Modifying redox potential and its impact on metabolic fluxes in *Saccharomyces cerevisiae*

by

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DECLARATION

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SUMMARY

The production of glycerol by *Saccharomyces cerevisiae* under anaerobic conditions is essential for maintaining the intracellular redox balance thereby allowing continuous energy generation through conversion of sugars into ethanol. In addition, glycerol can act as an osmolyte and is synthesized to maintain turgor pressure under hyperosmotic conditions. The production of ethanol from sugars can be a redox-neutral process, where the NAD^+ (nicotinamide adenine dinucleotide) that is consumed during the glycolytic conversion of glyceraldehyde-3-phosphate to pyruvate is later regenerated by the reduction of acetaldehyde to ethanol. However, in particular the redirection of metabolic flux of pyruvate to biomass formation leads to excess NADH formation. The intracellular redox balance in these conditions is then primarily maintained through formation of glycerol which is control by two main enzymes, namely Gpd1p and Gpd2p. Deletion of the genes coding for these two proteins leads to accumulation of NADH and renders the cells incapable of maintaining their fermentative ability and growth under anaerobic conditions.

The goal of this study was to investigate the growth, fermentative ability and metabolite synthesis of various *gpd1*Δ*gpd2*Δ double mutant (DM) strains in which the redox balancing potential was partially restored through expression of native or heterologous genes. Strains were constructed by introducing alternative NADH oxidizing pathways or manipulating existing pathways to favour the oxidation of excess NADH. More specifically, the modifications included (i) sorbitol formation; (ii) establishing a pathway for propane-1,2-diol formation; and (iii) increasing ethanol formation. Apart from genetically manipulating the *gpd1*Δ*gpd2*Δ double mutant, the addition of pyruvate during growth was also investigated. The experiments were carried out under oxygen limited conditions in a high sugar medium and the fermented product was analyzed for total sugar consumed, biomass and primary and secondary metabolites formed by the different strains. The relationships between sugar consumption, growth and metabolite production by different strains were investigated by comparing the data generated from the different strains by using multivariate data analysis tools. Analysis of the pathways involved in the production of primary (acids, ethanol and other metabolites) and secondary metabolites (aroma compounds) were also carried out in order to establish flux modification in comparison to the wild type (WT) strain.

The results revealed that these manipulations improved the fermentative capacity of the *gpd1*Δ*gpd2*Δ double mutant, suggesting a partial recovery of NAD^+ regeneration ability, albeit not to the extent of the WT strain. As expected a significant correlation was found between sugar consumption and ethanol and biomass formation. Ethanol yields but not final concentrations were increased by the genetic manipulations. Sorbitol by DM(*srlD*) and DM(*SOR1*) strains and propane-1,2-diol by DM(*gldA*, *GRE3*, *mgsA*) strain were formed in significant amounts although at lower molar yields than glycerol. Furthermore, by genetic manipulation the yield of secondary metabolites (isobutanol, isoamyl alcohol, 2-phenyl ethanol and isobutyric acid) was increased whereas the ethyl acetate concentration and yield decreased. The results indicate that aroma compound properties of wine yeasts could be favourably changed by manipulating the glycerol synthesizing pathway. The addition of pyruvate during the growth of *gpd1*Δ*gpd2*Δ double mutant contributes to excess NADH re-oxidation through additional ethanol formation.

OPSOMMING

Die produksie van gliserol deur *Saccharomyces cerevisiae* onder anaërobiese toestande is noodsaaklik vir die onderhouding van die intrasellulêre redoksbalans en maak dus ononderbroke energie-ontwikkeling tydens die omsetting van suikers in etanol moontlik. Daarbenewens kan gliserol as ‘n osmoliet optree en word dit gesintetiseer om turgordruk onder hiperosmotiese toestande te onderhou. Die produksie van etanol uit suikers kan ‘n redoksneutrale proses wees, waar die NAD^+ (nikotinamiedadenien-dinukleotied) wat tydens die glikolitiese omskakeling van gliseraldehied-3-fosfaat na piruvaat verbruik word, later deur die reduksie van asetaldehied na etanol regenereer word. Die nasending van die metaboliese vloeiing van piruvaat na biomassavorming lei egter na ‘n oormaat NADH-vorming. Onder hierdie toestande word die intrasellulêre redoksbalans dan hoofsaaklik deur die vorming van gliserol onderhou. Laasgenoemde word veral deur twee ensieme beheer, naamlik Gpd1p en Gpd2p. Die delesie van die gene wat vir hierdie twee proteïene enkodeer, lei tot ‘n akkumulasie van NADH en veroorsaak dat die selle nie hulle gistingsvermoë en groei onder anaërobiese toestande kan onderhou nie.

Die doelwit van hierdie studie was om die groei, gistingsvermoë en metabolietsintese van verskeie *gpd1*Δ*gpd2*Δ dubbelmutant (DM) rasse te ondersoek waarin die redoksbalanseringspotensiaal gedeeltelik herstel is deur die uitdrukking van inheemse of heteroloë gene. Rasse is gekonstrueer deur alternatiewe NADH-oksiderende weë in te voer of deur bestaande weë te manipuleer om die oksidasie van oormaat NADH te bevoordeel. Meer spesifiek het die modifikasies die volgende ingesluit: (i) sorbitolvorming; (ii) die vestiging van ‘n weg vir propaan-1,2-diol-vorming; en (iii) die verhoging van etanolvorming. Buiten die genetiese manipulering van die *gpd1*Δ*gpd2*Δ dubbelmutant, is die byvoeging van piruvaat tydens groei ook ondersoek. Die eksperimente is onder suurstofbeperkte toestande in ‘n hoë-suiker medium uitgevoer en die gegiste produk is ondersoek vir totale suikerverbruik, biomassa en primêre en sekondêre metaboliete wat deur die verskillende rasse gevorm is. Die verhoudings tussen suikerverbruik, groei en metabolietproduksie deur die verskillende rasse is ondersoek deur die data wat deur die verskillende rasse gegeneer is deur middel van meerveranderlike data-analise te vergelyk. Analise van die weë wat in die produksie van primêre (sure, etanol en ander metaboliete) en sekondêre metaboliete

(aromaverbindings) betrokke is, is ook uitgevoer om die verandering in vloei te bepaal in vergelyking met die wildetipe (WT) ras.

Die resultate het gewys dat hierdie manipulasies die gistingsvermoë van die *gpd1*Δ*gpd2*Δ-dubbelmutant verbeter het, wat ‘n gedeeltelike herstel van NAD^+-regenerasievermoë voorstel, hoewel nie tot dieselfde mate as in die WT-ras nie. Soos verwag, is ‘n beduidende korrelasie tussen suikerverbruik en etanol- en biomassavorming gevind. Etanolopbrengs is deur genetiese manipulasies verhoog, maar nie die finale konsentrasies van etanol nie. Sorbitol is in beduidende hoeveelhede deur die DM(*srlD*) en DM(*SOR1*)-rasse gevorm en propaan-1,2-diol deur die DM(*gldA*, *GRE3*, *mgsA*) -rasse, hoewel teen laer molare opbrengste as gliserol. Verder is die opbrengs van sekondêre metaboliete (isobutanol, iso-amielalkohol, 2-fenieletanol en isobottersuur) deur genetiese manipulasie verhoog, terwyl die etielasetaatkonsentrasie en -opbreng verlaag is. Die resultate dui aan dat die aromaverbindingseienskappe van wyngiste voordelig verander kan word deur die gliserolsintetiseringsweg te manipuleer. Die byvoeging van piruvaat tydens die groei van die *gpd1*Δ*gpd2*Δ-dubbelmutant dra by tot uitermate NADH-reoksidasie tydens die bykomende vorming van etanol.

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BIOGRAPHICAL SKETCH

Vishist Kumar Jain was born of 22 November 1980 in the small town of Gaya in the state of Bihar, India. He matriculated in 1997 from Delhi Public School, Bokaro Steel City, India. He completed his Bachelor's degree in Biochemical Engineering and Biotechnology in 2003 at the Indian Institute of Technology (IIT), Delhi, India. He continued his Master's degree in Biochemical Engineering and Biotechnology at the same institution during 2003-2004. He enrolled for his PhD at the Institute for Wine Biotechnology, Stellenbosch University in 2005.

PREFACE

This dissertation is presented as a compilation of six chapters. Each chapter is introduced separately and is written according to the style of the journal “Microbiology”.

Chapter 1 **General Introduction and project aims**

Chapter 2 **Literature review**
Role of redox balance systems in *Saccharomyces cerevisiae* survival and metabolism.

Chapter 3 **Research results I**
Yeast sorbitol dehydrogenase and bacterial sorbitol-6-phosphate dehydrogenase partially restore fermentative ability in *Saccharomyces cerevisiae* mutants defective in glycerol formation.

Chapter 4 **Research results II**
Introduction of propane-1,2-diol formation pathway into a defective glycerol synthesizing *Saccharomyces cerevisiae* mutant can partially restore growth and ethanol formation.

Chapter 5 **Research results III**
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CHAPTER 7 GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Chapter 1

GENERAL INTRODUCTION AND PROJECT AIMS

1.1 Introduction

One of the most important metabolic challenges for *Saccharomyces cerevisiae* growing under fermentative conditions is to maintain the balance between the oxidized and reduced forms of NAD. NAD^+ and NADH are used in many biological reactions and are required for biomass formation and enzymatic oxidations and reductions (Bakker *et al.*, 2001). As only catalytic amounts of NAD are present in the cell, any accumulation of the reduced or oxidized form must be avoided so that biological processes continue without disruption (Bakker *et al.*, 2001). In wine fermentation, glycolysis is the main energy generating pathway. The pathway requires the presence of NAD^+ to oxidize glyceraldhyde-3-phosphate (G3P) to 1,3-biphosophoglycerate, generating $NADH+H^+$ in the process. During respiration, the necessary re-oxidation of $NADH+H^+$ takes place via the electron transport chain. However, under fermentative conditions, most NAD^+ is regenerated through ethanol production. However, not all pyruvate produced during glycolysis is converted to ethanol, and excess NADH is converted to NAD^+ through the formation of glycerol. Glycerol formation is catalyzed by Gpd1p (Albertyn *et al.*, 1994) and Gpd2p (Eriksson *et al.*, 1995) and accounts by far for the majority of the re-oxidation of excess NADH formed in the cytoplasm and mitochondria (Gancedo *et al.*, 1968; Ansell *et al*., 1997). In anaerobic conditions, the deletion of the *GPD1* and *GPD2* genes results in an inactive cell as NADH cannot be re-oxidized and growth ceases (Albertyn *et al*., 1994; Ansell *et al*., 1997)

Insertion of gene/genes that can replace the NADH oxidizing functions of *GPD1* and *GPD2* genes in a *gpd1*Δ*gpd2*Δ double mutant (DM) under anaerobic conditions has been investigated previously (Bjorkqvist *et al.*, 1997; Costenoble *et al.*, 2003 Liden *et al.*, 1996; Nissen *et al.*, 2001). Other strategies, such as the addition of external electron acceptors such as xylose and acetoin that can accept electrons from NADH and promote growth of the DM have also been investigated (Liden *et al.*, 1996). These studies focussed on the phenotypic analysis of the DM (Bjorkqvist *et al.*, 1997; Liden *et al.*, 1996; Nissen *et al.*, 2001) or the production of alternative metabolites such as xylitol, sorbitol and mannitol to replace glycerol (Liden *et al.*, 1996; Shen *et al*., 1999 Costenoble *et al.*, 2003). However, information on growth properties of the DM and strains overexpressing heterologous or native gene/genes in the DM under fermentative conditions is lacking. Moreover, the effect of the insertion of new pathways or modification of the existing pathways for NAD^+ regeneration on overall metabolic fluxes

in the DM is only known to a limited extent. NADH and NAD^+ are involved in the reduction and oxidation of many metabolites. Therefore changes in the production or consumption of these cofactors may lead to widespread alteration of metabolism thereby affecting the flux towards primary and secondary metabolites as end-products. Formation of secondary metabolites such as higher alcohols, esters and fatty acids is desirable in beverages such as wine and beer for their contribution to aroma. Furthermore, the presence of some primary metabolites such as organic acids is also desired as they impart unique taste to wine (Hufnagel & Hofmann, 2008). Therefore the purpose of this thesis was to investigate the impact of manipulation of some NADH/NAD^+ pathways on the growth and flux of primary and secondary metabolites in *S. cerevisiae.*

1.2 Project aims

The aims of the project were as follows:

1. Construction of a *S. cerevisiae* DM and selection of genes encoding enzymes that might be able to complement the redox imbalance created by the deletion of *GPD1* and *GPD2.*

2. Cloning and overexpression of native or heterologous gene/genes encoding enzymes involved in redox reactions in the DM and the strategies used to achieve this aim were:
   (i) Overexpression of native sorbitol dehydrogenase (*SOR1*) and expression of bacterial sorbitol-6-phosphate dehydrogenase (*srlD*) in the DM (chapter 3).
   (ii) Expression of bacterial glycerol dehydrogenase (*gldA*) and methylglyoxal synthase (*mgsA*) and overexpression of native aldose reductase (*GRE3*) simultaneously in the DM (chapter 4).
   (iii) Overexpression of native pyruvate decarboxylase (*PDC1*) and deletion of alcohol dehydrogenase (*ALD6*) in the DM (chapter 5).

3. Cultivation of the genetically manipulated strains in a high sugar medium (100 g/l) under fermentative conditions.

4. Analysis of the sugar consumed and biomass and primary and secondary metabolites formed by the genetically manipulated strains and relating their formation to the NADH formed and NAD^+ regenerated in all the genetically manipulated strains including the wild type (WT) strain.

5. An investigation of sugar consumption, growth and metabolite production by WT *S. cerevisiae* and genetically manipulated strains using principal component analysis (PCA) in order to establish patterns in metabolite production (chapter 6).

6. Analysis of the pathways involved in production of primary and secondary metabolites in order to establish flux regulation.

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Chapter 2

LITERATURE REVIEW

Role of redox balance systems in *Saccharomyce cerevisiae* survival and metabolism

2.1 Introduction

Saccharomyces cerevisiae is one of the most extensively studied eukaryotic microorganisms and an abundance of knowledge is available on this yeast with respect to its physiology, biochemistry and genetic constitution. This is in part due to its industrial importance as well as the fact that it is one of the simplest eukaryotic organisms. It is therefore frequently studied as a model towards a better understanding of more complex organisms including *Homo sapiens*.

S. cerevisiae is one of the few yeasts which is capable of significant growth under near-anaerobic conditions (Visser *et al.*, 1990). A good example where this character of *S. cerevisiae* is exploited is during the production of ethanol and alcoholic beverages such as beer and wine. Particularly during the winemaking process, *S. cerevisiae* has to confront a very high sugar concentration. In this kind of environment, *S. cerevisiae* ferments even in the presence of oxygen (Fiechter *et al.*, 1981). When exposed to such fermentative conditions, *S. cerevisiae* confronts specific metabolic challenges regarding energy generation, redox balancing and biomass formation.

Oxidations and reductions of metabolites are some of the most common reactions in biological systems. One of the basic life requirements of all such systems is therefore to maintain a balance between oxidative and reductive equivalents, a concept refered to as redox balance, to ensure continuous growth and metabolism. The intracellular redox status of the cell is largely dependent on the ratios of two pyridine nucleotides or cofactor systems, nicotinamide adenine dinucleotide ($NADH/NAD^+$) and nicotinamide adenine dinucleotide phosphate ($NADPH/NADP^+$) (Bakker *et al.*, 2001). The yeast genome database (www.proteome.org) lists approximately 100 open reading frames that encode established or putative pyridine-nucleotide-dependent oxidoreductases. These cofactors are essential to complete a large number of biochemical reactions. As a consequence, manipulating the pathways involving these cofactors by overexpressing or deleting the pyridine-nucleotide-dependent oxidoreductases may have drastic effects on overall metabolic networks. Furthermore, yeast metabolism is also strongly affected by the catalytic amount of these cofactors present inside the cell.

During the metabolism of sugars by yeasts, NADH and NADPH play distinct roles. NADH may be regarded as a predominantly catabolic reducing equivalent, whereas NADPH is mainly involved in anabolic processes where energy in the form of ATP is

consumed for synthesizing complex biomolecules from simpler components (Bakker *et al.*, 2001; van Dijken & Scheffers, 1986). Since catabolic and anabolic pathways share the same initial reactions of sugar metabolism, NADH is also formed during the assimilation of sugars to cell material. The formation of NADH during assimilation is even higher than anticipated on the basis of comparison between the reduction levels of the sugar and the biomass formed (van Dijken & Scheffers, 1986). This is due to the fact that the NADH produced during the formation of intermediates of glycolysis and tricarboxylic acid (TCA) cycle is not the principal reductant for the conversion of these intermediates into the building blocks of cell polymers (van Dijken & Scheffers, 1986). In fact, the principal reductant is NADPH which is required for most reductive anabolic reactions.

The principal aim of this review is to provide an overview of the current knowledge of pyridine nucleotides cofactor systems in *S. cerevisiae*. Special emphasis will be on the biosynthesis of NAD^+, one of the main pyridine nucleotides and its consumption and regeneration during respiratory and fermentative conditions. In addition, this review will focus on the genes playing a major role in the regeneration of NAD^+ under fermentative conditions. Finally, alcoholic fermentation during winemaking will serve as an example to illustrate a biological system rendered fermentative because of the presence of high sugar concentration in grape juice and the Crabtree effect. The important metabolic sinks under such stressful condition will be described.

2.2 The redox cofactors and their impact on the overall metabolic network in *S. cerevisiae*

2.2.1 NAD^+ as a conserved moiety in the living organism

NAD^+ is an essential cofactor for cellular redox reactions and energy metabolism. The biosynthesis of NAD^+ is tightly regulated so that its consumption and biosynthesis in the cell is in balance. There are two major pathways for NAD^+ biosynthesis in both prokaryotic and eukaryotic systems (Lin & Guarente, 2003a; Panozzo *et al.*, 2002). In one pathway, known as the *de novo* pathway, NAD^+ is synthesized from tryptophan (Kucharczyk *et al.*, 1998) (Fig. 2.1). The other pathway is known as the salvage pathway or Preiss-Handler pathway (Fig. 2.1), in which NAD^+ is generated by recycling degraded NAD^+ products such as nicotinamide (Nam) (Smith *et al.*, 2000). In yeast, the

de novo pathway consists of six enzymatic steps catalyzed by the Biosynthesis of Nicotinic Acid (*BNA*) genes and one non-enzymatic reaction (Lin & Guarente, 2003b). The last enzymatic reaction is catalyzed by a quinolinate phosphoribosyl transferase, encoded by the *BNA6/QPT1* gene, which converts quinolinic acid to nicotinic acid mononucleotide (NaMN) (Lin & Guarente, 2003b). At this point, the *de novo* pathway converges with the salvage pathway. During the salvage pathway, the nicotinic acid (Na) may come from the hydrolysis of nicotinamide (Nam) or from the extracellular medium if present. Hydrolysis of Nam to Na is catalyzed by a nicotinamidase, encoded by the *PNC1* gene (Ghislain *et al.*, 2002). A nicotinate phosphoribosyl transferase, encoded by the *NPT1* gene (Anderson *et al.*, 2003), converts the nicotinic acid to nicotinic acid mononucleotide (NaMN), which is then converted to deamido-NAD (NaAD) by a nicotinate mononucleotide adenylyl transferase, encoded by the *NMA1* and/or *NMA2* genes (Emanuelli *et al.*, 1999). An NAD^+ synthase encoded by *QNS1* (Suda et al., 2003) converts NaAD to NAD^+, which completes the final step in NAD^+ synthesis.

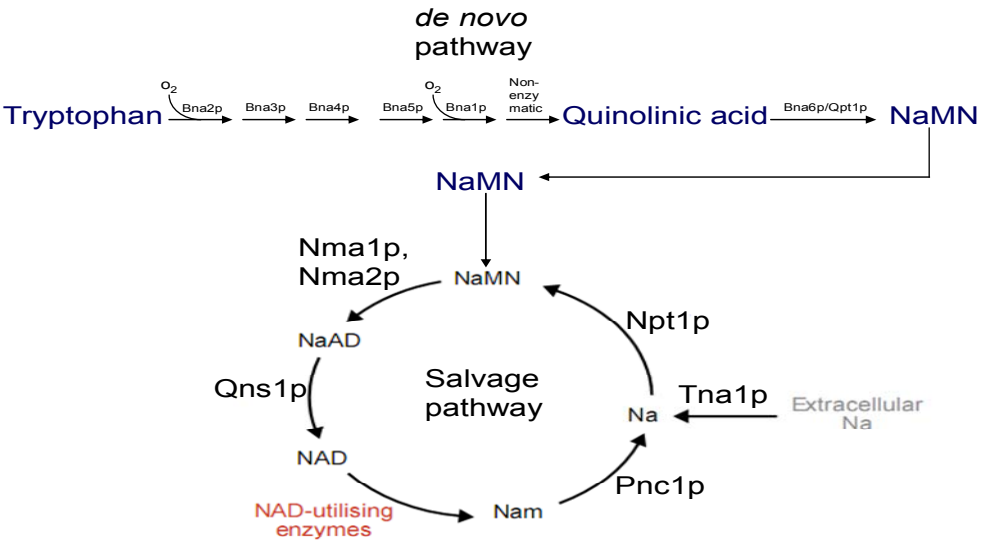

FIG. 2.1 Biosynthesis of NAD^+ by *de novo* and salvage pathway. Adapted from Lin & Guarente, (2003b).

In yeast, both above-mentioned pathways play essential roles (Lin *et al.*, 2000). Studies performed in this field have shown that deleting the *NPT1* gene decreases the NAD^+ level by 2.5-fold, whereas deleting the *QPT1* gene has no effect on the NAD^+ level

(Panozzo *et al.*, 2002; Sandmeier *et al.*, 2002; Smith & Boeke, 1997). These studies suggest that under respiratory conditions, the salvage pathway plays a more important role in NAD^+ synthesis. Moreover, under respiratory conditions, both pathways are functional. However, during fermentative conditions, the *de novo* pathway is not functional because the first and fifth steps in the conversion of tryptophan to quinolinic acid require oxygen. As a result, the sole salvage pathway synthesizes NAD^+ from nicotinic acid under anaerobic conditions. Nicotinic acid is imported into the cell from the extracellular medium through nicotinic acid permease, encoded by the *TNA1* gene. Thus, *S. cerevisiae* is a nicotinic acid auxotroph under fermentative conditions (Panozzo *et al.*, 2002).

2.2.2 Biosynthesis of $NADP^+$ from NAD^+ and formation of NADPH and NADH in *S. cerevisiae*

Once formed, NAD^+ can act as the main precursor for the biosynthesis of $NADP^+$. The conversion of NAD^+ to $NADP^+$ is governed by the ATP-NAD^+ kinase encoded by *UTR1* (Kawai *et al.*, 2001; Shi *et al.*, 2005a) which phosphorylates both NADH and NAD^+.

Once $NADP^+$ is biosynthesized from NAD^+, it can be used for the formation of NADPH in any enzymatic reaction requiring $NADP^+$ as cofactor. The main route for the formation of NADPH is through the oxidative part of the pentose phosphate pathway where the first and third steps are catalyzed by the glucose-6-phosphate dehydrogenase and the 6-phosphogluconate dehydrogenase encoded by *ZWF1* (Nogae & Johnston, 1990) and *GND1*/*GND2* (Sinha & Maitra, 1992) respectively. These enzymes use $NADP^+$ as cofactor and convert it to NADPH. The other major routes are through the $NADP^+$ linked isocitrate dehydrogenase encoded by *IDP1* (Minard *et al.*, 1998) and the $NADP^+$ linked aldehyde dehydrogenase encoded by *ALD6* (Grabowska & Chelstowska, 2003; Saint-Prix *et al.*, 2004).

The major route for the formation of NADH from NAD^+ is through catabolic reactions such as glycolysis and the tricarboxylic acid (TCA) cycle. NAD^+-dependent dehydrogenases such as Ald2p/Ald3p (Navarro-Avino *et al.*, 1999; White *et al.*, 2003) can also contribute to NADH formation, as well as generation of biomass. Once formed, NADH can be used in the electron transport chain for ATP generation.

2.2.3 The NAD^+ :NADH ratio determines yeast "performance"

The NAD^+/NADH ratio reflects the intracellular redox state and is often considered as an indication of the metabolic state. Many metabolic enzymes are regulated by the NAD^+:NADH ratio, such as the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase and the pyruvate dehydrogenase complex, which converts pyruvate to acetyl-CoA, a substrate for the TCA cycle. It has been well documented that the NAD^+:NADH ratio fluctuates in response to a change in metabolism (Nissen *et al.*, 2001). This means that biosynthesis and consumption of NAD^+ must be tightly regulated. As mentioned earlier, NAD^+ is consumed in several metabolic processes by the cell and NADH is formed. To maintain a proper redox state, NADH needs to be re-oxidized constantly. This is achieved via several mechanisms. During respiratory growth, both cytosolic and mitochondrial NADH are re-oxidized primarily by the respiratory chain (Bakker *et al.*, 2001). The mitochondrial inner membrane is virtually impermeable to pyridine nucleotide coenzymes (von Jagow & Klingenberg, 1970). Consequently, the cellular redox balance dictates that reduced coenzymes must be re-oxidized in the compartment where they have been generated.

In contrast to NADPH turnover, which occurs predominantly in the cytosol (Minard *et al.*, 1998), NADH turnover occurs at high rates in the cytosol as well as in the mitochondrial matrix. Several shuttle systems exist to transfer permeable redox equivalents across this barrier. The two main shuttles functional in the yeast under both respiratory and fermentative conditions are the glycerol-3-phosphate shuttle and the ethanol-acetaldehyde shuttle.

2.2.4 NADPH as principal reductant in yeast

Unlike prokaryotes, the investigations carried out so far on *S. cerevisiae* have confirmed that this yeast lacks transhydrogenase activity that might catalyze the conversion of NAD^+ and NADPH to NADH and $NADP^+$ and *vice versa* (Bruinenberg et al., 1983; Camougrand et al., 1988). Thus, yeast cannot directly couple the oxidation or reduction of these cofactors using a transhydrogenase enzyme. The fact that NADH cannot be used for the reductive steps of biomass synthesis makes the situation more difficult in terms of NADH re-oxidation. In fact, NADPH, and not NADH, acts as the principal reductant in biomass formation (van Dijken & Scheffers, 1986) (Fig. 2.2). The specific

requirement for NADPH in assimilation of sugars to cell material, in combination with the absence of transhydrogenase activity necessitates the conversion of part of the sugar exclusively for the purpose of generating reducing power in the form of NADPH. The oxidative part of the pentose phosphate pathway, a $NADP^+$-linked isocitrate dehydrogenase and the $NADP^+$-dependent acetaldehyde dehydrogenase have been suggested to be the major routes of NADPH formation in yeasts. Thus, there must also be a way by which yeast can convert excess NADH back to NAD^+. This aspect will be covered later in this literature review.

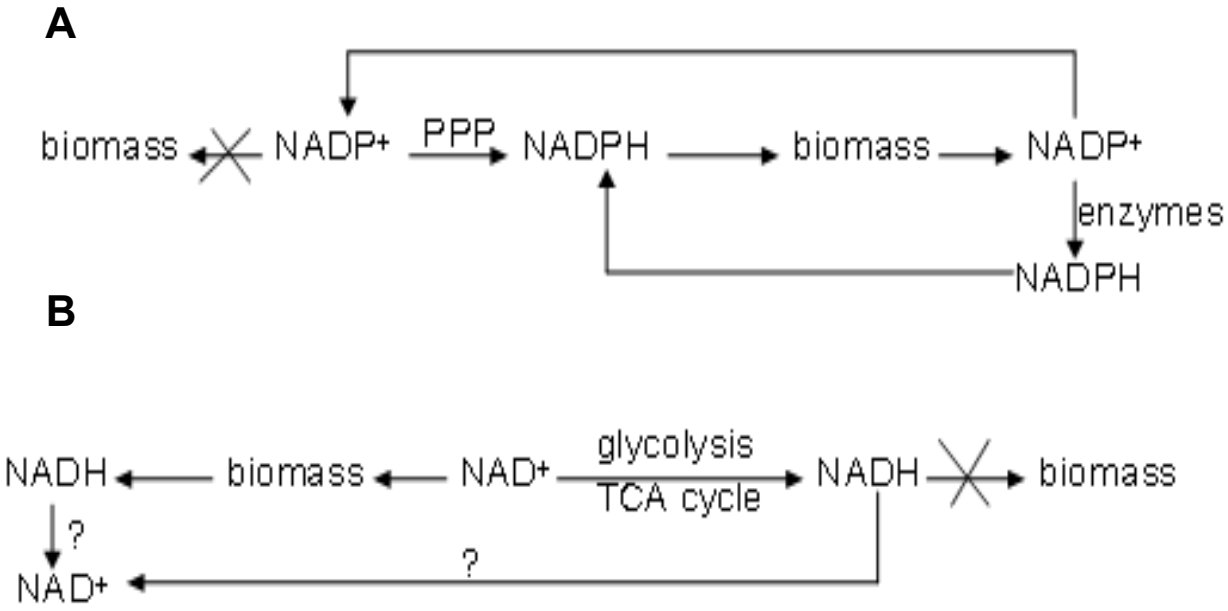

FIG. 2.2 Cofactor requirement for biomass formation by *S. cerevisiae* where NADPH is utilized for biomass formation and not $NADP^+$ (A) and NAD^+ is used for biomass formation and not NADH (B). PPP: Pentose Phosphate Pathway.

