. It means that the production of NADPH depends on the requirement of biosynthesis. Furthermore, it was concluded by Bruinenberg *et al.* (1986) that the NADPH requirement for biomass formation in yeast strongly depends on carbon sources used for growth. The conclusion was made from the studies on *C. utilis*. However, it has been proposed that the conclusion is also correct for *S. cerevisiae* because the activity of the PPP in *S. cerevisiae* was similar to that in the yeast *C. utilis* when they were grown under the same conditions (Bruinenberg, 1986). The carbon sources as glucose, acetate and methanol require the same amount of NADPH for biomass formation. This similarity is based on the fact that the conversion of these compounds to a common precursor for syntheses, e.g. triose phosphates, does not involve NADP(H)-linked

oxidation-reduction reactions. In contrast, a twofold increase of NADPH is required when xylose is used as carbon source, resulting from the initial NADPH-linked reduction of xylose to xylitol.

Bruinenberg *et al.* (1983) suggested that the NADPH requirement for biomass formation in yeast also depends on nitrogen sources. A fourfold increase in the NADPH requirement has been observed when nitrate was used instead of ammonia. The explanation of this finding is that NADPH is needed to reduce NO_3^- to NH_4^+ by cytoplasmic nitrate and nitrite reductase. This might explain the significantly higher expression of all the enzymes of the PPP pathway when cells were grown on glucose with nitrate instead of ammonia.

2. Improvement of the pentose-fermentation in *S. cerevisiae* as an example for the important role of redox balancing in metabolic engineering

Production of bioethanol is of world-wide interest because of its significant role in replacing petroleum for an internal combustion engine. Starches and sugars, the carbon sources of ethanol fermentation, are abundant in many crops, but expansion of ethanol production as an automotive fuel in the future will require feedstocks that do not complete for food or fibre (Wheals *et al.,* 1999). Such feedstocks include lignocellulosic by-product residues from agriculture. Lignocellulose contains five major sugars including three hexoses (D-glucose, D-mannose, D-galactose) and two pentoses (D-xylose, L-arabinose). D-xylose is the second most abundant sugar after D-glucose, averaging about 5-20 % of carbohydrate fraction and L-arabinose is about 1-5% (Aristidou and Penttilä, 2000). However, *S. cerevisiae*, the main producer of bioethanol, can ferment hexose rapidly and efficiently, but cannot ferment D-xylose and L-arabinose (Hahn-Hägerdal *et al*., 2001). Therefore, the inability of *S. cerevisiae* to ferment D-xylose and L-arabinose is one main reason limiting ethanol productivity from lignocellulose. Some other yeasts as *Pichia stipitis*, *Candida shehatae* and *Pachysolen tannophylus* are able to ferment xylose, but unfortunately, these yeasts have a low tolerance to ethanol (Jeffries, 1985). Therefore, they are not a good choice for industrial applications. Some Gram-negative bacteria as *Escherichia coli* or *Klebsiella oxytoca* can also

ferment pentose to ethanol. However, fermentation by bacteria only can reach a low yield, since bacteria have low tolerance with ethanol. Moreover, since fermentation by yeast is not susceptible to contamination by bacteria and viruses, ethanol processors retain an interest in yeast.

Many studies over the last decade aimed at modifying *S. cerevisiae* to become D-xylose-fermenting. For detailed information, the reader is referred to the excellent reviews by Jeffries and Shi (1999), Hahn-Hägerdal *et al*. (2001) and Jefferies and Jin (2003). In contrast to the case of D-xylose, studies on improving the utilization of L-arabinose in *S. cerevisiae* are at the beginning steps. However, certain successes have been obtained (Jefferies and Jin, 2004).

2.1 Improvement of D-xylose fermentation in *S. cerevisiae*

2.1.1 D-xylose metabolism in yeast and bacteria

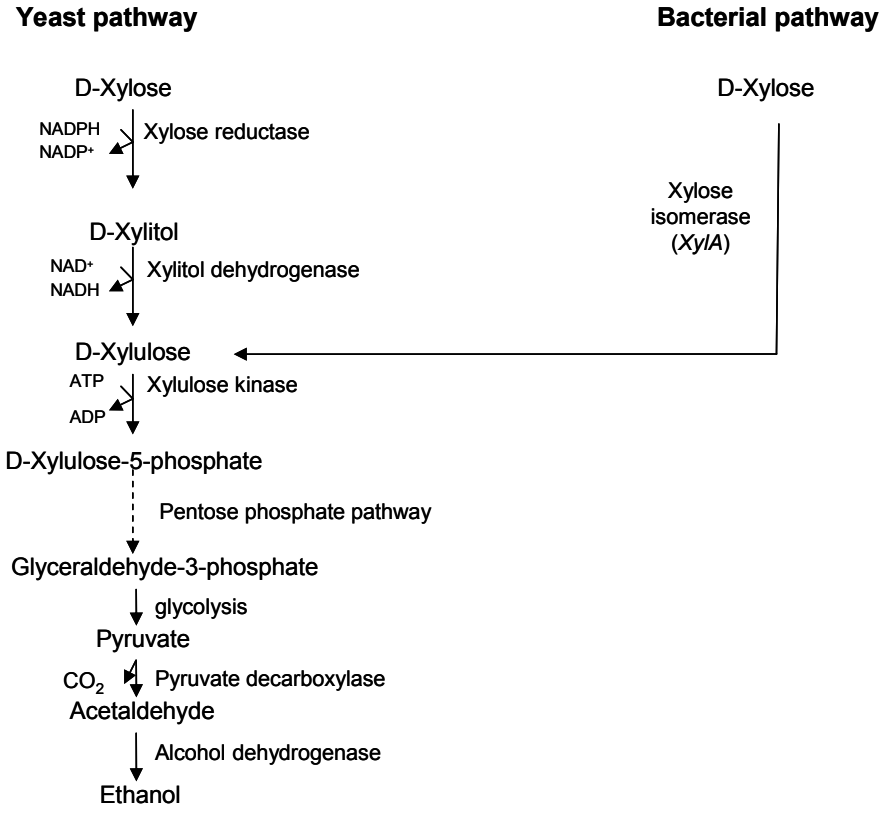


Fig. 4 D-xylose fermentation by yeast and bacteria.

The pathway of xylose utilisation in natural xylose-fermenting yeasts is shown in Fig. 4. D-xylose first is converted to xylitol by xylose reductase (XR). In most yeasts and fungi which do not or only slowly ferment xylose anaerobically, this enzyme is NADPH-linked and they either lack or exhibit low NADH-linked reductase activity. In contrast, the corresponding enzymes in the native xylose-fermenting yeasts are capable of functioning with both NADH and NADPH. However, the *in vitro* activities of XR differ strongly between strains when using NADH or NADPH as cofactor. For example, while the enzymes from *P. stipitis* and *Pachysolen tannophilus* prefer NADPH over NADH (NADH/NADPH ratio 0.7) (Verduyn *et al*., 1985; Rizzi *et al*., 1988; Metzger and Hollenberg, 1995), the enzyme from *Candida boidinii* prefers NADH (Winkelhausen and Kuzmanova, 1998).

The second step is carried out by xylitol dehydrogenase (XDH) which converts xylitol to xylulose (Fig. 4). Unlike xylose reductase, this enzyme is always specific for NAD.

It was supposed that *S. cerevisiae* has no ability to convert xylose to xylulose (Gong *et al*., 1983). However, according to later reports, *S. cerevisiae* can produce all the enzymes necessary for xylose utilization. In fact, the activities of xylose reductase (XR) and xylitol dehydrogenase (XDH) at low levels have been detected in *S. cerevisiae* (Batt *et al*., 1986). More recently, the gene coding for a *S. cerevisiae* XDH enzyme was discovered (Richard *et al*., 1999). These facts might explain why a slow rate of aerobic xylose metabolism has been observed in *S. cerevisiae* (van Zyl *et al*., 1989).

Even though *S. cerevisiae* can metabolise D-xylose at a slow rate, it cannot ferment it under anaerobic conditions. However, it can grow aerobically and anaerobically on its isomer, xylulose (Wang and Schneider, 1980; Ueng *et al*., 1981; Chiang *et al*., 1981), an intermediate of the xylose pathway. D-xylulose generated from xylitol is further phosphorylated to D-xylose-5-phosphate by xylulose kinase (XKS) which is encoded by the *XKS1* gene. This enzyme has been reported to be present in *S. cerevisiae* (Rodriguez-Pena *et al*., 1998). D-xylose-5-phosphate channels into glycolysis via the non-oxidative pentose phosphate pathway and finally is converted to ethanol.

The xylose pathway in bacteria is different from the yeast pathway only at the initial steps converting xylose to D-xylulose. Instead of the two redox reactions in yeast, D-xylose is converted directly to D-xylulose by xylose isomerase encoded by the *XylA* gene. The steps downstream of D-xylulose to ethanol are the same with those of the yeast pathway.

2.1.2 Redox imbalance of a recombinant *S. cerevisiae* strain expressing XR and XDH of *P. stipitis*

P. stipitis is a native xylose-fermenting yeast which can produce significant amounts of ethanol and has been studied intensively (for review, see Jeffries and Jin, 2000). As XR from *P. stipitis* can use both NADH and NADPH, the redox equivalents produced during xylitol oxidation could partially be used for xylose reduction. Therefore, the redox balance seems to be kept much better equilibrated under oxygen limited conditions than in other yeasts. In *P. stipitis,* XR and XDH are encoded by *XYL1* and *XYL2* genes, respectively (Verduyn *et al*., 1985; Rizzi *et al*., 1988; Kötter, *et al*., 1990). Several attempts to introduce *XYL1* and *XYL2* in *S. cerevisiae* single and combined have been carried out. A considerable amount of enzyme activity was observed constitutively when *XYL1* has been expressed. Even though this recombinant *S. cerevisiae* could convert xylose to xylitol with a high yield (95%), it could not neither grow on xylose as the sole carbon source nor produce ethanol from it (Takuma *et al*, 1991; Hallborn *et al*. 1991, Amore *et al*., 1991). As xylose uptake and accumulation of xylitol were observed, it was supposed that the conversion of xylitol to xylulose was the limiting step. This was probably due to insufficient XDH of *S. cerevisiae*.

The *S. cerevisiae* strain in which both *XYL1* and *XYL2* genes from *P. stipitis* were expressed was able to grow on xylose as sole carbon source. However, the amounts of ethanol formed by this strain were relatively low (10 mM, Kötter *et al.,* 1990). Besides, a large amount of xylitol was secreted, especially under oxygen limited conditions (Tantirungkij *et al*., 1993; Tantirungkij *et al*., 1994; Walfridsson *et al*., 1995). This could result from the redox imbalance when NADH produced from the reduction of xylitol is not reoxidised sufficiently, in spite of the fact that xylose reductase of *P. stipitis* is also able to use NADH for the reaction converting xylose to xylitol (Meinander *et al*., 1996). The xylitol excretion could also

be ascribed to the thermodynamic equilibrium for the xylose reductase (XR) and xylitol dehydrogenase (XDH) reactions which favour xylitol formation (Rizzi *et al*., 1988, 1989). Walfridsson *et al*. (1997) have regulated the expression level of *XYL1* and *XYL2* genes by setting these genes under the control of different strong promoters (*ADH1* and *PGK1*) in the engineered *S. cerevisiae* strain. It was shown that strains with a low XR/XDH ratio formed less xylitol than strains with a high ratio. However, *P. stipitis* produces less xylitol than *S. cerevisiae* despite a much higher ratio of XR/XDH (du Preez *et al*., 1989; Skoog and Hahn-Hägerdal, 1990). A mathematical core model of the ratio of the XR, XDH and XK enzymes indicated that cofactor concentrations might influence the formation of xylitol more than the activity ratios of the enzymes do (Eliasson *et al*., 2001). These results propose that the levels and ratios of NADPH and NADH are regulated differently in *P. stipitis* than in *S. cerevisiae*.

Very recently, Salusjärvi *et al*. (2003) have reported the first investigation at proteome level of xylose-fermenting recombinant *S. cerevisiae* containing the *XYL1* and *XYL2* genes from *P. stipitis*. Two-dimensional gel electrophoresis has been performed to detect and compare the differences when this strain was grown on xylose and glucose under aerobic and anaerobic conditions. Interestingly, it was found that the major differences observed in cells on xylose compared to glucose centralise around the redox-balancing reactions. The proteins significantly increased are those involved in the glycerol metabolism (glycerol 3-phosphatase, Gpp1p), ethanol consumption (alcohol dehydrogenase, Adh2p) and mitochondrial metabolism (Acetaldehyde dehydrogenase, Ald4p and Ald6p). These results indicated that to maintain the growth on xylose, the engineered *S. cerevisiae* has combated its redox imbalance mainly by using the glycerol and acetate pathways.

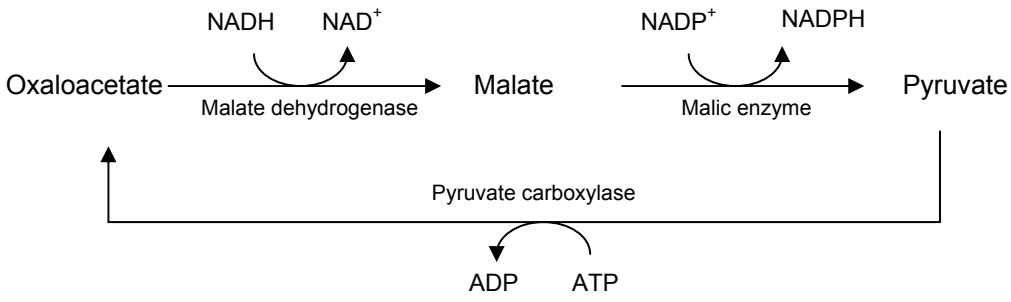
2.1.3 Introducing artificial transhydrogenase cycles in the recombinant xylose fermenting *S. cerevisiae*

As discussed above, different cofactor specificities of XR and XDH lead to an accumulation of NADH and a depletion of NADPH in the xylose fermenting recombinant *S. cerevisiae* strains. This redox imbalance seems to be a major reason which limits the utilization of xylose. To overcome this problem, one could think about a heterologous

expression of transhydrogenase which can directly convert NADH to NADPH. The transhydrogenases of bacteria, e.g. *E. coli* (Anderlund *et al*., 1999) or *Azotobacter vinelandii* (Nissen *et al*., 2000, 2001), have been introduced in *S. cerevisiae*. Interestingly, glycerol production, the major route of the reoxidation of cytosolic NADH under anaerobic conditions, and the formation of 2-oxoglutarate, a substrate which is converted to glutamate using NADPH as cofactor, considerably increased in the recombinant strains. This led to the conclusion that the heterologous transhydrogenase reaction in *S. cerevisiae* occurred in the opposite direction other than expected, i.e. it converted NADPH and NAD^+ into $NADP^+$ and NADH. It implies that these transhydrogenases are not helpful in balancing the redox equivalents when engineered xylose metabolizing *S. cerevisiae* cells are grown on xylose.

Aristidou *et al*. (1999, 2000) have introduced two artificial transhydrogenase cycles in xylose-fermenting *S. cerevisiae*. One based on the simultaneous overexpression of *GDH1* and *GDH2* genes encoding for the glutamate dehydrogenase 1 and 2 which are NADPH-dependent and NAD-dependent, respectively. In this case, the excess NADH generated from xylulose production reaction can be reoxidised to NAD^+ in the reaction converting 2-oxoglutarate to glutamate by GDH2. GDH1, however, uses $NADP^+$ to convert glutamate back to 2-oxoglutarate, releasing NADPH which is needed for the reduction of xylose. The enzyme pair GDH1/GDH2 acts as a transhydrogenase system, thus successfully counteracts the redox imbalance.

The other artificial transhydrogenase cycle established was the overexpression of the gene coding for malic enzyme. Malic enzyme was expressed without its mitochondrial targeting sequences, thus the enzymes of the cycle operate in cytosol where the reaction of xylose pathway occurs. The combination of the three enzymes: malic enzyme, malate dehydrogenase, pyruvate carboxylase, works as a transhydrogenase system and is driven by ATP. This may be explained by the following sequence of reactions that converts substantial parts of excess NADH and $NADP^+$ into NAD^+ and NADPH:



The resulting recombinant *S. cerevisiae* strains have been shown to utilise xylose at high rates and produce a considerable yield of ethanol.

Recently, Verho *et al*. (2002) have found the first fungal NADP-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) isolated from *Kluyveromyces lactis,* encoded by *GDP1* gene. This enzyme has been shown to use both NADP and NAD as cofactors and therefore can serve as a transhydrogenase. In fact, the discovery of this enzyme was based on a screening system for NADP(H)-linked oxidoreductases of *S. cerevisiae* (Boles *et al*., 1993). The deletion of phosphoglucose isomerase encoded by the *PGI1* gene in *S. cerevisiae* causes a phenotype for which glucose is toxic. It was ascribed to the redox imbalance resulting from the excess of NADPH produced from the oxidative steps of PPP. However, the similar deletion in *K. lactis* (*rag2*Δ) does not lead to this phenotype. It has been explained that the mitochondria of *K. lactis* enables the oxidation of cytosolic NADPH (Gonzáles Siso *et al*., 1996; Overkamp *et al*., 2002) while *S. cerevisiae* does not have this possibility. However, by transforming the genomic library of *K. lactis* into *pgi1*Δ *S. cerevisiae* and searching for the survived mutant, the NADP-dependent GAPDH mentioned above has been identified.
Expression of this enzyme in the D-xylose recombinant *S. cerevisiae* strain has been done very recently (Verho *et al.,* 2003). The recombinant enzyme could contribute to generate more NADPH for the reduction of xylose and to reduce the accumulation of the NADH by competing GAP with the NAD-dependent GAPDH. As expected, a higher yield of ethanol and lower level of the unwanted by-product xylitol in comparison to the corresponding wild type has been observed in this engineered strain.

2.1.4 Lowering the NADPH concentration by engineering the oxidative steps of PPP to improve the use of NADH by XR

Several studies have focussed on manipulating the oxidative steps of the PPP. It was expected that a strain with a low flux through PPP would produce less NADPH. Hence, a greater fraction of xylose would be then reduced in xylose reaction using NADH, since XR uses both NADPH and NADH. The consumption of NADH in this XR step will be compensated by the NADH production in the XDH step. Therefore, the xylose conversion to xylulose is balanced with respect to cofactors.

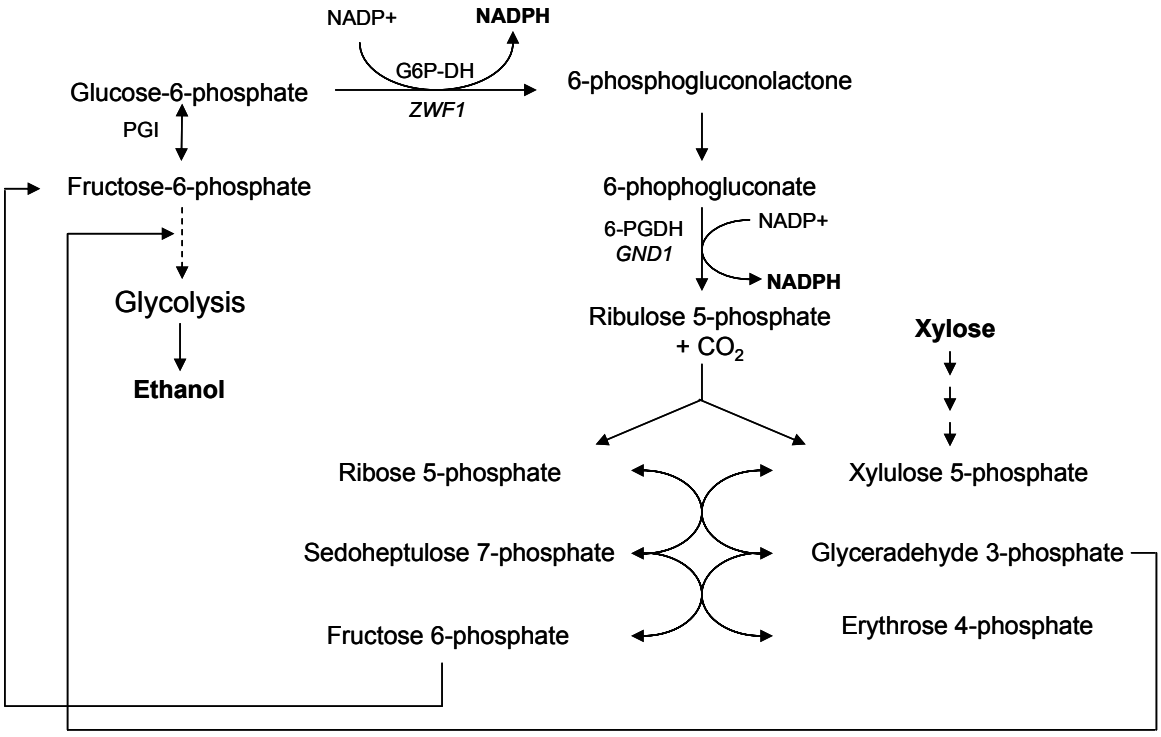


Fig. 5 Pentose phosphate pathway and the relevant enzymes have been engineered in order to reduce the production of NADPH.

The oxidative stage of the PPP includes the first three steps converting glucose 6-phosphate to ribulose 5-phosphate and producing NADPH. The non-oxidative stage contains the reactions downstream of ribulose 5-phosphate. It has been suggested that the PPP in

S. cerevisiae may play a role in regulating the efficiency of xylose/ xylulose fermentation (Ciriary and Porep, 1986).

The branch point metabolite between glycolysis and the PPP, glucose 6-phosphate, can be reversibly converted to either fructose 6-phosphate by phosphoglucose isomerase (PGI) or to 6-phosphogluconolactone by glucose 6-phosphate dehydrogenase. 6-phosphogluconolactone is further converted to ribulose-5-phosphate by 6-phosphogluconate dehydrogenase (6-PGDH). In fact, ethanol production from xylulose has been improved by reducing PGI activity or by deleting the *GND1* gene, one of the isogenes of 6-PGDH (Eliasson *et al*., 2000). It was ascribed to the loss of carbon in the form of carbon dioxide (about 20%) in the PPP which was prevented in the deleted mutants. The reduction of the flux through the NADPH-producing PPP in the recombinant xylose-fermenting *S. cerevisiae* by lowering PGI activity, by deleting the *GND1* or *ZWF1* gene (which encodes glucose 6-phosphate dehydrogenase) also leads to a higher ethanol yield and lower xylitol yield (Jeppsson *et al*., 2002). It has been reported that *zwf1*Δ mutant is the strain produced the highest ethanol yield (0.41g ethanol/ g xylose) and formed the lowest xylitol yield (0.05g xylitol/ g xylose) to date. However, the xylose consumption rate in this mutant was strongly reduced because of limited NADPH-mediated xylose reduction. Overexpression of the *XYL1* gene could restore fully the xylose consumption in this mutant (Jeppsson *et al.*, 2003). However, a high concentration of glycerol was observed during fermentation because dihydroxyacetone phosphate (DHAP) acted as a substrate for XR (Verduyn *et al*., 1985; Lidén *et al*., 1996).

The deletion of *PGI1* was combined with the expression of the NADP-dependent GAPDH from *K. lactis* (see I.2.4) in the xylose-recombinant *S. cerevisiae* strain (Verho *et al.*, 2003). In this engineered strain, generation of NADPH by PPP was replaced by the oxidation of GAP which was not directly coupled to the loss of CO_2. Furthermore, the use of NADP-dependent GAPDH could eliminate a stoichiometric amount of NADH production. As a result, ethanol was produced as main product under anaerobic conditions in this recombinant strain. However, it is difficult to compare the effect in this strain with the effect of *ZWF1* deletion alone because Verho *et al*. used batch cultures while Jeppsson *et al.* used continuous cultures.

2.1.5 Introducing the bacterial xylose isomerase in *S. cerevisiae*

Bacteria can directly convert xylose to xylulose by xylose isomerase (XI). This enzyme is encoded by *xylA* gene. Unlike yeasts, the conversion of xylose to xylulose in bacteria does not contain redox reactions. Therefore, some studies have tried to use the bacterial enzyme instead of the two redox reaction steps of the yeast' initial xylose pathway. *XylA* genes from several bacteria have been cloned and expressed in *S. cerevisiae*, including *xylA* from *Bacillus subtilis* (Amore *et al*., 1989), *Actinoplanes missouriensis* (Amore *et al*, 1989), *Clostridium thermosulfurigenes* (Moes *et al*., 1996), *E. coli* (Ho *et al*., 1983; Sarthy *et al*.,1987) and *Streptomyces rubiginosus* (Schrunder and Gunger, 1996). However, the gene products from these bacteria have been shown to be inactive in the recombinant *S. cerevisiae* strains. It was ascribed to the changes in internal pH of yeast which probably leads to an improper posttranslation modifications, inter- and intramolecular disulfide bridge formation and thus, caused the misfolding of the protein (Walfridsson *et al*., 1996). The only functional *xylA* gene product in *S.cerevisiae* known so far is from *Thermus thermolyticus* (Walfridsson *et al*., 1996). Unfortunately, the recombinant enzyme showed the highest activity at 85°C and a yield of only 0.13 g ethanol/ g xylose was observed. In spite of this, the work has demonstrated a new functional metabolic pathway in *S. cerevisiae* which converts xylose to ethanol under oxygen-limited conditions. Recently, random PCR mutagenesis has been applied to the *xylA* gene of *T. thermolyticus* to obtain a recombinant enzyme working effectively at a lower temperature (Lonn *et al.*, 2002). However, the mutated *xylA* genes have not yet been expressed in *S. cerevisiae*.

2.2 Improvement of L-arabinose fermentation in *S. cerevisiae*

Although L-arabinose is not the most abundant hemicellulose sugar, a significant amount of it is included in cellulosic biomass, such as corn fiber and many herbaceous crops (MacMilan, 1994; Hespell, 1998). Therefore, in addition to modify *S. cerevisiae* to be a xylose-fermenting yeast, engineering the *S. cerevisiae* to have ability utilising L-arabinose is also necessary to improve the yield and productivity of ethanol production from lignocellulose feedstock.

In fact, the fungal L-arabinose pathway has been described for the moulds of *Penicillium chrysogenum* and *Aspergillus niger* by Chiang and Knight (1961) and Witteveen *et al*. (1989), respectively. It was supposed that this pathway also exists in yeasts. The fungal L-arabinose pathway has similarities to the D-xylose pathway. However, the conversion of L-arabinose to D-xylose 5-phosphate goes through two reducing steps which are NADPH-linked and two oxidizing NAD-linked steps (see Fig. 4). Like D-xylose metabolism, the process is redox neutral but the different redox equivalents, NADPH/NADP and NAD/NADH, acting in the reactions of the L-arabinose pathway is not recycled. That means NADPH and NAD used in the reactions must be regenerated in other metabolic pathway.

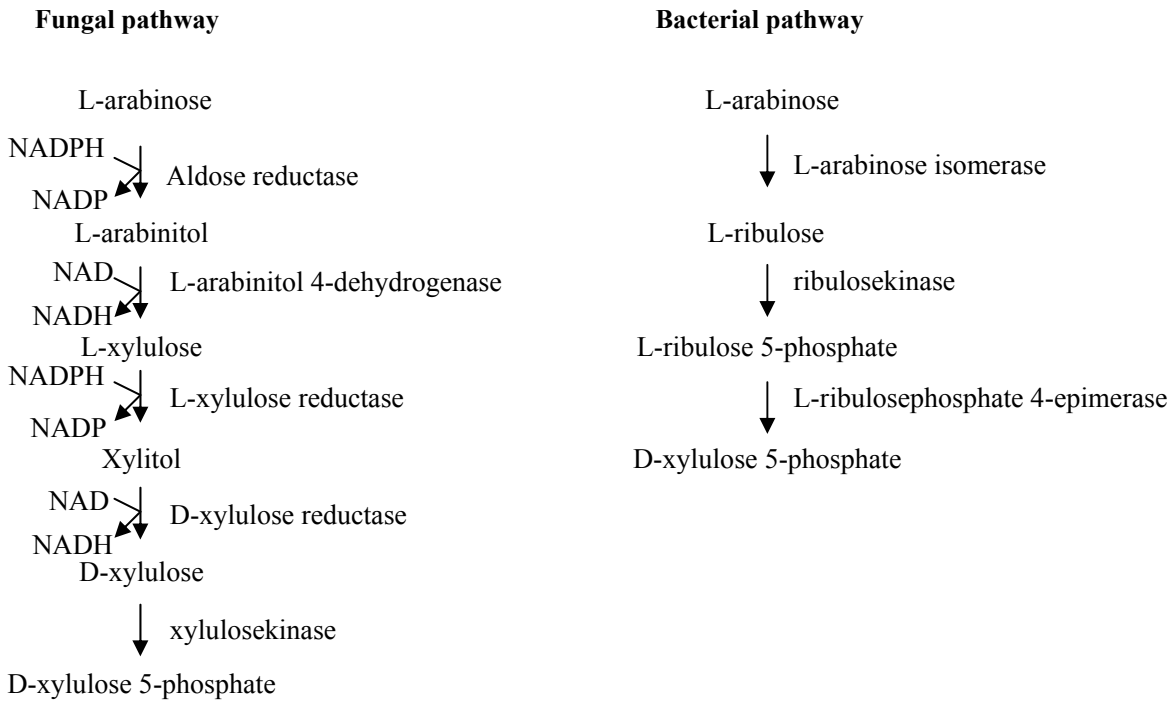


Fig. 6 The L-arabinose pathway of fungi and bacteria (Richard *et al*., 2002)

The first enzyme of the L-arabinose pathway is aldose reductase. This enzyme has been purified and characterised in *S. cerevisiae* (Kuhn *et al*., 1995). The enzyme which is encoded by *GRE3* gene (Garray-Arroyo and Covarrubias, 1999) is unspecific and has similar activities with both D-xylose and L-arabinose. The two involved enzymes, xylitol dehydrogenase (D-

xylulose reductase), xylulosekinase and their corresponding genes have been known in several yeasts including *S. cerevisiae* (Richard *et al*., 1999; Ho and Chang 1989). Very recently, genes coding for fungal L-arabinitol 4-dehydrogenase or L-xylulose reductase have been identified from the mould of *Trichoderma reesei (Hypocrea jecorina)* (Richard *et al*., 2001, 2002). Overexpression of all genes of the pathway has been done in *S. cerevisiae* (Richard *et al*., 2003). The recombinant *S. cerevisiae* strain showed activities of all enzymes of the pathway and could utilise L-arabinose. However, the growth on L-arabinose either aerobically or anaerobically has shown a very low rate. Ethanol production was observed but the yield was also very low. It was ascribed to the imbalance of redox cofactors when NADH produced is accumulated and the needed NADPH for the reductive steps is lacked. Besides, the ineffective transport of L-arabinose into the cell could possibly also be a reason.

Another strategy is to introduce the genes of the bacterial L-arabinose pathway in *S. cerevisiae.* The pathway of L-arabinose metabolism is well established in bacteria (Lee *et al*., 1986). L-arabinose is converted to L-ribulose, L-ribulose-5-P, and D-xylulose-5-P by using L-arabinose isomerase (AraA), L-ribulokinase (AraB), L-ribulose 5-P 4-epimerase (AraD) respectively, hence does not use the redox reactions as fungal pathway (Fig. 4). Nevertheless, attempts to introduce the *E. coli* pathway in *S. cerevisiae* failed to either grow on L-arabinose or ferment it to ethanol (Sedlak and Ho, 2001). In fact, L-arabinose isomerase activity was not detected in this recombinant *S. cerevisiae* strain, i.e. the *araA* gene from *E. coli* was not active in *S. cerevisiae* (Becker and Boles, 2003). In contrast, the *araA* genes of *B. subtilis* and *Mycobacterium smegmatis* have been shown to be active in yeast. Becker and Boles (2003) have overexpressed the *araA* gene from *B. subtilis* and *araB* and *araD* genes from *E. coli* in *S. cerevisiae*. In addition, the *GAL2* gene encoding for yeast galactose permease (Gal2) which is known to enable the transport of L-arabinose was simultaneously overexpressed in order to improve L-arabinose uptake. The resulting recombinant *S. cerevisiae* strain not only grows fast on L-arabinose but also ferments and produces ethanol. It was shown that the initial regulatory step of the L-arabinose pathway is the phosphorylation by L-ribulokinase. This is consistent with the need of tight control of catabolic pathways which first utilise and later generate an excess of ATP (Teusink *et al*.,

1998). The second prerequisite is enhancement of transaldolase activity which is shown previously to be insufficient for the efficient utilization of pentose phosphate pathway metabolites (Walfridsson *et al*. 1995). The overexpression of a transporter is not necessary for growth on L-arabinose but can improve it.

3. Conclusion

In addition to ATP, cells have a second currency, reducing power. It is represented by NAD(H) and NADP(H). It was generally accepted that NAD(H) is preferentially used in assimilatory pathways and NADP(H) is dominantly used in dissimilatory pathways. However, as discussed in this review, since some central metabolism pathways as glycolysis and TCA cycle are amphibolic, these two processes cannot be simply separated. As assimilation and dissimilation are two sides of cell metabolism which are tightly involved with each other as well as with energy metabolism, metabolism of the NAD(H) and NADP(H) plays a crucial role in regulating the overall cellular metabolism in every living organisms. To keep the cells functioning, each cofactor, NAD(H) or NADP(H), has to be maintained in balance not only in cytosol but also in each organelle of the cell, since yeast do not have transhydrogenase and the membranes of the organelles are not permeable to NAD(H) or NADP(H).

Changes of redox balance in *S. cerevisiae* can lead to redirection of metabolic pathways. Therefore, studying the mechanisms of the regulation, production and consumption of redox equivalents not only can help to explain metabolism in different cultivation conditions but also expand the useful knowledge which can be applied in metabolic engineering to improve strains in a desired fashion. Improving the fermentation of pentoses, including D-xylose and L-arabinose, by regulating the redox metabolism is a good example for this. The main goal of this work is the sustainable ecological production of bio-ethanol from the lignocellulose feedstock which contains not only hexose but also a large amount of pentose. To this aim, heterologous genes encoding for enzymes of the functional D-xylose and L-arabinose pathways of the pentose fermenting yeasts or bacteria have been introduced in *S. cerevisiae*. However, the process of D-xylose or L-arabinose metabolisation often leads to an imbalance

of redox equivalents in which NADPH is depleted and NADH is accumulated. This imbalance is the main reason of inhibition of the pentose fermentation, accumulating xylitol (when D-xylose is used as carbon source), an unwanted side product, reducing the yield of ethanol in the recombinant *S. cerevisiae* strains. To overcome these difficulties, with respect to improvement of xylose utilization, several strategies have been applied:

i) regulation of the activity and cofactor-specificity of the reductase and dehydrogenase enzymes to get an optimized level;
ii) introduction of artificial transhydrogenase cycles;
iii) reducing the flux through PPP in order to reduce production of NADPH;
iv) expression of the cofactor-independent bacterial xylose isomerase instead of redox factor dependent yeast pathway.

The obtained engineered *S. cerevisiae* strains are able to grow on xylose as sole carbon source and ferment it to produce ethanol. Some strains have even been applied for industrial production. However, more studies need to be done in order to further improve the rate and productivity of ethanol production.

II. Experimental Part

"Engineering of *Saccharomyces cerevisiae* for the production of L-glycerol 3-phosphate"

1. Introduction

1.1 L-G3P - a promising starting material for pharmaceuticals

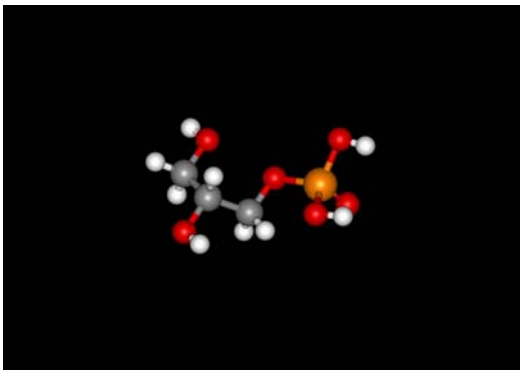


Fig. 7 Three-dimensional structure of L-glycerol 3-phosphate.

The L-enantiomer of glycerol 3-phosphate (L-G3P) is a small chiral compound having a three carbons backbone with a phosphate residue at C3 and two hydroxyl groups located at C1 and C2 (Fig. 7). L-G3P is a natural stereoenantiomer in all organisms. Furthermore, it has a high stability with temperature.

L-G3P is a potential starting material for the enzymatic synthesis of monosaccharides and glycerophospholipids. Monosaccharides can be synthesised from L-G3P via dihydroxyacetone phosphate (DHAP) taking advantage of the stereospecific aldol condensation performed by aldolases (Takayama *et al.*, 1997; Koeller and Wong, 2001). In addition, L-G3P is a precursor in the biosynthesis of phosphatidic acid, the key intermediate for the formation of all glycerophospholipids, the main component of biological membranes,

e.g. lecithine and phosphatidylcholin. Monosaccharides and glycerophospholipids are of growing interest for the development of novel therapeutics and vaccines (Dove 2001; Koeller and Wong, 2000; Maeder, 2002). As an example, on the surfaces of cells (see Fig. 8A), sugars decorate many proteins and lipids and participate in many biological processes as immunity and cell-to-cell communication (Maeder 2002). Carbohydrate-based drugs, therefore, can be used as vaccines or as inhibitors of tumor growth. For instance, vaccines used in combating cancer (Fig. 8B) are designed to incorporate with unusual sugars on the surface of tumor cells. The vaccine induces the immune system to produce antibodies which facilitate the destruction of tumor cells.

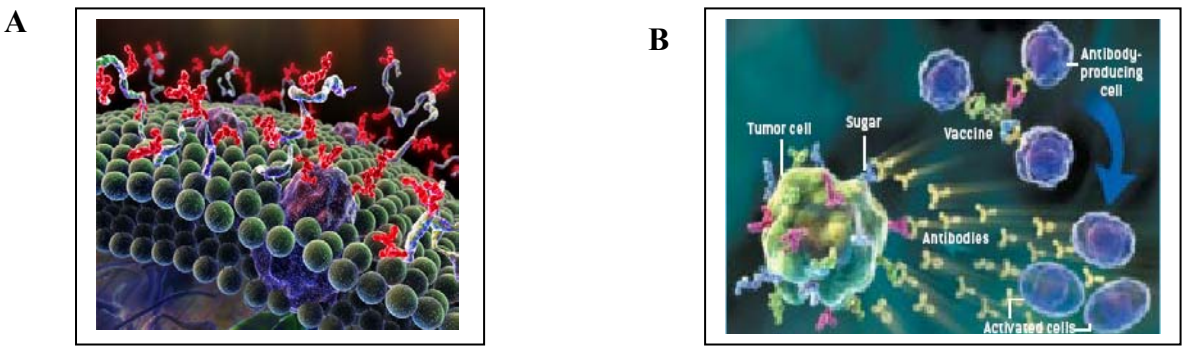


Fig. 8 Sugar coat on cell surface (A) and the incorporation of sugars in a vaccine for combating cancer (B). Pictures adapted from Maeder (2002).

Glycosylation itself is also a tempting target for novel antiviral drugs (Dove, 2001). For example, hemagglutinin of influenza virus mediates target cell recognition by specifically binding to cell-surface receptors bearing terminal sialic acid residues and thus causing infection. Analogues of sialic acid compete and permanently occupy the binding sites of the viral protein and thus prevent the infection. Another kind of antiviral drugs based on a small molecular inhibitor of cellular glycosylation enzymes leads to mis-glycosylation and subsequent misfolding of the matrix protein of viruses. The host cell proteins are also mis-glycosylated when the drug is given, but while the cells have mechanisms to eliminate the

defective proteins, the virus cannot do so. This drug has been used for hepatitis B virus and even HIV treatment.

In conclusion, L-G3P is a stable and potential precursor of pharmaceuticals. Since it is naturally synthesized in the living organisms, it is an attractive substance of biotechnological approach.