2.2.5 Flavoproteins and their prosthetic group FAD^+

Flavoproteins are ubiquitous proteins that use flavins (FAD^+) as prosthetic groups. The common flavin cofactors are flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD^+) (Fig. 2.3) which are synthesized *in vivo* from riboflavin (vitamin B1) by the action of riboflavin kinase (Rajeswari *et al.*, 1999) and FAD^+ synthetase (Manstein & Pai, 1986). The redox active isoalloxazine moiety of the flavin cofactor may undergo one or two electron transitions but these electrons enter the isoalloxazine ring

one at a time (Massey, 2000). This property and the ability to catalyze a wide range of biochemical reactions (Ghisla & Massey, 1989b; Manstein *et al.*, 1988) place flavoproteins at the crossroads of cellular redox chemistry. During the past 60 years, an impressive number of flavoproteins has been characterized and their catalytic and structural features have been determined (Fraaije & Mattevi, 2000; Hefti *et al.*, 2003).

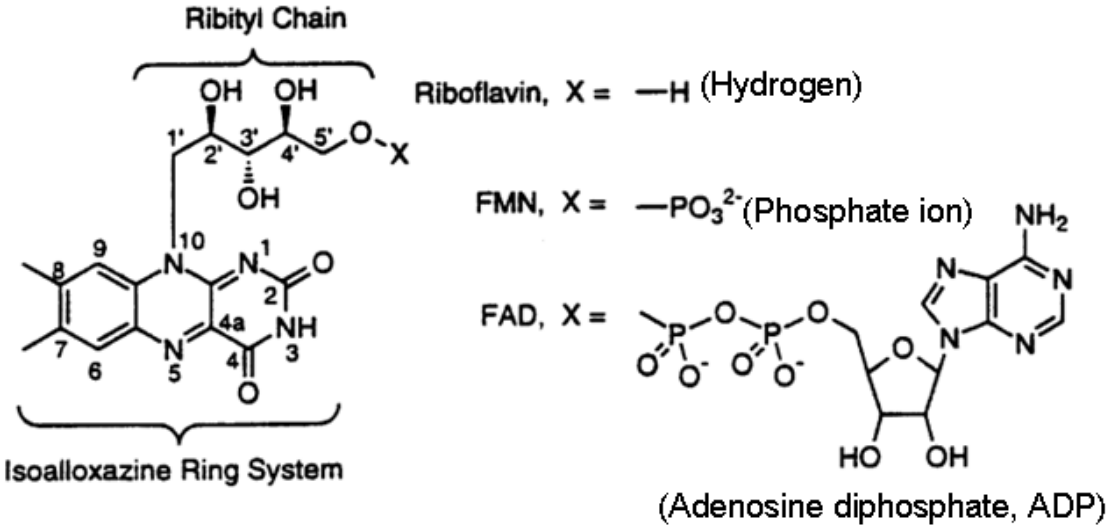

FIG. 2.3 Structure of isoalloxazine ring system with ribityl chain where X = -H represents riboflavin, where X = $-PO_3^{2-}$ represents flavin mononucleotide and where X = -ADP represents FAD^+ (Fraaije and Mattevi, 2000).

2.2.6 Mechanism of flavin catalyzed reaction

Most biological oxidation reactions involve the rupture of at least one organic substrate with a hydrogen bond, with concomitant transfer of two electrons to a suitable acceptor. In the case of oxidation reactions catalyzed by simple pyridine nucleotide linked enzymes, the acceptor is NAD^+ or $NADP^+$ and the resultant NAD(P)H needs to be re-oxidized by coupling with a second pyridine nucleotide-linked enzyme. In the case of flavoproteins, the flavin is tightly bound to the protein. Therefore, a second substrate serves to re-oxidize the reduced flavin to complete the catalytic cycle (Ghisla & Massey, 1989a; Hefti *et al.*, 2003). Thus, catalysis by flavoprotein enzymes always involve a reductive half reaction, where the enzyme bound flavin is reduced, and an oxidative half reaction, where the reduced flavin is re-oxidized (Ghisla & Massey, 1989a) (Fig. 2.4).

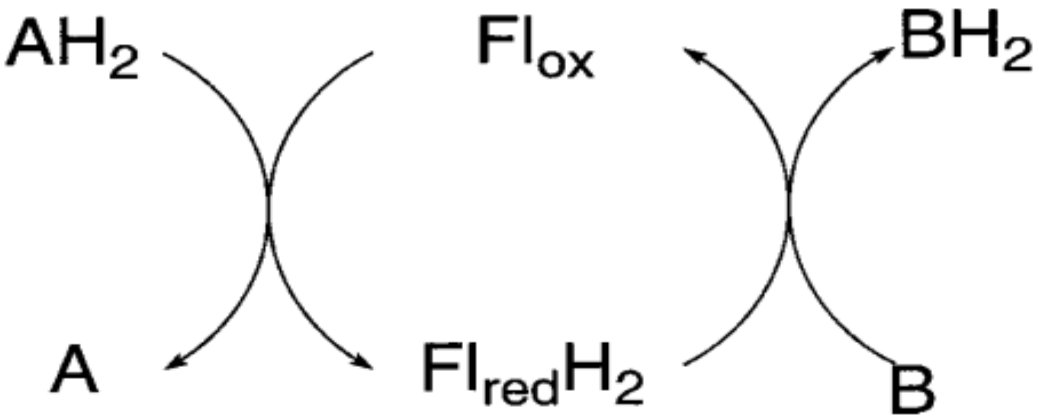

FIG. 2.4 Reductive and oxidative half reactions of flavoproteins. AH_2, a reduced substrate passes electrons to oxidized flavoprotein (FL_{ox}). Similarly reduced flavoprotein ($FL_{red}H_2$) passes the electrons to the other oxidized substrate (B) which can then be reduced to (BH_2). In this process, FL_{ox} is regenerated and cycle continues (Fraaije & Mattevi, 2000).

2.3 Yeast growth under respiratory conditions

2.3.1 Pasteur and Crabtree effects

S. cerevisiae metabolizes simple sugars such as glucose and fructose to carbon dioxide (CO_2) and water by the process of aerobic respiration. Two properties that regulate respiratory metabolism depend upon the availability of oxygen and the concentration of glucose in the medium, and these are known as the Pasteur Effect and Crabtree Effect, respectively. The Pasteur Effect can be defined as the suppression of fermentation (conversion of sugars to ethanol and CO_2) in the presence of air (Laws & Stickland, 1958). However, this property is only observable when glucose concentrations in the medium are low (*e.g*. below ~9 g/l in *S. cerevisiae*) (Lagunas, 1979; Piskur *et al.*, 2006; Goddard, 2008). The Crabtree Effect, also known as the "Glucose Effect" or "contre-effect Pasteur" was defined as the suppression of respiration by high glucose concentration (above ~9 g/l). Respiration is the sole route of glucose catabolism when the concentration of glucose is very low. Under conditions of vigorous aeration and low carbohydrate concentration, the glucose is primarily metabolized by the glycolytic pathway to pyruvate. Due to the presence of oxygen, pyruvate can be further oxidized by the tricarboxylic acid cycle (TCA) to CO_2 and water. These key metabolic pathways

serve three major functions in the cell: the generation of ATP, the regeneration of NAD^+ and the formation of intermediates for the biosynthesis of cellular material.

2.3.2 NADH metabolism in yeast under aerobic conditions

When grown on hexoses as carbon source, the major source of NADH production in the cytosol is the glycolytic pathway where it is formed during the conversion of glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate (Fig. 2.5). Furthermore, when the intermediates of glycolysis are withdrawn as the precursor of biomass, excess NADH is generated in the cytosol (van Dijken & Scheffers, 1986). Moreover, formation of acetic acid might also play minor role in the formation of NADH in the cytosol under aerobic conditions (Nissen *et al.*, 1997; van Dijken & Scheffers, 1986).

In the mitochondrion, the major source of NADH production is the formation of acid intermediates by the TCA cycle in the mitochondrion (Fig. 2.6). Under aerobic conditions, these intermediates are cycled to continuously regenerate NADH, which is then used for energy generation. The acid intermediates can however also be used for the biosynthesis of amino acids when required. 30-50% of the total NADH that is generated in the mitochondrial matrix mainly come from synthesis of 2-oxoglutarate, regarded as the main acid intermediate of the TCA cycle. 2-oxoglutarate is a precursor of glutamate (Albers *et al.*, 1996; Bruinenberg *et al.*, 1983). Glutamate is regarded as the crossroads of amino acid synthesis (Albers *et al.*, 1996). Cells indeed drive the reactions leading to the formation of 2-oxoglutarate, which is a precursor of glutamate, thereby generating large amounts of NADH in the formation of 2-oxoglutarate. After formation of glutamate, it can be converted to alanine and aspartate by transamination (Albers *et al.*, 1996). All the remaining amino acids can than be synthesized from glutamate or aspartate, by transamination of these two amino acids with a 2-keto acid (Albers *et al.*, 1996). These amino acids are then used as precursor for biomass formation and therefore biomass formation also indirectly contributes to NADH production (Nissen *et al.*, 2000a). Generation of NADH also takes place in mitochondrial matrix when pyruvate is oxidized to acetyl-CoA using the pyruvate dehydrogenase complex (Pronk *et al.*, 1996).

Due to impermeability of the mitochondrial inner membrane to pyridine nucleotide coenzymes, the re-oxidation of reduced coenzymes must occur in the compartment

where they are generated (von Jagow & Klingenberg, 1970). In contrast to NADPH turnover, which occurs predominantly in the cytosol through the pentose phosphate pathway, NADH turnover occurs at high rates in the cytosol as well as in the mitochondrial matrix (Albers *et al.*, 1996).

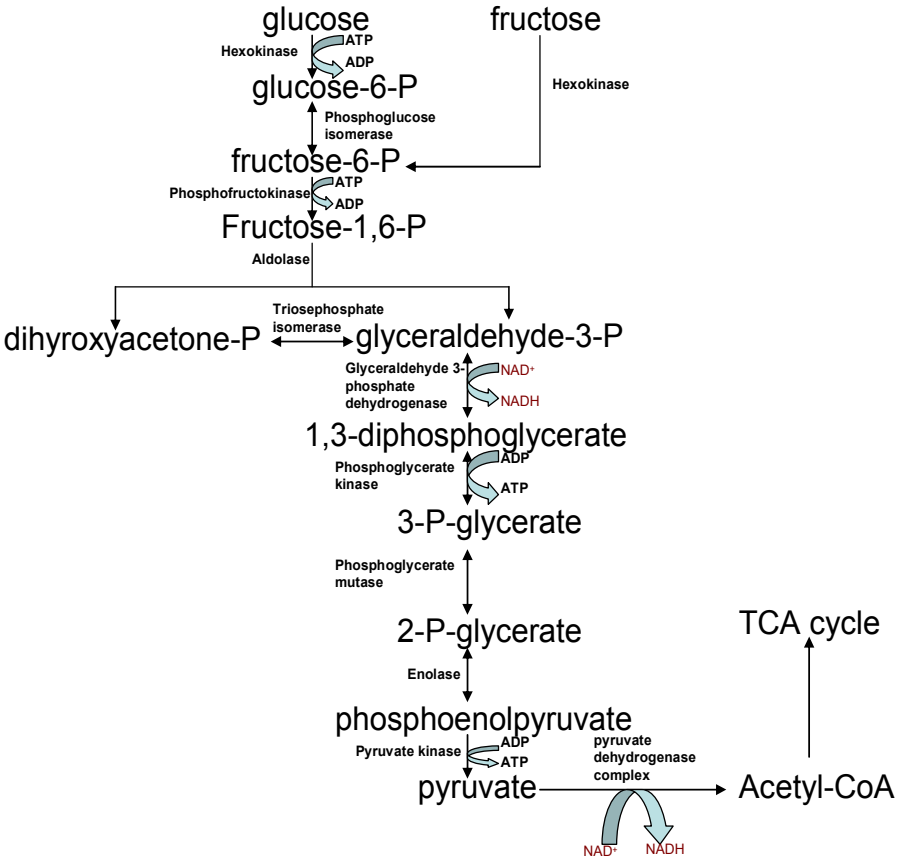

FIG. 2.5 Glycolytic pathway under respiratory growth of *S. cerevisiae*. Adapted from Lehninger *et al*., (2000).

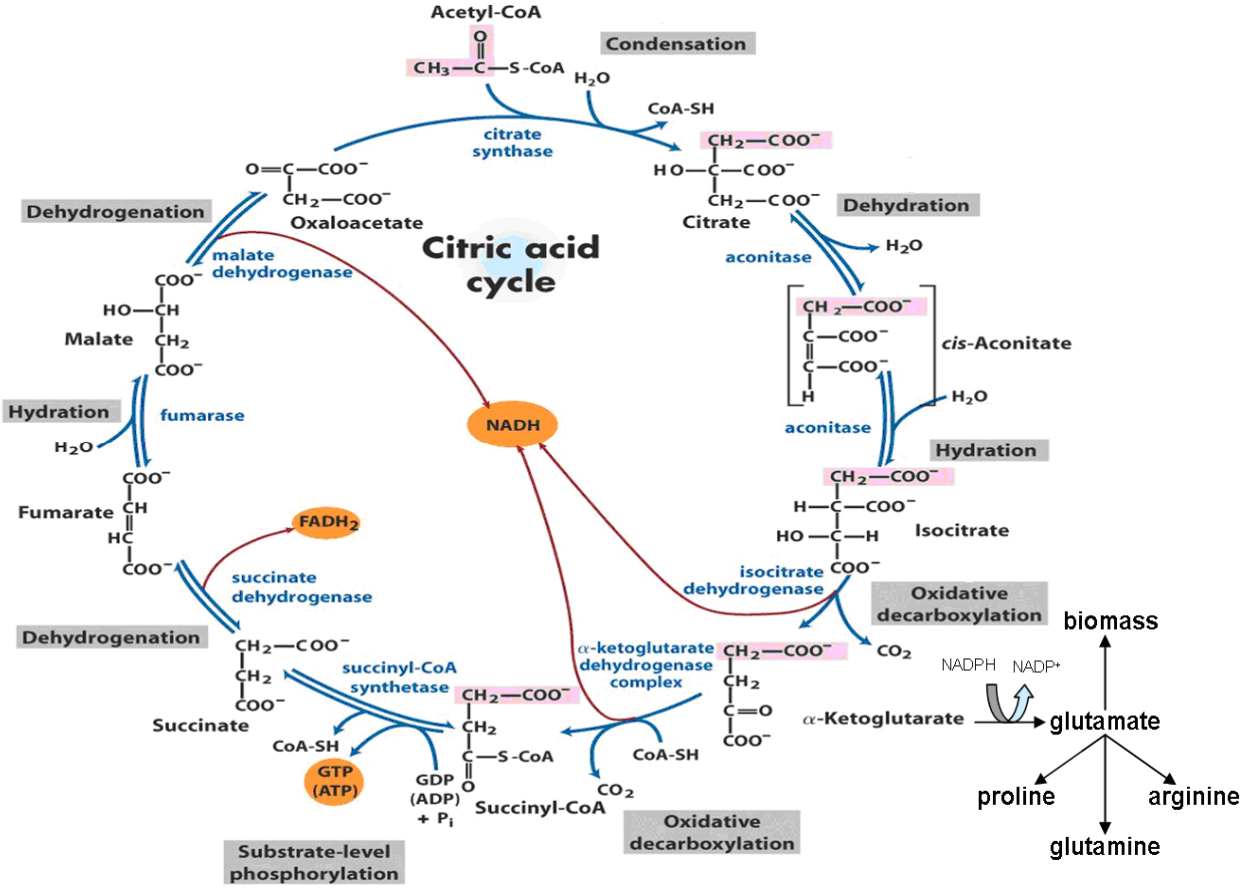

FIG. 2.6 Tricarboxylic acid cycle under respiratory growth of *S. cerevisiae*. Adapted from Lehninger *et al*., (2000).

2.3.3 The redox balance in *S. cerevisiae* under aerobic conditions

2.3.3.1 Internal NADH dehydrogenases: Physical and catalytic properties

In contrast to many eukaryotic cells, *S. cerevisiae* lacks the multi-subunit complex I-type NADH dehydrogenase (Nosek & Fukuhara, 1994). Instead, *S. cerevisiae* contains a single subunit NADH:ubiquinone oxidoreductase, which couples the oxidation of intramitochondrial NADH to the respiratory chain (Bakker *et al.*, 2001). This enzyme, referred to as the internal NADH dehydrogenase, catalyzes the transfer of two electrons from the intramitochondrial NADH to ubiquinone (de Vries & Grivell, 1988; Marres *et al.*, 1991).

The unique nuclear gene *NDI1* encodes a 57-kDa precursor protein Ndi1p (de Vries *et al.*, 1992), with a 26-amino acid N-terminal targeting sequence that is cleaved off upon

import into the mitochondrion (de Vries *et al.*, 1992). Ndi1p contains non-covalently bound FAD^+ as the sole prosthetic group (de Vries & Grivell, 1988).

Ndi1p is localized in the inner mitochondrial membrane, with its active site facing the mitochondrial matrix (Marres *et al.*, 1991) (Fig. 2.7). In contrast to complex I-type NADH dehydrogenases, Ndi1p itself does not pump protons (Bakker *et al.*, 2001). As a consequence, the transfer of electron from NADH to the ubiquinone pool does not contribute to the generation of a proton-motive force across the mitochondrial inner membrane (de Vries & Marres, 1987). This has the interesting effect on growth energetics in *S. cerevisiae* which is visible when considering the P/O ratio (number of ATP molecules produced per pair of electrons donated to the electron transport system) (Famili *et al.*, 2003) of the mitochondria which has been estimated to be close to one (Verduyn *et al.*, 1991). Thus, the textbook value of the ATP yield of completely respiratory dissimilation of glucose (36 moles ATP per mol of glucose) does not seem correct for *S. cerevisiae*. With as estimated *in vivo* P/O ratio of 1, a value of 16 ATP/mol of glucose would be more realistic (Bakker *et al.*, 2001).

2.3.3.2 Physiology of *ndi1*Δ mutants

In aerobic, glucose-limited cultures, *S. cerevisiae* exhibits exclusive respiratory growth. Mitochondria isolated from cultures grown under this condition were tested for oxygen consumption rates by providing different substrates like ethanol, pyruvate and malate (Bakker *et al.*, 2000; Overkamp *et al.*, 2000). It was found that mitochondria isolated from *S. cerevisiae ndi1*Δ mutants do not oxidize these substrates because they generate NADH in the mitochondrial matrix (Bakker *et al.*, 2000). This confirmed that Ndi1p was the only internal NADH dehydrogenase in *S. cerevisiae* (Bakker *et al.*, 2000).

To check whether deletion of *NDI1* affects the biomass yield on glucose, Bakker *et al.* (2000) grew WT and *ndi1*Δ mutants in aerobic, glucose-limited chemostat cultures at the dilution rate of 0.10 /h. WT *S. cerevisiae* did not exhibit alcoholic fermentation at low dilution rates. This was concluded from the absence of ethanol in the culture supernatant. The biomass yield on glucose was 0.49 g/g and the respiratory quotient (the ratio of the specific rates of CO_2 production and oxygen consumption) was close to one. At the same dilution rate, the biomass yield on glucose of *ndi1*Δ strain (0.43 g/g) was slightly lower than that of the WT. Respiratory quotient was also close to one

indicating that the growth of *ndi1*Δ strain was almost completely respiratory (Bakker *et al.*, 2000). This experiment clearly showed that the growth kinetics of *S. cerevisiae* is not affected by the deletion of *NDI1* and that the yeast has other systems for the re-oxidation of mitochondrial NADH formed in the TCA cycle when *NDI1* is deleted.

Exclusive respiratory growth of *S. cerevisiae* on glucose requires the activity of the TCA cycle (Bakker *et al.*, 2000) (Fig. 2.6). Usually the TCA cycle is thought to take place inside the mitochondrion. In that case, all NADH would be produced in the mitochondrial matrix. However, cytosolic isoenzymes of isocitrate dehydrogenase (Loftus *et al.*, 1994) and malate dehydrogenase (Minard & McAlister-Henn, 1991) have been identified in *S. cerevisiae*. Furthermore, although the pyruvate-dehydrogenase complex is confined to the mitochondrion, there is a cytosolic bypass via pyruvate decarboxylase (Flikweert *et al.*, 1996; Pronk *et al.*, 1996), acetaldehyde dehydrogenase (Saint-Prix *et al.*, 2004), and acetyl-coenzyme A synthetase (Pronk *et al.*, 1996). 2-Oxo-glutarate dehydrogenase, however, is an exclusively mitochondrial enzyme in *S. cerevisiae* (Repetto & Tzagoloff, 1991), implying that at least in this reaction, intramitochondrial NADH is produced. For respiratory growth of the *ndi1*Δ mutant, it is required that this NADH be shuttled to the cytosol, where it can be oxidized by the external NADH dehydrogenases or by the glycerol-3-phosphate shuttle (Bakker *et al.*, 2000).

2.3.3.3 Function of ethanol-acetaldehyde shuttle

It has been reported by Bakker *et al.* (2000) that the respiratory growth of the *ndi1*Δ mutant is possible due to the activity of the putative ethanol-acetaldehyde shuttle, which transfers redox equivalents from the mitochondrion to the cytosol (von Jagow & Klingenberg, 1970) (Fig. 2.7). In this shuttle, acetaldehyde formed in the cytosol diffuses into the mitochondrion where it is converted to ethanol by the action of Adh3p (Young & Pilgrim, 1985). In this reductive process, NADH formed in the mitochondrion can be converted back to NAD^+. The formed NAD^+ can again be used for the functioning of the TCA cycle. The ethanol which is formed can diffuse to the cytosol where instead of being secreted outside the cell, it is converted back to acetaldehyde. This conversion is catalyzed by Adh2p which uses NAD^+ and converts it back to NADH. The resulting NADH can be then channelled to the respiratory chain by external NADH dehydrogenase (Bakker *et al.*, 2001).

2.3.3.4 External NADH dehydrogenases: Physical and catalytic properties

Yeast mitochondria do not only contain an internal mitochondrial NADH dehydrogenase, but also possess external NADH dehydrogenase activity in the intramitochondrial space (von Jagow & Klingenberg, 1970) (Fig. 2.7). Like the internal NADH dehydrogenase (Ndi1p), the external iso-enzymes do not pump protons (von Jagow & Klingenberg, 1970). *S. cerevisiae* possesses two genes encoding external NADH dehydrogenase iso-enzymes, *NDE1* and *NDE2* (Luttik *et al.*, 1998; Small & McAlister-Henn, 1998). Both genes were identified on the basis of their homology to *NDI1*. The amino acid sequence of Nde1p is 48% identical to that of Ndi1p and the Nde2p sequence is 46% identical to that of Ndi1p, over the whole length of the proteins (Luttik *et al.*, 1998; Small & McAlister-Henn, 1998).The identity of the peptide sequences of Nde1p and Nde2p to each other is 62% (Luttik *et al.*, 1998; Small & McAlister-Henn, 1998). These isoenzymes catalyze the transfer of cytosolic NADH to the respiratory chain.

2.3.3.5 Physiology of *nde1/2*Δ mutants

Under low dilution rates in glucose limited chemostat culture, the primary mode of yeast growth is respiratory unlike in batch culture on glucose where alcoholic fermentation is predominant because of the glucose repression of the respiratory enzymes (Entian *et al.*, 1984). Luttik *et al.* (1998) isolated mitochondria from a culture grown at low dilution rate in aerobic, glucose-limited medium and tested it for oxygen consumption rates by providing exogeneous NADH. They found that mitochondria isolated from *S. cerevisiae nde1*Δ*nde2*Δ s do not show any NADH-dependent oxygen consumption (Luttik *et al.*, 1998). This result confirmed that Nde1p and Nde2p are the only external NADH dehydrogenase in *S. cerevisiae*.

Nevertheless, at low dilution rates, the growth of the *nde1*Δ*nde2*Δ double mutant was almost completely respiratory (Luttik *et al.*, 1998). This is surprising because it was assumed that these two enzymes are the only external NADH dehydrogenases which couple the oxidation of cytosolic NADH to the respiratory chain. This indicates that, at low dilution rates, the role of the external NADH dehydrogenases can be completely taken over by the glycerol-3-phosphate shuttle (Luttik *et al.*, 1998).

2.3.3.6 Function of glycerol-3-phosphate shuttle

The glycerol-3-phosphate shuttle is an indirect mechanism to oxidize cytosolic NADH and to transfer the electrons to the respiratory chain (Larsson *et al.*, 1998) (Fig. 2.7). The dihydroxyacetone phosphate formed by glycolysis is converted to glycerol-3-phosphate by the action of Gpd1p and Gpd2p. These isoenzymes use NADH as cofactor. The resulting glycerol-3-phosphate then enters into the mitochondria where it is converted back to dihydroxyacetone phosphate by the action of mitochondrial glycerol-3-phosphate dehydrogenase (*GUT2*) (Larsson *et al.*, 1998). This enzyme uses FAD^+ as the cofactor and during the conversion of glycerol-3-phosphate back to dihydroxyacetone phosphate, $FADH_2$ is formed (Larsson *et al.*, 1998). The $FADH_2$ then joins the electron transport chain by passing on the electrons to ubiquinone oxidoreductase. The dihydroxyacetone phosphate returns to the cytosol and the shuttle continues (Larsson *et al.*, 1998).

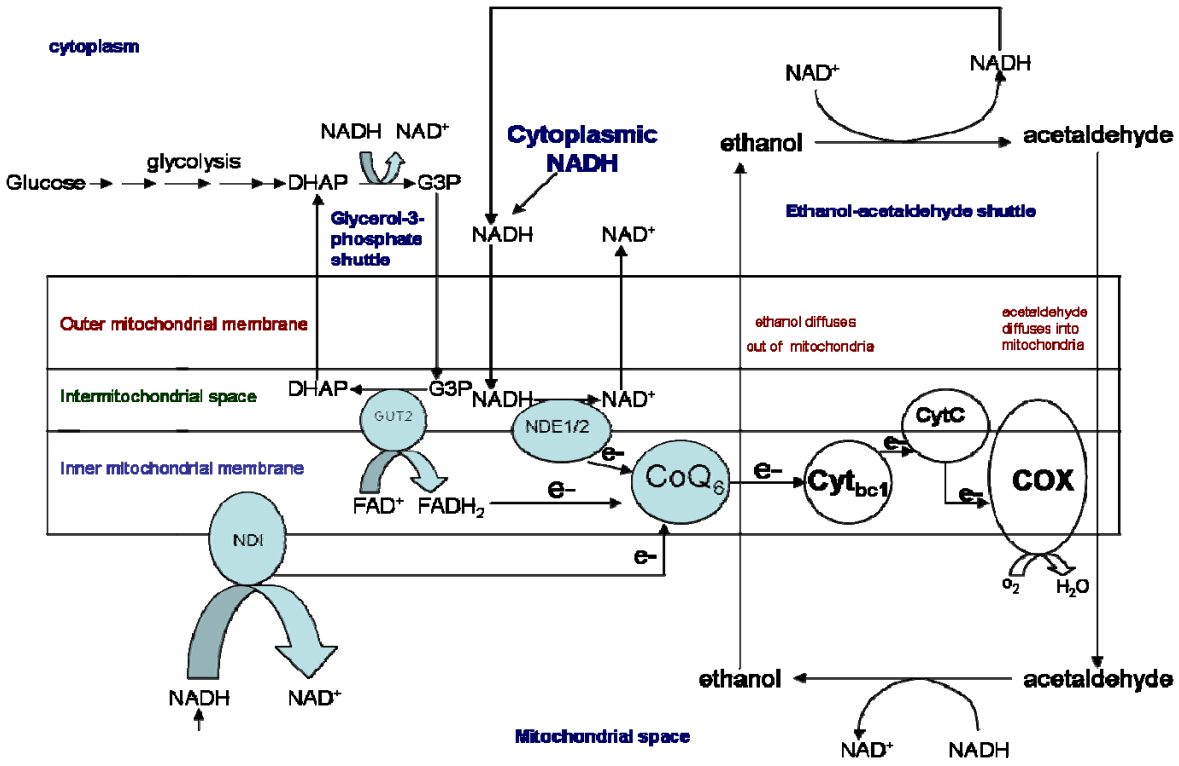

FIG. 2.7 Redox balance scheme in *S. cerevisiae* under aerobic condition. The respiratory chain of *S. cerevisiae* showing the function of Ndi1p, Nde1p, Nde2p and Gut2p. The function of the ethanol-acetaldehyde shuttle and glycerol-3-phosphate shuttle are also shown. Adapted from Bakker *et al.*, (2001).