1.2 Conventional and biotechnological approaches for synthesis of L-G3P

Several methods to produce L-G3P have been used in the past. Each of them has disadvantages: they are either very laborious, expensive or produce racemic mixtures. A stereoselective method is the chemical synthesis from D-acetone glycerol. This process involves many protection and deprotection steps and is thus very laborious (Baer and Fischer, 1939). Simple phosphorylation of glycerol by polyphosphoric acid leads to complex mixtures of G2P, D- and L-G3P (Cherbiliez and Weniger, 1946). An enzymatic method, the phosphorylation of glycerol by glycerol kinase, yields only L-G3P but requires an expensive ATP regenerating system, e.g. acetyl phosphate or phosphoenolpyruvate (Crans and Whitesides, 1985). The catalysis can also be carried out by phosphatases using pyrophosphate as a substrate, but this procedure also leads to racemic mixtures of D- and L-G3P (Pradines *et al*., 1988; Schoevaart *et al*., 2000).

An alternative method could be the biotechnological production using microbial fermentation, e.g. *S. cerevisiae*. The usage of microorganisms to produce L-G3P has several significant advantages: First, only the desired L-enantiomer of L-G3P will be generated. Second, the lower price of L-G3P could be attained since different renewable carbon sources, e.g. molasses, can be metabolized. Also because of this fact, this is an environmentally sustainable method to produce L-G3P in bulk.

However, similar to all other intermediates of carbon metabolism, L-G3P is at a very low concentration in microorganisms. Metabolic engineering, therefore, has to be done to increase it.

1.3 Metabolism of L-G3P – an intermediate of the glycerol biosynthetic pathway in *S. cerevisiae*

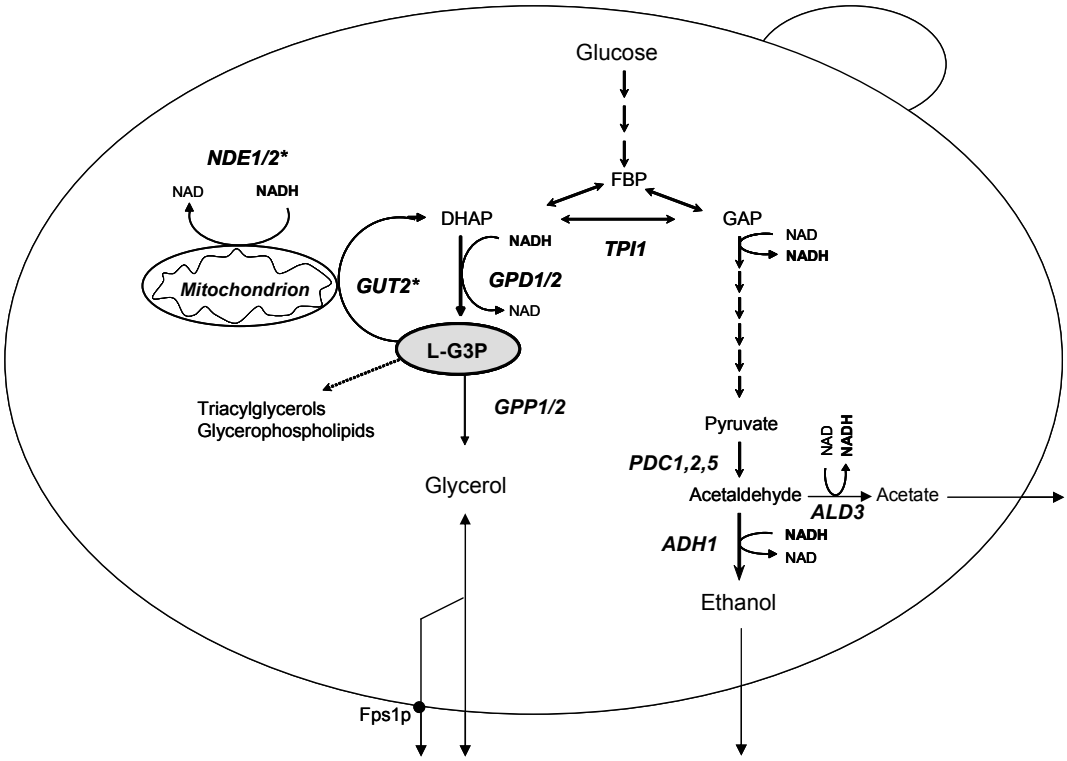


Fig. 9 Schematic presentation of L-G3P metabolism in *S. cerevisiae*: pathways and enzymes relevant in this context. Abbreviations: L-G3P, L-glycerol 3-phosphate; DHAP, dihydroxyacetone phosphate; GAP, glyceraldehyde 3-phosphate; FBP, fructose 1,6-bisphosphate; *GPP1/2*, cytosolic glycerol 3-phosphatase; *GPD1/2*, glycerol 3-phosphate dehydrogenase; *GUT2*, mitochondrial FAD-dependent glycerol 3-phosphate dehydrogenase; *PDC2*, positive regulator of pyruvate decarboxylase; *NDE1/2*, mitochondrial NADH dehydrogenase; *ALD3*, Aldehyde dehydrogenase, Fps1p, glycerol facilitator.

* These enzymes transmit reducing equivalents to the mitochondrial respiratory chain hence only being relevant in the presence of oxygen.

In *S. cerevisiae*, L-G3P is an intermediate of glycerol metabolism, formed from sugars by the reduction of the glycolytic intermediate dihydroxyacetone phosphate (DHAP) concomitant with NADH oxidation via cytosolic glycerol 3-phosphate dehydrogenase (GPD) (E.C.1.1.1.8). This enzyme has been purified and characterized by Albertyn *et al*. (1992) and Cai *et al*. (1994). It is encoded by two highly homologous isogenes *GPD1* and *GPD2*

(Albertyn *et al*., 1994; Eriksson *et al*., 1995). The subsequent dephosphorylation of L-G3P leads to the formation of glycerol. This reaction is catalysed through the activity of glycerol 3-phosphatase (GPP) (E.C. 3.1.3.2.1); (Fig. 9). GPP has been purified and characterized by Norbeck *et al*. (1996). The enzyme is also encoded by the two isogenes *GPP1* and *GPP2*.

It has been shown that the expression of the isogenes of GPD and GPP is differentially regulated in response to altered growth conditions. While the transcription of *GPD1* and *GPP2* is stimulated by an increase in external osmolarity (Albertyn *et al*., 1994; Hirayama *et al*., 1995; Ansell *et al*., 1997; Hohmann 2002), the expression of *GPD2* and *GPP1* is stimulated under oxygen limited conditions (Norbeck *et al*., 1996; Ansell *et al*., 1997; Påhlman *et al.*, 2001). L-G3P can be converted back to DHAP by the L-G3P shuttle (see I.1.4). The reaction occurs between the inner and outer membranes of mitochondria by the mitochondrial FAD-dependent GPD which is encoded by the *GUT2* gene (Rønnow and Kielland-Brandt, 1993) and transmits the reducing equivalents to the mitochondrial respiratory chain. Hence, this enzymatic reaction is only relevant in the presence of oxygen.

The intermediate L-G3P also serves as a precursor for the biosynthesis of triacylglycerols, the energy reservoirs in eukaryotic cells, and glycerophospholipids, the major lipid components of biological membranes (Fig. 9). The first step towards these lipids is the acylation catalysed by L-G3P acyltransferase (Kent, 1995).

1.4 Significance of glycerol production in *S. cerevisiae* and metabolic engineering approaches to improve it

Glycerol production plays a critical role when yeast cells grown in hyperosmotic stress or under anaerobic conditions (for reviews see Hohmann, 2002; Bakker *et al.,* 2001).

It is well established that polyols, in particular glycerol, have a role as compatible solute to adjust the intracellular osmolarity when yeast cells are grown under osmotic stress conditions, which is normally done by adding salt (e.g. NaCl) or sugars (e.g. sorbitol, saccharose) into the cultivation medium (Nevoigt and Stahl, 1997). There are two distinct transmembrane proteins, Sln1p and Sho1p, which are located on the surface of the cells and participate in sensing osmotic stress (Maeda *et al*., 1994; Posas and Saito, 1997). Both of

them interact with a specific osmoresponsive signal transduction pathway, the high osmolarity glycerol (HOG) mitogen-activated protein kinase (MAPK) pathway, to mediate the responses of cells caused by the alterations in external osmolarity (for reviews see Hohmann, 2002; Mager and Siderius, 2002; O´Rourke *et al*., 2002). The HOG MAPK pathway, also called HOG MAPK cascade, is composed of three sequentially acting kinases. In response to hyperosmotic stress, a MAP kinase kinase kinase (MAPKKK) phosphorylates and activates a MAPKK; this then phosphorylates and activates a MAPK. It has been confirmed that the activation of Hog1p, the sole terminal MAPK, stimulates the transcription of the *GPD1* gene (Albertyn *et al*., 1994). The *GPP2* gene is also known to be induced and regulated by the HOG pathway (Norberg *et al*., 1996). Beside these two genes, the expression of at least 300 genes involved in glycerol formation, stress protective proteins and the readjustment of carbohydrates, amino acids, lipid and redox metabolism, are changed upon osmotic shock in *S. cerevisiae* (Gasch *et al*., 2000; Rep *et al*., 2000).

Moreover, glycerol production in *S. cerevisiae* plays a crucial role in maintaining the redox balance under anaerobic conditions (van Dijken and Scheffers, 1986) since it is the only way to reoxidise the cytosolic NADH generated by biomass production (see I.1.4).

Engineering *S. cerevisiae* to improve glycerol production has been done since early 20th century. The first success was the sulfite process that was invented by Neuberg and Reinfurth (1918). In this process, sulfite was added into *S. cerevisiae* culture in order to trap acetaldehyde, the electron acceptor for the reoxidation of glycolytic NADH. Glycerol production was improved since more NADH was available for the reduction of DHAP. Later, other approaches using recombinant DNA technology have focused on minimizing NADH consumption in the ethanol production pathway, simulating the classical sulfite process by reducing or completely blocking the activities of the key enzymes of alcoholic fermentation. The first key enzyme is ADH encoded by four isoenzymes. Three of them, ADHI-ADHIII, have been described in section I.1.4. ADHIV is another type of yeast ADH (Paquin and Williamson, 1986). It differs in many biochemical properties from ADHI through ADHIII and was proposed to be not involved in fermentation of glucose by laboratory strains (Drewker and Ciriacy, 1988). Deletion of all four ADH isoenzymes resulted in the formation of glycerol

as a major fermentation product (Drewke *et al.*, 1990). The second key enzyme is pyruvate decarboxylase (PDC) - a crucial enzyme for the growth of *S. cerevisiae* on glucose (Flikweert *et al*., 1996). It is encoded by three structural genes, *PDC1* (Kellermann *et al*., 1986; Schmitt *et al*., 1983; Hohmann, 1991a), *PDC5* (Hohmann and Cederberg, 1990; Seeboth *et al*., 1990) and *PDC6* (Hohmann 1991b). The expression of *PDC1* is six times stronger than of *PDC5* during glucose fermentation. However, the deletion of only *PDC1* did not lead to a decrease in PDC activity because *PDC5* was stimulated to substitute for *PDC1*. *PDC6* does not seem to contribute to the total PDC activity of the cell during growth on glucose. Its expression is only activated with the presence of *PDC1* or a nonfermentable carbon source. Deletion of both *PDC1* and *PDC5* or all three structural genes leads to undetectable activity of PDC. Full expression of *PDC1* and *PDC5* requires the presence of a functional *PDC2* gene, which encodes a positive transcription regulator (Schmidt *et al.,* 1983, Wright *et al.,* 1989; Hohmann, 1993). Deletion of *PDC2* led to a reduction of 80% of the activity of PDC. Nevoigt and Stahl (1996) reported that the *pdc2*Δ mutant produced 4.7 times more glycerol than the wild type.

Glycerol production has also been improved by overexpressing a truncated Fps1p, the channel protein that mediates glycerol export in *S. cerevisiae*. This modification led to constitutive glycerol efflux which triggered a 2- to 2.5-fold increase in final glycerol concentration (Remize *et al*., 2001).

Other approaches focused directly on manipulating the enzymes of glycerol biosynthesis (Nevoigt and Stahl, 1996; Michnick *et al*., 1997; Remize *et al*., 2001; Nevoigt *et al.*, 2002). In fact, overexpression of *GPD1*, the major isogene of GPD led to a 6.5fold increase of glycerol yield (Nevoigt and Stahl, 1996). It has been shown by Remize *et al*. (2001) that the overexpression of *GPD2* has the same effect as the overexpression of *GPD1*. Interestingly, the overproduction of GPP did not increase glycerol production (Påhlman *et al*., 2001; Remize *et al*., 2001).

The combination of different above approaches has also been done. Simultaneous deletion of *PDC2* and overexpression of the *GPD1* gene have led to an 8.1 times higher yield of glycerol (Nevoigt and Stahl, 1996). Overexpression of *GPD1* in the strain expressing the

truncated Fps1p increased the amount of glycerol in comparison to the strain expressing truncated Fps1p only but led to a pronounced growth defect (Remize *et al*., 2001).

Glycerol production was also strongly improved by deleting the *TPI1* gene encoding triosephosphate isomerase. This enzyme acts at an important branch point of the glycolytic backbone. Its deletion leads to an accumulation of DHAP and therefore increases glycerol production (Compagno *et al*., 1996; 1998; 2001). Nevertheless, *tpi1*Δ mutants are unable to grow on glucose as carbon source because of the accumulation of DHAP. Recently, deletion of the *TPI1* gene has been combined with the deletion of the *ADH1* gene (Cordier and François, 2003). Disruption of the *ADH1* gene has the same effect as the deletion of the *PDC2* gene, i.e. cells of the mutant can save the cytosolic NADH for DHAP reduction. Furthermore, in this work, *GPD1* and *ALD3*, encoding an isoenzyme of aldehyde dehydrogenase (ALD) catalysing the conversion of acetaldehyde to acetate, have been individually or in combinaion overexpressed in the *tpi1*Δ, *adh1*Δ single mutants or *tpi1*Δ *adh1*Δ double mutant. It should be mentioned that ALD uses NAD^+ as cofactor and thus, the overexpression of *ALD3* could lead to an increase of cytosolic NADH, which is needed for glycerol production. Different recombinant strains have been created as a result of these genetic modifications. It has been shown that the strains *tpi1*Δ *adh1*Δ + *GPD1* and *tpi1*Δ *adh1*Δ + *GPD1* + *ALD3* produced 44-51 times more glycerol than wild type. Furthermore, the overexpression of *GPD1* could partially restore the impaired growth of the *tpi*Δ mutant by consuming excess of DHAP in these mutants.

The highest glycerol yield obtained so far by metabolic engineering of *S. cerevisiae* resulted from a combined deletion of *TPI1* gene with the deletion of three other genes *NDE1, NDE2* and *GUT2* (Overkamp *et al*., 2002). The simultaneous disruption of these three genes could completely block the mitochondrial reoxidation of cytosolic NADH and thus, render it available for DHAP reduction. The glycerol yielded from the quadruple mutant *tpi1*Δ *nde1*Δ *nde2*Δ *gut2*Δ almost reached the maximum theoretical yield expected.

2. Aim of work

This thesis aims at engineering the relevant enzyme activities in *S. cerevisiae* to create a strain that accumulates L-G3P. A step by step scenario reflected by the following key questions was designed to find out the most suitable solution.

(1) Is it possible to accumulate L-G3P, an intermediate of glycerol biosynthesis, by simultaneously enhancing its biosynthesis and inhibiting its consumption?

The activity of GPD was increased by multicopy expression of *GPD1* in mutants whose GPP activity was partially reduced (*gpp1*Δ) or completely inhibited (*gpp1*Δ *gpp2*Δ) (Påhlman *et al*., 2001).

(2) How do the genetic modifications affect the growth of S. cerevisiae cells?

The growth of engineered *S. cerevisiae* strains was recorded and the exponential growth rate (μ) was calculated.

(3) How can the cultivation conditions be optimised in order to increase the intracellular L-G3P accumulation?

The two conditions which are well known to enhance glycerol production in *S. cerevisiae*, osmotic stress and oxygen limitation, were tested whether they also cause an increase in the genetically engineered strains.

(4) Could a Neuberg-like approach improve the accumulation of intracellular L-G3P?

To this aim, PDC activity was diminished by deleting the *PDC2* gene in the *gpp1*Δ *gpp2*Δ mutant. Afterwards, the *GPD1* gene was overexpressed resulting in the strain *gpp1*Δ *gpp2*Δ *pdc2*Δ + *GPD1*.

(5) Do cells of the genetically engineered strains release L-G3P into the cultivation medium?

The supernatants of yeast cultures were analysed by measuring L-G3P.

3. Materials and methods

3.1 Materials

3.1.1 Equipments

Autoclave	Varioklav 500 EV (H+P Labortechnik, Oberschleissheim)
Balances	Type 1907 (Sartorius, Göttingen)
Centrifuges	Sorvall RC-5B (for SS34- and GSA-Rotors) (Sorvall DuPont, Bad Homburg); Microrapid/K (Hettich, Tuttlingen); DW41 (Qualitron, Korea)
Clean bench	Uniflow UVUB1200 (UniEquip, Martinsried)
Electrophoresis chambers	Mini-Sub and Wide Mini-Sub DNA-Cell (Biorad)
Homogenizer	Micro-Dismembrator; Braun, Melsungen
Hybridization oven	Compact Line OV4 (Biometra, Göttingen)
Incubators	Typ B6420 & FunctionLine Typ B20 (Heraeus Instruments, Hanau)
Microscope	Labophot (Nikon, Japan)
PCR equipment	GeneAmp 9600 (PerkinElmer, Norwalk, USA)
pH-electrode	IntLab 412 (Mettler-Toledo, Urdorf, Schweiz)
pH meter	Digital-pH-Meter (Knick)
Pipetting equipment	Gilson Pipetman P10, P20, P200, and P1000 (Abimed, Langenfeld)
Shaker	Certomat U (BBraun Biotech, Melsungen);Polymax 1040T (Heidolph, Kelheim)
Spectrophotometer	UV-160A (Shimadzu, Japan)
Vacuum equipment	Laborota 4000 (Heidolph, Kelheim)
Water baths	Lauda A100 (Wobser, Lauda-Königshofen)
X-ray cassettes	Kodak X-Omatic with intensifying screen; Kodak, Berlin

3.1.2 Enzymes, chemicals and kits

Agarose	Seakem LE, GTG & Gold, Incert^{R} Agarose (FMC Bioproducts, Denmark)
Biochemicals/ chemicals	Kanamycin, Phenol (BioMol, Hamburg); Agar-Agar, Yeast extract, Peptone, Triptone, Glucose, Sorbitol, Yeast Nitrogen Base, Hydrazine Sulphate (Merck, Darmstadt; Serva, Heidelberg; Difco, MI, USA); Dihydroxyacetone phosphate, Imidazol, Thiaminpyrophosphate (Sigma, St. Louis, USA); NAD, NADH (Roche Diagnostic, Mannheim); Ethanol, Chloroform, Isopropanol (Roth, Karlsruhe)
Enzymes	Restriction enzymes (New England Biolabs, Schwalbach); Glycerol 3-phosphate dehydrogenase (Roche Diagnostic, Mannheim; Fluka, Neu-Ulm); Alcohol dehydrogenase (Roche Diagnostic, Mannheim);
Kits	BioRad Protein-Assay (BioRad, München); DIG-system for filter hybridization (non-radioactive) (Roche Diagnostic, Mannheim); PCR Purification Kit (Qiagen, Düsseldorf), Plasmid Maxi Kit (Qiagen, Hilden); NucleobondR AX500 (Macherey, Nagel, Düren); D-Glucose Kit (Boehringer Mannheim)
Membranes	Microdialysis membranes-0.025 μm (Millipore, Eschborn); Cellulose Acetate filter - 0.45 μm (Satorius, Goettingen)
Nucleic acid	λ-DNA (Roche Diagnostic, Mannheim); GeneRuler TM DNA Ladder Mix (MBI Fermentas, Litauen)
Oligonucleotide	Metabion, Berlin
PCR-reagents	Deoxynicleoside-Triosephosphate Set, Taq-polymerase (Roche Diagnostic, Mannheim)

All chemicals not listed above were obtained from the following firms: Fluka, Merck, Roche Diagnostic, Sigma, Serva, Pharmacia, and were of analytical grade or better quality.