In conclusion, it can be deduced that during respiration, *S. cerevisiae* has several mechanisms to regenerate NAD^+. Although the main roles for the re-oxidation of excess NADH are played by Ndi1p in the mitochondrion and Nde1p/Nde2p in the cytosol, *S. cerevisiae* has different shuttle systems which can re-oxidize excess NADH in both the mitochondria and the cytosol in the absence of Ndi1p and Nde1p/Nde2p respectively. In the next section, focus will be on the fermentative mode of growth of *S. cerevisiae* and NAD^+ regeneration under such condition.

2.4 NADH metabolism in yeast under anaerobic condition

2.4.1 Implication of absence of oxygen under anaerobiosis

Due to absence of oxygen under anaerobic conditions, NADH cannot be converted back to NAD^+ by the electron transport chain. Under aerobic conditions, pyruvate which is considered as the central metabolite can be further oxidized in the TCA cycle

because of the presence of oxygen as the final electron acceptor. However under anaerobic condition, it must be converted into some metabolite which can accept electrons from NADH. It is well known that pyruvate under anaerobic conditions is converted to acetaldehyde which accepts electrons from NADH formed during the glycolysis and gets converted into ethanol (Nissen *et al.*, 1997). The process of conversion of glucose into ethanol is referred to as alcoholic fermentation. The conversion of glucose to ethanol is a redox neutral process (van Dijken & Scheffers, 1986). It implies that the NAD^+ which is consumed in the glycolytic pathway is regenerated when ethanol is produced. However, when the intermediates of glycolysis are withdrawn as precursors for synthesis of cellular material, this balance is disturbed since the surplus of NADH is not converted back into NAD^+. Furthermore, the excess NADH is not only formed during biomass formation but also during the production of by-products such as acetate (Gancedo *et al.*, 1968a).

2.4.2 NADH formation in the mitochondrion

Under respiratory conditions, NADH is generated in the TCA cycle during the synthesis of intermediates which act as the precursors for the biosynthesis of biomass. It is reported in literature that most of the isoenzymes of the TCA cycle are also present in the cytosol (Bakker *et al.*, 2000). Therefore, under fermentative conditions, when there is no oxygen as the final electron acceptor, the TCA cycle can partially function in the cytosol. Nevertheless, it was observed that mitochondria are partly active during fermentative condition and residual TCA pathway activity is maintained during fermentation (Camarasa *et al.*, 2003). This activity primarily fuels biosynthetic reactions by supplying the cells with C_4 compounds (oxaloacetate and 2-ketoglutarate), the precursors of aspartate and glutamate, respectively. TCA pathway activity during fermentation also leads to excretion of organic acids such as citrate, malate and succinate. These compounds are of major interest to the food industry and in wine production, in particular, because they affect the organoleptic balance of wines (Camarasa *et al.*, 2003).

It is widely accepted that during alcoholic fermentation, the TCA pathway does not operate as a cycle, but as two branches culminating at succinate (Camarasa *et al.*, 2003) (Fig. 2.8). Due to the functioning of TCA cycle under fermentative conditions, NADH is formed in the mitochondria during the conversion of 2-ketoglutarate to

succinyl-CoA because the enzyme 2-ketoglutarate dehydrogenase which catalyzes this reaction is exclusively mitochondrial and uses NAD^+ as cofactor (Repetto & Tzagoloff, 1991). Succinyl-CoA is then converted to succinate and ATP is generated in this reaction through substrate level phosphorylation (Fig. 2.8). The formation of succinate via acetyl-CoA is known as the oxidative pathway of the TCA cycle. Succinate cannot be converted to fumarate because the succinate dehydrogenase complex which catalyzes the conversion of succinate to fumarate does not operate during fermentation (Camarasa *et al.*, 2003). Moreover succinate can also be formed via oxaloacetate through a succession of reactions known as reductive pathway. This pathway allows regeneration of NAD^+, since oxaloacetate is reduced to malate by malate dehydrogenase. The following step of this pathway uses fumarate reductase which catalyzes the irreversible conversion of fumarate to succinate (final step of the reductive branch of the TCA cycle) and replaces the activity of succinate dehydrogenase under fermentative condition. This phenomenon can explain the presence of a higher amount of succinate under fermentative conditions as compared to respiration. Furthermore, strong evidence now exists which suggests that the reductive branch of the TCA cycle is the main pathway for succinate formation during anaerobic fermentation (Camarasa *et al.*, 2003). 2-Ketoglutarate of the oxidative pathway is mainly used for ammonium assimilation and therfore little if any goes to succinate (Fig. 2.8). Thus, succinate is nearly exclusively formed in the reductive branch of the TCA cycle.

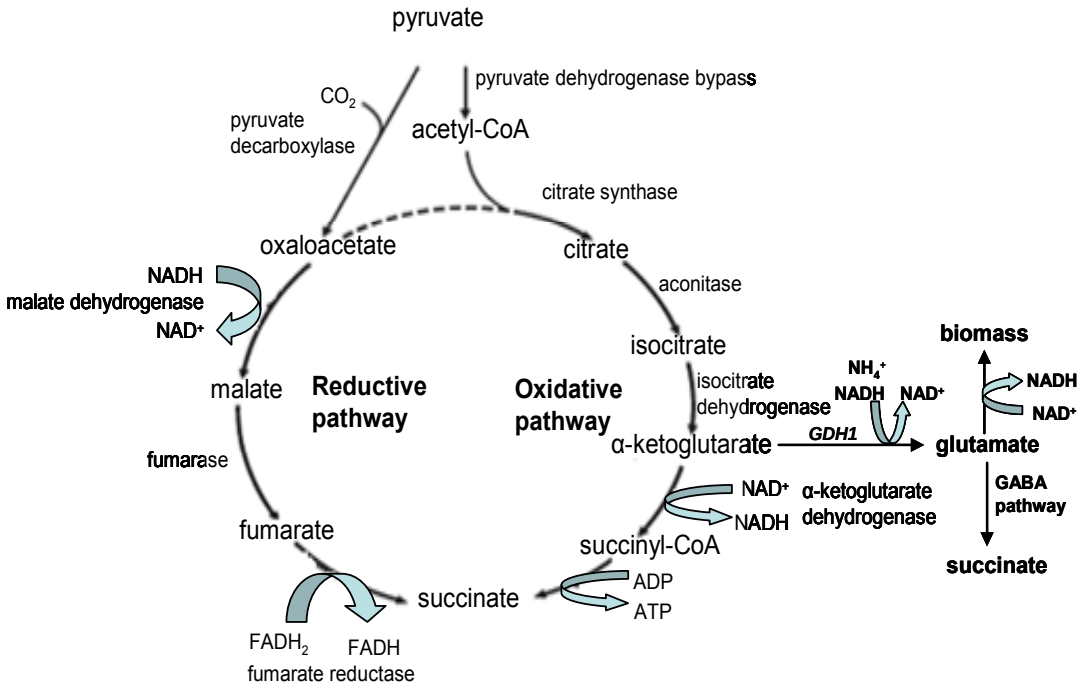

FIG. 2.8 Tricarboxylic acid cycle of *S. cerevisiae* under fermentative growth conditions and NADH formation and regeneration in mitochondria. Reduction of α-ketoglutarate to glutamate using NADH dependent glutamate dehydrogenase (*GDH1*) and formation of succinate from glutamate via γ amino butyric acid pathway are also shown. Adapted from Coulter *et al*., (2004).

2.4.3 NADH formation in cytoplasm

As described above, the anaerobic conversion of glucose to ethanol by *S. cerevisiae* is a redox neutral process. However, when intermediates in the pathway are withdrawn as precursors for the synthesis of cellular material, this balance is disturbed since the surplus of NADH produced is not converted back to NAD^+ (Fig. 2.9). Surplus NADH is formed not only through biomass formation but also by the formation of acids such as acetate (Fig. 2.9). The external NADH dehydrogenases encoding *NDE1* and *NDE2* and internal NADH dehydrogenase encoding *NDI1* are aerobic genes (*i.e.* these genes are transcribed and their corresponding enzymes are active under aerobic conditions) (Bakker *et al.*, 2001), and therefore other processes are required for the regeneration of NAD^+, otherwise metabolism eventually stops.

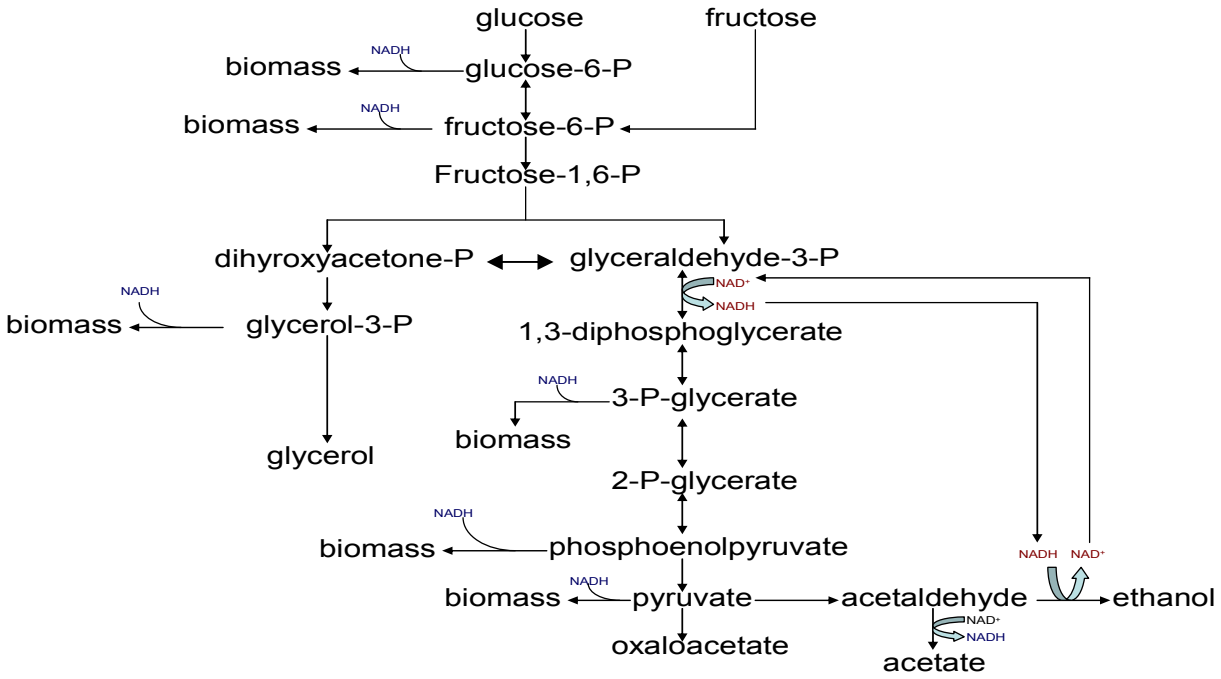

FIG. 2.9 NADH formation in cytoplasm under fermentative growth of *S. cerevisiae.* Adapted from Lehninger *et al*., (2000).

Furthermore, the redox equivalent of NADH which is formed in the mitochondria due to the activity of the 2-oxoglutarate dehydrogenase is shuttled to the cytoplasm by ethanol-acetaldehyde shuttle (Bakker *et al.*, 2000) as described earlier. For the functioning of ethanol-acetaldehyde shuttle, acetaldehyde has to enter the mitochondria. This will further disturb the redox neutral process of ethanol production in the cytoplasm because all the NADH which is formed by the Embden-Meyerhof-Parnas (EMP or glycolysis) pathway is not oxidized as some of the acetaldehyde entering the mitochondria is not reduced to ethanol.

Many different reactions therefore lead to the accummulation of NADH in the cytoplasm. To maintain a closed cytosolic redox balance, a reduced product must be formed thus reoxidizing excess NADH back to NAD^+. The principal reduced product is glycerol (Gancedo *et al.*, 1968b), whch accounts for the re-oxidation of most of the NADH which is formed under anaerobic conditions (Gancedo *et al.*, 1968b).

2.4.4 Glycerol: The most important by-product of anaerobic yeast growth in terms of NAD^+ regeneration

Under anaerobic conditions, the principal redox sink is the NADH coupled reduction of dihydroxyacetone-phosphate (DHAP) to glycerol-3-phosphate (G3P) (Fig. 2.10). This was first demonstrated by redox and carbon balance studies and given firm genetic evidence by mutant studies. The DHAP reduction is catalyzed by NADH dependent glycerol-3-phosphate dehydrogenase encoded by the two iso-genes, *GPD1* (Albertyn *et al*., 1994) and *GPD2* (Eriksson *et al*., 1995). The produced G3P is further dephosphorylated via a highly specific phosphatase encoded by the iso-genes *GPP1* and *GPP2* (Pahlman *et al*., 2001). The isoenzymes of the glycerol pathway appear to have distinct physiological roles. The genes *GPD1* and *GPD2* will be discussed more extensively in the next section.

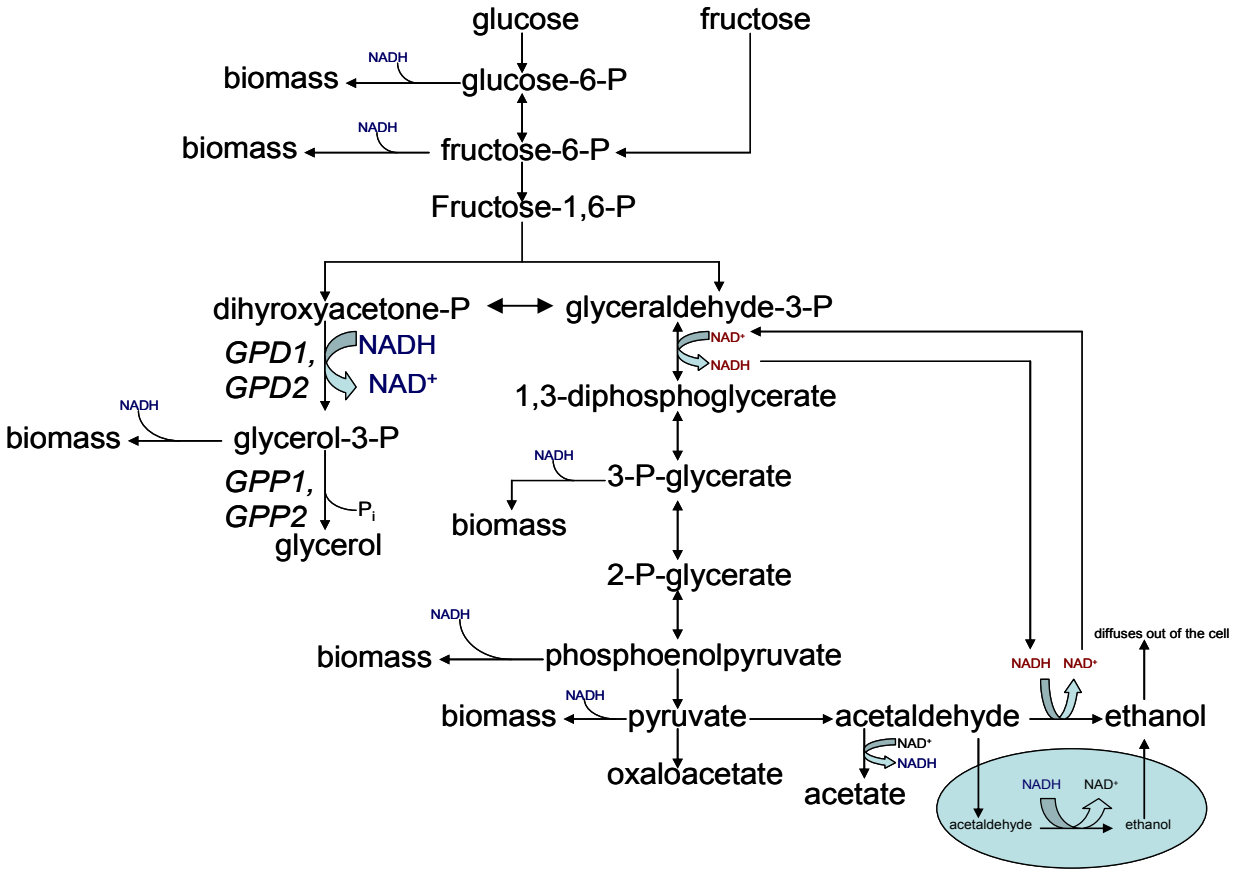

FIG. 2.10 Glycerol formation under fermentative growth of *S. cerevisiae.* Adapted from Lehninger *et al*., (2000).

2.4.5 Other important functions of glycerol in the cell

2.4.5.1 Osmoregulation

Glycerol plays an essential role as a compatible solute during osmoregulation in yeasts (Blomberg, 2000). In response to decreased extracellular water activity, *S. cerevisiae* greatly increases its rate of glycerol formation which then protects the yeast from losing intracellular water and helps to maintain turgor pressure (Albertyn *et al.*, 1994). The intracellular accumulation of glycerol in *S. cerevisiae* is also controlled by the Fps1p-channel (Luyten *et al.*, 1995). Under conditions of hyperosmotic stress (high sugar medium), the channel is closed thereby conserving the glycerol within the cell in order to maintain an osmotic equilibrium with the external environment. In the absence of hyperosmotic stress, the channel remains open and glycerol freely permeates from the cell (Hohmann *et al.*, 2000; Tamas *et al.*, 1999).

2.4.5.2 Maintaining the ratio of free to bound phosphate level in the cytosol

In *S. cerevisiae*, glycerol formation is partially responsible for balancing the ratio of free to bound phosphate level in the cytosol (Krallish *et al.*, 2007; Luyten *et al.*, 1995). Phosphate is an essential nutrient for all organisms. It plays a pivotal role in cell functioning, being involved in most metabolic energy transductions, and serves as an intermediate in the biosynthesis of numerous metabolites. It is therefore essential for organisms to have regulatory mechanisms for the acquisition, storage and release of this molecule (Martinez *et al.*, 1998; Persson *et al.*, 1998).

2.4.5.3 Lipid biosynthesis

In lipid biosynthesis, the formation of triacylglycerol and glycerophospholipids mainly proceeds through acylation of an intermediate of glycerol formation, G3P. The enzyme which catalyzes the reaction is glycerol-3-phosphate acyltransferase, encoded by the *GAT1* gene (Athenstaedt & Daum, 1997; Racenis *et al.*, 1992). The same enzyme can also use dihydroxyacetone phosphate as substrate (Athenstaedt *et al.*, 1999). The acyl dihydroxyacetone phosphate that is formed by this reaction can be reduced in a NADPH-coupled reaction catalyzed by acyl DHAP reductase, encoded by the *AYR1* gene (Athenstaedt & Daum, 2000). These reactions can provide a functional route for

the biosynthesis of triacylglycerol, when the synthesis of G3P is blocked by deletion of the *GPD1* and the *GPD2* genes (Ansell *et al.*, 1997; Bjorkqvist *et al.*, 1997).

2.4.6 *GPD1* and *GPD2*

In *S. cerevisiae*, the isoenzymes of G3P dehydrogenase have been identified and studied extensively. They were shown to be encoded by two isogenes, *GPD1* and *GPD2*. The amino acid sequences of Gpd1p and Gpd2p are 69% identical and the kinetic properties of the two isoenzymes are similar (Eriksson *et al.*, 1995). The K_m of Gpd2p for dihydroxyacetone phosphate (86 μM) is higher than that of Gpd1p (37-54 μM), whereas both enzymes exhibit a K_m of 18 μM for NADH (Ansell *et al.*, 1997). They are regulated in different manners in the cell (Albertyn *et al.*, 1994; Ansell *et al.*, 1997; Bjorkqvist *et al.*, 1997; Larsson *et al.*, 1998). *GPD1* is induced under osmotic stress in order to enhance the production of glycerol, the main osmoregulatory in *S. cerevisiae*. Its expression is regulated by the high-osmolarity glycerol (HOG) response pathway (Albertyn *et al.*, 1994; Blomberg, 2000; Reynolds *et al.*, 1998; Van Wuytswinkel *et al.*, 2000). *GPD2* is induced to maintain cytoplasmic redox balance (Eriksson *et al.*, 1995). The two enzymes encoded by *GPD1* and *GPD2* can substitute each other depending on the growth conditions (Ansell *et al.*, 1997).

2.4.6.1 Deletion studies of *GPD1* and *GPD2*

Various studies have been conducted on the mutants *gpd1*Δ, *gpd2*Δ and *gpd1*Δ*gpd2*Δ cultivated under both aerobic and anaerobic conditions with glucose as the sole carbon and energy source (Bjorkqvist *et al.*, 1997; Nissen *et al.*, 2000a). Bjorkqvist *et al.* (1997) carried out an experiment in which they grew *gpd1*Δ, *gpd2*Δ and *gpd1*Δ*gpd2*Δ DM in aerobic batch culture with continuous sparging air in the bioreactor. They discovered that no glycerol was formed by the *gpd1*Δ*gpd2*Δ DM strain as expected and the glycerol yield of *gpd1*Δ and *gpd2*Δ strains were 69% and 54% lower compared to the WT.

To monitor the growth of these strains under strict anaerobic condition, aerobic-anaerobic step change experiments were also conducted (Bjorkqvist *et al.*, 1997). The most dramatic effect observed in these experiments was that both the growth and the metabolic activity (monitored through CO_2 evolution rate, CER) of the *gpd1*Δ*gpd2*Δ DM strain completely stopped under strict anaerobic conditions. However, the WT and the

*gpd1*Δ mutant showed very similar responses in terms of CER. The CER of the *gpd1*Δ mutant and the WT strain demonstrated a low-amplitude, short-duration decrease. However, the decrease in the CER of the *gpd2*Δ mutant was much larger and lasted longer.

The glycerol yield of the *gpd2*Δ strain (0.07 g/g of glucose) was lower when compared to the glycerol yield of *gpd1*Δ and WT which was 0.1 g/g and 0.09 g/g of glucose respectively. However, earlier findings suggested that the *GPD1* gene is responsible for the formation of glycerol in response to hyperosmosis (Albertyn *et al.*, 1994) and the *GPD2* gene for the regeneration of NAD^+ under anaerobic condition (Ansell *et al.*, 1997). Bjorkqvist *et al* (1997) pointed out that the immediate preparedness to sustain anaerobic condition is apparently not as good in strain *gpd2*Δ as compared to strain *gpd1*Δ.

A study similar to that performed by Bjorkqvist *et al.* (1997) was carried out by Nissen *et al* (2000). In this case, there was no transfer from aerobic to anaerobic conditions but the system was anaerobic throughout the study. The glycerol yield measured in the anaerobic cultivations was 8-10% higher than reported by Bjorkqvist *et al.* (1997). Nissen *et al.* (2000) explained this result by mentioning that in the earlier study, the growth medium was supplemented with amino acids such as leucine, histidine, tryptophan, uracil and adenine which may reduce their need for *de novo* biomass precursor synthesis, resulting in a reduced production of NADH in the biosynthetic reactions and hence in a lower glycerol yield.

2.4.6.2 Metabolic engineering of glycerol production

From an industrial point of view, glycerol can be used in many products, ranging from cosmetics to lubricants (Overkamp *et al*., 2002). Furthermore, there has been considerable interest in modifying glycerol metabolism in *S. cerevisiae* because of its sensory importance in the wine industry. Although glycerol does not contribute directly to wine aroma, it can have a positive influence on the taste and mouthfeel of wines (Lubbers *et al*., 2001). Nieuwoudt *et al*. (2002) did extensive research on the effect of glycerol on the quality of white and red South African wine. In contrast to Lubbers *et al*. (2001), they did not find any clear correlation between the quality of dry red wines and the glycerol concentration. However, the data showed that in noble late harvest wines,

glycerol might improve the quality of wine because these wines are sweeter and glycerol may improve the smoothness on the mouth. It was also found that high glycerol concentrations in wines with high percentage volume of ethanol may also be beneficial because glycerol reduces the burning sensation in these wines (Dr Hélène Nieuwoudt 2008, pers. comm., November). The establishment of the precise nature of the contribution of glycerol to wine quality through descriptive aroma analyses, as well as by means of an investigation of the possible indirect effects of glycerol on wine quality through physical/chemical interactions between glycerol and other flavour constituents in wine requires further investigation.

Since the beginning of the 20th century, the role of glycerol in redox metabolism has been exploited for glycerol production from glucose. One of the first methods used to achieve higher yields was by chemically trapping acetaldehyde by adding sulphite to the fermenting *S. cerevisiae* cultures (Wang *et al*., 2001). Sulphite forms adduct with acetaldehyde, thus making the latter compound unavailable as an electron acceptor for the re-oxidation of glycolytic NADH (Wang *et al*., 2001). Instead, NADH is re-oxidized by glycerol production.

Over the past decades, research on glycerol production in *S. cerevisiae* has shifted to true metabolic engineering (*i.e*. the application of recombinant DNA technology) for a rational reprogramming of cellular metabolism (Bailey, 1991). Several approaches aimed at minimizing the reduction of acetaldehyde to ethanol by mimicking the sulphite process. Before attempting to reduce the carbon flux from acetaldehyde to ethanol, a metabolic engineering strategy was used that involved the deletion of the *TPI1* gene encoding for triose phosphate isomerase (Compagno *et al.*, 1996; Compagno *et al.*, 2001). This strategy introduced a stoichiometrically forced equimolar DHAP and glyceraldehyde-3-phosphate (GAP) formation. The maximum theoretical yield of this process is 1 mol of glycerol per mole of glucose consumed if all NADH formed during glycolysis is re-oxidized via *GPD1* and *GPD2.* However, *S. cerevisiae tpi1*Δ mutants are unable to grow on glucose as the sole carbon source (Compagno *et al.*, 1996; Compagno *et al.*, 2001). The inability of *tpi1*Δ mutant to grow on glucose has been recently explained by its inositol-defective phenotype. The deficiency is due to the potent inhibition of the MIP (myo-inositol-1-phosphate) synthase by DHAP and G3P. These compounds accumulate to very high levels in a *tpi1*Δ mutant (Cordier *et al.*, 2007; Shi *et al.*, 2005b). This inability of *tpi1*Δ to grow on glucose as the sole carbon

source was overcome by removing the cytosolic NADH re-oxidation by other genes such as *NDE1*, *NDE2* and deletion of *GUT2* which is involved in the re-oxidation of cytosolic NADH by the G3P shuttle (Overkamp *et al.*, 2002). This strategy ensured that all the NADH produced in the cytosol must be re-oxidized via Gpd1p and Gpd2p so that DHAP does not accumulate in the cells and is completely reduced to glycerol-3-phosphate and subsequently to glycerol. In other words, elimination of all competing reactions for re-oxidation of cytosolic NADH can provide Gpd1p and Gpd2p with sufficient NADH for the production of glycerol. Indeed, this strategy has been relatively successful since it raised the glycerol production from 0.25 g/g glucose consumed obtained by the sulphite process (Petrovska *et al.*, 1999) to 0.42 g/g glucose consumed (Overkamp *et al.*, 2002).

Building on the study of Overkamp *et al.* (2002), Geertman *et al*. (2006) succeeded in increasing the glycerol yield up to 0.50 g/g glucose by using another genetic engineering strategy which required the disruption of genes encoding respiratory chain linked NADH dehydrogenase and pyruvate decarboxylase. The major pyruvate decarboxylase genes encoded by *PDC1*/*5*/*6* were deleted to reduce the conversion of pyruvate to acetaldehyde. The authors also tried to increase the availability of cytosolic NADH by providing formate in the growth medium. In *S. cerevisiae*, formate is oxidized to CO_2 and does not serve as a carbon source, but does provide cytosolic NADH (Casal & Leão, 1995; Gellissen & Hollenberg, 1997). In addition, Geertman *et al.* (2006) showed that simultaneous overexpression of formate reductase encoded by *FDH1* and glycerol-3-phosphate dehydrogenase encoded by *GPD2* stimulates the efficient coupling of formate oxidation and glycerol production in such cultures.

In a recent study, Cordier *et al*. (2007) achieved a glycerol yield of 0.46 g/g glucose consumed. Although this yield is lower than the yield obtained by Geertman *et al*. (2006), the strategy highlighted many cellular targets that can be responsible for restraining higher glycerol productivity by *S. cerevisiae*. The strategy of Cordier *et al*. (2007) comprised the overexpression of *GPD1* encoding glycerol-3-phosphate dehydrogenase and disruption of *TPI1* and *ADH1*, which encode the triose phosphate isomerase and the major alcohol dehydrogenase respectively. In addition, they attempted to increase NADH availability by overexpressing NAD^+ dependent aldehyde dehydrogenase encoded by *ALD3.* It was shown that glycerol efflux is rate-limiting in glycerol hyperproducing strains and that the rate-limiting step of glycerol production was

no longer at the level of *GPD* genes. This was concluded by the observation that in none of the engineered strains DHAP and glycerol-3-phosphate concentrations returned to those in the WT strain. This finding suggests that although DHAP and glycerol-3-phosphate are completely converted to glycerol in these engineered strains, glycerol is not secreted. It was indeed observed that engineered strains accumulated large amounts of intracellular glycerol which points to glycerol efflux being the major rate-limiting step in this process. However, the over-expression of the *FPS1* gene encoding the major glycerol facilitator (Luyten *et al.*, 1995) had minor or no effect on glycerol efflux. Cordier *et al*. (2007) explained this phenomenon by the recent finding that high intracellular content of glycerol can mediate the closure of the channel (Karlgren *et al.*, 2005). Alternatively, it is possible that other aquaglyceroporins are also needed for complete glycerol efflux (Luyten *et al.*, 1995). Furthermore, accumulation of glycerol can trigger a Hog1-dependent osmoprotective pathway (Albertyn *et al.*, 1994; Siderius *et al.*, 2000), which may lead to a slow-down of glycolysis and growth rate by an as yet uncharacterized mechanism (Blomberg, 2000). Taken together, these effects may also explain why the over-expression of *FPS1* resulted in a weak increase in glycerol production. Ultimately, it appears that the removal of glycerol during its synthesis could be a target to enhance glycerol yields.

Most of the above mentioned studies (Cordier *et al.*, 2007; Geertman *et al.*, 2006; Overkamp *et al.*, 2002; Wang *et al.*, 2001) were conducted to improve the glycerol yield were to provide tools for the production of the pure metabolite. For this reason, they were carried out in batch cultures with continuous aeration. The aim of these strategies was to allow some respiratory dissimilation of glucose thereby increasing the availability of ATP for high biomass formation and ultimately high glycerol yield. On the other hand, the situation is different in winemaking where the system is oxygen limited and ATP formation is low when compared to aerobic cultures. Unlike aerobic conditions with low glucose concentration where glycerol is formed only for the purpose of regenerating NAD^+, during winemaking it is also formed as a compatible solute due to hyperosmotic stress imposed by the high sugar concentration in grape juice. In this process, adjustment of glycerol production would rather aim at achieving a better balance of glycerol versus ethanol production, and would not aim at increasing yields to the same degree. Consequently many attempts to increase the glycerol yield during fermentation have been made by for example overexpressing *GPD1* and *GPD2* (Cambon *et al.*, 2006; Eglinton *et al.*, 2002; Michnick *et al.*, 1997; Remize *et al.*, 1999; Remize *et al.*,

2001) or by using breeding techniques. The common finding from all the above mentioned studies was that glycerol overproduction leads to formation of acetate. Excessive production of acetate (above 1 g/l) has a major negative impact on wine since the maximum amount desirable below 0.6 g/l (Remize *et al.*, 1999). The high formation of acetate in strains that overexpress *GPD1* and *GPD2* was due to the increased formation of NAD^+.

Cambon *et al.* (2006) rectified the problem of high acetate by deleting the *ALD6* gene (Grabowska & Chelstowska, 2003) which encodes for aldehyde dehydrogenase and catalyzes the oxidation of acetaldehyde to acetate. However, even this strategy was not without side-effects. While *GPD1* overexpression largely affects central metabolism particularly at the acetaldehyde node, additional changes are triggered by *ALD6* deletion. Due to the deletion of *ALD6*, acetaldehyde accumulated in the cells which then condensed with active acetaldehyde to form acetoin (Remize *et al.*, 1999). Acetoin is then reduced to 2,3-butanediol by the *BDH1* gene (Gonzalez *et al.*, 2000) encoding for 2,3-butanediol dehydrogenase. Both acetoin and 2,3-butanediol are undesirable in wines. However, a major possibly positive effect of this strategy was the lower ethanol content which was due to major carbon flux directed towards glycerol. Low alcohol wines are usually desirable because of health concerns.

These results highlight the potential of strategies linked to glycerol metabolism for producing low alcohol wines. However, it is clear that any such strategy requires the balancing of redox equivalents in ways that would not negatively impact on food or beverage quality, such as accumulation of acetate, acetoin or 2,3-butanediol. At this stage, the formation of undesirable by-products limits the extent to which carbon flux can be diverted to glycerol. Further improvement of these strains requires new efforts to minimize the formation of undesirable compounds, in particular at the acetaldehyde branch point (Cambon *et al.*, 2006).

2.4.6.3 Substituting *GPD1* and *GPD2* in double deletion mutant by overexpressing heterologous gene/genes

Over the past decade, several authors aimed to substitute *GPD1* and *GPD2* in the *gpd1*Δ*gpd2*Δ DM by transforming it with heterologous gene/genes. Their objective was to check if glycerol-free anaerobic fermentation could be sustained in *S. cerevisiae* by

incorporating an alternative internal redox sink to glycerol and to analyze how the metabolism during anaerobic growth of *S. cerevisiae* might be altered due to the incorporation of new pathways for NADH re-oxidation (Costenoble *et al.*, 2003; Heux *et al.*, 2006; Liden *et al.*, 1996; Nissen *et al.*, 2001; Zhang & Chen, 2008). A further objective of these studies was to examine if a blocked glycerol formation pathway would enhance some other osmolyte or other valuable metabolite production (such as xylitol and mannitol) because of decreased competition for NADH. Liden *et al*. (1996) transformed a *gpd1*Δ*gpd2*Δ DM with a plasmid containing the xylose reductase gene (*XYL1*) taken from *Pichia stipitis* (Verduyn *et al.*, 1985). *P. stipitis* is xylose fermentating yeast and Xyl1p converts xylose to xylitol which after a succession of reactions enters the pentose phosphate pathway (Nevoigt, 2008). *XYL1* from *P. stipitis* has dual cofactor specificity so the reduction of xylose to xylitol may take place with either NADH or NADPH (Verduyn *et al.*, 1985). Therefore, in the *gpd1*Δ*gpd2*Δ DM, Xyl1p was supposed to convert extracellularly added xylose to xylitol by using excess NADH in the *gpd1*Δ*gpd2*Δ DM thereby regenerating NAD^+. Aerobic-anaerobic step change experiments were carried out by Liden *et al*. (1996). The authors observed that for the *gpd1*Δ*gpd2*Δ DM, growth as well as CO_2 evolution abruptly stopped when the mutant was subjected to anaerobic conditions. This observation is in accordance with previous observation for the *gpd1*Δ*gpd2*Δ DM strain (Bjorkqvist *et al.*, 1997). After aerobic growth, a step-wise change from aerobic to strict anaerobic conditions was made and 3 h later xylose addition to the growth medium started. The effect of xylose addition was visible only after addition of substantial amount of xylose. Addition of up to 4 g/l xylose produced barely detectable changes. However, after addition of 50 g/l xylose to the culture medium, the anaerobically measured CER and ethanol formation rate were again comparable to the aerobically measured ones. The specific xylitol formation rate under anaerobic conditions was calculated to be 0.38 g/g biomass /h. This experiment clearly showed that the alternative redox sink provided by the reduction of xylose to xylitiol can compensate partly for the lost capability of glycerol formation and make anaerobic growth possible in the *gpd1*Δ*gpd2*Δ DM strain. The explanation for the need to add a high concentration of xylose (50 g/l) to the growth medium before detecting any change in CER and ethanol formation was that xylose reductase has a low affinity for xylose, with a reported K_m value of 63 mM (Thestrup & Hahn-Hagerdal, 1995). Moreover, the *XYL1* gene of *P. stipitis* encodes an active transport system for xylose (Boles & Hollenberg, 1997) and is therefore able to accumulate xylose against a concentration gradient in that yeast. However, *S. cerevisiae* lacks this active uptake

system for xylose which is otherwise taken up via the glucose uptake system (Liden *et al.*, 1996). This system is a facilitated system and does not allow accumulation against a gradient. Furthermore, there will be competition for the carriers between glucose and xylose. High concentrations of xylose are therefore needed to obtain sufficiently high concentrations for the Xyl1p to operate efficiently.

Liden *et al*. (1996) also observed that there are other redox sinks, such as acetoin which may also serve the purpose of regeneration of NAD^+. Six hours after a step change from aerobicity to anaerobicity, acetoin was added to the growth medium. The authors found that even small amount of acetoin (0.5 g/l) were in fact more effective than xylose (4 g/l) in restoring metabolic acitivity of the *gpd1*Δ*gpd2*Δ DM strain. Extracellularly added acetoin is converted to 2,3-butanediol catalyzed by the enzyme 2,3-butanediol dehydrogenase (*BDH1*) (Nordling *et al.*, 2002) and NAD^+ is regenerated in this reaction. The probable reason for this observation could be related to poor transport of xylose and dual cofactor specificity of *XYL1* as discussed earlier.

Unlike the experiment carried out by Liden *et al*. (1996) where xylose was provided as a carbon and energy source, Costenoble *et al*. (2003) investigated if the growth of *S. cerevisiae gpd1*Δ*gpd2*Δ DM can continue under anaerobic conditions by reduction of substrates derived from glucose. They transformed the *gpd1*Δ*gpd2*Δ DM with a plasmid containing the mannitol-1-phosphate dehydrogenase gene (*mtlD*) taken from *E. coli* (Teschner *et al.*, 1990). *E. coli* can use mannitol as carbon and energy source where mannitol first enters the cell and gets phosphorylated to mannitol-1-phosphate which is then oxidized to fructose-6-phosphate using NAD^+ as the cofactor. The hypothesis was that in the transformed *gpd1*Δ*gpd2*Δ DM, this enzyme would catalyze the conversion of fructose-6-phosphate to mannitol-1-phosphate which is the reverse of the normal reaction in *E. coli* thereby re-oxidizing the excess NADH back to NAD^+. In an aerobic-anaerobic step change experiment Costenoble *et al*. (2003) observed that for both *gpd1*Δ*gpd2*Δ DM and *gpd1*Δ*gpd2*Δ DM transformed with *mtlD*, the CER decreased abruptly and at the same time, glucose consumption and ethanol production stopped. However, the *gpd1*Δ*gpd2*Δ DM transformed with *mtlD* produced some mannitol which means that this transformed strain actually converted some NADH back to NAD^+. Mannitol formation clearly shows that redox imbalance was the driving force for the mannitol formation in the mutant but the reason for the failure to grow under anaerobic conditions was not immediately obvious. The lack of mannitol export causing osmotic

problems was suggested to lead to cessation of growth under anaerobic condition. A micrograph of the cells of *gpd1*Δ*gpd2*Δ DM transformed with *mtlD* indicated cells having large vacuoles and an improvement of mannitol efflux was apparently necessary to obtain anaerobic growth together with a sustained mannitol production.

Nissen *et al*. (2001) isolated a gene encoding the pyridine nucleotide cytoplasmic transhydrogenase from *Azotobacter vinelandii*. This enzyme catalyzes the interconversion between the two cofactor systems of $NADH/NAD^+$ and $NADPH/NADP^+$ according to the reaction:

$$NADH + NADP^+ \longrightarrow NAD^+ + NADPH$$

The hypothesis was that overexpressing this gene in a *gpd1*Δ*gpd2*Δ DM could potentially represent an alternative route for re-oxidation of NADH to NAD^+ enabling the strain to grow under anaerobic growth conditions. Unfortunately, the overexpression of the transhydrogenase gene did not result in growth under anaerobic condition probably due to the localization of the enzyme in a membrane without an appropriate proton gradient (Nissen *et al.*, 2000a). After this failure, Nissen *et al*. (2001), changed strategy and overexpressed transhydrogenase gene in the WT *S. cerevisiae* T2-3D strain. The hypothesis was that the overexpression of this gene in the WT strain may result in a decrease in both glycerol formation and the carbon flux through the pentose phosphate pathway, where there is loss of carbon in the form of CO_2. This reduction in the carbon flux towards “waste” components could be redirected towards formation of ethanol, leading to higher yields. This strategy was also not successful although the transhydrogenase was functionally expressed in *S. cerevisiae* T2-3D strain. They suggested that instead of converting excess NADH to NAD^+ according to the above mentioned reaction, the heterologous transhydrogenase catalyzed the reverse reaction, *i.e.* the transfer of electrons from NADPH to NAD^+ in anaerobic cultures. This conclusion was supported by the observation that overexpression of transhydrogenase caused a significant increase in formation of 2-ketoglutarate (due to generation of $NADP^+$ through reverse reaction) and glycerol (due to generation of NADH through reverse reaction) (Fig. 2.6) (Nissen *et al.*, 2000b).

Overall, these experiments suggest that to replace glycerol by some other metabolite (such as mannitol and 2,3-butanediol) and to regenerate NAD^+ by some other means

(by overexpressing transhydrogenase) is a difficult task. This is probably due to the requirement for a very tight regulation of the redox balace to ensure normal growth, meaning that any engineered system would have to meet very specific flux requirements in order not to lead to undesirable side effects. However, these strategies were partly successful in terms of producing metabolites other than glycerol and regenerating NAD^+. Moreover, no reports have yet appeared where native yeast genes would have been used for restoring both redox balance and metabolite production in *S. cerevisiae gpd1*Δ*gpd2*Δ DM.

2.5 Other redox-maintenance factors in *S. cerevisiae*

All reports to date show that a *S. cerevisiae gpd1*Δ*gpd2*Δ DM strain cannot grow under anaerobic conditions unless provided with an external redox sink such as acetoin or xylose to the medium. While the data clearly demonstrate that maintaining the balance of NAD^+ and NADH is essential for growth, other redox-related factors, and in particular FAD^+, also play an important role (Arikawa *et al.*, 1998).

The yeast *S. cerevisiae* possesses both mitochondrial and cytosolic soluble fumarate reductases, encoded by the *OSM1* (Muratsubaki & Enomoto, 1998) and *FRDS1* (Enomoto *et al.*, 1996) genes, respectively. These enzymes non-covalently bind $FADH_2$ or $FMNH_2$, which serve as the electron donors to oxidized substrates (Muratsubaki & Enomoto, 1998). As for the NAD^+/NADH balance, oxidized flavins accept electrons from reduced substrate and convert back to the reduced form.

The *osm1*Δ*frds1*Δ cannot grow under strict anaerobic conditions, similar to the *gpd1*Δ*gpd2*Δ DM. To account for this phenotype, it was proposed that under anaerobiosis, the fumarate reductase might provide the only way for yeast to regenerate the FAD/FMN prosthetic group of the flavin enzymes that are required for growth (Camarasa *et al.*, 2007). It is also interesting to note that Frds1p and Osm1p can functionally substitute for each other just like Gpd1p and Gpd2p (Camarasa *et al.*, 2007).

In conclusion, in addition to reduction of NAD^+, yeast must continuously regenerate FAD/FMN for the metabolic activity to continue. Similar to the studies done earlier to

replace *GPD1* and *GPD2* in the *gpd1*Δ*gpd2*Δ DM, it would be interesting to see if heterologous gene/genes can replace *OSM1* and *FRDS1* in the *osm1*Δ*frds1*Δ DM.

2.6 Alcoholic fermentation in wine as an example of anaerobic growth in high sugar concentration medium

Wine making is perhaps the oldest form of alcoholic beverage production in human history. Wine is made from grape juice which has very high concentration of sugars (mixture of glucose and fructose) ranging from 180 to 230 g/l (Erasmus *et al.*, 2003). Due to the presence of such high concentrations of sugars, grape juice in the presence of suitable yeast undergoes alcoholic fermentation (Wejnar, 1970) even in the presence of oxygen, referred to as the Crabtree effect as explained earlier in this chapter. High sugar concentration in the grape must also creates other problems for the wine yeast. To survive in such high sugar medium, *S. cerevisiae* produces glycerol as an osmoprotectant (Gancedo *et al.*, 1968a). The two main acids which are also present in wine are acetic acid and succinic acid. Acetate production is actually an indirect effect of glycerol formation. Production of glycerol leads to high NAD^+ formation which is then used by the aldehyde dehydrogenases and then acetate is produced. Succinate is also thought to be usually produced in the mitochondria to maintain redox balance (Camarasa *et al.*, 2007).

The most mysterious aspect of wine is the endless variety of flavours that stem from a complex, completely non-linear system of interactions among hundreds of compounds. In its widest sense, wine flavour refers to the overall impression of both aroma and taste components. Aroma is usually associated with odorous volatile compounds such as esters and higher alcohols.

2.6.1 Higher alcohols and their biosynthesis

Higher alcohols contribute significantly to wine aroma and some higher alcohols such as isobutanol, amyl alcohol and isoamyl alcohol when present in appropriate concentrations are considered beneficial for wine aroma. Branched-chain amino acids (BCAAs) are key substrates in the formation of these higher alcohols (Fig. 2.11). The starting precursor for the formation of isobutanol, amyl alcohol and isoamyl alcohol are L-valine, L-isoleucine and L-leucine respectively. The first step in the catabolism of

branched-chain amino acids is transamination to form the respective α-keto-acids (α-ketoisocaproic acid from leucine, α-ketoisovaleric acid from valine, and α-keto-β–methylvaleric acid from isoleucine). This step is catalyzed by a branched-chain amino acid transaminase (BCAAT) named as Bat1p (Eden & Benvenisty, 1998; Kispal *et al.*, 1996; Schoondermark-Stolk *et al.*, 2005a) and Bat2p (Schoondermark-Stolk *et al.*, 2005a). An α-keto-acid decarboxylase *PDC1,5,6* (Dickinson *et al.*, 2003; Pronk *et al.*, 1996) for α-ketoisovaleric, *THI3* (Dickinson *et al.*, 2000; Nishimura *et al.*, 1992) or *ARO10* (Iraqui *et al.,* 1999*)* for α-ketoisocaproic and *PDC1,5,6*, *THI3* or *ARO10* for α-keto-β–methylvaleric convert the resulting α-keto-acids to the corresponding branched-chain aldehydes with one less carbon atom, and then alcohol dehydrogenase (*ADH1/5, SFA1*) catalyzes the NADH-dependent reduction of this aldehyde to the corresponding higher alcohol. The metabolic pathway for the production of higher alcohol has been proposed by Ehrlich and therefore the pathway is named after him as Ehrlich pathway (Hazelwood *et al.*, 2008). Apart from these higher alcohols, higher alcohols such as propanol and butanol may also contribute to the wine aroma (Atsumi *et al.*, 2008). The biosynthesis of these higher alcohols is shown in Fig. 2.11.

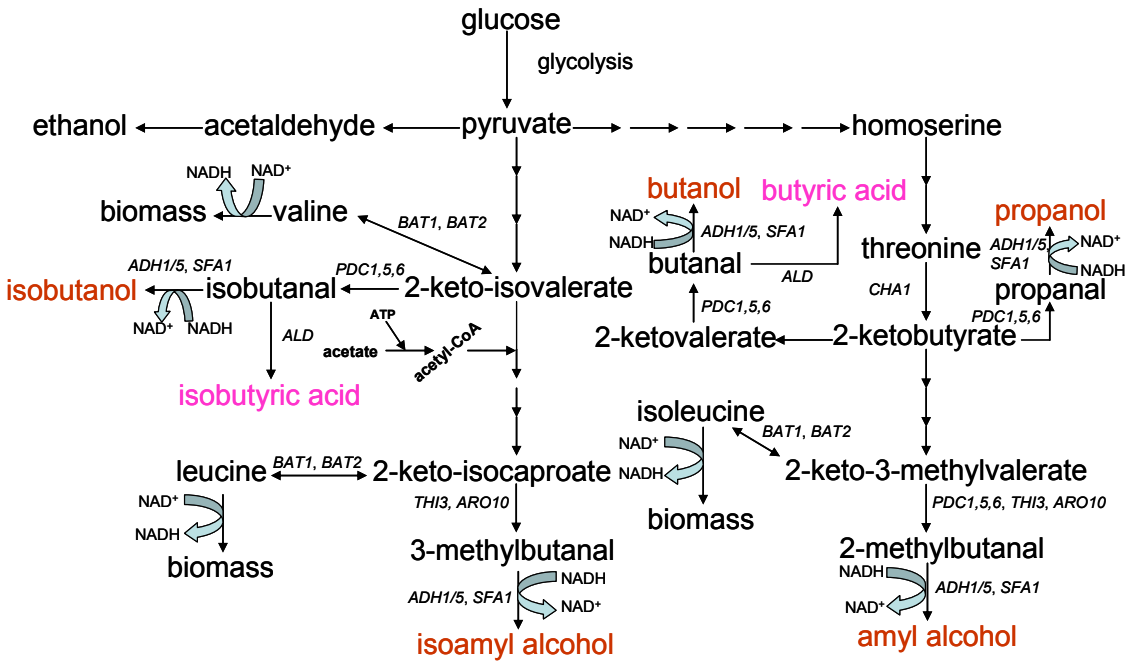

FIG. 2.11 Biosynthesis of branched chain higher alcohols such as isobutanol, isoamyl alcohol and amyl alcohol from their corresponding amino acids valine, leucine and isoleucine respectively via the Ehrlich pathway in *S. cerevisiae*. Simultaneously, biosynthesis of amino acids from pyruvate are also shown. It can be seen that 2-keto-isovalerate, 2-keto-isocaproate, and 2-keto-3-methylvalerate can either convert to their corresponding amino acids by transamination or can be diretly decarboxylated to their corresponding aldehyde using α-keto-acid decarboxylase (Atsumi *et al.*, 2008). Amino acids can also be used for biomass formation. Formation of other higher alcohols such as propanol and butanol from threonine and formation of fatty acids such as isobutyric acid and butyric acid from their corresponding aldehydes are also shown. The genes encoding enzymes in the biosynthesis are *ADH*= alcohol dehydrogenase, *PDC*= pyruvate decarboxylase, *SFA*= alcohol dehydrogenase, *BAT*= branched chain amino acid transaminase, THI= α-ketoisocaproate decarboxylase, *ARO10*= aromatic amino acid decarboxylase, *CHA1*= threonine dehydratase.

An aromatic higher alcohol, phenylethanol, is commonly formed by *S. cerevisiae* under fermentative condition. Its biosynthesis can either start from glucose or from phenylalanine. Formation of phenylethanol using both of these pathways combines at phenylpyruvate. Glucose is converted to phosphoenolpyruvate and erythrose-4-phosphate using glycolysis and pentose phosphate pathway respectively. Phosphoenolpyruvate and erythrose-4-phosphate combine to form phenylpyruvate. Phenylpyruvate can also be formed directly from the amino acid phenylalanine through transamination using aromatic amino acid transferases (*ARO8/9*) (Iraqui *et al.*, 1998). Phenylpyruvate is then converted to phenylacetaldehyde using enzymes encoded by *PDC1/5/6* or *ARO10*. Phenylacetaldehyde is ultimately reduced to phenylethanol using

alcohol dehydrogenase encoded by enzymes *ADH1/5* or *SFA1*. The biosynthesis of phenylethanol from phenylalanine and glucose is shown in Fig. 2.12.

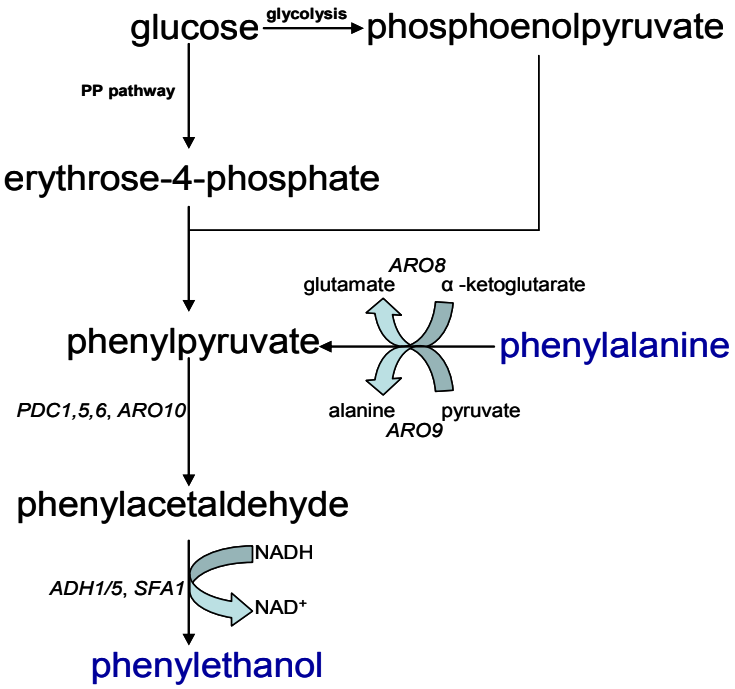

FIG. 2.12 Biosynthesis of phenylethanol in *S. cerevisiae* from glucose or from amino acid phenylalanine. Glucose is converted to phosphoenolpyruvate using glycolysis and erythrose-4-phosphate using pentose phosphate pathway (PP pathway). Phosphoenolpyruvate can then combine with erythrose-4-phosphate to form phenylpyruvate. Phenylpyruvate can also be formed directly from amino acid phenylalanine through transamination using aromatic amino acid transferases (*ARO8/9*). Phenylpyruvate is decarboxylated to phenylacetaldehyde using aromatic acid specific decarboxylase (*ARO10*) or using unspecific decarboxylases (*PDC1/5/6*). Phenylacetaldehyde is then converted to phenylethanol using unspecific alcohol dehydrogenases encoded by genes such as *ADH1/5* or *SFA1* (Kobayashi *et al*., 2008).

The reason for the formation of higher alcohol as proposed by Schoondermark *et al*. (2005) is that *S. cerevisiae* usually synthesize these higher alcohols as a way to regenerate NAD^+ (Schoondermark-Stolk *et al.*, 2005b). The last step in the Ehrlich pathway (Fig. 2.11) is catalyzed by an alcohol dehydrogenase which uses NADH as the cofactor and regenerate NAD^+ (Schoondermark-Stolk *et al.*, 2005b). Moreover, higher alcohols are formed mostly under anaerobic conditions when there is depletion of NAD^+ and the only way by which yeast can regenerate NAD^+ is through glycerol formation. Thus, it can be concluded by stating that higher alcohol formation can serve as a way to regenerate "extra" NAD^+ under fermentative conditions.

2.6.2 Biosynthesis of esters

Esters are a group of volatile compounds that impart mostly pleasant smell in wine. Most esters found in alcoholic beverages are produced by yeasts during fermentation as secondary products of sugar metabolism and constitute one of the largest and most important groups of compounds affecting flavour. The two most common esters found in wine are ethyl acetate (fruity) and isoamyl acetate (banana, pear). The biosynthesis of these esters is shown in Fig. 2.13. The last step in the ester biosynthesis is catalyzed by alcohol acetyltransferase encoded by *ATF1/2* (Mason & Dufour, 2000).

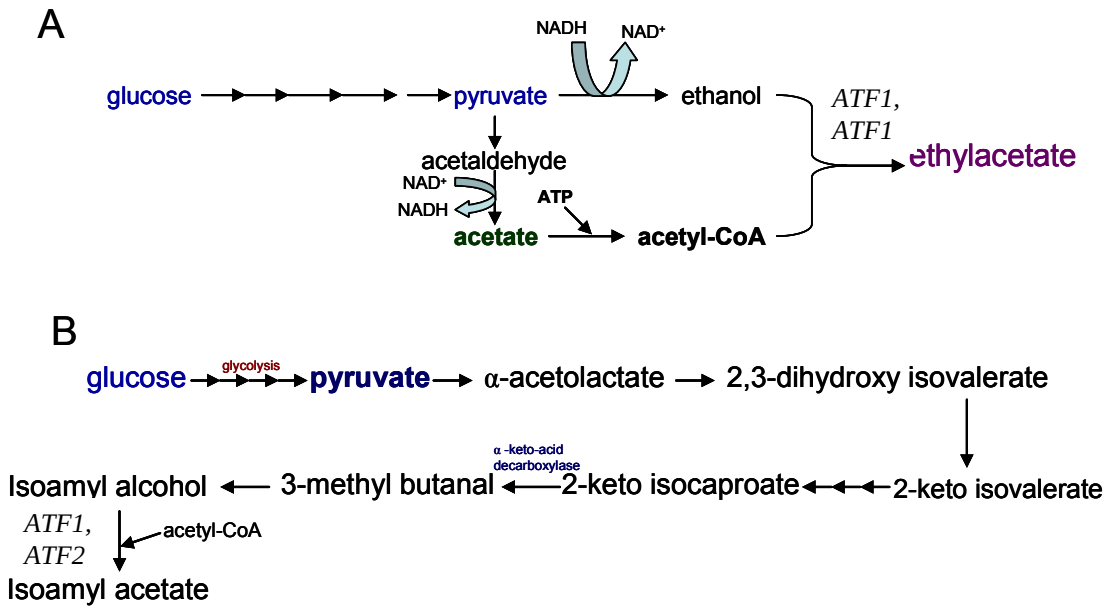

FIG. 2.13 Biosynthesis of ethyl acetate (A) isoamyl acetate (B) in *S. cerevisiae* (Adapted from Fraile *et al.*, 2000; Mason & Dufour, 2000; Santillan-Valverde & Garcia-Garibay, 1998; Schreier, 1979).