3.1.3 Strains

Table 2. *S. cerevisiae* strains used in this study

Strain	Genotype	Source or reference
S. cerevisiae W303-1A*	MAT**a**	Thomas and Rothstein (1989)
YA101*	MATα *gpp1*Δ::kanMX4	Pahlman *et al*. (2001)
YA103*	MAT**a** *gpp1*Δ::kanMX4 *gpp2*Δ::*HIS3*	Pahlman *et al*. (2001)
YA104*	MAT**a** *gpp1*Δ::kanMX4 *gpp2*Δ::*HIS3gut2 ::URA3*	Pahlman *et al*. (2001)
YHN1*	MATα *gpp1*Δ::kanMX4 *pdc2*Δ::*TRP1*	This study
YHN2*	MATα *gpp1*Δ::kanMX4*gpp2*Δ::*HIS3pdc2*Δ::*TRP1*	This study
YSH306	MAT**a** *leu2-3/112 trp1-92 ura3-52 pdc2*Δ::*TRP1 SUC GAL mal*	Hohmann *et al*. (1993)
E. coli	*SupE44, ΔlacU169(ϕ80lacZΔM15), hsdR17, recA1, endA1, gyrA96, thi-1, relA1*	

** These strains harbour additional mutations as follows: leu2-3/112 ura3-1 trp1-1 his3-11/15 ade2-1 can1-100 GAL SUC2 mal0*

3.1.4 Medium and culture conditions

E. coli

E. coli strains were cultivated in LB medium (1% bactotryptone, 0.5% yeast extract, 1% NaCl, pH 7.5) at 37°C. Recombinant *E. coli* strains were selected in LB medium with 100 μg/ ml of Ampicilline. Solid LB medium was obtained by adding 1.5% agar. To keep the stock cultures, 1 ml of liquid culture was mixed with 0.3 ml glycerol. The cultures then were stored at – 70°C.

S. cerevisiae

Yeast cells were grown in Erlenmeyer flasks at 30°C on a rotary shaker at 200 rpm in YEPD medium (2% peptone, 1% yeast extract, 2% glucose). Strains containing plasmids

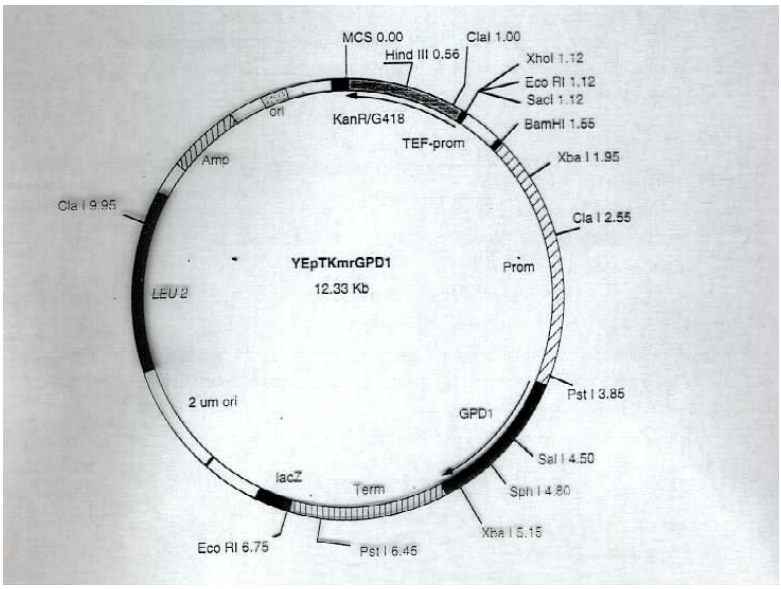
YEpKm^R*GPD1* or YEpKm^R also harbour the *LEU1* gene as a genetic marker and were grown in a synthetic yeast nitrogen base YNB medium (Difco, USA) supplemented with 2% glucose and histidin, adenine, tryptophane and uracil (final concentration of 120 μg/ml each). This medium is referred to as YNB (Glc, leu$^-$). The *pdc2*Δ mutants containing YEpKm^R*GPD1* or YEpKm^R which grow very slowly on glucose were first cultivated in YEPEG medium (2% peptone, 1% yeast extract, 2% (v/v) glycerol, (v/v) 2% ethanol) supplemented with 200 μg/ml Geneticin G418 until exponential phase was reached. Cells were harvested and transferred to YNB (Glc, leu$^-$) medium for batch fermentation.

Batch fermentation under oxygen limitation was performed in Erlenmeyer flasks closed with air-locks which ensures the exclusion of oxygen but allowed the release of gases. The cultures were stirred continuously at 350 rpm.

3.1.5 Plasmids

Table 3. Plasmids used in this study

Plasmid	Description	Reference
YepTkm^R*GPD1*	*E. coli/ S. cerevisiae* shuttle vector, containing Amp$^+$, lacZ, *LEU2* as markers and *GPD1* gene under the control of its natural promotor	Nevoigt and Stahl, 1996
YepTkm^R	Reference plasmid (without *GPD1* gene)	



Map of the plasmid YEpTKm^{R}*GPD1* (Nevoigt *et al.*, 1996)

3.1.6 Oligonucleotides

The oligonucleotides used in this work were synthesized by Metabion (Berlin)

Primers used to amplify *PDC2*::*Trp1* cassette (from YSH306 strain):

P1 (Forward primer): 5′- CGAACGTCCTCTGTCCCACTAT -3′

P2 (Reverse primer): 5′- AGCCTGTGTTACCAGGTAAG - 3′

Primers used to verify the *PDC2* deletion:

P3 (Forward primer): 5′- CTGGAGTCCAATGATGCAGCC - 3′

P4 (Reverse primer): 5′- CAGGATAGATCAAAGAGCAGTC - 3′

Primers used to verify the *GPP2* deletion:

P5 (Forward primer): 5′- ACACAGCCCAATCCTGTGAT - 3′

P6 (Reverse primer): 5′- AGATTTCTGCAGTGTTCCGT - 3′

3.2 Methods

3.2.1 Transformation of *E. coli*

Transformation of *E. coli* was carried out by the heat shock method according to Himeno *et al*., (1984) and Miler (1987). Competent cells were prepared by $CaCl_2$ treatment. The *E. coli* cells were first precultured in 20 ml LB-medium from a frozen stock culture (-70°C) for

16 hours at 37°C. To start the main culture, 1 ml of preculture was transferred into an Erlenmeyer flask with 100 ml of LB-medium. The cells were grown until an optical density (OD) at a wavelength of 600 nm of 0.4 was reached. The culture was cooled down on ice for 1 hour and cells are collected by centrifugation at 4°C for 5 minutes with 4000 rpm. The supernatant was decanted and the cells were washed in 100 ml of ice cold solution I (0.1 M $MgCl_2$; 0.01 M Tris-HCl, pH 7.6). The cells were collected as last step. The pellet was taken in 50 ml of ice cold solution II (0.1 M $CaCl_2$; 0.01 M Tris-HCl, pH 7.6). The suspension was centrifuged again. The pellet was resuspended in 4 ml of ice cold solution II and the suspension was kept on ice in 30 minutes. The competent cells were directly used for transformation or stored at -70°C with glycerol (15% at final concentration). For transformation, approximately 50 ng DNA were mixed with 100 ml of ice cold solution III (1 mM EDTA; 0.2 mM NaCl; 8 % PEG; 10 mM Tris-HCl, pH 7.6). This mixture of DNA then was added to 2 ml Eppendorf tube containing 240 µl of competent cells. The cells were kept on ice for 30 minutes and subjected to a heat shock by incubation for 90 seconds at 42°C. After that, 1 ml of LB medium was added. The tube was incubated with agitation at 37°C for 1 hour. Afterwards, aliquots were plated on LB plates containing ampicilline (150 µg/ml).

3.2.2 Transformation of *S. cerevisiae*

Yeast cells were transformed by electroporation method which slightly modified from the method of Becker and Guarente (1991). An Erlenmeyer flask with 100 ml of YEPD-medium was shaken at 200 rpm with 1 ml of a 20 ml overnight preculture. The cells were grown at 30°C until the OD_{600} of 1.3 - 1.5 (ca. 1 x 10^8 cells/ ml) was reached. The culture was cooled down on ice for 5 - 10 minutes and kept on ice during the following steps. The cells were collected by centrifugation for 5 minutes by 7000 rpm at 4°C and were washed with 100 ml of ice cold distilled water and centrifuged as mentioned above. The pellet was resuspended in 20 ml of ice cold 1 M sorbitol then centrifuged. The pellet was suspended in a volume as little as possible of 1 M sorbitol so the cells suspension could be taken with a pipette (1-2 x 10^{10} cells/ ml). For transformation, 40 µl of cell suspension was gently mixed with 100-200 ng of plasmid DNA in a maxium of 5 µl of distilled water, which was previously cleaned by micro-

dialysis-membrane. The mixture was incubated on ice for 5 minutes then transferred to a pre-cold electroporation cuvette and electroporated at 1.5 kV; 25 mF; 200 W with a Gene Pulser (BIO-RAD). After pulsing, 1 ml of ice cold 1 M sorbitol solution was immediately added to the cuvette. The electroporated cells were plated directly on YNB (Glc, leu^-) agar medium.

3.2.3 DNA isolation

3.2.3.1 Isolation of DNA plasmid from *E. coli* (minipreparation)

The plasmid DNA was isolated from *E. coli* following the alkaline method of Birnboim and Doly (1979). Cells were grown from a single colony over night in a tube containing 3 ml of LB medium supplemented with ampicilline (150 mg/ ml) at 37°C by shaking at 200 rpm. Pellets from 1.5 ml of the culture were harvested by centrifuging for 30 seconds at 13,000 *g*. The pellets were frozen for 30 minutes in -20°C to favour resuspendation. To solve the pellet, 100 µl of solution I (10 mM EDTA; 25 mM tris-HCl, pH 8.0; 50 mM glucose) was added and the mixture was shaken well. The tube was kept at room teparature for 5 minutes and 200 µl of solution II (200 mM NaOH; 1 % SDS) was added. The cells were mixed carefully by inverting the tube five times. The tube was kept again at room temparature for 5 minutes and then transferred to the ice bath and cooled down for 2 minutes before the addition of 150 µl of solution III (3 M Na/ 5 M K-acetate, pH 5.2). The tube was shaken strongly, kept on ice for 30 minutes and centrifuged for 15 minutes at 13,000 *g*, 4°C. The supernatant was transferred to a new tube and the DNA was precipitated by 0.7 volumes of isopropanol. The DNA pellet was washed with 70% ethanol and dried well before resuspending in 50µl of distilled water.

3.2.3.2 Isolation of DNA of *S. cerevisiae* for back transformation of *E. coli* (minipreparation)

Genomic DNA was isolated from *S. cerevisiae* following the method of Hoffman and Winston (1987). Yeast cells were pre-cultured over night in 20 ml YEPD medium. A pellet of 1.5 ml of the culture was harvested by centrifugation for 5 minutes at 13,000 *g* and was briefly shaken with the residual medium. To disrupt the cells, 200 µl of lysis buffer (2% triton X-100; 1% SDS; 100 mM NaCl; 10 mM tris-HCl, pH 8.0; 1 mM EDTA), 200 µl of

phenol:chloroform:isoamyl alcohol (25:24:1) and 300mg of glass bead (0.45-0.5 mm) were added to the cell suspension. The tube was shaken for 2 minutes and centrifuged for 5 minutes at 13,000 *g*, 4°C. The upper phase was transferred to a new tube and DNA was precipitated by isopropanol. The DNA was washed with 70% ethanol before being solved in 30 µl of distilled water.

3.2.4 Agarose DNA gel electrophoreses

Depending on the size of the DNA fragments to be separated, the agarose concentration varied from 0.8-1.5 %. The gel was prepared by adding agarose to 1x TBE buffer and boiling for 3 minutes. DNA samples were mixed with 1/5 volume of loading buffer (20mM EDTA, pH 8.0, 0.025% bromophenol blue, 60% saccharose). The electrophoresis was run in 1xTBE buffer (89 mM Tris; 89 mM Boric acid; 2 mM EDTA, pH 8.0) with the currency of 50-80 mA. λDNA digested with *Hind*III and *Eco*RI were used as a size marker. The gels were stained in 0.5 mg/ ml EtBr solution for 20 min. The DNA fragments in agarose gels were visualised under UV light (λ = 254nm).

3.2.5 PCR

Reactions were performed in GeneAmp9600. PCR-programs were used depending on the primer length (annealing temperature), fragment length (elongation time) and target used (plasmid or genomic DNA). In general, the following program was applied: after 5 min pre-treatment at 94°C, 25 (plasmid DNA as target) or 30 (genomic DNA as target) cycles followed: annealing for 90s at 56-64°C, elongation for 120s at 72°C, and denaturation for 60s at 94°C. End-elongation was usually carried out for 10 min at 72°C. Annealing temperature was determined by the G/C rule:

$T_{annealing} = \sum$ (4°C for every G or C) + (2°C for every A or T).

PCR reactions were performed in 50µl volume in 0.2ml (GeneAmp9600) reaction tubes. The reaction mix contained 0.25 mmol/ 1 of each dNTP, 1.5 µmol/l of each primer, 1-100 ng template DNA, 1-2 U Taq-polymerase, 5 µl of 10 x reaction buffer, and H_2O up to 50 µl.

For subsequent transformation in yeast, the PCR products were purified using the QIA-PCR purification kit.

3.2.6 Mating, sporulation and spore analysis

Mating was carried out using the standard protocol of Sprague (1991). Cells of two opposite mating types were mixed and applied to a small area of the surface of a YEPEG agar plate. The plate was incubated over night at 37°C and the cells were harvested and transferred to a 100 ml Erlenmeyer flask containing 20 ml of YEPD medium and incubated (without shaking) over night. The cells were collected by centrifugation and washed twice with sterile water before being spread on the sporulation medium (1% potasium acetate, 0.1% yeast extract; 0.05% glucose, 2% agar) (Sherman, 1991). After 7 days, when the zygotes were detected under microscope, all the cells on the sporulation agar plate were washed with 10 ml of distilled water. The pellet from 6 ml of the obtained mixture was resuspended in 500 µl of distilled water. After transferring 100 µl to a new tube, 5 µl Glucuronidase were added and incubated for 20 min at 37°C. After centrifugation (2 min, 10000 rpm) the pellet was washed in 0.5 ml distilled water, centrifuged again and resuspended in 0.5 ml distilled water. The suspension was shaken at highest speed for 2 min. This step makes the spores stick to the hydrophobic walls of the tube, while the less hydrophobic cells stay in the solution. The cell-solution was carefully taken away. The remaining spores were washed 3 times with 0.5 ml distilled water (add water, carefully shake tube and pipette water). The spores were resuspended in 1 ml detergent-solution and isolated under ultrasonic waves (position 10%, 1-3 min, the tube was kept on ice). Immediately after the isolation, the spores were spread on 2% YEPEG media-plates in different concentrations: not diluted, 1:10 and 1:100). The plates were incubated for 4 days at 27°C. Then master plates (2% YEPEG) were prepared and incubated for 2 days. The master plates were stamped on selective media-plates (2% YNBEG + 25% amino acids) and incubated for one week. Single colonies were transferred on new selective media-plates and incubated for one week. The strains then were checked by mating-type-testing and PCR.

3.2.7 Extraction of intracellular L-G3P

Extraction of intracellular L-G3P from *S. cerevisiae* was done following the method of Boles et al (1993) which was slightly modified. An aliquot of cultures containing approximately 1.5 x 10^9 cells was mixed with the same volume of pre-cold methanol (-70°C) to quench metabolism. The cells were rapidly collected on cellulose acetate filters (pore size 0.45 μm, Sartorius, Göttingen, Germany). The filter membrane with the collected cells was transferred quickly into a round-bottomed flask containing a mixture of 5 ml EtOH/ 3 ml chloroform/ 0.15 ml formic acid which was pre-cold at -70°C. The flask was briefly shaken until the cells were separated from the filter and stored at -20°C for at least two hours. Cells were completely dried in a rotary evaporator at 60°C for 5-10 minutes. The dry residues were resuspended in 50 mM imidazole buffer whose pH was adjusted to 9.0 with NaOH. The resulting suspension was cleared by centrifugation at 13000 *g* for 10 min and cells were harvested for the determination of L-G3P.

3.2.8. Determination of L-G3P concentration

L-G3P was determined by an enzymatic assay which is slightly modified from the described method by Bergmeyer (1974). The basic of the measurement is the specific reaction which oxidizes L-G3P to DHAP by GPD in the presence of NAD. However, L-G3P is practically quantitatively oxidized if the reaction products are removed. For this reason, hydrazine sulphate buffer (0.4 M Hydrazine sulphate; 1 M glycine; 5mM EDTA, pH 9.8) was used to trap DHAP.

The equation for the spectrophotometric determination of L-G3P is:

$$\text{L-G3P} + \text{NAD}^+ + \text{Hydrazine} \xrightarrow[\text{pH 9.8}]{\text{GDH}} \text{DHAP-hydrazone} + \text{NADH} + \text{H}_2\text{O} + \text{H}^+$$

The increase of NADH concentration measured by the change in absorbance at wavelength of 340 nm is proportional to the amount of L-G3P. The enzymatic reaction

contained 0.189 M hydrazine, 0.47 M glycine, 2.7 mM EDTA, 2.3 mM NAD, 1 mM ATP and 0.9 mM $MgSO_4$, 1000 U/l GPD, and up to 200 μM of L-G3P.

3.2.9 Determination of yeast dry weight

Dry weight was determined by filtering 50 ml of the culture using pre-weighed nitrocellulose filters (pore size 0.45 μm). The filters were washed with distilled water and dried until the weight reached a stable value.

3.2.10 Determination of glucose consumption in yeast cultures

To determine the consumption of glucose in yeast culture, the concentrations of glucose in the medium before and after fermentation were measured by using the D-glucose kit. The samples were diluted 1:100 for the assay.

3.2.11 Determination of specific enzyme activity

3.2.11.1 Preparation of cell extracts

Cells from main culture were harvested and disrupted by shaking them together with glass beads (Ciriacy 1975) and 0.2 mM PMSF for 15 min at 4°C. Depending on the measurement of the specific enzyme activity (GPD or PDC), different amount of cells and buffers were used.

To measure the specific GPD activity, approximately 3 x 10^9 cells (30-50 ml of culture at OD_{600} of 1.2-1.5) were homogenized by shaking together with 2 ml of TRED buffer (10 mM TrEA, pH 7.5; 1 mM EDTA, 1 mM DTT (fresh) and 0.5 g of glass beads. To measure PDC activity, cells were grown in YEPEG medium until OD_{600} of 1 reached and transferred to fresh YEPD medium 6 hours. About 5 x 10^8 cells (5-10 ml of culture with the OD_{600} of 1.2-1.5) were harvested and mixed with 1 ml of imidazol buffer (50 mM imidazol, pH 6.8; 0.1 M EDTA; 10 mM $MgCl_2$, 100 mM KCl) and 0.5 g of glass beads. The suspensions were centrifuged at 12,000 x *g* for 15 min at 4°C. The supernatant was either used directly for the

PDC assay or desalinized with the help of a Sephadex-G-25 column before measuring the GPD activity.

3.2.11.2 *In vitro*-enzyme assays

Enzyme activities were measured based on the test developed by Otto Warburg in 1936. Since reduced NAD and NADP absorb light of wave lengths between 338.5 and 340.5 nm whereas the oxidized electron carriers do not absorb light of these frequencies, the shift from reduced to oxidized states can be measured.

Pyruvate decarboxylase (PDC)

PDC catalyzes the decarboxylation of pyruvate to form acetaldehyde. Acetaldehyde is then quantitatively converted to ethanol by ADH added to the assay. The amount of the reacted substrate and the activity of PDC can be measured via the oxidation of NADH.
The measures were conducted by Seehaus (1986) using imidazole buffer with pH 6.8. Under these conditions, PDC has a very sharp stability optimum while the highest activity is seen at pH 5.6.