Several authors have proposed reasons for ester production under fermentative condition by *S. cerevisiae* (Fredlund *et al.*, 2004; Mason & Dufour, 2000). Firstly ester synthesis has been proposed to act as a detoxification mechanism used by *S. cerevisiae* to remove the toxic compounds such as acetate, medium chain fatty acids and some aldehydes from the cells (Fredlund *et al.*, 2004). Secondly, ester synthyesis has been suggested to be required to optimise the recirculation of free CoA (CoA-SH), which is liberated in the condensation reaction between acetyl-CoA and ethanol (Mason & Dufour, 2000). The molar amount of ethyl acetate is usually below 0.5% of ethanol produced, and thus the removal is not significant. However, the removal of acetate may

be important under oxygen limitation, if the cell is more sensitive to intracellular organic acids under these conditions. However, the second hypothesis is more likely since the ester production is induced during oxygen limitation when the lipid synthesis and the resulting release of CoA-SH are reduced (Fredlund *et al.*, 2004).

Over the past decade, many studies have contributed to a better understanding of the genes and their enzymes involved in higher alcohol and ester production and how their expression is regulated (Mason & Dufour, 2000; Schoondermark-Stolk *et al.*, 2005b). The data show that the various pathways are strongly interlinked and constitute a complex network of shared intermediates. While this network is reasonably well mapped, its regulation and the specific contribution of individual genes is poorly understood (Rossouw *et al*., 2008). An outstanding question in this regard is how changes in the redox balance impact on the flux of metabolites within this complex network. Such understanding is important for the application of metabolic engineering strategies to improve the aroma of alcoholic beverages. Indeed, a desirable trait in beer and wine fermentation is to achieve high levels of branched-chain and aromatic alcohol production in combination with low levels of off-flavors. The growing understanding of the key components of the Ehrlich pathway and their regulation will aid in the design of strains exhibiting specific flavour profiles in foodstuffs as well as in the metabolic engineering of yeast strains for the production of individual Ehrlich pathway products. In conclusion there is still huge potential to improve the aroma profile of wine by a better understanding of known and unknown pathways that led to the production of aroma components in wine or any alcoholic beverages.

2.7 Overall conclusion

It is evident from this literature review that fermentation confronts *S. cerevisiae* with several different challenges. Of all these challenges, NAD^+ regeneration seems to be one of the most important. The data strongly suggest that anaerobic conversion of glucose to ethanol by *S. cerevisiae* without formation of glycerol is not possible unless an externally added redox sink is provided. It is a challenge for the yeast research scientific community to design a strategy where a *gpd1*Δ*gpd2*Δ DM can be engineered to grow in anaerobic conditions, and to fully understand the regulations involved. Furthermore, it is important to understand how the redox balance impacts on other metabolic pathways of biotechnological relevance such as the network of aroma-

producing pathways. Little research has thus far been conducted in this direction. Data generated in this field might also be useful for the bio-ethanol industry because the carbon which was directed towards glycerol could be redirected towards ethanol. This could increase the yield of ethanol, thereby rendering its production more cost effective for the industry.

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Chapter 3

RESEARCH RESULTS I

Yeast sorbitol dehydrogenase and bacterial sorbitol-6-phosphate dehydrogenase partially restore fermentative ability in *Saccharomyces cerevisiae* mutants defective in glycerol formation

ABSTRACT

All biological systems have to maintain a balance of redox equivalents for growth. In *S. cerevisiae* growing under fermentative conditions NAD^+ is required for glycolytic activity. Under these conditions, *S. cerevisiae* re-oxidizes excess NADH through glycerol production which involves NADH-dependent glycerol-3-phosphate dehydrogenase (Gpd1p and Gpd2p). Deletion of the genes encoding these two enzymes renders the cells incapable of maintaining glycolytic activity and growth under anaerobic condition due to accumulation of NADH. The possibility of converting this excess NADH to NAD^+ by overexpressing either a native yeast gene (*SOR1*) encoding a NADH-dependent sorbitol dehydrogenase or a heterologous gene (*srlD*) from *Escherichia coli* encoding a NADH-dependent sorbitol-6-phosphate dehydrogenase in a *S. cerevisiae gpd1*Δ*gpd2*Δ double mutant (DM) was investigated. The strains were grown under fermentative conditions in high sugar medium (5% glucose, 5% fructose) and the rate of fermentation was monitored by weight loss. It was found that overexpression of these genes in the DM resulted in partial improvement of the fermentative ability but did not reach the efficiency of the WT strain. Similar to the WT strain, the mutant strains utilized glucose more rapidly than fructose. This observation was surprising in the *SOR1* strain since Sor1p should specifically convert fructose to sorbitol. Genetic manipulation of the DM strain also affected the overall metabolic fluxes significantly. A greater yield of sorbitol was found in the strain over-expressing *srlD* than *SOR1* whereas the WT and DM strains produced no sorbitol. The genetic modifications also influenced the yield of tricarboxylic acid intermediates such as succinate and malate. Furthermore, the formation of the secondary metabolites isobutanol, isoamyl alcohol, propanol, butanol, isobutyric acid, ethyl acetate and 2-phenyl ethanol were modified in comparison to the wild-type and DM strains indicating a general redirection of flux through secondary metabolism.

3.1 Introduction

Glycerol is quantitatively the most important product of alcoholic fermentation after ethanol and CO_2 (Blomberg, 2000; Geertman *et al.*, 2006; Ohmiya *et al.*, 1995; Wang *et al.*, 2001). The anaerobic conversion of glucose into ethanol by *S. cerevisiae* is a redox neutral process, *i.e.* the NAD^+ consumed initially in the glycolytic pathway is regenerated by the formation of ethanol which ultimately diffuses out of the cell (Albers *et al.*, 1996; Bakker *et al.*, 2001; Nissen *et al.*, 1997; van Dijken & Scheffers, 1986). However, when the intermediates of glycolysis are withdrawn as precursors for synthesis of cellular material and also when several oxidized by-products such as pyruvate and acetate are formed and released, this balance is disturbed because these processes lead to a net production of NADH without directly associated regeneration of NAD^+ (Nissen *et al.*, 1997). This will eventually cause metabolism to stop unless the surplus NADH can be converted back to NAD^+. Under aerobic conditions, conversion of excess NAD^+ to NADH takes place via the electron transport chain where oxygen is the final electron acceptor from NADH and converted to water (de Vries *et al.*, 1992; Luttik *et al.*, 1998; von Jagow & Klingenberg, 1970). This is however not possible under anaerobic conditions. Instead, in these conditions, most of the re-oxidation of the cytosolic NADH occurs via the NADH-coupled reduction of dihydroxyacetone phosphate (DHAP) to glycerol-3-phosphate (G3P) which ultimately leads to the formation of glycerol (Albertyn *et al.*, 1994; Eriksson *et al.*, 1995; Pahlman *et al.*, 2001).

Under fermentative conditions, the tricarboxylic acid (TCA) cycle is partially active in the mitochondrion and also in the cytoplasm to provide TCA-cycle intermediates as precursors for biomass building blocks (Camarasa *et al.*, 2003; Visser *et al.*, 1994). Due to the functioning of TCA cycle under fermentative conditions, NADH may also be formed in the mitochondrion during the conversion of 2-ketoglutarate to succinyl-CoA. The enzyme 2-ketoglutarate dehydrogenase which catalyzes this reaction is exclusively mitochondrial and uses NAD^+ as cofactor (Bakker *et al.*, 2000; Repetto & Tzagoloff, 1991). The NADH formed in this way is thought to be oxidized by means of an acetaldehyde-ethanol redox shuttle involving the mitochondrial alcohol dehydrogenase Adh3p (Bakker *et al.*, 2000). The result of this process is also a net production of NADH in the cytosol. In this way, both cytosolic and mitochondrial excess NADH can lead to glycerol production. Therefore, formation of glycerol plays a central role in the

intracellular redox state of *S. cerevisiae* growing under anaerobic condition (van Dijken & Scheffers, 1986) (Fig. 3.1).

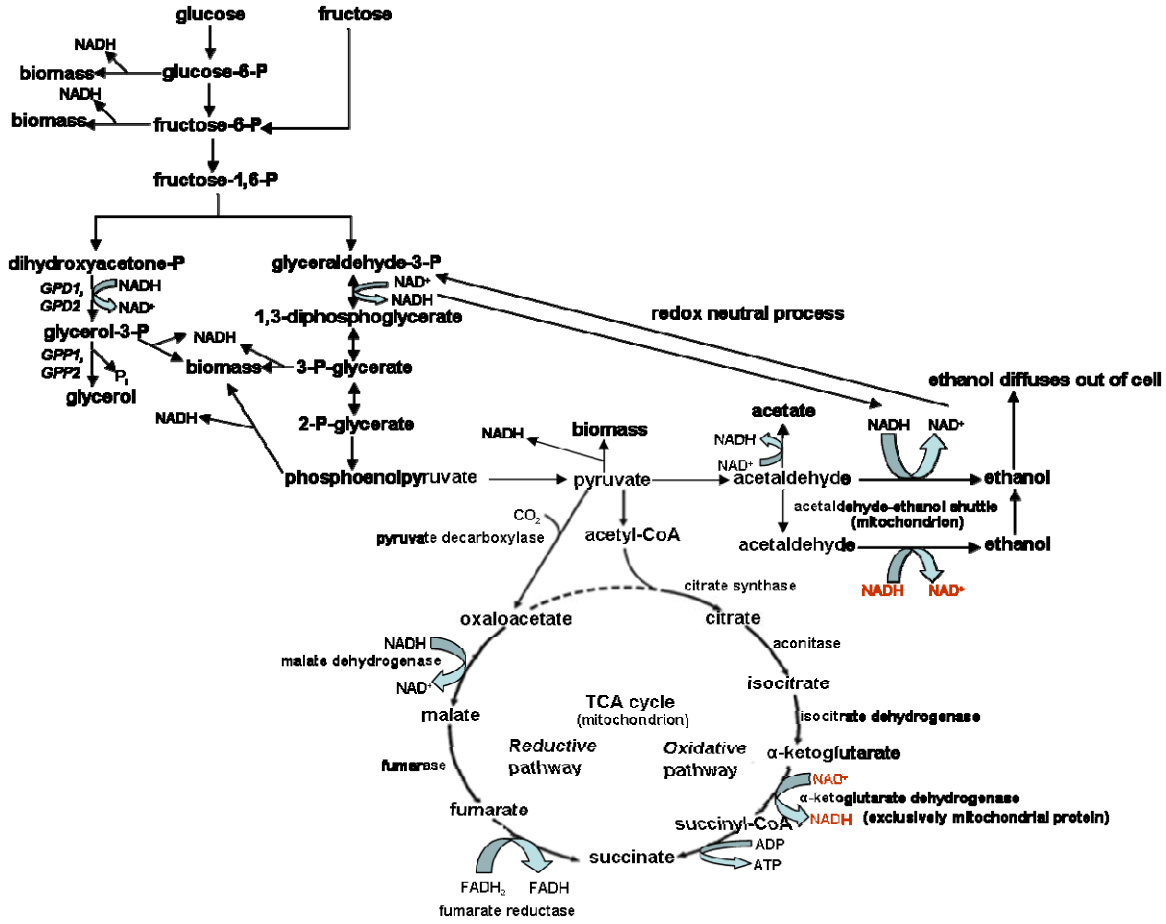

FIG. 3.1 Schematic representation of NADH production in the cytoplasm and mitochondrion, glycerol and major acids formation and of the acetaldehyde-ethanol shuttle in *S. cerevisiae* growing under fermentative conditions. NADH is formed during the conversion of α-ketoglutarate to succinyl-CoA in the oxidative pathway of the TCA cycle in mitochondrion in fermentatively growing *S. cerevisiae*. This conversion is catalyzed by α-ketoglutarate dehydrogenase which is exclusively mitochondrial protein and the NADH formed is converted back to NAD^+ during the conversion of acetaldehyde to ethanol in the acetaldehyde-ethanol shuttle taking place in the mitochondrion. The NADH formed in glycolysis is converted back to NAD^+ during the conversion of acetaldehyde to ethanol and this process is termed as redox neutral process because of no net regeneration of NAD^+. The genes *GPD1/2* and *GPP1/2* respectively encoding glycerol-3-phosphate dehydrogenase and glycerol-3-phosphate phosphatase respectively are involved in glycerol formation in the cytoplasm (Bakker *et al.*, 2000; Camarasa *et al.*, 2003; van Dijken & Scheffers, 1986).

The conversion of DHAP to G3P and subsequent dephosphorylation leads to formation of glycerol (Gancedo *et al.*, 1968a; Gancedo *et al.*, 1968b). The first reaction is catalyzed by a cytosolic NADH-dependent glycerol-3-phosphate dehydrogenase (Gpd).

The two known isogenes coding for Gpd in *S. cerevisiae* are *GPD1* and *GPD2* (Albertyn *et al.*, 1994; Eriksson *et al.*, 1995) (Fig. 3.1). *GPD1* is normally induced by high osmolarity (Albertyn *et al.*, 1994; Blomberg, 2000) and *GPD2* induction is governed by the ratio NADH/NAD^+ (Eriksson *et al.*, 1995). The genes and the gene products can partially replace each other (Ansell *et al.*, 1997).

A *gpd1*Δ*gpd2*Δ DM is unable to grow under strict anaerobic conditions because NAD^+ regeneration through glycerol production is no longer possible in this mutant and NADH starts to accumulate intracellularly (Ansell *et al.*, 1997). In principle, this excess NADH can be used to drive other NADH dependent reduction reactions. Some of these reactions occur intracellularly, such as the reduction of various aldehydes to higher alcohols. However, the low concentration of available precursors probably limits the efficiency of such native pathways. For this reason, it is tempting to consider the introduction of heterologous or native proteins which catalyze NADH-coupled reactions and may allow the DM to restore its NADH balance thereby allowing anaerobic growth, while directing metabolic flux towards other by-products than glycerol. Since NADH is a major factor controlling the activity of many oxido-reductases, the availability of excess NADH in the DM may indeed provide a useful tool to favour the production of many biotechnologically relevant products.

The objective of this study was to investigate the possibility of manipulating a *gpd1*Δ*gpd2*Δ DM of *S. cerevisiae* to regenerate NAD^+ through sorbitol production by the overexpression of the bacterial *srlD* and native *SOR1* genes. *SOR1*, a native gene of *S. cerevisiae*, encodes a sorbitol dehydrogenase (EC 1.1.1.14) that uses NAD^+ as a cofactor during the conversion of sorbitol to fructose (Sarthy *et al.*, 1994; Toivari *et al.*, 2004). However, the activity of the enzyme in the reverse direction has not been assessed. The sorbitol-6-phosphate dehydrogenase (*srlD*) (EC 1.1.1.140) gene in *E. coli* enables sorbitol assimilation via fructose-6-phosphate using NAD^+ as a cofactor (Novotny *et al.*, 1984). To our knowledge, this is the first report on the effect of the overexpression of *SOR1* and *srlD* on the growth of a *gpd1*Δ*gpd2*Δ DM under fermentative conditions. The impact of the overexpression of the yeast *FPS1* gene encoding an osmolyte transporter on the anaerobic growth capability of DM(*SOR1*), DM(*srlD*), DM(*FPS1*) and WT(*FPS1*) mutants was also investigated using plate assay. The general redirection of carbon flux by all the transformed yeast strains was assessed by comparison to the WT strain.

3.2 Materials and methods

3.2.1 Yeast strains and genotypes

The WT strain used in this study was *S. cerevisiae* BY4742. DM was created by deleting *GPD2* gene from the *gpd1*Δ BY4742 strain from the Euroscarf deletion library (www.uni-frankfurt.de/fb15/mikro/euroscarf/yeast.html) (Wilson *et al.*, 2003). The genotypes of the WT and genetically manipulated strains are listed in Table 3.1.

TABLE 3.1 Yeast strains and their genotypes

Strains	Genotype	Reference
WT	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	Brachmann *et al.*,1998
DM	BY4742; *gpd1*::*KanMx*; *gpd2*::*URA3*	This study
WT(*FPS1*)	WT[PGK_P-*FPS1*-PGK_T]	This study
DM(*FPS1*)	DM[PGK_P-*FPS1*-PGK_T]	This study
DM(*srlD*)	DM[PGK_P-*srlD*-PGK_T]	This study
DM(*SOR1*)	DM[PGK_P-*SOR1*-PGK_T]	This study
DM(*srlD*, *FPS1*)	DM[PGK_P-*srlD*-PGK_T, PGK_P-*FPS1*-PGK_T]	This study
DM(*SOR1*, *FPS1*)	DM[PGK_P-*SOR1*-PGK_T, PGK_P-*FPS1*-PGK_T]	This study

3.2.2 Primers and PCR templates

Primers for amplifying the *URA3* gene with a 50 bp region homologous to the 5' and 3' parts of *GPD2* were designed. The forward primer was 5'-ATGCTTGCTGTCAGAAGATTAACAAGATACACATTCCTTAAGCGAACGCAATGTCGAAAGCTACATATAAGG-3' and reverse primer was 5'-CTATTCGTCATCGATGTCTAGCTCTTCAATCATCTCCGGTAGGTGTTCCATTAGTTTTGCTGGCCGCATCTTCT-3' (underlined parts of the primers are homologous to the *GPD2* gene). These primers were used to amplify the *URA3* gene from the Yep24 vector containing the *URA3* marker (ATCC culture collection) (Botstein *et al.*, 1979; Parent *et al.*, 1985). The PCR product was then integrated into the *gpd1*Δ BY4742 strain to obtain a *gpd1*Δ*gpd2*Δ DM. The *srlD* gene was amplified using the *E. coli* DH5α genomic DNA and the *SOR1* and *FPS1* genes were amplified using the *S. cerevisiae*

BY4742 genomic DNA. The 2μ origin of replication was amplified using the Yep24 vector. The following primers were used for polymerase chain reaction (PCR) amplification of the *srlD* gene: forward 5'-AGATCTATGAATCAGGTTGCCGTTGTCATCG-3' and reverse 5'-CTCGAGTCAGAACATCACCTGACCGCCG-3'. The primers used for PCR amplification of the *SOR1* gene were: forward 5'-GAATTCATGTCTCAAAATAGTAACCCTGCAG-3' and reverse 5'-CTCGAGTCATTCAGGACCAAAGATAATAGTC-3'. The primers used for PCR amplification of the *FPS1* gene were: forward 5'-GAATTCATGAGTAATCCTCAAAAAGCTCTAAACGAC-3' and reverse 5'-CTCGAGTCATGTTACCTTCTTAGCATTACCATAATGC-3'. The primers used for PCR amplification of the 2μ origin of replication gene were: forward 5'-GCCGGCATCCAATATCAAGGAAATGATAGC and reverse 5'-CAGCTGTTAACGAAGCATCTGTGCTTCA.

3.2.3 DNA manipulations, construction of plasmids and yeast and bacterial transformation

The *SOR1* and *srlD* gene products were cloned in pDMPM multicopy shuttle vector (PhD thesis, Malherbe, DF 2010, Stellenbosch University) with an ampicillin resistance marker (amp^r) and a leucine auxotrophic marker and a modified multiple cloning site flanked by the constitutive phosphoglycerate-kinase-1 gene promoter (*PGK1P*) and terminator (*PGK1T*) derived from pHVX2 (Volschenk *et al.*, 1997). The *FPS1* gene was first cloned into pSTAH (Gururajan *et al*., 2007) integrating vector containing a histidine auxotrophic marker and an ampicillin resistance marker (amp^r) resulting in pSTAH(*FPS1*) vector. To convert this vector into a multicopy shuttle vector, a 2μ yeast origin of replication gene was inserted into the pSTAH(*FPS1*) vector by digesting with *Nae*I and *Pvu*II and the 6241 bp band was ligated with the 2μ yeast origin of replication gene (1341 bp) having *Nae*I and *Pvu*II overhangs. All the genes were under the control of the constitutive *PGK* promoter and terminator (Gellissen & Hollenberg, 1997). The pDMPM(*SOR1*), pDMPM(*srlD*) and pSTAH(*FPS1*) plasmids were then transformed into DM and the pSTAH(*FPS1*) plasmid was also transformed into WT. The resultant strains obtained were DM(*srlD*), DM(*SOR1*), DM(*srlD*, *FPS1*), DM(*SOR1*, *FPS1*), WT(*FPS1*) and DM(*FPS1*). Prior to transformation, the genes were sequenced at the Central Sequencing Facility of Stellenbosch University. The sequence for all three genes was 100% homologous to the sequence obtained from their GenBank accession numbers.

The accession numbers for *SOR1*, *srlD* and *FPS1* genes are P35497, P05707 and P23900 respectively.

Subcloning in *E. coli* DH5α, yeast and bacterial transformations and isolation of genomic DNA from *E. coli* and *S. cerevisiae* was done using standard protocols (Gietz & Schiestl, 2007; Harju *et al.*, 2004).

3.2.4 Medium and fermentation conditions

The fermentations were conducted four-fold in 250 ml Erlenmeyer flasks with a 100 ml working volume and fitted with a fermentation cap filled with sterile water and rubber stopper to make the system air-locked (Fig. 3.2). The fermentations were monitored for 20 days and weight loss measurement, sampling (for metabolite analysis and sugar consumption) and biomass measurement occurred each alternate day starting from 0^{th} day (immediately after inoculation). The 20^{th} day sample was used to calculate yield of metabolites and total sugar consumption. This initial aerobic system becomes anaerobic with the release of CO_2 as the culture grows thereby replacing O_2 from the headspace of the flask and making the system a self anaerobic system. All cultures were grown without agitation and contained 10% total sugar (5% glucose and 5% fructose) and 6.7 g/l yeast nitrogen base (YNB) without amino acids. Media were supplemented with the nutrients required for the growth of these auxotrophic strains. The amino acids supplied were uracil, leucine, lysine and histidine to a final concentration of 24 mg/l, 72 mg/l, 36 mg/l and 24 mg/l respectively. pH and temperature were maintained at 3.5 and 30°C respectively to mimic fermentation conditions in food products containing high sugar concentrations such as grape juice. The medium were inoculated at an initial absorbance (OD_{600nm}) of 0.5 and fermentation was monitored by weight loss. The samples were then centrifuged at 5000 rpm for 10 min and the supernatants were filtered (0.2 µm membrane filter) and stored at 4°C in 100 ml glass vials until further analysis. Cells were resuspended in 1 ml water and centrifuged in 1.5 ml microcentrifuge tubes. The supernatant was removed and the microcentrifuge tubes containing cells were dried at 60°C overnight. The weight of the empty microcentrifuge tubes was subtracted from microcentrifuge tubes containing the cells and the dried biomass weight was calculated. The shift from aerobic to anaerobic conditions in the inoculated flasks was determined by adding methylene blue (2 mg/l) to the growth

medium and monitoring the change of the dye from dark blue colour indicating aerobic conditions to colourless showing strictly anaerobic conditions (Gordon & Dubos, 1970).

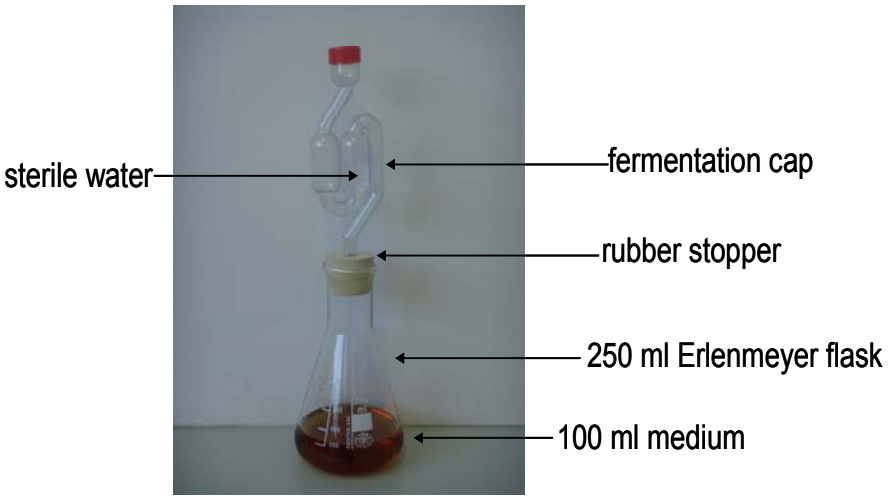

FIG. 3.2 Self anaerobic system used for the fermentation

3.2.5 Plate assays of strains under aerobic and anaerobic conditions

Plate assays were performed under aerobic and anaerobic conditions. Two sets of plates were prepared for each condition, one set containing 2% glucose, 6.7 g/l YNB without amino acids, 2% agar and the other set containing 5% glucose, 5% fructose, 6.7 g/l YNB without amino acids and 2% agar. For anaerobic plate assays the medium was supplemented with ergosterol and Tween 80 to the final concentrations of 10 mg/l and 420 mg/l respectively (Bro *et al.*, 2006). These components are necessary for anaerobic growth of *S. cerevisiae* (Verduyn *et al.*, 1990a). The plate assays were performed by inoculating 5 ml liquid medium (2% glucose, 6.7 g/l YNB without amino acids supplemented with amino acids as required) with a single yeast colony and growing under aerobic conditions at 30°C for 48 h. The culture was diluted to an OD_{600nm} value of 1 and 5 µl aliquots were spotted on the plates to achieve the dilutions of 1.0, 0.1, 0.01 and 0.001. Plates were incubated for respectively 8 and 20 days under aerobic and anaerobic conditions at 30°C. Anaerobic conditions were maintained in 3.5 l anaerobic jars using the OXOID Anaero*Gen*™ system. The sachet creates anaerobic conditions in the jar within 1 h. Anaerobic conditions were confirmed by placing a wet blue strip in the jar which turns white as the anaerobic conditions prevail.

3.2.6 Chemical analyses

Substrates consumed and metabolites formed were analyzed using a Waters HPLC system equipped with an Aminex HPX-87H column (BioRad, USA), connected to a refractive index (RI)-ultraviolet (UV) detector (RID-6A, Shimadzu, Japan). A mobile phase of 5 mM H_2SO_4 at a flow rate of 0.6 ml/min and a column temperature of 45°C were used. The components measured using a RI detector were glucose, fructose, ethanol, sorbitol and glycerol and the components measured using the UV detector were pyruvate, acetate, succinate and malate.

Higher alcohols, esters and minor acids formed by yeasts were determined with a gas chromatograph (GC) equipped with a flame ionization detector (FID) and Nukol free fatty acid phase fused-silica capillary column (DB-FFAP) (Hewlett Packard 6890 Plus) (Ng, 2002). The internal dimensions of the column were 60 m × 0.32 mm with a film thickness of 0.5 μm. The temperature, H_2 flow and air flow of the detector were maintained at 250°C, 30 ml/min and 350 ml/min respectively. The extraction of volatile compounds from the sample was performed as follows: 100 μl of an internal standard 4-methyl-2-pentanol was added to 5 ml of sample and extracted with 1 ml diethyl ether by placing the ether/sample mixture in an ultrasonic bath for 5 min. The sample/ether mixture was centrifuged at 4000 rpm for 3 min. The ether layer was removed and dried on Na_2SO_4. This extract was injected into the GC-FID column.

3.3 Results

3.3.1 Growth of strains on agar plates under aerobic and anaerobic conditions

All strains grew equally well under aerobic conditions on 2% glucose agar plates. However, on 5% glucose-5% fructose agar plates, the DM and DM(*FPS1*) grew poorly while DM(*SOR1*), DM(*srlD*), DM(*SOR1*, *FPS1*) and DM(*srlD*, *FPS1*) strains showed improved growth but less than the WT and WT(*FPS1*) strains (Fig. 3.3). Under anaerobic conditions on 2% glucose agar plates, growth of all strains was less than observed under aerobic conditions. Strains DM(*FPS1*), DM(*srlD*, *FPS1*) and DM(*SOR1*, *FPS1*) failed to grow under anaerobic conditions on both 2% glucose and 5% glucose-5% fructose agar plates. Surprisingly, DM showed slight growth on 2% glucose agar plate under anaerobic condition which might be due to the residual oxygen that was

present in the culture when being prepared under aerobic conditions. However, no growth was observed for DM on 5% glucose-5% fructose agar plates under anaerobic conditions. DM(*SOR1*) and DM(*srlD*) strains showed some growth on both 2% glucose and 5% glucose-5% fructose agar plates under anaerobic conditions albeit much less than WT but more than DM. These results reveal that, as expected, the deletion of *GPD1* and *GPD2* from WT led to a serious growth defect under both aerobic and anaerobic condition. However, transformation of DM with *SOR1* and *srlD* partially restores growth on 2% glucose and 5% glucose-5% fructose agar plates under anaerobic conditions and more completely under aerobic conditions. Overexpression of the *FPS1* gene in the DM, DM(*SOR1*) and DM(*srlD*) strains resulted in a no growth phenotype under anaerobic conditions on both 2% glucose and 5% glucose-5% fructose agar plates and also in a slight reduction of growth in the WT strain on 5% glucose-5% fructose agar plates under anaerobic conditions. Under aerobic conditions the overexpression of *FPS1* in the WT, DM, DM(*SOR1*) and DM(*srlD*) strains had no apparent negative effect on both 2% glucose and 5% glucose-5% fructose agar plates as growth was similar to the DM(*SOR1*) and DM(*srlD*) strains (Fig. 3.3).

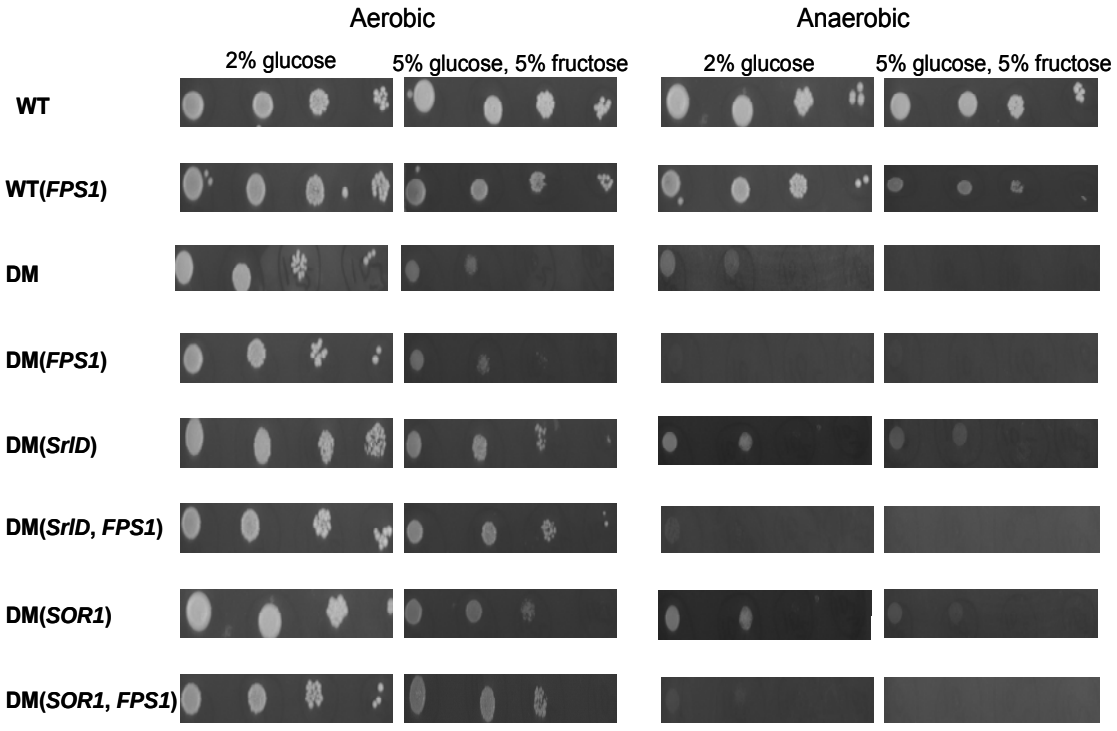

FIG. 3.3 Plate assays for the strains under aerobic and anaerobic conditions in 2% glucose and 5 % glucose-5 % fructose and 6.7 g/l YNB. Ten µl of ten-fold dilution series were spotted for each row, beginning with a culture of an OD_{600nm} of one (corresponding to 2.7 X 10^5 cells / 10 µl).

3.3.2 Shift from respirofermentative to strictly anaerobic growth of the strains in liquid medium

Methylene blue dye was added in the flasks inoculated with different strains to assess the time to attain the anaerobic conditions. It was found that the flask inoculated with the WT attained anaerobicity after 1.5 days whereas the flasks inoculated with DM, DM(*srlD*), DM(*SOR1*) took 2.5 days for the same to happen. This indicates that the growth of all the strains started in the presence of oxygen. However, gradually the consumption of oxygen and the release of CO_2 by the growing strains displaced O_2 from the headspace and made the system anaerobic. This phenomenon happened most rapidly in the WT strain due to rapid release of carbon dioxide (CO_2) while the genetically manipulated strains all took approximately the same time to reach anaerobicity as the rate of release of CO_2 was similar (Fig. 3.4).

3.3.3 Effect of *SOR1* and *srlD* overexpression on CO_2 release, total sugar consumption and biomass formation

The WT strain released CO_2 more rapidly than the mutants and the fermentation was almost completed within 6 days. After 20 days, the WT strain had released by far, the largest amount of CO_2 among the strains tested (Fig. 3.4). The DM strain released CO_2 more slowly and to a lesser amount than the WT. Overexpression of *SOR1* and *srlD* in DM improved CO_2 release but the release was neither as rapid nor to the same level as the WT.

The WT consumed all the sugars (100 g/l) whereas the DM only consumed 47% of the total sugar present initially in the medium. Overexpression of *srlD* and *SOR1* in the DM led to significant improvement in the sugar utilization albeit not reaching WT levels. The DM(*srlD*) and DM(*SOR1*) consumed 67% and 75% of the total sugar respectively (Table 3.2). The DM consumed less of both fructose and glucose when compared to WT, DM(*srlD*) and DM(*SOR1*) strains. Overexpression of *SOR1* and *srlD* in DM resulted in improved utilisation of both sugars. Neither DM(*SOR1*) nor DM(*srlD*) strain showed an improved fructose utilization relative to glucose suggesting that overexpression of *SOR1* and *srlD* in DM did not stimulate fructose utilization. Nevertheless, fructose utilization in DM(*SOR1*) was slightly more rapid than in the DM(*srlD*) strain (Fig. 3.5).

Biomass production was the highest in WT and the least in DM. Overexpression of *srlD* and *SOR1* in the DM increased biomass formation but not to the extent of the WT. However, the yield of biomass was the highest for DM(*srlD*) followed by DM(*SOR1*), WT and DM strains (Table 3.3). Biomass concentration remained constant in DM after ~2.5 days which correspond with the establishment of full anaerobic conditions. This result together with lack of growth on agar plates containing 5% glucose-5% fructose by DM (Fig. 3.3) confirms that aerobic conditions are necessary for any growth by DM strain.

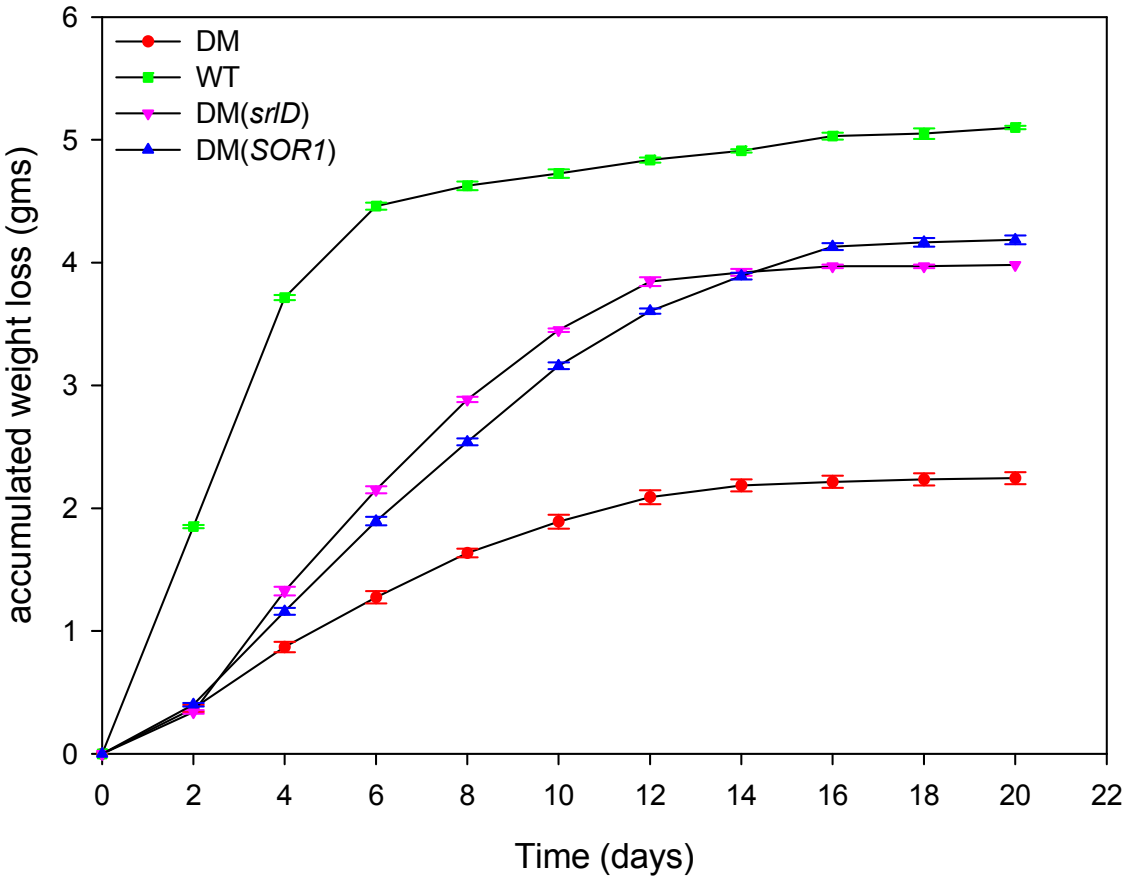

FIG. 3.4 Release of CO_2 by *S. cerevisiae* WT, *gpd1*Δ*gpd2*Δ DM and DM transformed with *srlD* and *SOR1* genes when cultivated in 5% glucose-5% fructose and 6.7 g/l YNB carried out in four replicates. Error bars indicated are the standard deviation of the four replicates for each strain. Weight loss is assumed to only originate from CO_2.

TABLE 3.2 Effect of overexpression of *SOR1* and *srlD* genes in *S. cerevisiae* *gpd1*Δ*gpd2*Δ strain on sugar consumption[a] cultivated in 5% glucose-5% fructose and 6.7 g/l YNB over 20 days.

Strains	Glucose used (g/l)	Fructose used (g/l)	Total sugar used (g/l)
WT	50 ± 0	49.85 ± 0.07	99.85 ± 0.07
DM	30.4 ± 0.81	17.01 ± 1.38	47.41 ± 1.95
DM(*srlD*)	40.4 ± 0.55	26.6 ± 0.79	67.00 ± 1.21
DM(*SOR1*)	45.23 ± 0.66	30.08 ± 1.14	75.30 ± 1.58

[a]Values represent the average and standard deviation of four independent cultivations.

TABLE 3.3 Effect of overexpression of *SOR1* and *srlD* genes in *S. cerevisiae* *gpd1*Δ*gpd2*Δ strain on product yield (mol/mol sugar utilized)[a] cultivated in 5% glucose-5% fructose and 6.7 g/l YNB during an oxygen limited fermentation. The values in brackets represent the metabolite or dry weight (for the biomass) concentrations (g/l).

	Strains			
Metabolite	**WT**	**DM**	**DM(*srlD*)**	**DM(*SOR1*)**
CO_2	1.74 (42.5)	1.91 (22.13)	1.80 (29.28)	1.88 (33.87)
ethanol	1.74 (44.79)	1.91 (23.15)	1.80 (30.89)	1.88 (36.25)
biomass[b]	0.072 (0.99)	0.06 (0.41)	0.096 (0.88)	0.091 (0.94)
glycerol	0.082 (4.20)	0	0	0
sorbitol	0	0	0.065 (4.39)	0.015 (1.17)
acetate	0.02 (0.66)	0.85[c] (0.014)	0.009 (0.212)	0.3[c] (0.01)
succinate	0.011 (0.73)	0.01 (0.31)	0.006 (0.28)	0.003 (0.16)
malate	0.005 (0.35)	0.015 (0.53)	0.006 (0.31)	0.008 (0.43)
pyruvate	0.004 (0.175)	0.008 (0.182)	0.017 (0.57)	0.023 (0.847)

[a]Yield was calculated from the 20th day sample of four independent cultivations
[b]Molecular formula of *Saccharomyces cerevisiae* is taken as **$CH_{1.8}O_{0.5}N_{0.2}$**
[c]mmoles/mole sugar utilized
Coefficient of variation (CV) for concentration and yield of metabolites was less than 12% for all the strains.

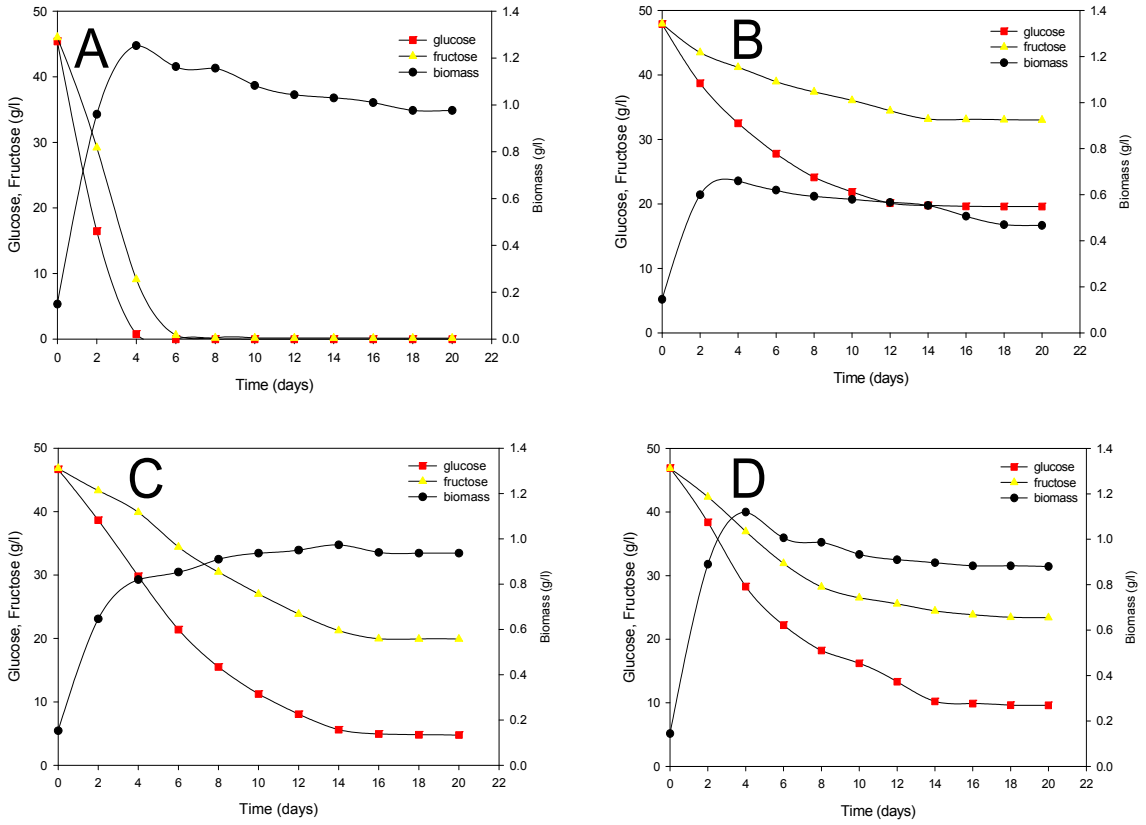

FIG. 3.5 Glucose and fructose consumption and growth (biomass: dry weight) by strains of *S. cerevisiae* WT (A), DM (B) transformed with *SOR1* (C) and *srlD* (D) when cultivated in 5% glucose-5% fructose and 6.7 g/l YNB in a representative experiment.

3.3.4 Ethanol, glycerol and sorbitol production

DM, DM(*SOR1*) and DM(*srlD*) strains did not produce glycerol due to the deletion of the *GPD1* and *GPD2* genes. The WT strain produced glycerol up to the 10th day and the concentration was constant thereafter (Fig. 3.6). Also as expected, the strains over-expressing *SOR1* and *srlD* produced sorbitol. There was a more rapid production of sorbitol by DM(*srlD*) than DM(*SOR1*) and the maximum sorbitol concentration achieved was also considerably higher for DM(*srlD*) compared to the DM(*SOR1*) strain. Ethanol formation varied greatly among the strains with WT producing the highest concentration and DM least. Among the manipulated strains, DM(*SOR1*) produced more ethanol as compared to DM(*srlD*) (Fig. 3.6). Interestingly, the highest ethanol molar yield was observed in DM whereas WT had the lowest ethanol yield (Table 3.3). This indicates that some glucose has been directed to glycerol formation in the WT strain whereas the other strains produced no glycerol due to the *gpd1*Δ*gpd2*Δ deletion. A higher molar yield of ethanol was achieved by the DM(*SOR1*) strain as compared with the DM(*srlD*) strain, whereas the molar yield of sorbitol was higher for the DM(*srlD*) than for the

DM(*SOR1*) strain (Table 3.3). The available hydrogen in each metabolite was calculated from each of the products (Table 3.4). Approximately 100% of the hydrogen moles from the sugars were accounted for in the products for all the strains (Table 3.4).

TABLE 3.4 Moles of available hydrogen present[a] in each metabolite formed by strains cultivated in 5% glucose-5% fructose and 6.7 g/l YNB under oxygen limited fermentation. The available hydrogen in one mole each of glucose and fructose is 24 thereby giving a total available 48 moles hydrogen.

	Strains			
Metabolite	**WT**	**DM**	**DM(*srlD*)**	**DM(*SOR1*)**
CO_2	0	0	0	0
ethanol	41.76	45.84	42.96	45.12
biomass[b]	0.69	0.58	0.92	0.87
glycerol	2.3	0	0	0
sorbitol	0	0	3.38	0.78
acetate	0.32	13.6[c]	0.144	4.8[c]
succinate	0.31	0.28	0.112	0.084
malate	0.12	0.36	0.144	0.192
pyruvate	0.08	0.16	0.34	0.46
Total	45.58	47.22	48	47.51
%Hydrogen recovered	**94.96**	**98.37**	**100**	**98.98**

[a]moles of available hydrogen present in each metabolite is calculated from the final day (20th day) molar yield of each metabolite.
[b]Molecular formula of *S. cerevisiae* is taken as $\mathbf{CH_{1.8}O_{0.5}N_{0.2}}$
[c]mmoles of H formed/48 moles of H consumed from glucose and fructose
Coefficient of variation for available hydrogen moles was less than 10% for all the strains.

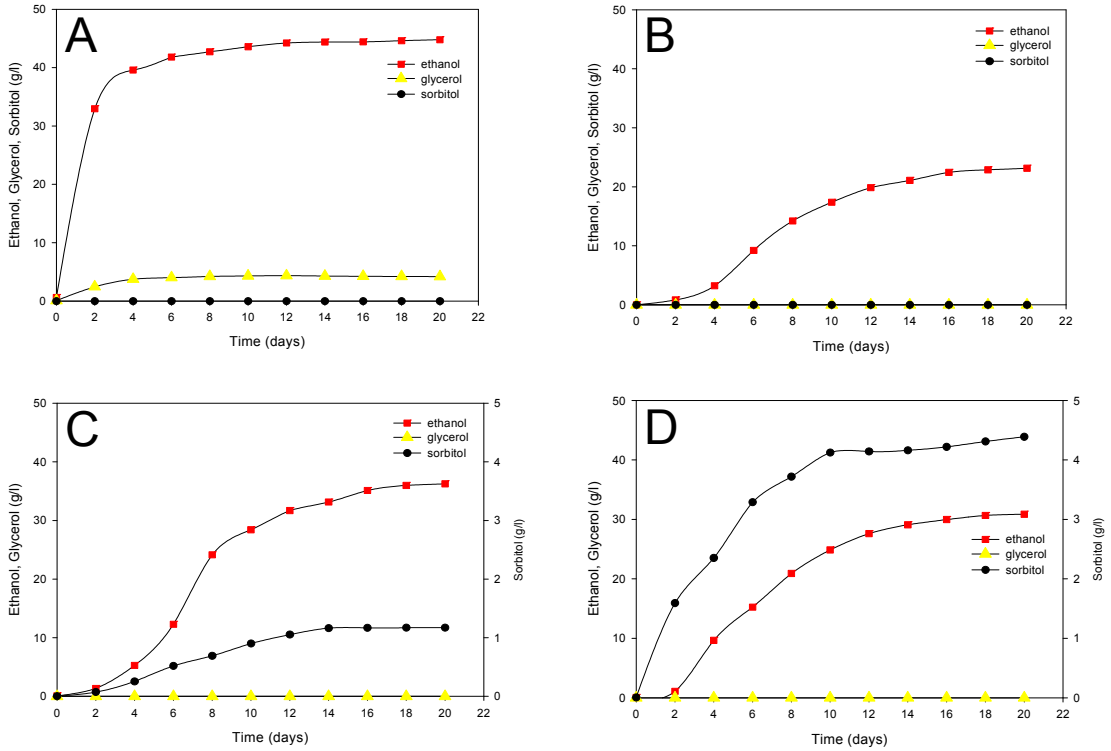

FIG. 3.6 Ethanol, glycerol and sorbitol production by strains of *S. cerevisiae* WT (A), DM (B) transformed with *SOR1* (C) and *srlD* (D) when cultivated in 5% glucose-5% fructose and 6.7 g/l YNB in a representative experiment.

3.3.5 Cytosolic and mitochondrial acid production

The acids pyruvate and acetate are formed in the cytoplasm whereas malate and succinate formation occurs in the mitochondrion (Bakker *et al.*, 2001; Camarasa *et al.*, 2003). WT and DM(*srlD*) strains produced much more acetate than DM and DM(*SOR1*) (Fig. 3.7). Interestingly, the highest concentration of malate was produced by DM when compared to the WT, DM(*SOR1*) and DM(*srlD*) strains. WT formed the highest concentration of succinate whereas the over-expression strains revealed very similar concentrations. Again, the DM formed slightly more succinate than the over-expressing strains. Similar amounts of pyruvate were found in WT and DM whereas in DM(*SOR1*), pyruvate accumulated rapidly for 12 days and then started to decline. For DM(*srlD*), pyruvate accumulated for 4 days and remained constant thereafter. However, the final concentration of pyruvate achieved after 20 days was higher in DM(*SOR1*) strain as compared to DM(*srlD*) strain. The molar yields of the metabolites are shown in Table 3.3.

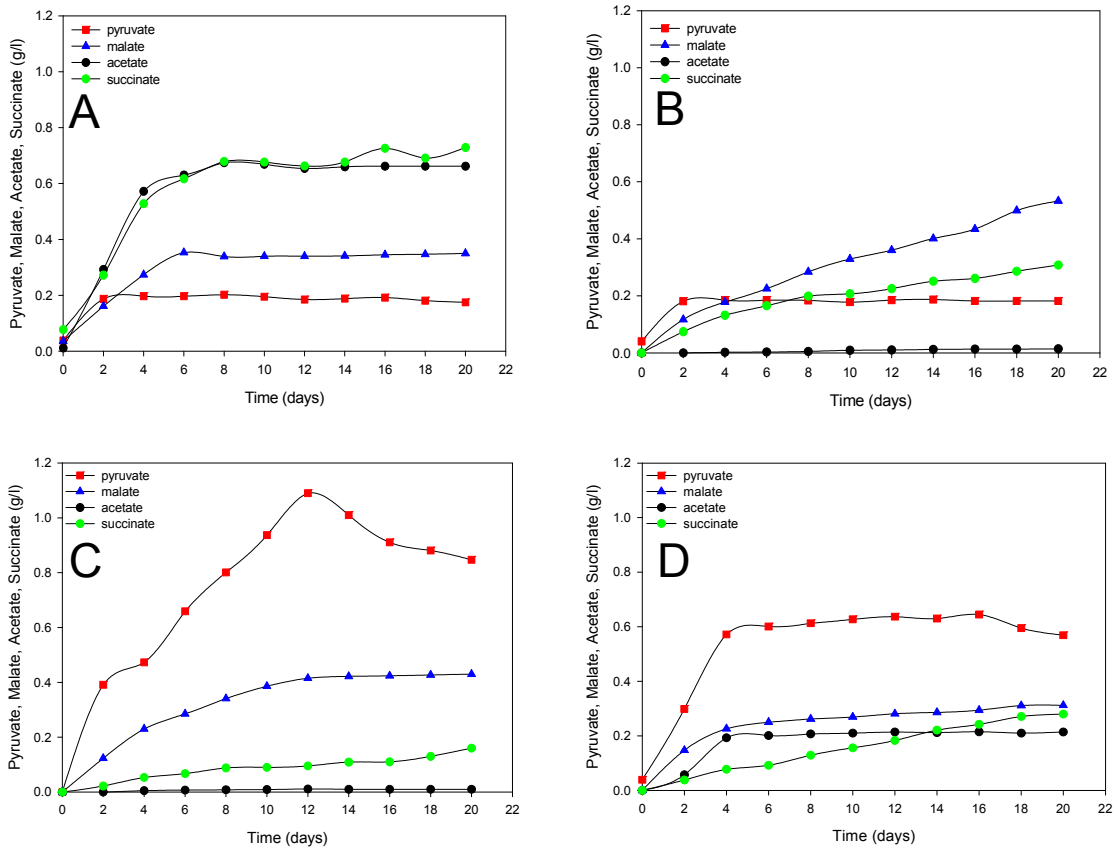

FIG. 3.7 Pyruvate, malate, acetate and succinate production by strains of *S. cerevisiae* WT (A), DM (B) transformed with *SOR1* (C) and *srlD* (D) when cultivated in 5% glucose-5% fructose and 6.7 g/l YNB in a representative experiment.

3.3.6 Higher alcohols, minor acids and esters production

The higher alcohols isobutanol, isoamyl alcohol, propanol, butanol and 2-phenyl ethanol and the esters ethyl acetate and isoamyl acetate were analyzed as they are usually the most common esters found when the yeast grows fermentatively in high sugar medium (Lilly *et al.*, 2006a). The highest isobutanol concentration was formed by DM followed by DM(*SOR1*), DM(*srlD*) and WT (Fig. 3.8). Overexpression of *SOR1* and *srlD* stimulated isoamyl alcohol formation with a slightly higher concentration of isoamyl alcohol found in DM(*SOR1*) strain than in DM(*srlD*) (Fig. 3.8). Similar concentrations of 2-phenyl ethanol were found in all the strains. Production of butanol and propanol after 10 days was observed in DM(*SOR1*) strain (Fig. 3.8).