Reactions were taken out in 1 ml final volume and contained 50 mM imidazole, pH 6.8; 0.1 mM EDTA, 10 mM $MgCl_2$, 100 mM KCl, 2 mM TPP, 0.26 mM NADH, 1U/ml ADH (from yeast) and 33 mM sodium pyruvate. 800 µl of test mix (contains all of the above substances, except sodium pyruvate, in 1.25 fold concentration) were mixed thoroughly with 100 µl cell extract and incubated for 5-10 min at room temperature. Then, the reaction was started by adding 100 µl of 330 mM sodium pyruvate, and the decrease in extinction by time was measured at 340 nm for 3 to 4 minutes.

Glycerol-3-phosphate dehydrogenase (GPDH)

Since NADH is oxidized when DHAP is converted to glycerol-3-phosphate, the GPDH activity can be measured via the decrease of NADH.

We used a method based on Gancedo *et al.* (1968). Measurements were performed in imidazole buffer (pH 7.0) which leads to higher activities than the cell extraction buffer (Andre *et al.*, 1991).

The reactions contained 20 mM imidazole (pH 7.0), 1 mM $MgCl_2$, 1mM DTE, 0.2 mM NADH and 1.2 mM DHAP. 800µl test mix (containing all of the above substances, except DHAP, in 1.25fold concentration) was mixed with 100µl cell extract and incubated for 5-10 min. Then, the reaction was started by addition of 100 µl of 12 mM DHAP and the decrease of extinction by time was measured.

All the enzyme activity measurements were carried out at room temperature (22-23°C). The decrease of extinction before adding the substrate (background activity) was always small enough to be neglected. The cell extracts were diluted with buffer to reach a decrease of extinction between 0.1 and 0.2. For calculation of the specific activity, the decrease of extinction was measured in the linear area of the curve (2-3 min. after starting the reaction).

3.2.11.3 Determination of protein content

The protein concentration in cell extracts was determined after Bradford (1976). The dye reagent was purchased from Bio-Rad and diluted 1:3 with distilled water. 800 µl of this solution was mixed with 200 µl cell extract and incubated for 5-10 min at room temperature. The extinction was then measured at a wave length of 595 nm against a reference sample. For each protein determination, a calibration curve of BSA with concentrations of 20-70 µg/ ml was carried out.

3.2.11.4 Calculation of specific enzyme activity

From the change of extinction per min, the volume activity (U/ml) was determined. The formula used for it is derived from the Lambert-Beer law:

$$\text{Volume activity } (U \times ml^{-1}) = \Delta E \times \Delta t^{-1} \times \varepsilon^{-1} \times d^{-1} \times V \times v^{-1} \times VF$$

$$= \Delta E \times \Delta t^{-1} \times 1.608\ (U \times ml^{-1})$$

$\Delta E \times \Delta t^{-1}$	Change of extinction per time	(min)
ε	Extinction coefficient of NADH (340 nm)	(6.22 ml x µmol-1 x cm-1)

d	The thickness of layer (width of the cuvette)	(1cm)
V	Measuring volume (cuvette contents)	(1ml)
v	Sample volume	(0.1 ml)
VF	Dilution factor of cell extracts	
Def.:	1 unit converts 1 µmol substrate in the minute	

By means of division of the volume activity by the appropriate protein concentration in the cell extracts, one receives the specific enzyme activity (U/ mg protein).

3.2.12 Determination of growth behaviour and exponential growth rate

To determine the growth curve, the strains were precultured for 48 hours in 20 ml medium then were dissolved in 100 ml main cultures starting with the OD_{600} of about 0.15. Samples were taken after each 2 hours for measurering the OD.

Exponential growth rate (µ) was calculated as following formula (Schlegel 1972)

$$\mu = \frac{\ln_{xt} - \ln_{xo}}{(tx - to)}$$

to and tx : the beginning and the ending point of the exponential phase

$\ln_{xt}$: natural logarithm of the OD_{600} at the time tx

$\ln_{xo}$: natural logarithm of the OD_{600} at the time to

4. Results

4.1 Accumulation of intracellular L-G3P by engineering the glycerol biosynthetic pathway

4.1.1 Overexpresion of the *GPD1* gene leads to an increase of intracellular L-G3P levels in mutants lacking one or both of the two isoenzymes of glycerol-3-phosphatase

The important role of glycerol-3-phosphatase in glycerol biosynthesis and yeast cell physiology under different cultivation conditions was deeply studied by Påhlman *et al.* (2001). By investigation of *S. cerevisiae* W303 mutants lacking one or both of the two isoenzymes (*gpp1*Δ, *gpp2* Δ or *gpp1*Δ *gpp2*Δ, respectively), they have showed that the reduction of GPP activity had positive effects on the accumulation of intracellular L-G3P.

Besides, it is known from the previous work (Nevoigt and Stahl, 1996) that the enhancement of GPD activity by overproducing the major isoenzyme GPD1 was an effective approach to increase glycerol production in *S. cerevisiae*. This approach, therefore, was applied to check whether or not it can further increase the L-G3P pool in the strains whose GPP has been inhibited. To this aim, the multicopy plasmid YEpTKm^R*GPD1* was transformed into the strains *gpp1*Δ, *gpp1*Δ *gpp2*Δ and the isogenic wild type. Besides, the empty vector YEpTKm^R, the vector from which YEpTKm^R*GPD1* was constructed (Nevoigt and Stahl, 1996), was transformed into the above strains as control. YEpTKm^R is a high copy plasmid based on Yep-lac161 (Gietz and Sugino, 1988). The leucine marker (*LEU2*) in this plasmid allows the transformants to grow on the synthetic medium without leucine (YNB Glc/ leu$^-$). The reason for using the strains with the reference plasmid as a control instead of the untransformed strains was to enable the same growth conditions and a direct comparison with the strains bearing the plasmid for *GPD1* overexpression (YEpTKm^R*GPD1*).

Intracellular L-G3P in these engineered strains was extracted and determined (Fig. 10). The result showed an L-G3P level in the wild type at a very low level (< 0.2 mg/ g YDW). Overexpression of *GPD1* did not bring about a higher L-G3P level in this strain despite an obvious increase in GPD activity, which was observed in all three *GPD1* overexpression strains, in comparison to the wild type (data not shown). Consistent with the results of

Påhlman *et al.,* the same level of L-G3P as in the wild type was observed in strain *gpp1*Δ and this level was 15fold increased in the strain *gpp1*Δ *gpp2*Δ. However, it was shown that the additional overexpression of the *GPD1* gene could further increase the L-G3P levels in both strains *gpp1*Δ and *gpp1*Δ *gpp2*Δ by the factor of 2.8 and 1.7, respectively. Furthermore, it can be seen that among these six engineered strains, the strain *gpp1*Δ *gpp2*Δ expressing *GPD1* (*gpp1*Δ *gpp2*Δ + *GPD1*) had the best capacity to produce L-G3P with the level of ca. 26fold higher than in the wild type.

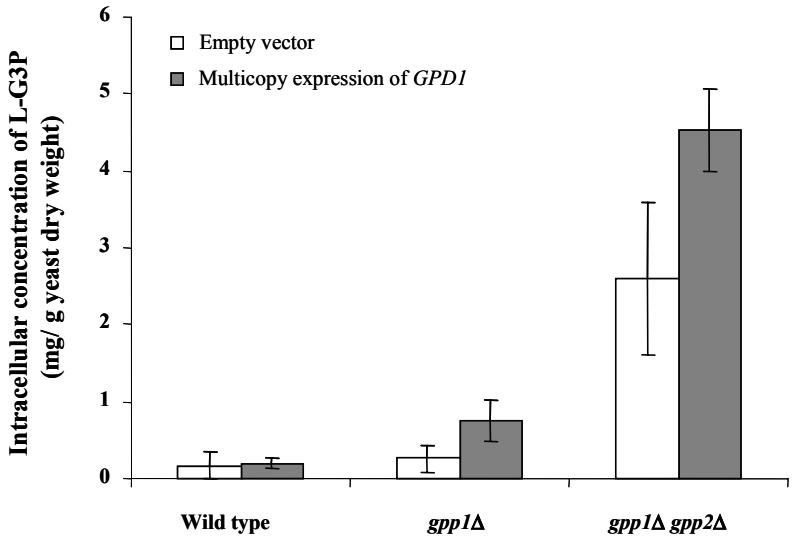


Fig. 10 Intracellular accumulation of L-G3P in wild type (W303-1A), *gpp1*Δ and *gpp1*Δ *gpp2*Δ mutants of *S. cerevisiae* transformed with either *GPD1* multicopy plasmid or the empty vector. Cells were grown in shake flasks, YNB (Glc/leu$^-$) medium and harvested for extraction of L-G3P at the mid-log phase. Values shown are the average values from three independent experiments, including standard deviations.

4.1.2 Influence of the genetic modifications on the growth of the engineered strains

To figure out whether the genetic changes have any influence on growth, growth curves of the engineered strains have been recorded. First, the growth behaviour of wild type and two strains *gpp1*Δ and *gpp1*Δ *gpp2*Δ with and without overexpressing *GPD1* was investigated. Both pre-cultures and main cultures were grown in YNB (Glc, leu$^-$) medium. As can be seen from Fig. 11, the *gpp1*Δ and *gpp1*Δ *gpp2*Δ mutants with the empty vector showed no obvious growth limitation in the comparison to the wild type also carrying the empty vector. In contrast, *GPD1* overexpression caused a slight decrease of growth in both the wild-type and *gpp1*Δ mutant strain. However, the growth of the strain *gpp1*Δ *gpp2*Δ + *GPD1* was strongly reduced. The lag phase was prolonged and the exponential growth rate was decreased to about 77% ($\mu \sim 0.20$) in comparison to the wild type ($\mu \sim 0.26$). In fact, this strain accumulated the highest amount of intracellular L-G3P when compared with the other five strains considered in Fig. 11.

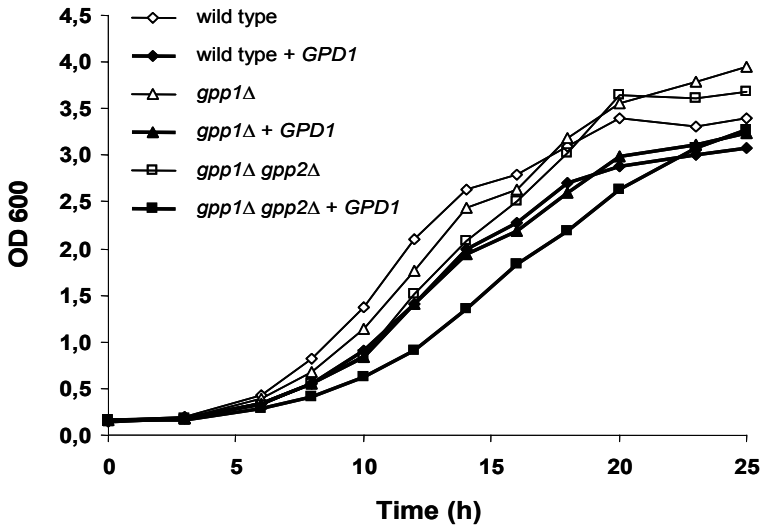


Fig. 11 Growth of the wild type (W303-1A), *gpp1*Δ single mutant (YA101) and *gpp1*Δ *gpp2*Δ double mutant (YA103) during shake-flask cultivation in YNB (Glc/leu$^-$) medium. The open symbols are referred to strains transformed with the empty vector. The closed symbols represent the same strains transformed with the *GPD1* multicopy plasmid.

4.1.3 Transfer of the *GPD1* multicopy expression vector into the triple mutant *gpp1*Δ *gpp2*Δ *gut2*Δ did not generate transformants

As mentioned in the section I.1.4, L-G3P can be converted to DHAP by the FAD-dependent mitochondrial glycerol 3-phosphate dehydrogenase which is encoded by the *GUT2* gene. It cannot be excluded that Gut2p causes a loss of accumulated L-G3P in the engineered yeast strains under the conditions used, i.e. in the presence of oxygen. To test if the deletion of *GUT2* can improve the level of L-G3P, the triple mutant *gpp1*Δ *gpp2*Δ *gut2*Δ (Påhlman *et al.,* 2001) was compared with the double mutant *gpp1*Δ *gpp2*Δ. However, as can be seen from Fig. 12, the strain *gpp1*Δ *gpp2*Δ *gut2*Δ did not show a visible increase in the level of intracellular L-G3P.

To check whether the increase of GPD activity could improve the L-G3P level in this *gpp1*Δ *gpp2*Δ *gut2*Δ mutant, we tried to overexpress the *GPD1* gene by transforming the multicopy plasmid YEpTKm^R*GPD1* into this triple mutant. Surprisingly, no transformant could be detected while the control experiment using the reference vector YEpTKm^R led to selectable transformants.

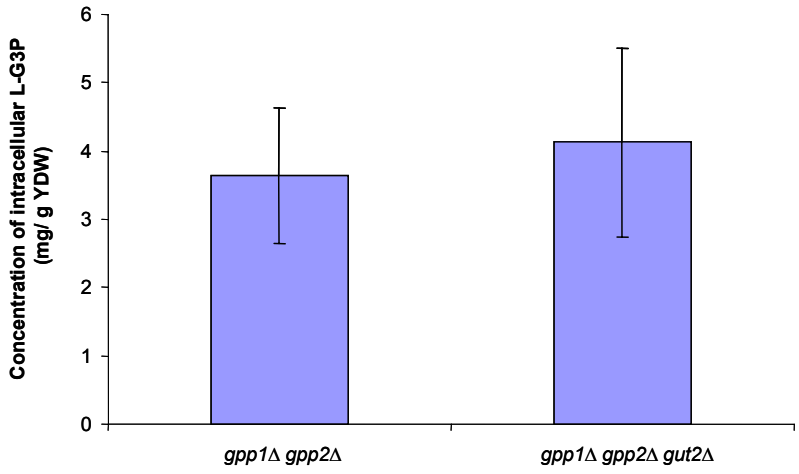


Fig. 12 Intracellular L-G3P accumulated in the strains *gpp1*Δ *gpp2*Δ and *gpp1*Δ *gpp2*Δ *gut2*Δ. Cells were grown in shake-flasks, YEPD medium and harvested at mid-log phase for extraction of L-G3P. Values shown are the average values from two independent experiments, including standard deviations.

4.2 Effects of osmotic stress and anaerobiosis on the accumulation of intracellular L-G3P in the strain *gpp1*Δ *gpp2*Δ + *GPD1*

Among the engineered strains investigated above, the strain *gpp1*Δ *gpp2*Δ + *GPD1* was the best L-G3P producer. Therefore, the next experiments will focus on this strain.

4.2.1 Osmotic stress led to an increase of GPD activity but did not cause a remarkable accumulation of intracellular L-G3P

Osmotic stress is well known to induce glycerol biosynthesis in *S. cerevisiae* mainly by increased transcription of *GPD1* but also by changes in the activity of other enzymes (for reviews see Nevoigt and Stahl, 1997; Blomberg, 2000; Hohmann, 2002). The strain *gpp1*Δ *gpp2*Δ + *GPD1,* which has high capacity to produce L-G3P, was focused to test whether the influence of osmotic stress could further increase the L-G3P pool. Osmotic stress conditions are often established by adding NaCl into the cultivation medium (Nevoigt and Stahl, 1997). It was reported that these conditions have negative effects on the growth of the *gpp1*Δ *gpp2*Δ double mutant (Påhlman *et al*., 2001). The growth of this strain was strongly reduced in the medium contained 0.5 M NaCl and was completely arrested at the concentration of 1M. However, there was no change in the accumulation of L-G3P by these two concentrations of NaCl. Therefore, the medium of 0.5 M NaCl was used in this experiment. In order to avoid the negative effect of salinity medium on growth, cells were first cultivated in non-salinity YNB (Glc/leu$^-$) medium until the mid-log phase then NaCl (0.5 M final concentration) was added into the cultivation medium. Cells were further incubated in salinity medium for 6 hours and harvested for extraction of L-G3P. Samples were taken at the same time for measuring of GPD activity. As expected, specific GPD activity was strongly induced by hyperosmotic stress (Table 4). Surprisingly, the increased GPD activity did not lead to a noticeable increase of L-G3P level.

4.2.2 Oxygen limited conditions increase the intracellular L-G3P level

As discussed extensively in the Literature Review (I.1.4) and Introduction Part (II.1.4), glycerol biosynthesis plays a crucial role in maintaining the redox balance in the cell under anaerobic conditions, and therefore is strongly enhanced.

In the following experiment, it was tested whether anaerobiosis is able to enhance the intracellular L-G3P concentration in the strain *gpp1*Δ *gpp2*Δ + *GPD1*.

As can be seen from Table 4, oxygen limitation caused an increase of about 60% of the intracellular L-G3P concentration despite a very slight increase in GPD activity in comparison to this under aerobic conditions. This small increase might be caused by the induction of the *GPD2* isogene which is known to be induced by anaerobiosis (Ansell *et al.*, 1997, Påhlman *et al.*, 2001) in contrast to *GPD1*, the abundant isogene in the multicopy transformant investigated here.

Table 4. Impact of hyperosmotic stress and oxygen limitation on specific activity of GPD and intracellular L-G3P accumulation in the *gpp1*Δ *gpp2*Δ mutant overexpressing *GPD1*. Cells were grown in shake-flasks, YNB (Glc/leu$^-$) until the cultures reached an OD_{600} of 0.8, switched to the conditions indicated in the table and incubated for 6 hours. Values shown are the average values from at least two independent experiments, including standard deviations.

Conditions of cultivation	Specific activity of GPD (U/ mg protein)	Intracellular concentration of L-G3P (mg/g YDW)
Reference[1)]	2.3 ± 0.8	4.5 ± 0.5
Hyperosmotic stress[2)]	9.0 ± 2.1	5.2 ± 0.7
Oxygen limitation[3)]	2.7 ± 0.3	7.2 ± 1.3

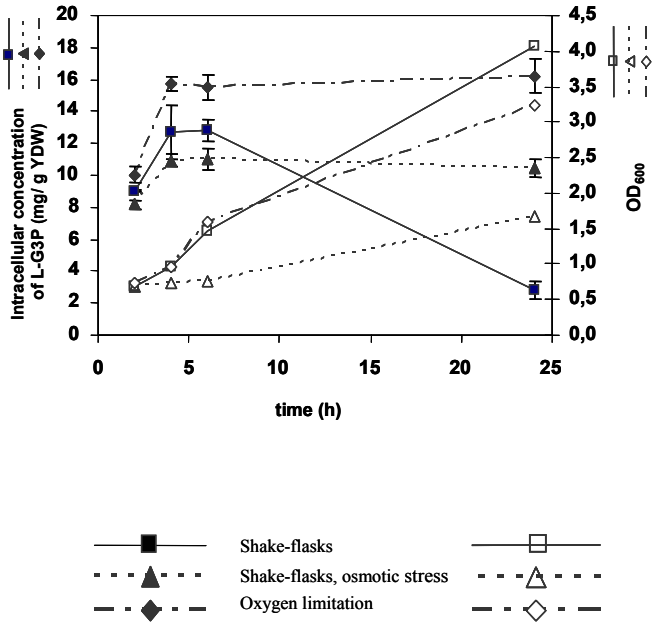
1) Glucose batch fermentation in shake flasks (aerobic conditions).
2) Glucose batch fermentation in shake flasks. Medium was supplemented with 0.5 M NaCl
3) Glucose batch fermentation under oxygen limitation was performed in Erlenmeyer flasks closed with air-locks which ensures the exclusion of oxygen but allowed the release of gases. The cultures were stirred continuously at 350 rpm.

4.2.3 Time-course of intracellular L-G3P level under osmotic stress and anaerobic conditions

To determine the change of L-G3P pool during cell growth, L-G3P was extracted and measured from cell cultures withdrawn after 2, 4, 6 and 24 hours of batch fermentation. This time-course of intracellular L-G3P level was recorded for three different conditions: osmotic stress (0.5 M NaCl), oxygen limitation and non-stress, aerobic conditions (as reference). Glucose concentration in the supernatant of these samples was also determined.

As demonstrated in Fig. 13A, the level of L-G3P increased between 2 and 4 hours of fermentation under aerobic conditions (shake-flask). The L-G3P level of about 13mg/g YDW stayed stable between 4 and 6 hours and dramatically dropped to about 3 mg/g YDW after 24 hours of the fermentation. In contrast, the level of L-G3P is stable after 6 hours of fermentation under both osmotic stress and oxygen limited conditions. However, as can be seen from Fig. 13B, cells grown in the salinity medium consumed glucose at a very low rate which can be partially explained by a slow growth in these conditions (Fig. 13A).

A



B

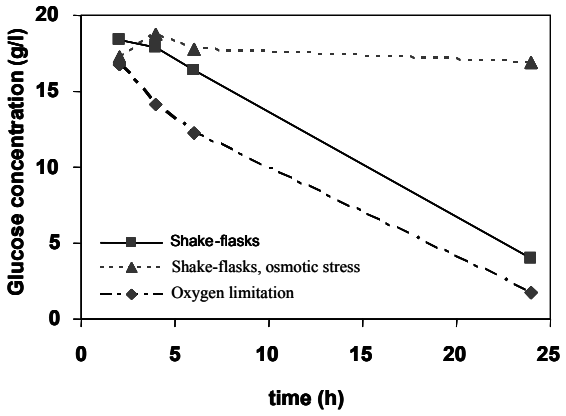


Fig. 13 Accumulation of L-G3P (A) and consumption of glucose (B) in the strain *gpp1*Δ *gpp2* Δ + *GPD1* during batch fermentation under different conditions. Cells were pre-grown in shake-flasks, YNB (Glc, leu$^-$) medium until cells reached mid-log phase (OD_{600} ~ 2), centrifuged, transferred to main cultures with fresh YNB (Glc, leu$^-$) medium. Main cultures with the start OD_{600} of 0.5 were incubated under conditions indicated in the legend of table 4.

4.3 Reduction of flux towards ethanol in L-G3P overproducing strains by simultaneously deleting the *PDC2* gene

Ethanol production is the main route of central carbon catabolism in *S. cerevisiae*. To redirect the carbon flux towards L-G3P production, one could think about the reduction of flux towards ethanol. To check this hypothesis, reduction of PDC, the key enzyme of alcoholic fermentation, was done by deleting the regulatory gene *PDC2*. Besides, a decrease in activity of PDC could provide more cytosolic NADH, the factor which was suggested from the results above (see II.2.2) to play an important role in L-G3P production, since it can minimize the competitive use of NADH through ADH (see I.1.4).

4.3.1 Construction of the triple mutant *gpp1*Δ *gpp2*Δ *pdc2*Δ

Deletion of the *PDC2* gene was done in the most productive of L-G3P accumulating strain *gpp1*Δ *gpp2*Δ + *GPD1*. To this aim, first we tried to delete directly the *PDC2* gene in the

*gpp1*Δ *gpp2*Δ mutant by using the *URA3* disruption cassette. However, no transformant was obtained from this experiment. The reason is likely that the growth of the *gpp1*Δ *gpp2*Δ *pdc2*Δ transformant on the selection minimal agar medium (YNB, ura$^-$) with ethanol and glycerol as carbon sources was so weak that the transfomants could not develop to detectable colonies. Therefore, we decided to follow another strategy: first, *PDC2* was deleted in the *gpp1*Δ single mutant (4.3.1.1). Second, the resulting *gpp1*Δ *pdc2*Δ mutant then was crossed with the *gpp1*Δ *gpp2*Δ double mutant thereby creating a diploid strain *gpp1*Δ/*gpp1*Δ *GPP2/ gpp2*Δ *PDC2/pdc2*Δ which was consequently used for isolating the haploid *gpp1*Δ *gpp2*Δ *pdc2*Δ strain after sporulation (4.3.1.2).

4.3.1.1 Deletion of the *PDC2* gene in the strain *gpp1*Δ

In fact, a *pdc2*Δ mutant already existed at the time when this work was started. This strain (YSH306) was kindly provided by the author (Hohmann *et al*., 1993). However, YSH306 has not the same genetic background as the other strains used in this study which are derivatives of W303-1A. Therefore, it was decided to not directly use the existing *pdc2*Δ mutant (YSH306) for the crossing experiment with *gpp1*Δ (YA101). Rather, the genomic DNA of this strain was used to amplify a *PDC2::TRP1* disruption cassette via PCR which was used to delete *PDC2* in the YA101 genetic background using the one-step gene disruption method (Rothstein, 1983). In the YSH306 strain, a 2.2 kb fragment of the *PDC2* coding region, starting with the ATG start codon, was replaced by a 1.4 kb *Bgl*II-*Sph*I fragment, cloned from vector YRp7 (Struhl *et al.*, 1979), carrying the *TRP1* gene (Fig. 14). PCR was carried out with primers P1 and P2 (see Materials and Methods) to amplify a 2.4 kb fragment cassette containing the 1.4 kb *TRP1* fragment with two 500 bp long homologous upstream and downstream flanking sequences (Fig. 14).

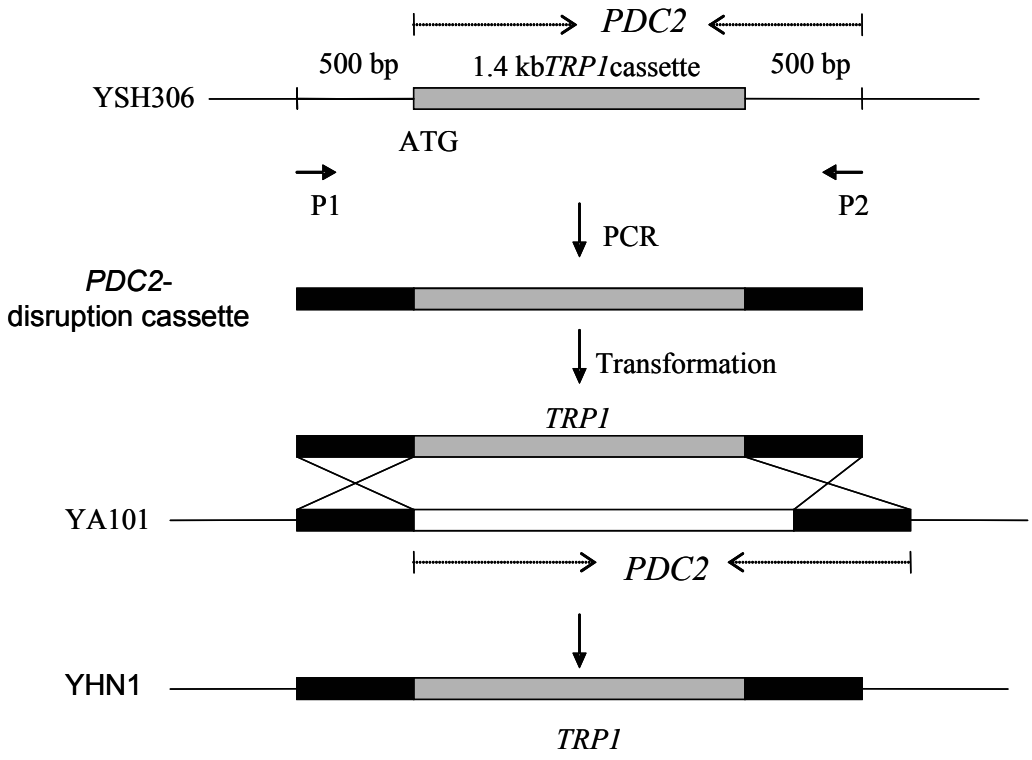


Fig. 14 Schematic presentation of the *PDC2* deletion in the *gpp1*Δ mutant by integration of *PDC2::TRP1* disruption cassette isolated from *pdc2*Δ strain YSH306.

The PCR product was introduced into the strain *gpp1*Δ (YA101) by electroporation. Transformants carrying the *TRP1* disruption cassette were selected on YNB agar medium supplemented with 2% ethanol and 2% glycerol as well as required amino acids and nucleic bases but lacking tryptophan. The reason to use EtOH/glycerol instead of glucose medium is that pdc- mutants cannot grow on defined medium with glucose as sole carbon source but with C_2 carbon substrates like ethanol or acetate (Pronk *et al*., 1996, Flikweert *et al*., 1996). In addition, growing the pdc2 mutant on ethanol medium can avoid the generation of revertants which often obtained by using glucose medium (Velmurugant *et al.,* 1997).

Seven randomly selected transformants were transferred to a new agar plate for further investigation. The deletion of *PDC2* in these transformants was confirmed by diagnostic PCR.

The primers P3 and P4 (see Materials and Methods) which were located 150bp upstream and downstream outside the *PDC2::TRP1* disruption cassette amplify a 2.7 kb DNA fragment in the positive transformants. In contrast, the untransformed strain *gpp1*Δ (YA101) led to an amplification of a 3.5 kb fragment in length (control). Fig. 15 revealed that among 7 clones checked, only 2, i.e. clone number 3 and 4 (lanes 4 and 5), were the correct *gpp1*Δ *pdc2*Δ transformants.

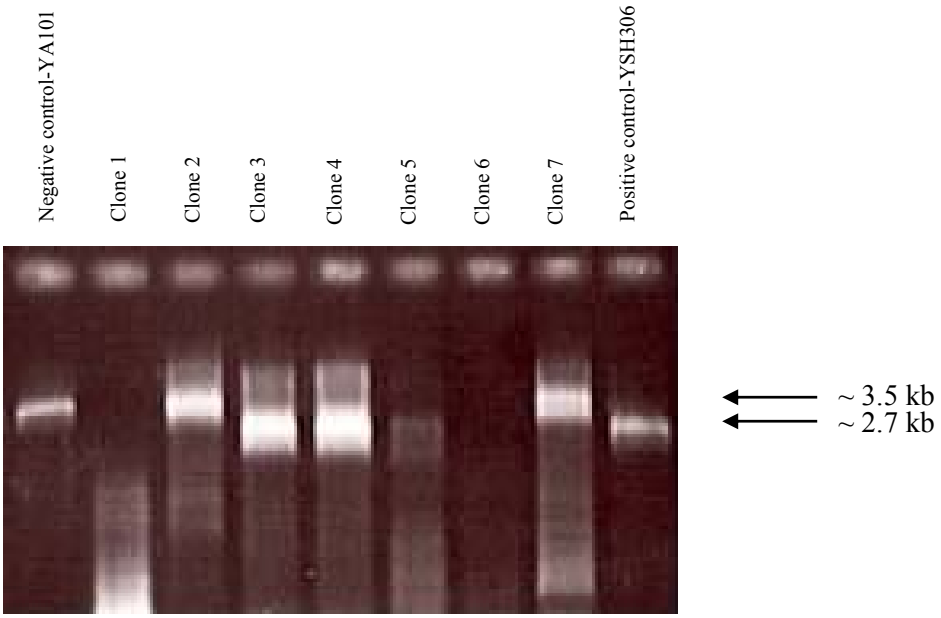


Fig. 15 Diagnostic PCR for checking the correct *gpp1*Δ *pdc2*Δ disruption strains in the selected transformants

The specific activity of PDC in these two clones was measured. It was shown that PDC activities in these clones reduced to about 30-40% in comparison to the reference strain (*gpp1*Δ) (Fig. 16), confirming once more the correct *PDC2* disruption. Clone number 3 (named YNH1) with lower PDC activity was chosen for further experiments.

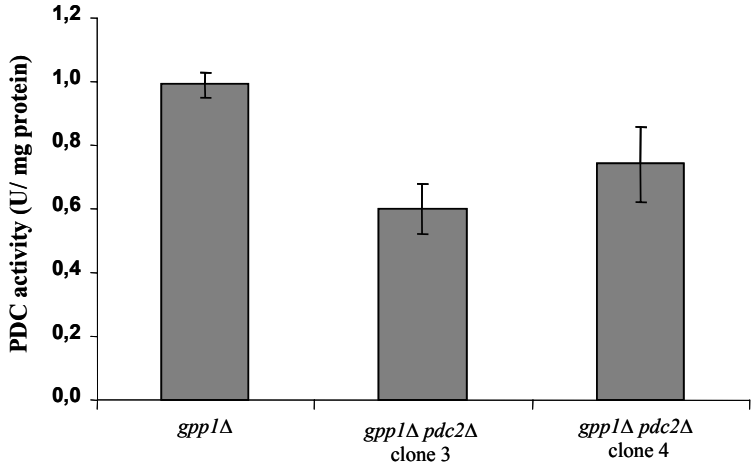


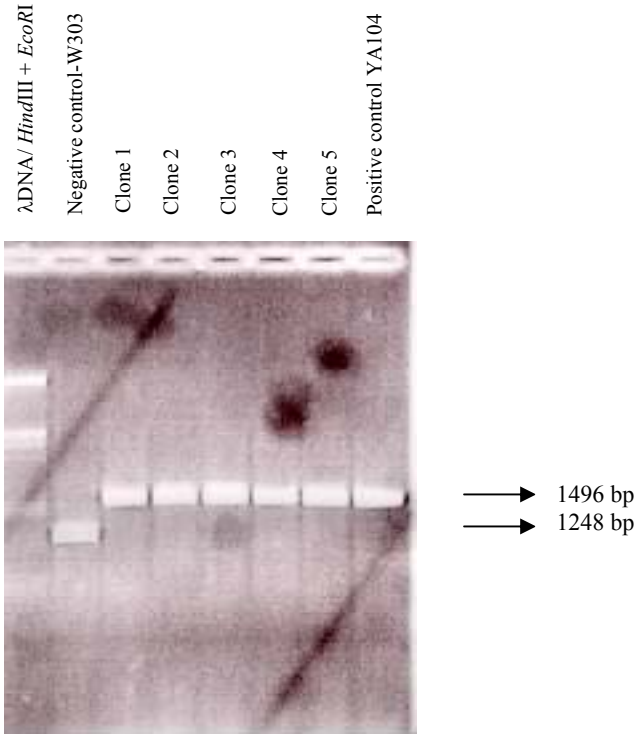
Fig. 16 PDC activity in the strain *gpp1*Δ and in the two selected tranformants *gpp1*Δ *pdc2*Δ carrying the *PDC2::TRP1* diruption cassette (clone 3 and 4)

4.3.1.2 Crossing, sporulating and spore analysis for construction of the triple mutant *gpp1*Δ *gpp2*Δ *pdc2*Δ

To obtain the triple mutant *gpp1*Δ *gpp2*Δ *pdc2*Δ (YNH2), the strain *gpp1*Δ *pdc2*Δ (YNH1, 3.1.1) was crossed with the strain *gpp1*Δ *gpp2*Δ (YA103). The mating was carried out as described in the Materials and Methods section (3.2.6). The resulting diploid strain (*gpp1*Δ/*gpp1*Δ *GPP2*/*gpp2*Δ *PDC2*/*pdc2*Δ) was sporulated and the spore mixture was spread on the minimal medium. Since *GPP2* and *PDC2* were deleted by the *HIS3* and *TRP1* disruption cassettes, respectively, in order to select the correct *gpp1*Δ *gpp2*Δ *pdc2*Δ strain, histidine and tryptophan were not included into this selection medium. However, the colonies which survived on this selective medium could split up in two groups. One group contained representatives of vegetative diploid cells which did not sporulate because they contained wild-type alleles of *GPP2* and *PDC2*. The other group contained the haploid strain *gpp1*Δ *gpp2*Δ *pdc2*Δ . To select the desired haploid strain, seven

colonies from the selective plate were checked for their haploid status (see Materials and Methods 3.2.6). The test showed that five of them were the desired colonies. Nevertheless, to avoid working with revertants and to confirm correct deletions of *PDC2* and *GPP2*, diagnostic PCRs were done (Fig. 17). The set of primer P3 and P4 (see 3.1.6) was used to check the *PDC2* deletion. The primer pair P5-P6 (see 3.1.6) was designed to check the *GPP2* deletion. A DNA fragment of 1284 bp containing the 1139 bp *HIS3* disruption cassette (Påhlman *et al.,* 2001) and 75 bp upstream and 70 bp downstream flanking sequences was amplified. A 1496 bp DNA fragment was expected when using the genomic DNA of the corresponding wild type W303 as a template. All five strains selected before were shown to carry both mutations. Cells of clone 1 were used for further experiments.

A



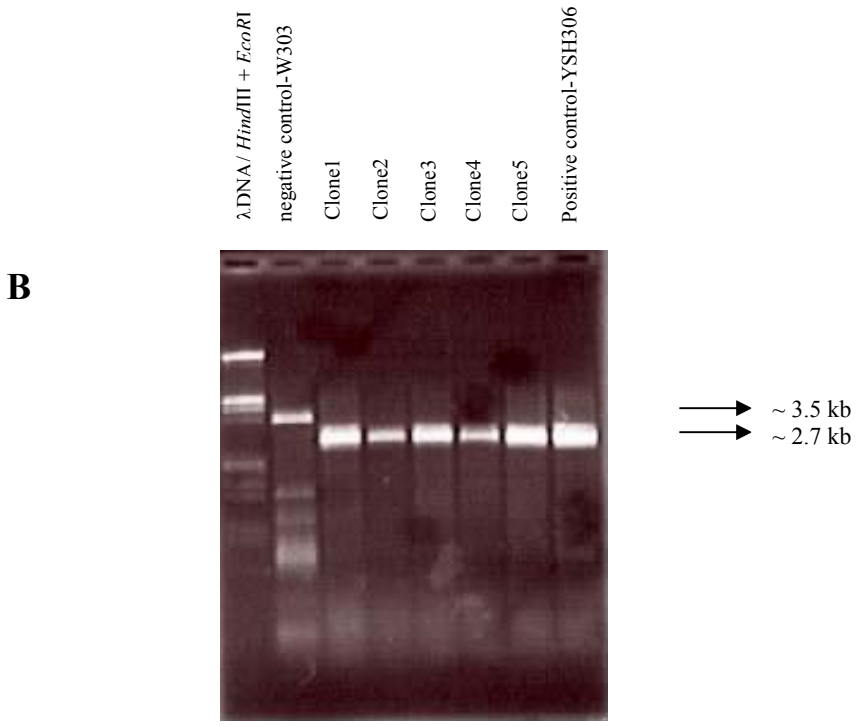


Fig. 17 Diagnostic PCRs to confirm the genotype *gpp1*Δ *gpp2*Δ *pdc2*Δ of the phenotypically selected clones. A. PCR to check *GPP2* disruption (Primer 5 and 6); **B.** PCR to check *PDC2* disruption (Primer 3 and 4).

4.3.2 Overexpression of *GPD1* gene in the triple mutant *gpp1*Δ *gpp*2Δ *pdc2*Δ

The next step was to overexpress *GPD1* in the obtained triple mutant *gpp1*Δ *gpp*2Δ *pdc2*Δ. Transformations were done with both YEpTKm^R*GPD1* and the reference plasmid YEpTKm^R. The transformants were selected on the YNB (EtOH/ Glycerol, leu$^-$). It was known that *S. cerevisiae* grows very slowly on minimal medium with EtOH/Glycerol as carbon sources. However, to avoid the development of *PDC2* revertants, this medium has been used. In fact, it took 15 days of incubation to detect the transformants of the *gpp1*Δ *gpp*2Δ *pdc2*Δ mutant overexpressing *GPD1*.

Transferring the transformants directly from agar plates into YNB (EtOH/ Glycerol, leu$^-$) liquid medium led to a non-detectable growth. Therefore, to propagate the transformants, they

were grown in YEPEG medium. This medium was supplemented with G418. It should be mentioned that besides the *LEU2* marker, the YEpTKm^R plasmid carries another marker, a kanamycin (Km) resistance gene which was set under the control of the *TEF1*-promoter to ensure the high transcription levels of the gene. The transformations bearing this gene enable the cells to resist to G418 (Lang-Hinrichs *et al*., 1989).

In fact, G418 (200 µg/ml) was added to the medium to select the plasmid. However, it should be mentioned that the strain *gpp1*Δ *gpp*2Δ *pdc2*Δ carried one copy of the Km resistance gene in the *GPP1* disruption cassette (Påhlman *et al.,* 2001). It was not clear whether G418 acted as a selection marker. Hence, using this marker is a compromise solution. When the concentration of the transformants was high enough (OD_{600} ~ 5), 200 µl of cell culture were transferred back into 20 ml of selective pressure medium for cells containing plasmid (YNB with EtOH/Glycerol, leu$^-$). Cells were able to grow under this condition, however, at a low rate, especially the strain *gpp1*Δ *gpp*2Δ *pdc2*Δ overexpressing *GPD1*.