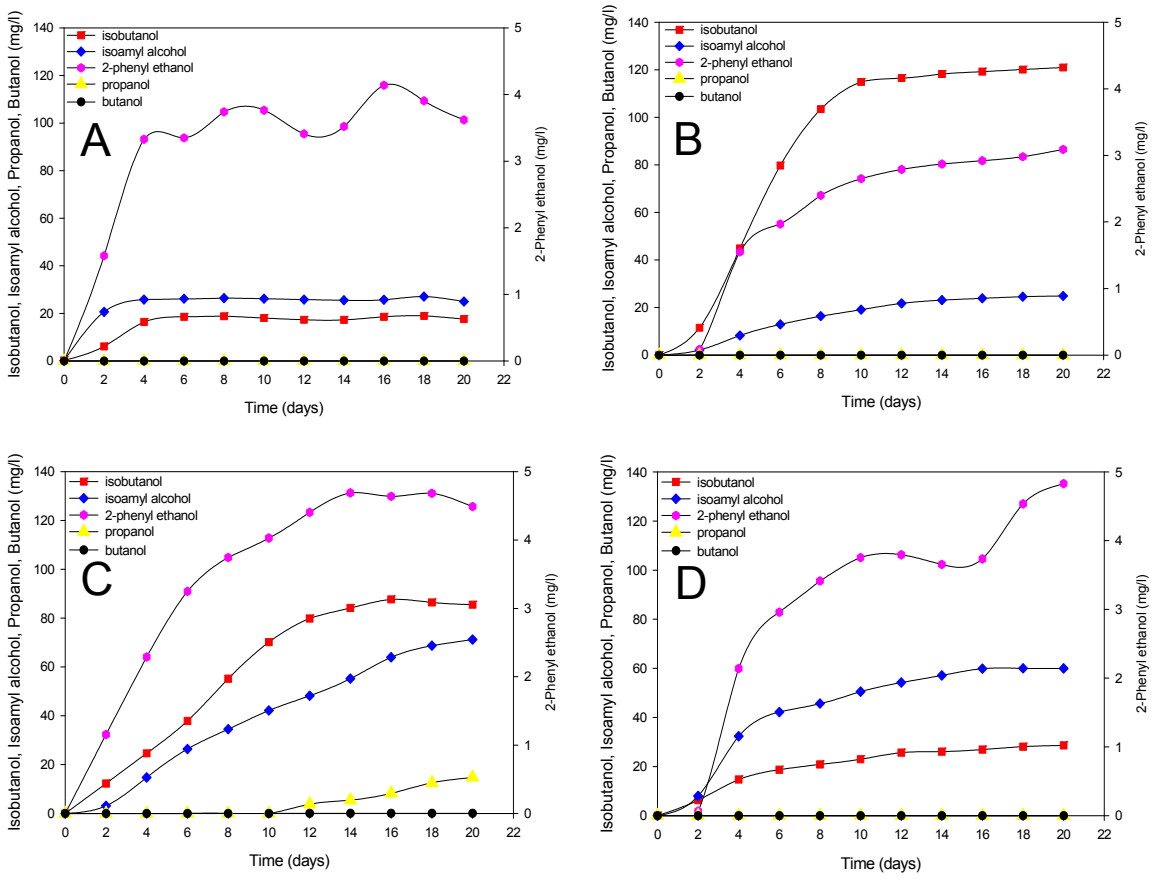

FIG. 3.8 Higher alcohol production by strains of *S. cerevisiae* WT (A), DM (B) transformed with *SOR1* (C) and *srlD* (D) when cultivated in 5% glucose-5% fructose and 6.7 g/l YNB in a representative experiment.

No strains produced isoamyl acetate (Fig. 3.9). The *GPD1* and *GPD2* deletion resulted in much lower ethyl acetate concentrations being produced compared to WT and overexpression of *SOR1* or *srlD* failed to restore levels back to those of the WT (Fig. 3.9). All strains failed to produce butyric acid whereas DM and DM(*SOR1*) formed high concentrations of isobutyric acid compared to WT and DM(*srlD*). The concentration and molar yields of higher alcohols, minor acids and esters are shown in Table 3.5.

TABLE 3.5 Effects of the overexpression of *SOR1* and *srlD* genes in *S. cerevisiae* *gpd1*Δ*gpd2*Δ strain cultivated in 5% glucose-5% fructose and 6.7 g/l YNB without amino acids on the concentration (mg/l) and the yield (mg/g sugar utilized; values in brackets) of higher alcohols, esters and minor acids during oxygen limited fermentation after 20 days.

	Strains			
Metabolite (mg/l)	**WT**	**DM**	**DM(*srlD*)**	**DM(*SOR1*)**
Isobutanol	20.4 (0.2)	123.22 (2.65)	28.63 (0.43)	85.52 (1.14)
Isoamyl alcohol	25.37 (0.29)	25.51 (0.60)	59.95 (0.89)	71.23 (0.95)
Propanol	0	0	0	14.09 (0.19)
Butanol	0	0	0	0.065 (0.001)
2-phenyl ethanol	3.66 (0.04)	3.17 (0.11)	4.83 (0.072)	4.49 (0.073)
Ethyl acetate	27.01 (0.29)	4.82 (0.10)	11.22 (0.17)	4.87 (0.07)
Isoamyl acetate	0	0	0	0
Butyric acid	0	0	0	0
Isobutyric acid	0.602 (0.008)	1.69 (0.04)	1.032(0.015)	2.86 (0.038)
Coefficient of variation for concentration and yield of metabolites was less than 8% for all the strains.				

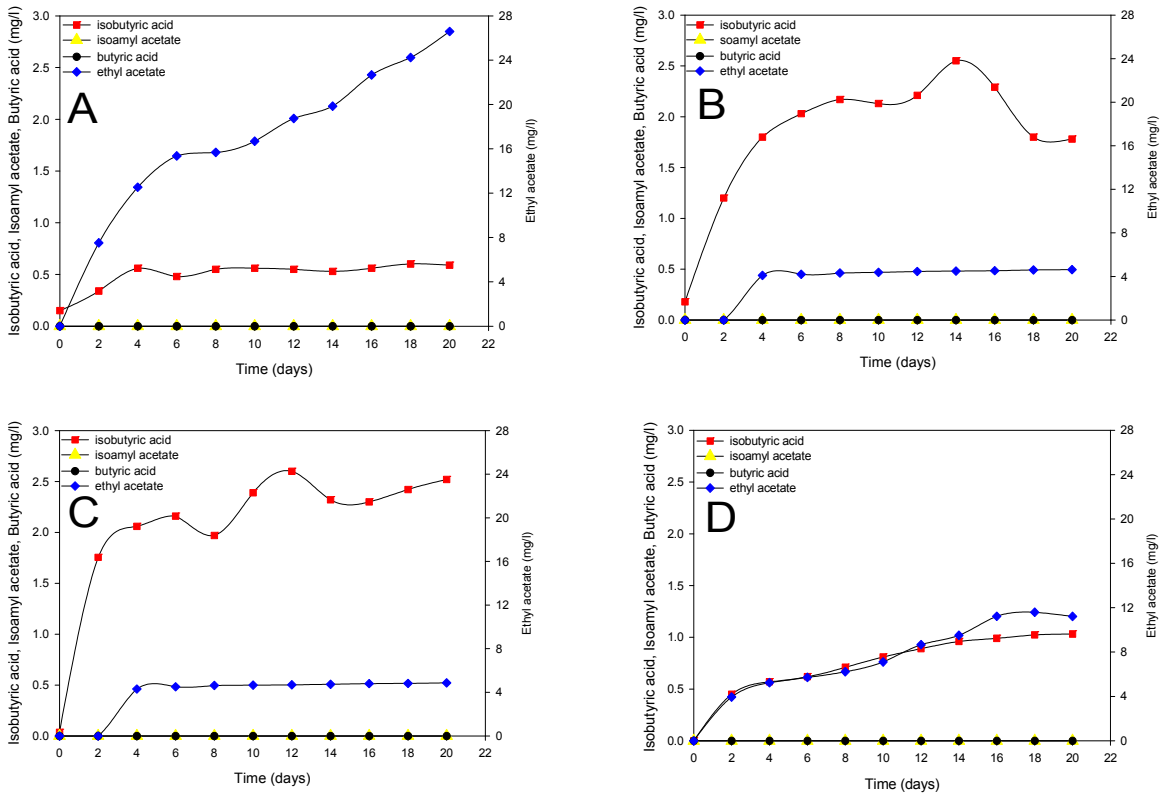

FIG. 3.9 Esters and minor acids production by strains of *S. cerevisiae* WT (A), DM (B) transformed with *SOR1* (C) and *srlD* (D) when cultivated in 5% glucose-5% fructose and 6.7 g/l YNB in a representative experiment.

3.4 Discussion

The data generated in this study indicate that the effect of the deletion of glycerol producing genes on yeast growth under fermentative conditions can be partially complemented by sorbitol producing genes. Moreover, this genetic manipulation has a marked impact on the concentration of many other metabolites formed.

3.4.1 Novel pathways for the regeneration of NAD^+ alter growth

The results presented here suggest that both genes overexpressed in DM lead to regeneration of NAD^+ thereby improving the growth and metabolic activity under fermentative conditions of a strain incapable of forming glycerol. The sorbitol dehydrogenase (Sor1p) therefore can apparently catalyze the conversion of fructose to sorbitol, while the sorbitol-6-phosphate dehydrogenase was able to catalyze the conversion of fructose-6-phosphate to sorbitol-6-phosphate (Fig. 3.10). Both of these

genes have been described as normally catalysing the reverse reaction, indicating that the availability of co-factor NAD^+ or NADH has a major impact on catalytic activity. To our knowledge, the impact of the overexpression of the *SOR1* and *srlD* genes on the restoration of the redox balance in *gpd1*Δ*gpd2*Δ DM has not been reported before, although other studies have reported that other heterologous genes can partially complement the DM. For example, when the aldose reductase gene from *Pichia stipitis* was overexpressed in a *gpd1*Δ*gpd2*Δ strain and cultivated in a medium containing 50 g/l xylose, anaerobic growth was partially achieved by reduction of xylose to xylitol generating NAD^+ from NADH (Liden *et al.*, 1996). In a similar study a *gpd1*Δ*gpd2*Δ strain transformed with the mannitol-1-phosphate dehydrogenase gene (*mtlD*) from *E. coli* converted fructose-6-phosphate to mannitol-1-phosphate thereby re-oxidizing the excess NADH back to NAD^+ under anaerobic conditions and partially complementing the deletion (Costenoble *et al.*, 2003). Mannitol was slowly exported from the cell suggesting that mannitol accumulation might have hindered growth. These reports together with the present study indicate that an alternative NADH oxidizing system might not be as efficient as glycerol-3-phosphate dehydrogenase in maintaining the redox balance in *S. cerevisiae* under strictly anaerobic conditions.

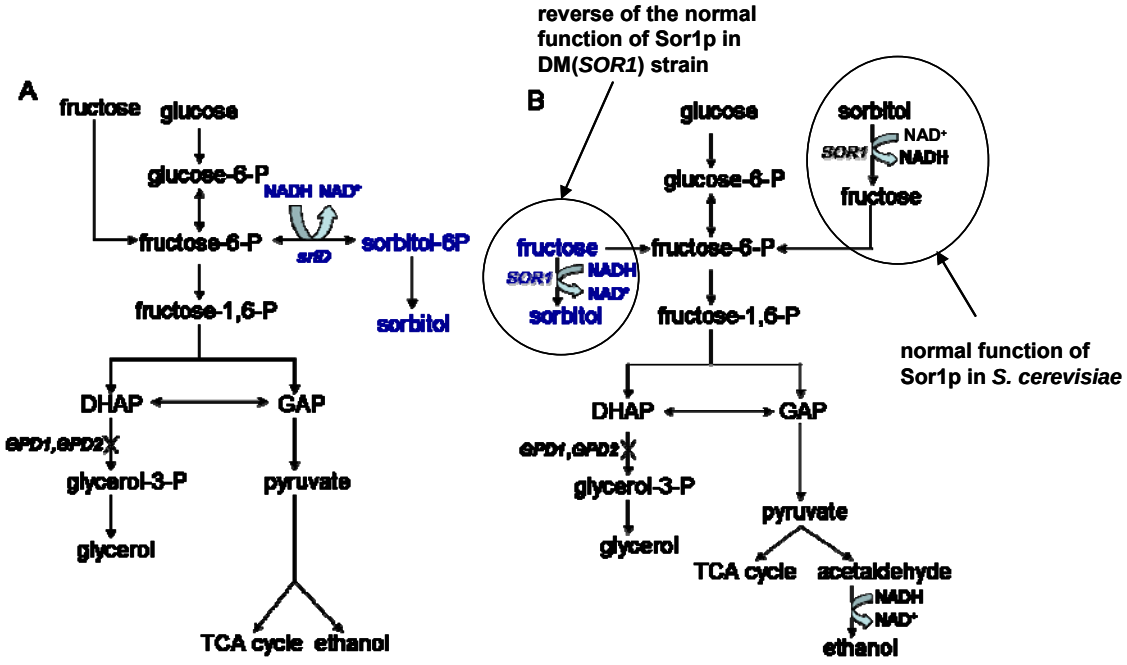

FIG. 3.10 Pathways for the regeneration of NAD^+ by *S. cerevisiae gpd1*Δ*gpd2*Δ transformed with *srlD* (A) and *SOR1* (B) genes. *srlD* catalyzes the conversion of fructose-6-phosphate to sorbitol-6-phosphate thereby regenerating NAD^+. Sorbitol-6-phosphate then gets dephosphorylated to sorbitol using unknown phosphatase (Akinterinwa *et al.*, 2008) (A). Normal function of Sor1p catalyzing the conversion of sorbitol to fructose which ultimately joins the glycolysis by converting to fructose-6-phosphate (B). In the genetically manipulated strain, DM(*SOR1*), the reverse activity of Sor1p which is conversion of fructose to sorbitol and regenerating NAD^+ is checked (B).

As reported previously, the *gpd1*Δ*gpd2*Δ DM was able to grow under aerobic conditions in 2% glucose medium despite the fact that the pathway producing glycerolipids via the precursor glycerol-3-phosphate is blocked (Ansell *et al.*, 1997). Presumably, the acyl dihydroxyacetone pathway enzymes of *S. cerevisiae* provide sufficient activity for alternative biosynthesis of phosphatidic acid (Racenis *et al.*, 1992). Despite the growth of *gpd1*Δ*gpd2*Δ in 2% glucose under aerobic conditions, the DM struggled to grow under aerobic conditions in the 5% glucose-5% fructose medium. This could be because glucose represses respiration in *S. cerevisiae* above ~9 g/l allowing alcoholic fermentation, even under fully aerobic conditions (Crabtree effect) (Lagunas, 1979; Piskur *et al.*, 2006; Goddard, 2008). The growth of the DM in 2% (20 g/l) glucose might be because of the decreasing Crabtree effect and increasing respiration at lower glucose concentration. Surprisingly under anaerobic conditions, slight growth for DM in 2% glucose medium was observed although no observable growth on plates (Fig. 3.3) or in broth (Fig. 3.5B) was apparent in the higher sugar concentration under anaerobic

conditions. This observation is in contrast to an earlier study where the DM failed to grow in 2% glucose (Ansell *et al.*, 1997). The absence of *GPD1* and *GPD2* genes stops alcoholic fermentation as regeneration of NAD^+ is not possible (Royt & MacQuillan, 1979). The possible reason for this observation could be due to the strain differences or different growth conditions. The improved growth by the strains overexpressing the *SOR1* and *srlD* genes on both 5% glucose-5% fructose medium under aerobic conditions and 2% glucose and 5% glucose-5% fructose medium under anaerobic conditions points to the re-oxidation of the excess NADH. However poorer growth compared to the WT indicates that these genes are unable to completely complement the deletion of the *GPD1* and *GPD2* genes.

Overexpression of *FPS1* gene in the strains WT, DM, DM(*SOR1*) and DM(*srlD*) had no apparent negative effect on growth on 5% glucose-5% fructose agar plates under aerobic conditions (Fig. 3.3). However, a serious growth defect was observed under anaerobic conditions on both 2% glucose and 5% glucose-5% fructose agar plates for the strains DM(*FPS1*), DM(*SOR1*, *FPS1*) and DM(*srlD*, *FPS1*) (Fig. 3.3). For the WT(*FPS1*) strain, there was no visible growth defect on 2% glucose agar plates under anaerobic conditions but the growth defect was visible on 5% glucose-5% fructose agar plates under anaerobic conditions. The objective behind overexpressing the *FPS1* gene in the various strains producing sorbitol was to establish whether such overexpression could increase the release of sorbitol via this channel protein (Cordier *et al.*, 2007). Fps1p in *S. cerevisiae* and other yeasts acts as an polyol transporter (Hohmann *et al.*, 2000; Karlgren *et al.*, 2005; Tang *et al.*, 2005) although it is not clear whether sorbitol can diffuse through Fps1p. Surprisingly, overexpression of *FPS1* gene leads to intracellular accumulation of glycerol which can trigger a Hog1-dependent osmoprotective pathway (Siderius *et al.*, 2000). This ultimately leads to a reduction of glycolysis and growth rate by a yet uncharacterized mechanism (Blomberg, 2000).

3.4.2 Effect of genetic manipulation on sugar consumption and metabolite production

Transformation of DM with *SOR1* and *srlD* improved general sugar consumption but did not significantly alter the relative consumption of glucose and fructose. This was expected in the DM(*srlD*) strain as sorbitol-6-phosphate dehydrogenase restores the redox balance by catalyzing fructose-6-phosphate derived from glucose and fructose. However, in the DM(*SOR1*) strain, NAD^+ is regenerated by directly converting non-

phosphorylated fructose to sorbitol. A possible outcome could therefore have been a faster consumption of fructose relative to glucose, which is a target of current strain improvement programs in the wine industry since stuck fermentations tend to occur in conditions characterised by high levels of fructose and low levels of glucose (Berthels *et al.* 2006). In *S. cerevisiae*, dehydrogenases and other similar enzymes may also be active in the reverse direction although the catalytic efficiency and substrate affinity may differ (Anderlund *et al.*, 1999; Bakker *et al.*, 2001; Nissen *et al.*, 1997; Nissen *et al.*, 2000). Whether Sor1p is efficient in converting fructose to sorbitol has not been investigated. The structural and functional properties of a yeast xylitol dehydrogenase (*XDH*), a Zn^{2+}-containing metalloenzyme has been shown to be similar to yeast sorbitol dehydrogenase (Sor1p) (Lunzer *et al.*, 1998). Interestingly, the affinity (K_m) of *XDH* for sorbitol is approximately 40 times lower than the affinity for fructose indicating that sorbitol is a much better substrate than fructose for *XDH* (Lunzer *et al.*, 1998). However, similar studies are needed to establish functional and structural properties of Sor1p to enable a better understanding of catalysis of fructose to sorbitol. Unavailability of free fructose inside the cell can be another possible reason for the lower utilization of fructose by strain DM(*SOR1*) than expected. Usually the hexoses such as glucose and fructose are phosphorylated immediately after they enter the cell by kinases bound to the cell membrane (Bisson & Fraenkel, 1983; Clifton *et al.*, 1993; Moriya & Johnston, 2004). The form of the fructose substrate to produce sorbitol by DM(*SOR1*) strain can not be derived from the data generated in this study. Dephosphorylation of glycolytic intermediate fructose-6-phosphate to fructose might enable reduction to sorbitol using Sor1p (Furuya *et al.*, 1982). Moreover, dephosphorylation of sorbitol-6-phosphate to sorbitol by unknown mechanism has been proposed earlier (Akinterinwa *et al.*, 2008) and this might also hold true for fructose-6-phosphate dephosphorylation. The higher molar yield of sorbitol by DM(*srlD*) (0.07 moles/mole of sugar consumed) than DM(*SOR1*) (0.023 moles/mole of sugar consumed) might suggest that the dephosphorylation process of hexose phosphates is not very efficient in *S. cerevisiae*. Since fructose-6-phosphate is a glycolytic intermediate and conversion of fructose-6-phosphate to fructose might be limited, most fructose is probably utilized in a similar fashion in DM(*srlD*) and DM(*SOR1*) strains. However, DM(*SOR1*) strain produced some sorbitol points to the availability of free fructose inside the cell.

The molar yield of metabolites was used to analyze the total NADH production and NAD^+ regeneration. The major amount of NADH is generated through biomass and

acetate production and the major amount of NAD^+ is assumed to be regenerated through glycerol/sorbitol, succinate, malate and ethanol production in WT and genetically manipulated strains (Table 3.6). NADH formed and NAD^+ regenerated through metabolite production appear to be in equilibrium in WT strain and ethanol formation was actually a redox neutral process where NAD^+ regenerated through ethanol production was used in the glycolysis. Higher sugar consumption in the strains was correlated to higher ethanol and biomass formation which indicates that the generation of higher amount of ATP through ethanol formation leads to higher biomass production in the strains. Furthermore, higher biomass production should lead to higher NADH production. Therefore, the least amount of biomass formed by DM strain might be a way to limit overproduction of NADH through biomass formation as this strain lacked a mechanism to reoxidize NADH unlike other strains. Higher molar yields of ethanol were observed in all the genetically manipulated strains. Moreover, in the DM and DM(*SOR1*) strains, a much higher ethanol yield as compared to WT and DM(*srlD*) was observed pointing to a higher need in NAD^+ regeneration. Higher ethanol yield in these circumstances should result in a lower biomass yield, a correlation that the data confirms. In the DM strain, glycolysis appears to be functional even in the absence of *GPD1* and *GPD2* genes through ethanol formation. This observation is supported by the continued weight loss due to CO_2 evolution until the 14^{th} day (Fig. 3.4) in spite of the absence of growth after 2.5 days (Fig. 3.5). In the case of DM(*SOR1*) strain, the situation was different since both sugar consumption and biomass generation continued much longer than in the DM strain. Therefore, NAD^+ is probably regenerated by some other means in DM(*SOR1*) to maintain the redox balance. Indeed, Sor1p might be able to catalyze the conversion of acetaldehyde to ethanol reaction (de Smidt *et al.*, 2008; Gonzalez *et al.*, 2000; Jornvall *et al.*, 1981; Nordling *et al.*, 2002; Sarthy *et al.*, 1994; van Iersel *et al.*, 2000) as normally carried out by Adh1p. Assuming that 0.015 moles of NAD^+ formed by the DM(*SOR1*) strain through sorbitol formation is used in glycolysis during the conversion of glyceraldehyde-3-P to 1,3-diphosphoglycerate, more pyruvate would be formed as an end product of glycolysis simultaneously consuming more glucose. Pyruvate can be further reduced to ethanol, simultaneously regenerating NAD^+. The higher number of moles of NAD^+ formed through ethanol formation by DM(*SOR1*) strain indicates that this strain uses increased ethanol formation as a way to regenerate NAD^+. The yields of ethanol and pyruvate by the DM(*SOR1*) were also higher than in DM(*srlD*) as well as in WT. A search for NAD^+ dependent, zinc-containing alcohol dehydrogenase in *S. cerevisiae* revealed a gene (open reading frame

YDL246C) coding for a protein nearly 99% identical to the product of the *SOR1* gene (Gonzalez *et al.*, 2000). This together with other alcohol dehydrogenases might be involved in generating NAD^+. However, further investigation of the specificity of Sor1p towards different substrates such as linear and branched chain aldehydes leading to the formation of ethanol and higher alcohols is required. Sor1p might be less efficient in converting fructose to sorbitol than sorbitol to fructose and this might push regeneration of NAD^+ by ethanol formation.

TABLE 3.6 NADH formed and NAD^+ regenerated for WT, DM, DM(*srlD*) and DM(*SOR1*) strains respectively through metabolite formation. NAD^+ regenerated in WT through ethanol formation is taken as zero by assuming that its formation is the result of redox neutral process. In other strains, NAD^+ regenerated through ethanol formation are "extra" moles of ethanol formed as compared to moles of ethanol formed by WT strain.

	Strains							
	WT		**DM**		**DM(*srlD*)**		**DM(*SOR1*)**	
Metabolite	NADH formed	NAD^+ regenerated	NADH formed	NAD^+ regenerated	NADH formed	NAD^+ regenerated	NADH formed	NAD^+ regenerated
Biomass	0.072	0	0.063	0	0.096	0	0.091	0
Acetate	0.02	0	0.001	0	0.009	0	0.0003	0
Glycerol	0	0.082	0	0	0	0	0	0
Sorbitol	0	0	0	0	0	0.065	0	0.015
Succinate	0	0.011	0	0.01	0	0.006	0	0.003
Malate	0	0.005	0	0.015	0	0.006	0	0.008
Total*	**0.092**	**0.098**	**0.064**	**0.025**	**0.105**	**0.077**	**0.091**	**0.026**
Ethanol	0	1.74-1.74 = **0**	0	1.91-1.74=**0.17**	0	1.80-1.74=**0.06**	0	1.88-1.74=**0.14**
Total	**0.092**	**0.098**	**0.064**	**0.195**	**0.105**	**0.137**	**0.091**	**0.166**

*Without taking into account the NAD^+ regenerated through ethanol production

Coefficient of variation for NADH formed and NAD^+ regenerated was less than 12% and 10% respectively for all the strains.

Formation of acetic acid is dependent on the availability of NAD^+ in *S. cerevisiae*. Higher availability of NAD^+ than required for cell biosynthesis usually favours acetate formation. Genetic manipulation such as overexpression of the *GPD1* gene (Remize et al., 1999) and expression of a NADH oxidase (Heux *et al.*, 2006) in *S. cerevisiae* has been shown to enhance acetate formation due to higher NAD^+ availability than normal. Contrary to this, deletion of *GPD* genes should theoretically decrease the formation of acetic acid as compared to strains containing *GPD* genes. Indeed, this study has shown

that all the genetically manipulated strains produced much lower concentration of acetate as compared to the WT strain which was probably due to lack of regeneration of NAD^+. The slight formation of acetate in the genetically manipulated strains could be because of the $NADP^+$ dependent aldehyde dehydrogenase (*ALD6*) (Saint-Prix *et al.*, 2004). The highest formation of acetate in the DM(*srlD*) strain as compared to other genetically manipulated strains could indicate NADPH dependent activity of the *srlD* gene thereby increasing the availability of $NADP^+$ in this strain and leading to higher acetate formation using $NADP^+$ dependent Ald6p. NADPH dependent sorbitol-6-phosphate dehydrogenases from plants have been purified and characterized (Hirai, 1981; Tao *et al.*, 1995). However, the presence of such enzymes in *E. coli* has not been investigated yet and further research is needed to resolve this issue.

Under fermentative conditions, yeast form succinate and malate in the mitochondrion which is later secreted in the medium. The formation of these acids is influenced by the redox balance in the cell (Camarasa *et al.*, 2003; Camarasa *et al.*, 2007). The highest yields of malate were found in the DM followed by DM(*SOR1*) DM(*srlD*) and WT strains. A similar relationship was found for ethanol where DM had the highest yield of this metabolite followed by DM(*SOR1*) pointing to the regeneration of NAD^+ through malate and ethanol formation. Later, malate is secreted into the medium in DM, DM(*SOR1*) and DM(*srlD*) strains because this compound cannot go further to form succinate due to deletion of *GPD1* and *GPD2* genes leading to disruption in the reductive pathway of the TCA cycle. Formation of succinate is linked to the G3P shuttle in *S. cerevisiae* because the conversion of G3P to DHAP regenerate $FADH_2$ which is then used for the reduction of fumarate to succinate in the reductive pathway of the TCA cycle using fumarate reductase (Fig. 3.11) (Camarasa *et al.*, 2003; Larsson *et al.*, 1998). As reductive pathway of the TCA cycle is the main branch to form succinate (Camarasa *et al.*, 2003) during alcoholic fermentation, therefore the highest succinate concentration produced by the WT strain could be probably due to succinate synthesis via reductive pathway of the TCA cycle. In the genetically manipulated strains, succinate formation via oxidative branch of the TCA cycle, if at all possible, would also be limited due to deficit of NAD^+ needed for its formation (Fig. 2.8). Moreover, the availability of 2-keto glutarate, an intermediate of the oxidative pathway of the TCA cycle and needed to form succinate would also be limited since it will be withdrawn from the pathway and converted to glutamate using NADH dependent glutamate dehydrogenase thereby regenerating NAD^+ (Fig. 2.8). Glutamate can subsequently either go to biomass formation or

degraded to succinate using γ amino butyric acid (GABA) pathway (Bach *et al.*, 2009). Therefore the fate of glutamate depends on the availability of NAD^+ since formation of biomass needs NAD^+. Indeed, the highest yield of succinate by DM (0.66 g/g biomass formed) (Table 6.4) clearly shows that most of the available glutamate might have been converted to succinate and not to biomass probably due to the highest NAD^+ deficiency in this strain.