4.3.3 Accumulation of L-G3P in the *gpp1*Δ *gpp*2Δ *pdc2*Δ mutant with and without overexpression of *GPD1*

To investigate the impact of *PDC2* deletion on L-G3P accumulation, the *gpp1*Δ *gpp2*Δ double and the *gpp1*Δ *gpp2*Δ *pdc2*Δ triple mutant with and without overexpression of *GPD1* were pre-grown in medium containing non-fermentable carbon sources (YEPEG) supplemented with G418 in order to enable growth of the *pdc2*Δ deletion mutants. Subsequently, batch fermentations were initiated in minimal glucose medium under oxygen limitation, the condition which caused the highest accumulation of L-G3P so far recognized in this study (4.2.2). After six hours of fermentation, samples were taken for the extraction and measurement of intracellular L-G3P. Results shown in Fig. 18 demonstrated that, an additional deletion of *PDC2* caused an increase of the L-G3P concentration by the factors of 1.5 and 2.0 in the *gpp1*Δ *gpp2*Δ mutant background with and without overexpression of *GPD1*, respectively.

The level of L-G3P achieved in the strain *gpp1*Δ *gpp2*Δ *pdc2*Δ overexpressing *GPD1* (~ 17 mg/g YDW) was the highest level accumulated by the engineered strains studied here and was about 100 times higher than that in the isogenic wild type (see Fig. 10). Interestingly, no significant raise of intracellular L-G3P level by *PDC2* deletion was observed under aerobic conditions (data not shown).

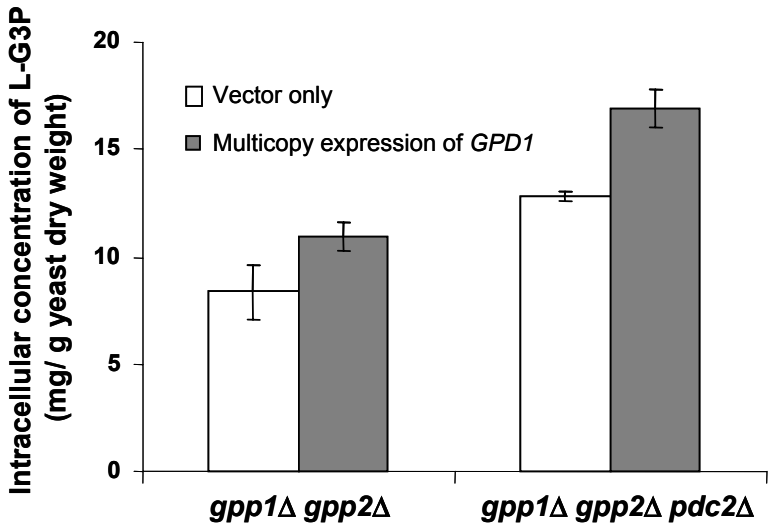


Fig. 18 Intracellular accumulation of L-G3P in the strains *gpp1*Δ *gpp2*Δ and *gpp1*Δ *gpp2*Δ *pdc2*Δ transformed with either *GPD1* overexpression plasmid or the reference plasmid. Cells were pregrown in YEPEG (G418) medium until mid-log phase, centrifuged and transferred to fresh YNB (Glc/ leu$^-$) medium with the oxygen limited conditions. Starting OD_{600}s of the main cultures were 0.6-0.8*. Samples were taken after 6 hours incubation in glucose medium for extraction and measurement of L-G3P. Values shown are the average values from two independent experiments, including standard deviations.

*It should be mentioned that growth of the strain *gpp1*Δ *gpp2*Δ *pdc*2Δ overexpressing *GPD1* was not detected when the main culture was started at a low OD_{600} (ca. 0.3).

4.3.4 Growth of the *gpp1*Δ *gpp2*Δ *pdc*2Δ background strains was severely reduced

The effect of the additional deletion of *PDC2* on growth was also investigated. The double mutant *gpp1*Δ *gpp2*Δ was used as control. Cells were pregrown on YEPEG medium until exponential phase and transferred into YNB (Glc) medium supplemented with leucine for recording the growth curve. As can be seen from Fig. 19, growth of the triple mutant *gpp1*Δ *gpp2*Δ *pdc*2Δ in glucose medium was severely inhibited in comparison to the double mutant *gpp1*Δ *gpp2*Δ. Calculations of exponential growth rate showed that the growth of the strain *gpp1*Δ *gpp2*Δ *pdc*2Δ reduced to ca. 17% in comparison to the strain *gpp1*Δ *gpp2*Δ. The growth of the strain *gpp1*Δ *gpp2*Δ *pdc*2Δ + *GPD1* was completely arrested in the minimal medium if the value of the starting OD_{600} was small (see legend of Fig. 18)

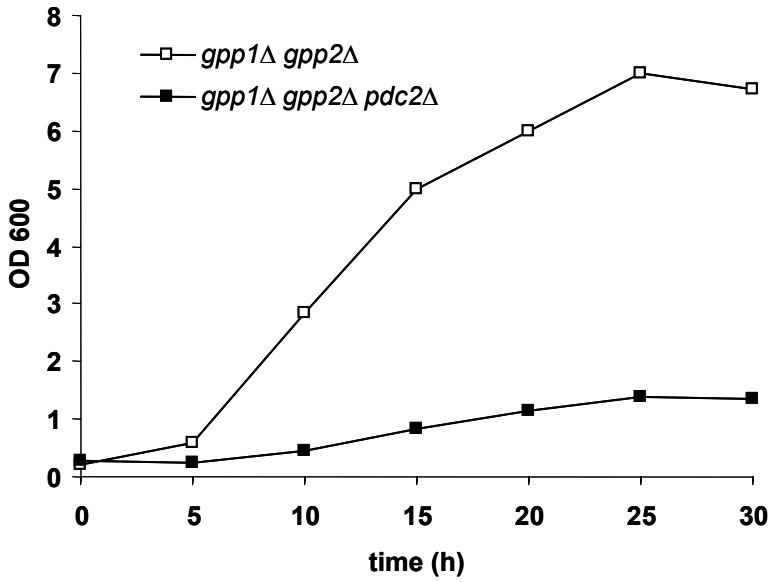


Fig. 19 Growths of the strains *gpp1*Δ *gpp2*Δ and *gpp1*Δ *gpp2*Δ *pdc2*Δ. Cells were pre-grown in shake-flasks, YEPEG medium until mid-log phase, then transferred to YNB (Glc, leu^+) with an initial OD_{600} of 0.2 for recording the growth curves.

4.4 L-G3P was detected in the supernatant of the culture

As shown above, intracellular L-G3P was accumulated in the engineered strains to high levels. The question arose, whether some L-G3P was released into the cultivation medium. To clarify this question, the concentration of L-G3P in the supernatant of a *gpp1*Δ *gpp2*Δ + *GPD1* culture was measured. The cultivation conditions used correspond to those used in the experiment described in section 4.2.3. Indeed, some extracellular L-G3P was first detected after 6 hours (0.5 mg/l). The level increased up to 20 mg/l when measured after 24 hours of fermentation (Fig. 20).

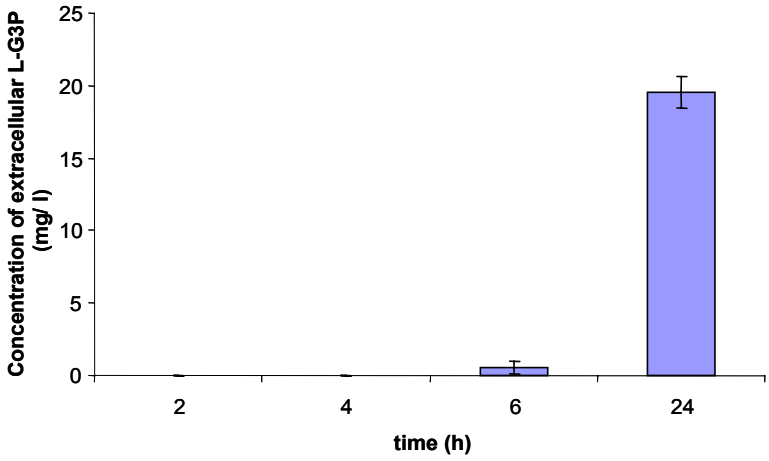


Fig. 20 Concentration of the extracellular L-G3P measured from culture supernatant of *gpp1*Δ *gpp2*Δ + *GPD1* strain. Cells were pre-grown in shake-flasks, YNB (Glc, leu$^-$) medium until cells reached mid-log phase, centrifuged, transferred to main cultures with fresh YNB (Glc, leu$^-$) medium. Main cultures with the start OD_{600} of 0.5 were incubated under oxygen limited conditions as indicated in the legend of table 4.

A rough calculation has been done to compare the intracellular and extracellular concentration of L-G3P (Table 5). Data of L-G3P concentrations measured after 24 hour of fermentation were taken into account. The results showed that the concentration of intracellular L-G3P was ca. 208 times higher than the extracellular L-G3P concentration.

Table 5. Intracellular and extracellular L-G3P of the strain *gpp1*Δ *gpp2*Δ + *GPD1* measured after 24 hours of fermentation. Growth conditions were indicated in the legend of Fig.20. Calculations were done based on the fact that:

* Yeast dry weight occupies 20% of yeast wet weight. In other words, 1 g YDW is equivalent with 4 g or 4 ml liquid (rough calculation).

** 1litre of culture contains ca. 0.8 g YDW.

L-G3P	Concentration measured	Concentration calculated in mM*	Amount calculated in mg/ l cell culture**
Intracellular	16.2 mg/ g YDW ± 1.0	23.6	**13**
Extracellular	19.5 mg/ l ± 1.1	0.1	**13**
Sum			**26**

However, when the total amount of intra- and extracellular L-G3P was taken into account, it can be clearly seen that the amount of L-G3P in the supernatant of cultivation medium was the same as the amount of L-G3P accumulated inside the cells (Table 5). In other words, half of the L-G3P produced was released into the medium by an unknown mechanism.

5. Discussion

While the production of glycerol by microbial fermentation using *S. cerevisiae* and other microorganisms has been extensively studied and improved for a long time (for review, see Wang *et al*., 2001), the metabolic precursor of glycerol, L-G3P, has been exclusively produced by chemical or enzymatic synthesis so far. This work aimed at the development of a cheap and environmentally sustainable production method for L-G3P using bakers' yeast as a whole-cell biocatalyst and sugar-containing media as starting material.

Accumulation of intracellular L-G3P in *S. cerevisiae* by metabolic engineering can take advantage from former approaches to enhance glycerol production which are mostly based on genetic modifications of enzymes directly or indirectly influencing the glycerol biosynthetic pathway (for details, see Introduction section II.1.4). In this thesis, a few of these approaches of genetic engineering were combined with the deletion of glycerol-3-phosphatases to accumulate L-G3P in *S. cerevisiae*. It was shown that overexpression of the *GPD1* gene in a strain completely lacking GPP activity (*gpp1*Δ *gpp2*Δ) leads to a remarkable increase in the L-G3P concentration in comparison to the wild type. Oxygen limited conditions, but not osmotic stress, strongly enhanced the production of L-G3P. The additional deletion of the *PDC2* gene could further improve the level of L-G3P in the modified yeast. Unfortunately, the latter genetic modification had a severe negative impact on growth.

An unexpected result was that the intracellularly accumulated L-G3P was partly released into the cultivation medium. However, the concentration of extracellular L-G3P was much lower than the concentration of intracellular L-G3P.

5.1 Accumulation of intracellular L-G3P by genetic modifications of enzymes directly involved in its metabolism

The strains *gpp1*Δ and *gpp1*Δ *gpp2*Δ have been constructed by Påhlman *et al.* (2001) who studied the biological role of these two isoenzymes in glycerol biosynthesis of *S. cerevisiae*. The deletion of *GPP1* alone caused a 75% decrease in GPP activity and no activity at all was

detected when both *GPP1* and *GPP2* were simultaneously disrupted. An accumulation of L-G3P by factors of about 2 and 20 in comparison to the isogenic wild type was observed in the *gpp1*Δ and *gpp1*Δ *gpp2*Δ strains, respectively (Påhlman *et al.,* 2001). These results demonstrated that complete inhibition of GPP activity is necessary to accumulate intracellular L-G3P to a noteworthy extent.

To address the question of whether an increased activity of GPD can further increase the intracellular L-G3P level in these mutants, they were transformed with the multicopy plasmid carrying the *GPD1* gene. Intracellular L-G3P levels measured in the strains *gpp1*Δ and *gpp1*Δ *gpp2*Δ without *GPD1* overexpression, i.e. strains carrying the reference plasmid (Fig. 10), were consistent with the results of Påhlman *et al*. However, the levels of L-G3P in both *gpp1*Δ single and *gpp1*Δ *gpp2*Δ double mutant could be significantly improved by multicopy expression of *GPD1*. This implies that the increase of GPD activity effectively enhanced the production of L-G3P. This finding clearly proved once more the rate-limiting role of GPD in the glycerol biosynthetic pathway of *S. cerevisiae* (Nevoigt and Stahl, 1996; Michnick *et al*., 1997; Remize *et al*., 2001; Nevoigt *et al.*, 2002).

No accumulation of L-G3P could be detected in the wild type overexpressing *GPD1*, indicating that wild-type GPP activity is sufficient to metabolise surplus L-G3P resulting from increased GPD activity. This observation matches the finding of Remize *et al.* (2001), that GPP activity is not rate limiting for glycerol production in *S. cerevisiae*.

The mitochondrial GPD, which is encoded by *GUT2* gene, catalyses the aerobic conversion of L-G3P back to DHAP. However, the deletion of *GUT2* in the mutant *gpp1*Δ *gpp2* did not cause an increase in the level of the intracellular L-G3P under aerobic conditions (Fig. 12). In fact, *GUT2* is repressed by glucose at least when the concentration of is higher than 0.2 % (Grauslund and Rønnow, 2000). This enzyme is activated only when glucose is depleted or when cells grow on other carbon sources, e.g. ethanol, glycerol, or lactate. Therefore, the result obtained here seems reasonable because L-G3P was extracted from cells harvested at the mid-log phase, i.e. when a non-limiting concentration of glucose was still in the medium. However, if the deletion of *GUT2* does not contribute to the accumulation of intracellular L-G3P under these experimental settings, it remains unclear why

no transformant was obtained when *GPD1* was overexpressed in the strain *gpp1*Δ *gpp2*Δ *gut2*Δ, while the control experiment with the reference plasmid (without *GPD1* gene) led to detectable transformants.

A negative correlation between L-G3P accumulation and the growth rate was observed in the engineered strains (Fig. 11). Påhlman *et al*. (2001) also observed a severe growth defect of the strain *gpp1*Δ *gpp2*Δ under anaerobic conditions. These cells accumulated a high level of intracellular L-G3P. Thus, the authors have added acetaldehyde, the electron acceptor for the reoxidation of glycolytic NADH, to the cell culture. The observed correlation between the decrease of about 50% in L-G3P level and the resumed growth caused by the addition of acetaldehyde led to the assumption that the high accumulation of intracellular L-G3P is responsible for the growth inhibition of strain *gpp1*Δ *gpp2*Δ.

However, the increase of intracellular L-G3P pool cannot be the only reason for the decreased growth rate of the engineered strains constructed in this work: the *gpp1*Δ *gpp2*Δ double deletion strain showed a considerably higher intracellular accumulation of L-G3P, but a similar growth rate and a higher biomass formation than the *gpp1*Δ single deletion strain overexpressing *GPD1*. In fact, there is an additional negative impact of *GPD1* overexpression on the growth of all strains investigated (Fig. 11). This result can be explained by a net loss of ATP (about 50%) when the production of glycerol from glucose, an ATP-consuming process, is improved by the overexpression of *GPD1* (Nevoigt and Stahl, 1996).

Moreover, a build up of DHAP could be a consequence of the accumulation of intracellular L-G3P. DHAP in turn could lead to an increase of methylglyoxal, a cytotoxic compound, that at millimolar concentration, arrests cell growth by reacting with various biological compounds, such as proteins, DNA and RNA, and inactivating them (Inoue *et al*., 1998; Zhu *et al*., 2001; Martin *et al.,* 2001). This could also be an explanation for the reduction of growth.

In conclusion, the strain *gpp1*Δ *gpp2*Δ overexpressing *GPD1* was the best L-G3P overproducing strain among the engineered strains investigated here, which were modified in

genes coding for cytosolic GPD and mitochondrial GPD as well as GPP, the enzymes directly involved in the metabolism of L-G3P.

5.2 Minor effects of the intracellular L-G3P accumulation on lipid synthesis

As L-G3P is a key intermediate in the biosynthesis of all glycerolipids (Fig. 9), accumulation of triacylglycerol (TAG) and/or alterations in the phospholipid pattern caused by an increased intracellular L-G3P pool was expected. To check this, the pattern of neutral lipids, i.e. TAG, and glycerophospholipids has been recoreded in the *gpp1*Δ *gpp2*Δ double mutant strain overexpressing *GPD1* and the isogenic wild type bearing the empty vector. These experiments have been carried out by Dr. Athenstaedt at the Technische Universität Graz, Institut für Biochemie.

The results did not show differences concerning the concentration of TAG. This is probably due to the lack of a concomitant excess of fatty acids, the second component needed for TAG synthesis. Påhlmann *et al.* (2001) assumed that despite the accumulation of L-G3P in *S. cerevisiae* strains deleted in *GPP1* and *GPP2*, the flux of this compound towards glycerophospholipids is rather low. However, this hypothesis has never been experimentally proven before. Indeed, the analysis of the phospholipid patterns in wild type and the *gpp1*Δ *gpp2*Δ mutant overexpressing *GPD1* demonstrates that accumulation of L-G3P has only a slight, but significant, influence on cardiolipin in *gpp1*Δ *gpp2*Δ double mutant strain overexpressing *GPD1* (5.8 $\pm$ 1.1 % of total phospholipids) compared to the wild type (4.2 $\pm$ 0.7 % of total phospholipids). Interestingly, both cardiolipin as well as the enzymes responsible for its biosynthesis are only found in mitochondrial membranes. In fact, there is a specific acyltransferase in yeast mitochondria using DHAP as a substrate to synthesise phosphatidic acid, the precursor of all phospholipids (Athenstaedt *et al.*, 1999). Furthermore, the inner mitochondrial membrane is the site where the FAD dependent glycerol 3-phosphate dehydrogenase (Gut2p) is located (Janssen *et al.*, 2002), which probably oxidises the accumulated L-G3P to DHAP (Fig. 9), directly providing the starting substrate for the mitochondrial cardiolipin synthesis. Furthermore, the synthesis of cardiolipin, in contrast to

all other glycerophospholipids, consumes three molecules of L-G3P instead of only one and therefore is more sensitive to the increased level of L-G3P.

It can be concluded from these findings that lipid biosynthesis is not influenced by the accumulation of the important precursor L-G3P. Hence, it seems to be not reasonable to engineer the glycerol-3-phosphat acyltransferase for further improving the L-G3P level.

5.3 Different cultivation conditions are hints for the limiting factors of the L-G3P production by the strain *gpp1*Δ *gpp2*Δ overexpressing *GPD1*

It is well known that both osmotic stress and oxygen limitation lead to a strong enhancement of glycerol biosynthesis in wild-type cells of *S. cerevisiae* because of different reasons (see introduction II.1.4). Therefore, it was assumed that these conditions could also enhance the accumulation of L-G3P in strains which show no activity of glycerol-3-phosphatases. Experiments were carried out using the strain *gpp1*Δ *gpp2*Δ overexpressing *GPD1*.

Osmotic stress was applied by adding 0.5 M NaCl to the growth medium. As expected, these conditions caused a marked increase of the already high GPD activity in the *GPD1* multicopy transformant (see Introduction II.1.3). In fact, the plasmid used in this study for overexpression of *GPD1* contains the natural promoter of *GPD1,* known to be induced by hyperosmotic stress (Ansel *et al.,* 1997, Påhlman *et al.,* 2001). Surprisingly, the pool of L-G3P was nearly unchanged despite a 4fold higher GPD activity under these conditions (Table 4). This implied that other factors apart from specific GPD activity hampered further increase of the L-G3P concentration. In fact, the activity of GPD was more than 3fold lower under oxygen limited conditions in comparison to osmotic stress. However, the concentration of L-G3P was raised 1.4fold under oxygen limited conditions. Under aerobic conditions, cytosolic NADH can be reoxidised by external NADH dehydrogenases and the L-G3P/ DHAP shuttle, both mechanisms are coupled to the respiratory chain (Fig. 9). These mechanisms do not work under anaerobic conditions and thus, NADH must be consumed by GPD to maintain the redox balance. Production of L-G3P thereby was enhanced. This result

proposed that the availability of NADH, the cofactor of GPD, seems to be an important factor for the synthesis of L-G3P in the strain *gpp1*Δ *gpp2*Δ overexpressing *GPD1*.

Time-courses of intracellular L-G3P level under osmotic stress, oxygen limited and reference (aerobic, non-stress) conditions were also recorded. Intracellular L-G3P was accumulated at highest level after 6 hours of fermentation under all three conditions. Furthermore, the L-G3P pool was strongly improved under all three conditions tested (2.9fold under aerobic, non-stress conditions and 2.1fold under osmotic stress and under oxygen limited conditions) in comparison to the first set of experiments (see Table 4 and Fig. 13A). However, the accumulated L-G3P under osmotic stress conditions was lower than under aerobic conditions, which is contradictory with the former results (Table 4). In fact, the cultivation conditions of these two sets of experiments were different (see legends of Table 5 and Fig. 13). A comparison of the results suggests that cells at logarithmic phase could probably be more potential to produce L-G3P.

Under osmotic stress and oxygen limited conditions, the level of L-G3P stayed stable after 6 hours of fermentation. It seems that the accumulation of L-G3P in this strain, under these conditions, was limited at these indicated levels. In fact, product inhibition of GPD, a process which happens with most of the enzymatic reactions, might play a role in regulating the level of intracellular L-G3P. In this process, the product L-G3P can bind to the active site of the GPD and compete with the substrate DHAP. The reaction, therefore, is reversible to DHAP when intracellular L-G3P concentration is high.

Surprisingly, under aerobic conditions, the level of L-G3P measured after 24 hours fermentation was markedly lower than after 6 hours. Consistent with this result, a sharp decrease of the accumulated L-G3P level in a culture of the same strain (*gpp1*Δ *gpp2*Δ + *GPD1*) was also observed when oxygen entry was allowed after 23 hours of anaerobic fermentation (Nevoigt, personal communication). These results suggest that the presence of oxygen caused a loss of L-G3P at the later phase of fermentation. The question arose: what happens with the accumulated L-G3P in the *gpp1*Δ *gpp2*Δ background strain when oxygen is present? It can be excluded that an increased synthesis of lipids is a main reason since nearly no difference in lipids pattern was observed in the aerobic culture of the strain *gpp1*Δ

*gpp2*Δ + *GPD1* in comparison to the wild type (5.2). The most plausible explanation is as follows: L-G3P can be converted to DHAP and further towards ethanol production. This explanation seems reasonable because the reversible reaction between DHAP and L-G3P could probably occur in the direction to DHAP when L-G3P is available and the cytosolic NADH needed for its production is depleted due to the preferable reoxidation by mechanisms coupled to the respiratory chain, e.g. external NADH dehydrogenase and L-G3P shuttle.

5.4 Decrease of the PDC activity by deleting the *PDC2* gene improved further the intracellular L-G3P concentration

The next step to increase the intracellular L-G3P level by genetic engineering was to reroute the carbon flux towards L-G3P production by preventing alcoholic fermentation, the main route of carbon metabolism. The activity of PDC, one of the two key enzymes of alcoholic fermentation catalysing the reduction of pyruvate to acetaldehyde, was reduced. The reduction of this enzyme could also provide more cytosolic NADH for the production of L-G3P (see Introduction II.1.4). To this aim, *PDC2* deletion was combined with the double deletion of *GPP1/GPP2,* resulting in triple mutant *gpp1*Δ *gpp2*Δ *pdc2*Δ. Afterwards, *GPD1* was overexpressed in this recombinant strain.

The disruption of *PDC2* causes a diminished transcription of the two structural genes *PDC1* and *PDC5,* thus, leading to a reduction in PDC activity (Raghuram *et al*., 1994). Values between 10-25% of wild type PDC activity have been observed in the YSH306 *pdc2*Δ mutant (Hohmann, 1993). However, the PDC activity measured in the *gpp1*Δ *pdc2*Δ mutant was about 60% of that in the strain *gpp1*Δ. This result could be due to the differences in genetic background between the YSH306 *pdc2*Δ strain and the strain used in this experiment (W303-1A). In fact, differences in protein expression between yeast strains with different genetic backgrounds have been recently confirmed by proteome analysis (Rogowska-Wrzesinska *et al.,* 2001). In addition, differences in cultivation conditions could also partially be responsible for the higher residual activity in our *gpp1*Δ *pdc2*Δ mutant. In this work, cells were pre-grown in complex medium with ethanol and glycerol, before glucose induction for 4

hours was carried out for measuring PDC activity, while Hohmann used ethanol alone for preculturing.

Nevertheless, the deletion of *PDC2* could indeed increase the L-G3P level in the *gpp1*Δ *gpp2*Δ background strain. This level was even improved when *GPD1* was overexpressed (Fig. 18). These results, in fact, were expected since a positive effect of a deletion of *PDC2* on glycerol production of strains with and without overexpression of *GPD1* have been observed by Nevoigt and Stahl (1996). In their studies, a *pdc2*Δ mutant overexpressing *GPD1* showed not only a higher glycerol yield but also a higher glycerol formation rate in comparison to the *pdc2*Δ strain without *GPD1* overexpression. The increases in levels of L-G3P in our experiment or of glycerol in the experiment of Nevoigt and Stahl (1996) were obtained when cells were grown under oxygen limited conditions. The L-G3P level measured in the strain *gpp1*Δ *gpp2*Δ *pdc2*Δ was not higher than in the strain *gpp1*Δ *gpp2*Δ when the cells were incubated under aerobic conditions (data not shown). Obviously, anaerobiosis is a necessary condition for the accumulation of L-G3P by the *pdc2*Δ mutant. In fact, the major mechanisms of the reoxidation of cytosolic NADH, i.e. through ADH, external NADH dehydrogenase, and the L-G3P shuttle, were inhibited and thus GPD is the only manner to reoxidise the excess NADH, maintaining redox balance inside the cells. This once more confirms the critical role of NADH in the accumulation of intracellular L-G3P.

In the strain *gpp1*Δ *gpp2*Δ *pdc2*Δ overexpressing *GPD1*, cells should have optimal conditions to accumulate L-G3P: block of its consumption, high activity of the enzyme responsible for the synthesis and abundance of the cofactor. As a result, the level of intracellular L-G3P in the strain *gpp1*Δ *gpp2*Δ *pdc2*Δ overexpressing *GPD1* was indeed the highest (~17 mg/ g YDW) among the engineered strains studied in this work.

Unfortunately, the growth of the *gpp1*Δ *gpp2*Δ *pdc2*Δ background strain was strongly diminished when cells were shifted from ethanol/ glycerol to the glucose medium. Growth of this strain with overexpression of *GPD1* was even not detected when a start OD_{600} of 0.3 was used. In fact, pyruvate decarboxylase negative (pdc^-) mutants are known to grow much slower than wild type (i.e. 20% residual growth rate) on glucose complex media (Hohmann, 1991) and their growth is even worse in defined mineral media with glucose as sole carbon source

(Flikweert *et al.,* 1996). One of the reasons could be that the NADH formed in glycolysis can no longer be reoxidised by alcoholic fermentation in Pdc^- mutants, so sugar metabolism becomes critically dependent on NADH-consuming respiration. However, the synthesis of many respiratory enzymes in *S. cerevisiae* is subjected to glucose repression (Entian, 1986; Gancedo, 1992; de Winde and Grevell, 1993). In fact, aerobic growth of a Pdc^- mutant on glucose complex medium was arrested when the respiratory inhibitor antimycin A was supplemented to the medium (Hohmann, 1991). Besides, the lack of cytosolic acetyl CoA, which is needed for lipids synthesis, could also contribute to the reduced growth of the pdc^- mutant on glucose medium (Pronk *et al*., 1996, Flikweert *et al*., 1999).

However, as discussed above (5.3), oxygen limited conditions are required to accumulate L-G3P at high levels. Therefore, the *gpp1*Δ *gpp2*Δ *pdc2*Δ background strains have to suffer difficult conditions for their growth to accumulate L-G3P. Furthermore, since the cultivation of these strains needs ethanol/ glycerol medium instead of glucose and includes a medium shift, the process implements higher cost and efforts. Therefore, it seems not possible to apply these strains in industrial L-G3P production.

5.5 Accumulated L-G3P was partially released into the cultivation medium

Extracellular L-G3P was investigated in fermentations with *gpp1*Δ *gpp2*Δ + *GPD1* under oxygen limited conditions. Interestingly, I could show that the amount of extracellular L-G3P was the same as the amount of intracellular L-G3P. In other words, half of the L-G3P produced by cells was released into the medium. The presence of L-G3P in the supernatant of the culture is possibly due to cell autolysis, since the high concentration of L-G3P in the medium was observed only after 24 hours of fermentation even though L-G3P could be detected at a very low concentration after 6 hours (Fig. 20). In fact, experiments checking viability of this strain showed that about 20% of cells die after 23 hours of fermentation (Nevoigt, personal comunication).

However, it could also be assumed that the excretion of L-G3P into the medium might be a regulatory mechanism of cells to diminish the accumulated L-G3P. However, it must be

mentioned that L-G3P cannot freely diffuse through the membranes because it is a phosphorylated and therefore charged intermediate. Hence, a transportation of L-G3P by a triosphosphate/ phosphate transporter could be a mechanism which is responsible for that. In fact, several L-G3P transporters have been described in other organisms, e.g. cTPT (chloroplast triose phosphate/ phosphate translocator) in plants (Flugge, 1998), GlpT (Glycerol 3-phosphate transporter) in *E. coli* (Hayashi *et al*., 1964; Larson *et al.,* 1982), Ugp (Uptake of glycerol phosphate) in bacteria (Argast *et al.,* 1978; Hengge *et al.,* 1983; Brzoska *et al.*, 1994). However, none of these transporters is able to secret the L-G3P out of the cell: cTPT is responsible for the export of triosphosphates from chloroplast to the cytosol, GlpT and Ugp are responsible for the uptake of L-G3P from medium. However, a transporter for triose phosphate/ phosphate in *S. cerevisiae* is not characterised so far, even though several open reading frames show similarity to the translocators of other organisms (Paulsen *et al.,* 1998).

L-G3P was released into the medium but the concentration of extracellular L-G3P was far beyond comparable to the concentration of intracellular L-G3P (Table 5). Improving the ability of cells to release L-G3P into the medium is a good solution to overcome product inhibition, which could be a bottle neck for the production of intracellular L-G3P. If the product L-G3P is released to the medium, the reaction could always happen towards L-G3P and the L-G3P pool is no longer limited by the cell boundary. Releasing of L-G3P into the medium could, possibly, also reduce the negative impact on growth, which is caused by the accumulation of intracellular L-G3P (5.1). Moreover, other genetic approaches to enhance the production of L-G3P will be more effective.

In addition, if L-G3P is selectively available in the medium, the cost for the whole extraction and purification process at high-scale fermentation can be reduced since no cell disruption is necessary.

5.6 Concluding remarks and outlook

This thesis reports the first results of work focusing on developing a method to produce L-G3P by using the yeast *S. cerevisiae* as a biocatalyst. The strain *gpp1*Δ *gpp2*Δ overexpressing *GPD1* produces about 100 times more L-G3P under oxygen limited conditions than the non-engineered wild type. Among the engineered strains investigated in this study, it is the best L-G3P overproducing strain growing on glucose medium and thus is suitable for practical application. The maximal yield of L-G3P, including both intra- and extracellular L-G3P, obtained from this strain was about 26 mg/ l culture (Table 5). When the consumption of glucose is taken into account, the yield is about 1.6 mmol L-G3P/ mol glucose. This level is about 300 times less than the yield of glycerol obtained from a strain overexpressing *GPD1* (Nevoigt and Stahl, 1996). That means a large amount of L-G3P was lost by unknown mechanisms. Furthermore, the L-G3P level produced by the strain *gpp1*Δ *gpp2*Δ overexpressing *GPD1* is much lower than the maximal theoretical yield of glycerol (1 mol/ mol glucose) (Overkamp *et al.*, 2002). Besides, this level is far to reach the industrial request (5 g/ l culture). Therefore, further work is needed to be done to improve the yield of L-G3P in this strain and to identify and circumvent the mechanisms which are responsible for the loss of L-G3P.

The yield of L-G3P per litre culture can be improved by increasing the number of cells (biomass) per litre culture. In fact, the strain *gpp1*Δ *gpp2*Δ overexpressing *GPD1* in our experiments produces only 0.8 g YDW/ l culture, while the maximal theoretical yield of biomass of *S. cerevisiae* culture growing on molasses is 50 times higher (40 g YDW/ litre culture, Dellweg 1987). Hence, by increasing the cell number, the yield of L-G3P could be improved to about 1.3 g/ l culture. To this aim, cells can be grown under aerobic conditions to optimise the production of biomass. Afterwards, they can be harvested and shifted to anaerobic conditions for production of L-G3P with a high cell density.

Further, it could be tested whether the benefits of anaerobiosis and osmotic stress could be combined to accumulate even more L-G3P. High concentrations of glucose can be used instead of NaCl to create an osmotic stress environment. By this way, the substance causing

osmotic stress is also the carbon source and thus, it could be more convenient for practical application.

The next step is to increase the yield of L-G3P per cell, since even if the yield of L-G3P reach the level of 1.3 g/ l culture as calculated above, it is still nearly 4 times lower than the level requested by the industry. A higher yield of L-G3P can be achieved by combining our approach with other methods which have been successfully been applied for the enhancement of glycerol biosynthesis.

Another important step is to overcome the product inhibition of GPD caused by the accumulation of L-G3P by improving the ability of cells to release L-G3P into the medium. To this aim, one strategy is to overexpress one of the known triose phosphate transporters, e.g. cTPT, Ugp or GlpT, in the strain *gpp1*Δ *gpp2*Δ overexpressing *GPD1*. The heterologous transporter then could be targeted to the plasma membrane to enable the export of L-G3P to the medium. Another strategy is to make a random mutagenesis in the strain *gpp1*Δ *gpp2*Δ overexpressing *GPD1* and then search for L-G3P leaky mutants.

Furthermore, since metabolites are the end products of cellular regulatory processes and their levels can be regarded as the ultimate response of biological systems to genetic or environmental changes, a comparative metabolome analysis of the L-G3P overproducing strain and the wild type can be carried out. Differences between their metabolomes could elucidate the unknown mechanism leading to the loss of L-G3P and other limiting factors of the production of L-G3P.

6. Summary

This thesis aimed at developing a microbial fermentation method to produce L-G3P, a promising starting material for pharmaceuticals, using *S. cerevisiae* as a biocatalyst. To accumulate L-G3P in cells, several approaches of engineering the activities of relevant enzymes were combined. Influences of the genetic modifications on growth and optimization of cultivation conditions of the engineered strains were investigated. The main results obtained are as follows:

1) Deletion of both isogenes *GPP1* and *GPP2*, encoding glycerol-3-phosphatases, had a strong positive effect on the accumulation of intracellular L-G3P (15fold higher than in the wild type). Simultaneously enhancing the biosynthesis of L-G3P by overexpressing the *GPD1* gene encoding the major isoenzyme of GPD could further increase the L-G3P level by a factor of 1.7.

2) Growth of the L-G3P overproducing strain *gpp1*Δ *gpp2*Δ + *GPD1* on minimal glucose medium was reduced by about 20% compared to the wild type.

3) Osmotic stress and oxygen limitation were known to enhance glycerol production. However, the experiments examining the influence of these conditions on the accumulation of L-G3P showed that only oxygen limitation could strongly enhance the production of L-G3P. Osmotic stress did not lead to an obvious increase of L-G3P level despite a remarkable increase in GPD activity.

4) Reduction of PDC activity by deleting *PDC2,* a gene encoding a positive regulator of PDC1 and PDC5, was done to check whether it can further improve the accumulation of intracellular L-G3P in the strain *gpp1*Δ *gpp2*Δ. The mutant *gpp1*Δ *gpp2*Δ *pdc2*Δ, indeed, produced 1.5 times more L-G3P than the strain *gpp1*Δ *gpp2*Δ. Overexpression of the *GPD1* gene in this triple mutant could further increase this level by a factor of 1.3. Unfortunately, the *gpp1*Δ *gpp2*Δ *pdc2*Δ strain did not grow on glucose as a sole carbon source.

5) Some L-G3P was detected in the culture medium of the strain *gpp1*Δ *gpp2*Δ overexpressing *GPD1* after 24 hours of fermentation. The total amount of extracellular L-G3P per litre was similar to that which was found intracellularly (about 13 mg/ l culture). However, the concentration of extracellular L-G3P was more than 200 times less than the intracellular L-G3P concentration.

6. Zusammenfassung

Ziel der Arbeit war die Entwicklung eines mikrobiellen Produktionsverfahrens zur Gewinnung von L-G3P. Durch rationales *Metabolic Engineering* wurde die Hefe *S. cerevisiae* in die Lage versetzt, das gewünschte Stoffwechselintermediat zu akkumulieren. Die wichtigsten Ergebnisse der Arbeit sind:

1) Die Deletion der Gene *GPP1* und *GPP2*, welche die beiden Isoenzyme der Glycerol-3-Phosphatase in *S. cerevisiae* kodieren, führte in *batch* Fermentationen in Glukosemedium zu einer 15fach höheren intrazellulären Konzentration von L-G3P im Vergleich zum Wildtyp. Die gleichzeitige Überexpression von *GPD1* erhöhte die erreichte L-G3P-Akkumulation nochmals um den Faktor 1.7.

2) Das Wachstum des resultierenden Stammes *gpp1*Δ *gpp*2Δ + *GPD1* auf Minimal-Glucosemedium war im Vergleich zum Wildtyp um 20% reduziert.

3) Osmotischer Stress und Sauerstofflimitierung steigern bekanntermaßen die Glycerolproduktion in *S. cerevisiae*. Die Konzentration von L-G3P wurde allerdings nur durch Sauerstofflimitierung und nicht durch osmotischen Stress beeinflusst, obwohl osmotischer Stress zu einem deutlichen Anstieg der GPD-Aktivität führte.

4) Um zu überprüfen ob eine Reduktion der PDC-Aktivität im Stamm *gpp1*Δ *gpp*2Δ zu einer weiteren Akkumulation von L-G3P führt, wurde *PDC2*, ein positiver Regulator der Strukturgene *PDC1* und *PDC5*, deletiert. Der resultierende Stamm *gpp1*Δ *gpp*2Δ *pdc*2Δ zeigte tatsächlich eine 1.5fach höhere L-G3P-Konzentation als der Ausgangsstamm. Eine Überexpression von *GPD1* in diesem Stamm führte zu einer weiteren 1.5fachen Steigerung der L-G3P Akkumulation. Die Dreifachmutante zeigte allerdings auf Glucose als einziger Kohlenstoffquelle kein Wachstum.

5) Im zellfreien Überstand des Stammes *gpp1*Δ *gpp*2Δ + *GPD1* konnten nach 24stündiger *batch* Fermentation etwa 13 mg L-G3P/l Zellkultur detektiert werden. Diese Menge an extrazellulärem L-G3P entsprach in etwa der Gesamtmenge an intrazellulärem L-G3P wenn letztere auch auf einen Liter Zellkultur bezogen wird. Nichtsdestotrotz war die extrazelluläre L-G3P-Konzentration an L-G3P ca. 200mal geringer als die intrazelluläre.

7. References

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