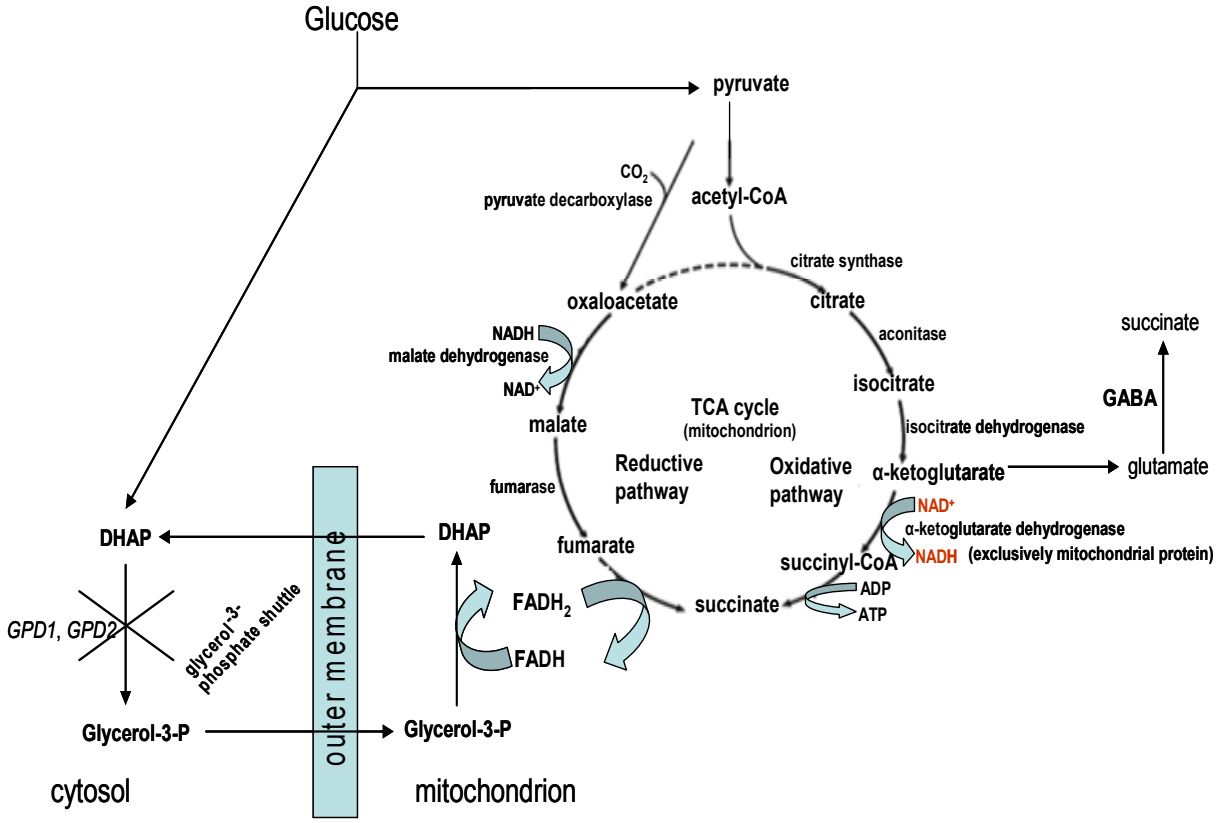

FIG. 3.11 Regeneration of NAD^+ through malate formation and accumulation of malate due to non functional glycerol-3-P shuttle in *S. cerevisiae gpd1*Δ*gpd2*Δ (Camarasa *et al.*, 2007; Larsson *et al.*, 1998).

Higher alcohols are minor metabolites (Atsumi & Liao, 2008) synthesized by *S. cerevisiae* as a way to regenerate NAD^+ (Schoondermark-Stolk *et al.*, 2005). The last step in the Ehrlich pathway (Schoondermark-Stolk *et al.*, 2005) is catalyzed by the *ADH* gene which uses NADH as the cofactor and regenerates NAD^+ (Fig. 2.11). Large differences were observed in the concentrations of these higher alcohols in the different strains used in this study. The higher alcohol which seemed to be most affected was isobutanol. The DM and DM(*SOR1*) strains produced much more isobutanol than WT and DM(*srlD*) strains. This points to the DM(*SOR1*) and DM strains regenerating extra NAD^+ through isobutanol as a result of the inability to regenerate NAD^+ through glycerol formation in DM strain or the poor efficiency of Sor1p in DM(*SOR1*). The effect of *SOR1*

on the production of isobutanol should also not be overlooked as this enzyme might act as an alcohol dehydrogenase (de Smidt *et al.*, 2008; Gonzalez *et al.*, 2000). Higher alcohol formation by alcohol dehydrogenase has been widely reported (Atsumi *et al.*, 2008; Atsumi & Liao, 2008; de Smidt *et al.*, 2008; Singh & Kunkee, 1976; Singh & Kunkee, 1977).

3.5 Potential implications for wine yeasts and wine production

This study has shown that the genetic manipulation of pathways regulating the redox balance can affect the growth and the overall metabolic flux of *S. cerevisiae*. As aroma is regarded as the main factor which determines the quality of wine, higher alcohols and esters may be regarded as the major yeast-derived compounds which can modify the aroma quality of wine (Carrau *et al.*, 2008; Garde-Cerdán & Ancín-Azpilicueta, 2008; Lilly *et al.*, 2006a; Lilly *et al.*, 2006b; Molina *et al.*, 2007; Rossouw *et al.*, 2008). This study carried out in the laboratory yeast strains has established that metabolite characteristics can be altered in ways that could be beneficial in wine production. Further experiments and investigation are necessary using classical and molecular techniques in attempts to improve the metabolite profiles in wine yeast strains.

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Chapter 4

RESEARCH RESULTS II

Introduction of propane-1,2-diol formation pathway into a defective glycerol synthesizing *Saccharomyces cerevisiae* mutant can partially restore growth and ethanol formation

ABSTRACT

Glycerol is a major by-product of alcoholic fermentation by *Saccharomyces cerevisiae* and typically 3-4% (0.06-0.08 moles/mole sugar) of the sugar fermented is converted to glycerol. Glycerol synthesis involves NADH-dependent glycerol-3-phosphate dehydrogenases (Gpd1p and Gpd2p) and deletion of these two genes renders the cells incapable of maintaining fermentative activity under anaerobic conditions due to lack of re-oxidation of NADH. Elimination of glycerol from the alcoholic fermentation and re-oxidizing the excess NADH through an alternative redox sink could lead to production of metabolites other than glycerol. One such metabolite that can replace glycerol as a redox sink in a *gpd1*Δ*gpd2*Δ double mutant (DM) could be propane-1,2-diol. Transformation of the DM with genes encoding *Escherichia coli* glycerol dehydrogenase (*gldA*) and methylglyoxal synthase (*mgsA*) and *S. cerevisiae* aldose reductase (*GRE3*) yielded a strain that produced 1.68 g/l propane-1,2-diol with a yield of 0.047 moles/mole sugar consumed in addition to 31.78 g/l ethanol with a yield of 1.81 moles/mole sugar consumed. The propane-1,2-diol producing strain had the lowest biomass molar yield (0.062 moles/mole sugar consumed) whereas biomass molar yield of WT and DM strains were 0.07 and 0.065 moles/mole sugar consumed respectively. The genetic modifications also influenced the yield of the tricarboxylic acid intermediates such as succinate and malate. Furthermore, the secondary metabolites concentrations were modified in comparison to the wild-type and DM strains which is an indication of general redirection of the carbon flux.

4.1 Introduction

Glycerol is quantitatively the most important by-product of alcoholic fermentation after ethanol both for its biotechnological and metabolic importance (*i.e*. maintaining intracellular redox status of the cell) (da Silva *et al.*, 2009; Wang *et al.*, 2001). NAD^+, a coenzyme used in several biological anabolic processes, is converted to NADH during the growth of *S. cerevisiae* on glucose or fructose as the carbon and energy source (Albers *et al.*, 1996; Bakker *et al.*, 2001; van Dijken & Scheffers, 1986). Regeneration of NAD^+ is required for yeast growth as NAD^+ is essential for glycolysis during the conversion of glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate. Most of the NAD^+ is regenerated through ethanol production (Nissen *et al.*, 1997; van Dijken & Scheffers, 1986). However, when the intermediates of glycolysis are withdrawn as the precursor of biomass, excess NADH is generated (Nissen *et al.*, 1997). Furthermore, when several oxidized by-products such as pyruvate and acetate are produced and secreted, additional NADH is formed. This will eventually cause metabolism to stop unless the surplus NADH can be converted back to NAD^+. Under aerobic conditions, conversion of excess NAD^+ to NADH takes place via electron transport chain where oxygen is the final electron acceptor from NADH and converted to water (de Vries *et al.*, 1992; Luttik *et al.*, 1998; von Jagow & Klingenberg, 1970). This is not possible under anaerobic conditions where re-oxidation of most cytosolic NADH occurs by NADH-coupled reduction of dihydroxyacetone phosphate (DHAP) to glycerol-3-phosphate (Albertyn *et al.*, 1994; Eriksson *et al.*, 1995; Pahlman *et al.*, 2001).

The intermediates of the partially active tricarboxylic cycle in the mitochondrion under fermentative conditions provide precursors for biomass building blocks (Camarasa *et al.*, 2003; Visser *et al.*, 1994). The NADH formed converting 2-ketoglutarate to succinyl-CoA is thought to be oxidized by means of an acetaldehyde-ethanol redox shuttle across the mitochondrial membrane involving a mitochondrial alcohol dehydrogenase Adh3p (Bakker *et al.*, 2000; Repetto & Tzagoloff, 1991). The process also adds to NADH production in the cytosol. Therefore, formation of glycerol plays a significant role in the intracellular redox state of *S. cerevisiae* growing under anaerobic condition (van Dijken & Scheffers, 1986) (Fig. 3.1).

The conversion of dihydroxyacetone phosphate to glycerol-3-phosphate and subsequent dephosphorylation leads to formation of glycerol in *S. cerevisiae* (Gancedo

et al., 1968a; Gancedo *et al.*, 1968b). The first reaction is catalyzed by two cytosolic NADH-dependent glycerol-3-phosphate dehydrogenases (Gpd1/2p) (Albertyn *et al.*, 1994; Eriksson *et al.*, 1995) (Fig. 3.1). A *gpd1*Δ*gpd2*Δ DM is unable to grow under strict anaerobic conditions because both cytosolic and mitochondrial excess NADH cannot be re-oxidized leading to intracellular accumulation of NADH (Ansell *et al.*, 1997). Therefore, in principle, this excess NADH can be used to drive other NADH-dependent reduction reactions. The introduction of heterologous or native proteins which catalyze NADH-dependent reactions could allow the DM to restore its NADH balance and permit anaerobic growth. Furthermore, such a DM strain is a potentially highly interesting biotechnological tool for the production of metabolites whose biosynthesis requires reduction by NADH since the imbalance between NAD^+ and NADH in the mutant should strongly favour such reactions.

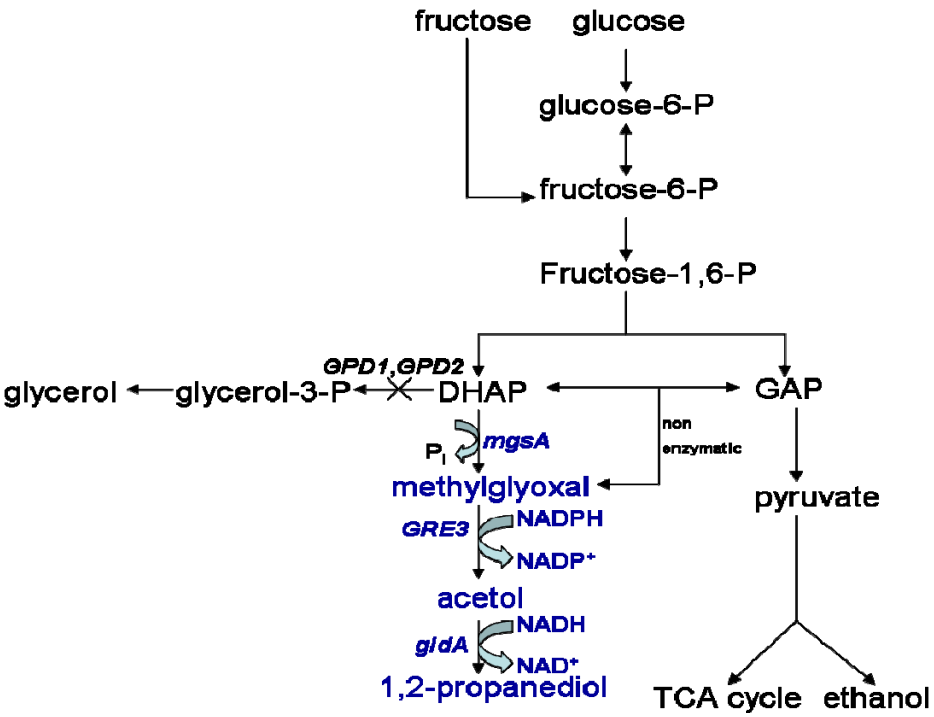

FIG. 4.1 Pathway showing the formation of propane-1,2-diol by DM(*gldA*, *GRE3*, *mgsA*) strain and regeneration of NAD^+ during the conversion of acetol to propane-1,2-diol using *E. coli* glycerol dehydrogenase (*gldA*). Non-enzymatic formation of methylglyoxal from the condensation of dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (GAP) is also indicated (Kuhn *et al.*, 1995; Martins *et al.*, 2001; Saadat & Harrison, 1998; Subedi *et al.*, 2008).

Diversion of carbon flux from glycerol to other valuable compounds has received consideration as excess glycerol is currently being produced in the world as a major

byproduct of the biodiesel industry. Consequently production of propane-1,2-diol as an alternative of glycerol by the DM(*gldA*, *GRE3*, *mgsA*) strain in order to create balance between NADH and NAD^+ can be beneficial for the food, chemical, pharmaceutical and cosmetic industries. The possibility of producing propane-1,2-diol in *S. cerevisiae* as an alternative to chemical synthesis was previously investigated (Cameron *et al*., 1998; Lee & Dasilva, 2006). Propane-1,2-diol was produced by genetically manipulating *S. cerevisiae* by overexpressing two key genes (*gldA* and *mgsA*) from *E. coli*. The *mgsA* gene product catalyzes the conversion of dihydroxyacetone phosphate to methylglyoxal and *gldA* gene product convert methylglyoxal to propane-1,2-diol. However, the propane-1,2-diol concentration produced by strains with these genetic manipulations were below 0.5 g/l. In another study, three genetic manipulations were conducted to improve propane-1,2-diol productivity (Jung *et al*., 2008) where the triosephosphate isomerase (*TPI1*) (to stop the interconversion between dihydroxyacetone phosphate and glyceraldehyde-3-phosphate) was deleted and *gldA* and *mgsA* genes in *S. cerevisiae* overexpressed. A total of 1.11 g/l of propane-1,2-diol was produced through these genetic manipulations in *S. cerevisiae* (Jung *et al*., 2008).

The objective of this study was to investigate the possibility of manipulating a *gpd1*Δ*gpd2*Δ DM of *S. cerevisiae* to regenerate NAD^+ through propane-1,2-diol production by the overexpression of genes encoding *E. coli* glycerol dehydrogenase (*gldA*) and methylglyoxal synthase (*mgsA*) and *S. cerevisiae* aldose reductase (*GRE3*) (Fig. 4.1). The *mgsA* gene (EC 4.2.3.3) of *E. coli* provides bacteria with an alternative to triosephosphate isomerase for metabolizing dihydroxyacetone phosphate (DHAP) since it converts DHAP to methylglyoxal and ultimately to lactate (Ferguson *et al.*, 1998; Saadat & Harrison, 1998). The normal function of *gldA* gene (EC 1.1.1.6) in *E.* coli is to catalyze the NAD^+-dependent oxidation of glycerol to dihydroxyacetone which allows the cell to utilize glycerol as a source of carbon under anaerobic conditions (Tang *et al.*, 1979; Truniger & Boos, 1994). However, an additional role of *gldA* is to regulate the intracellular level of dihydroxyacetone by catalyzing the reverse reaction, *i.e*. the conversion of dihydroxyacetone into glycerol (Gonzalez *et al.*, 2008; Subedi *et al.*, 2008). Moreover, this enzyme possesses a broad substrate specificity, since it is also able to reduce acetol into propane-1,2-diol (Subedi *et al.*, 2008). The *GRE3* gene encodes a NADPH-dependent aldose reductase (EC 1.1.1.14) from *S. cerevisiae* involved in metabolism of aldehyde substrates such as glyceraldehydes (Kuhn *et al.*, 1995) and may also be involved in the detoxification of methylglyoxal (Aguilera & Prieto,

2004). In the manipulated strain, methylgloxal synthase should catalyze the conversion of dihydroxyacetone phosphate (DHAP) to methylglyoxal which can in turn be converted to acetol by *S. cerevisiae* aldose reductase (Gre3p). Acetol is ultimately reduced to propane-1,2-diol by glycerol dehydrogenase (*gldA*) which uses NADH as the cofactor thereby regenerating NAD^+ (Fig. 4.1). The impact of the over-expression of these genes on the anaerobic and aerobic growth capability of DM(*gldA*, *GRE3*, *mgsA*) mutant was also investigated by plate assay and compared to WT and DM strains. The general direction of carbon flux by DM and DM(*gldA*, *GRE3*, *mgsA*) was compared to the WT strain.

4.2 Materials and methods

4.2.1 Yeast strains and genotypes

The WT strain used in this study was *S. cerevisiae* BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*). DM (BY4742; *gpd1*::*KanMx*; *gpd2*::*URA3*) strain was created as described in Chapter 3. The genotype of the mutant DM(*gldA*, *GRE3*, *mgsA*) is DM[PGK_P-*gldA*-PGK_T, PGK_P-*GRE3*-PGK_T, PGK_P-*mgsA*-PGK_T].

4.2.2 Primers and PCR templates

Primers and PCR template used to create DM are as described previously in Chapter 3. The *gldA* and *mgsA* genes were amplified using the *E. coli* DH5α genomic DNA and the *GRE3* gene was amplified using the *S. cerevisiae* BY4742 genomic DNA. The 2μ origin of replication gene was amplified using the Yep24 vector. The following primers were used for PCR amplification of the *gldA* gene: forward 5'-AGATCTATGCCGCATTTGGCACTACTCATCTC-3' and reverse 5'-CTCGAGTTATTCCCACTCTTGCAGGAAACGCT-3'. The primers used for PCR amplification of the *GRE3* gene were: forward 5'-GAATTCATGTCTTCACTGGTTACTCTTAATAACGG-3' and reverse 5'-CTCGAGTCAGGCAAAAGTGGGGAATTTAC-3'. The primers used for PCR amplification of the *mgsA* gene were: forward 5'-GAATTCATGGAACTGACGACTCGCACTTTACCTG-3' and reverse 5'-CTCGAGTTACTTCAGACGGTCCGCGAGATAACG-3'. The primers used for PCR amplification of the 2μ origin of replication gene were: forward 5'-

GCCGGCATCCAATATCAAGGAAATGATAGC and reverse 5'-CAGCTGTTAACGAAGCATCTGTGCTTCA.

4.2.3 DNA manipulations, construction of plasmids and yeast and bacterial transformation

The *gldA* gene was cloned in the pDMPM multicopy shuttle vector (PhD thesis, Malherbe, DF 2010, Stellenbosch University) having an ampicillin resistance marker (amp^r), a leucine auxotrophic marker and a modified multiple cloning site flanked by the constitutive phosphoglycerate-kinase-1 gene promoter (*PGK1P*) and terminator (*PGK1T*) derived from pHVX2 (Volschenk *et al.*, 1997). The *GRE3* gene was first cloned into pSTAH (Gururajan *et al*., 2007) integrating vector containing a histidine auxotrophic marker and an ampicillin resistance marker (amp^r) resulting in pSTAH(*GRE3*) vector. To convert this vector into a multicopy shuttle vector, 2µ yeast origin of replication was inserted into pSTAH(*GRE3*) vector by digesting with *NaeI* and *PvuII* and the 5215 bp band was ligated with the 2µ yeast origin of replication gene (1341 bp) having *NaeI* and *PvuII* overhangs.. To construct pSTAL(*mgsA*) vector, the *mgsA* gene was first cloned into pSTAH (Gururajan *et al*., 2007). The pSTAH(*mgsA*) vector was then digested with *Ssp*I restricticti on enzyme and the 1318 bp band containing *mgsA* gene with *PGK* promoter and terminator was gel-purified. This band was ligated with the Yip5 vector (ATCC culture collection) digested also with *Ssp*1 restriction enzyme. The resultant vector was digested with *Eco*RV and self-ligated. The self-ligated vector was then digested with *Eco*RV and *Nsi*I and the 4561 bp band was gel-purified. This band was then ligated to the YdpK vector digested with *Pvu*II and *Pst*I restriction enzymes. The 4600 bp DNA obtained after digestion of YdpK vector with *Pvu*II and *Pst*I restriction enzymes contains the *LYS2* gene with native promoter and terminator. The resulting vector was digested with *Sma*I and *Pvu*II and the 8544 bp band was ligated with the 2µ yeast origin of replication gene (1341 bp) with *Nae*I and *Pvu*II overhangs. The final vector thus obtained was pSTAL(*mgsA*) containing a lysine auxotrophic marker, the 2µ yeast origin of replication and an ampicillin resistance marker (amp^r). All the genes were under the control of the constitutive *PGK* promoter and terminator (Gellissen & Hollenberg, 1997). The pDMPM(*gldA*) plasmid was first transformed into DM to yield DM(*gldA*), followed by a further transformation with the pSTAH(*GRE3*) plasmid to yield DM(*gldA, GRE3*) and and finally transformed with the pSTAL(*mgsA*) plasmid to yield DM(*gldA*, *GRE3*, *mgsA*). Prior to transformation, the genes were sequenced at the

Central Sequencing Facility of Stellenbosch University. The sequence for all three genes was 100% homologous to the sequence obtained from their GenBank accession number. The accession numbers for *gldA*, *GRE3*, and *mgsA* genes are P0A9S5, P38715 and P0A731 respectively.

Subcloning in *E. coli* DH5α, yeast and bacterial transformations and isolation of genomic DNA from *E. coli* and *S. cerevisiae* was done using standard protocols (Gietz & Schiestl, 2007; Harju *et al.*, 2004).

4.2.4 Medium and fermentation conditions

The fermentations were conducted four-fold in 250 ml Erlenmeyer flasks with a 100 ml working volume as described in chapter 3.

4.2.5 Plate assays of strains under aerobic and anaerobic conditions

Plate assays were performed under aerobic and anaerobic conditions. The medium and growth conditions were as described in chapter 3.

4.2.6 Chemical Analyses

Substrates consumed (glucose, fructose) and metabolites formed (ethanol, glycerol, pyruvate, acetate, malate, succinate) were analyzed using a Waters HPLC system as described in chapter 3. Higher alcohols (isobutanol, isoamyl alcohol, propanol, butanol, 2-phenyl ethanol), esters (ethyl acetate, isoamyl acetate) and minor acids (isobutyric and butyric acid) formed by yeasts were determined with a gas chromatograph (GC) equipped with a flame ionization detector (FID) and Nukol free fatty acid phase fused-silica capillary column (DB-FFAP) (Hewlett Packard 6890 Plus) as described in chapter 3.

Propane-1,2-diol formed by WT, DM and DM(*gldA*, *GRE3*, *mgsA*) strains was determined with GC with a Nukol free fatty acid phase fused-silica capillary column (DB-FFAP) coupled to a mass spectrometer (MS) (Agilent 6890 GC coupled to a Agilent 5975C MSD). The internal dimensions of the column were 60 m $\times$ 0.25 mm with a film thickness of 0.5 μm. Helium was used as carrier gas at a constant flow of 1.8 ml/min

throughout the analysis. The injector was operated at 280ºC in the pulsed splitless mode (splitless time 3 min), applying a 350 kPa pressure pulse for 2 min after injection. The oven program was as follows: 50°C held for 2 min, ramped at 15°C/min to 240°C (held for 2 min). The transfer line to the MS was kept at 250ºC with the MS scanning from 35-300 m/z and the electron multiplier set to 1705 eV. The extraction of propane-1,2-diol from the sample was performed as follows: the 3-octanol internal standard (100 µl) was added to 5 ml of sample and extracted with 1 ml diethyl ether by placing the ether/sample mixture in an ultrasonic bath for 5 min. The sample/ether mixture was centrifuged at 4000 rpm for 3 min. The ether layer was removed and dried on Na_2SO_4. The extract 2 µl of the extract was injected into the GC-MS column.

4.3 Results

4.3.1 Growth of strains on agar plates under aerobic and anaerobic conditions

All strains grew equally well under aerobic conditions on 2% glucose agar plates. However, on 5% glucose-5% fructose agar plates, the DM grew poorly as reported in the previous chapter. The growth of the triple transformed DM(*gldA*, *GRE3*, *mgsA*) was better than of the untransformed DM albeit not to the extent of WT strain (Fig. 4.2). Under anaerobic conditions, the WT growth was similar to growth under aerobic conditions. To assess if the non-enzymatic formation of methylglyoxal (Fig. 4.1) is enough to restore the growth of DM under anaerobic conditions, single transformed DM(*gldA*) and double transformed DM(*gldA*, *GRE3*) strains were grown under anaerobic conditions on 2% glucose agar plates. However, no growth was observed by the strains under those conditions (data not shown). Assuming that this could be due to low availability of methylglyoxal in those strains and therefore a triple transformed DM(*gldA*, *GRE3*, *mgsA*) was constructed. On 5% glucose-5% fructose agar plates under anaerobic conditions, no growth of the DM and even the triple transformed DM(*gldA*, *GRE3*, *mgsA*) could be observed. This shows that although the over-expression of *gldA*, *GRE3* and *mgsA* in DM partially restored the growth defect on 5% glucose-5% fructose agar plates under aerobic conditions but the growth defect was not restored on both 2% glucose and 5% glucose-5% fructose agar plates under anaerobic conditions (Fig. 4.2).

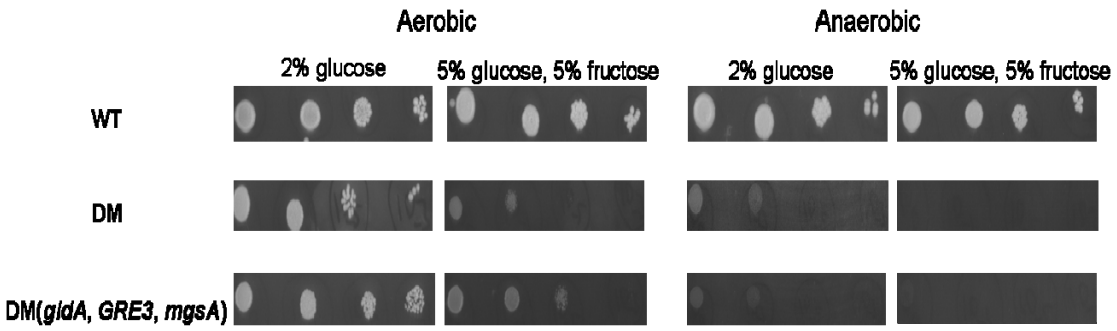

FIG. 4.2 Plate assays for the strains under aerobic and anaerobic conditions cultured on 2% glucose and 5% glucose-5% fructose and 6.7 g/l YNB. Ten µl of ten-fold dilution series were spotted for each row, beginning with a culture of an OD_{600nm} of one (corresponding to 2.7 X 10^5 cells / 10 µl).

4.3.2 Shift from respirofermentative to strictly anaerobic growth of the strains in liquid medium

Following the oxygen depletion of self-anaerobic cultures with methylene blue indicated that WT cultures were truly anaerobic after 1.5 days whereas the flasks inoculated with DM and DM(*gldA*, *GRE3*, *mgsA*) took 2.5 days. Initial growth of all the strains therefore started in aerobic conditions which gradually became anaerobic due to the consumption of oxygen and the release of carbon dioxide (CO_2) by the growing strains (Fig. 4.3).

4.3.3 Effect of *gldA*, *GRE3* and *mgsA* overexpression on CO_2 release, total sugar consumption and biomass formation

The CO_2 release, sugar consumption and biomass formation by WT and DM strains was same as reported in chapter 3. Sugar consumption ability of single transformed DM(*gldA*) strain was analyzed in 5% glucose-5% fructose liquid medium. The hypothesis was that methylglyoxal is formed non-enzymatically in *S. cerevisiae* grown in high sugar medium (Gomes *et al.*, 2005) (so no need to express *mgsA* gene) and *GRE3* is a native *S. cerevisiae* gene (Kuhn *et al.*, 1995) (so no need to overexpress *GRE3*). However, sugar consumption ability of DM(*gldA*) strain did not improved as compared to DM (data not shown). Assuming the need to overexpress *GRE3* gene in DM(*gldA*) background, a double transformed DM(*gldA*, *GRE3*) strain was constructed. However, even this strain was not able to restore the sugar consumption ability (data not shown) which could be due to low formation of methylglyoxal therefore leading to low acetol formation and ultimately low NAD^+ regeneration needed for glycolytic acitivity.

Therefore, a triple transformed DM(*gldA*, *GRE3*, *mgsA*) strain was ultimately constructed. Overexpression of *gldA*, *GRE3* and *mgsA* in DM led to significant improvement in the sugar utilization consuming 68% of the total sugar (Table 4.1) as compared to DM which consumed only 47% of the total sugar initially present in medium. This shows that overexpression of all the three genes in DM is required for improvement in the metabolic activity of DM in liquid medium. However, sugar utilization by the DM(*gldA*, *GRE3*, *mgsA*) was not to the extent of WT (Table 4.1). Overexpression of *gldA*, *GRE3* and *mgsA* in DM also improved CO_2 release but the gas release was neither as rapid nor to the same extent as the WT (Fig. 4.3).

Overexpression of *gldA*, *GRE3* and *mgsA* genes in DM increased biomass concentration but not to the extent of WT. Growth by DM(*gldA*, *GRE3*, *mgsA*) continued under anaerobic conditions and only ceased after 10 days (Fig. 4.4) in contrast to lack of growth on agar plates (Fig. 4.2). The molar yield of biomass was the highest for WT followed by DM and DM(*gldA*, *GRE3*, *mgsA*) strains (Table 4.2).

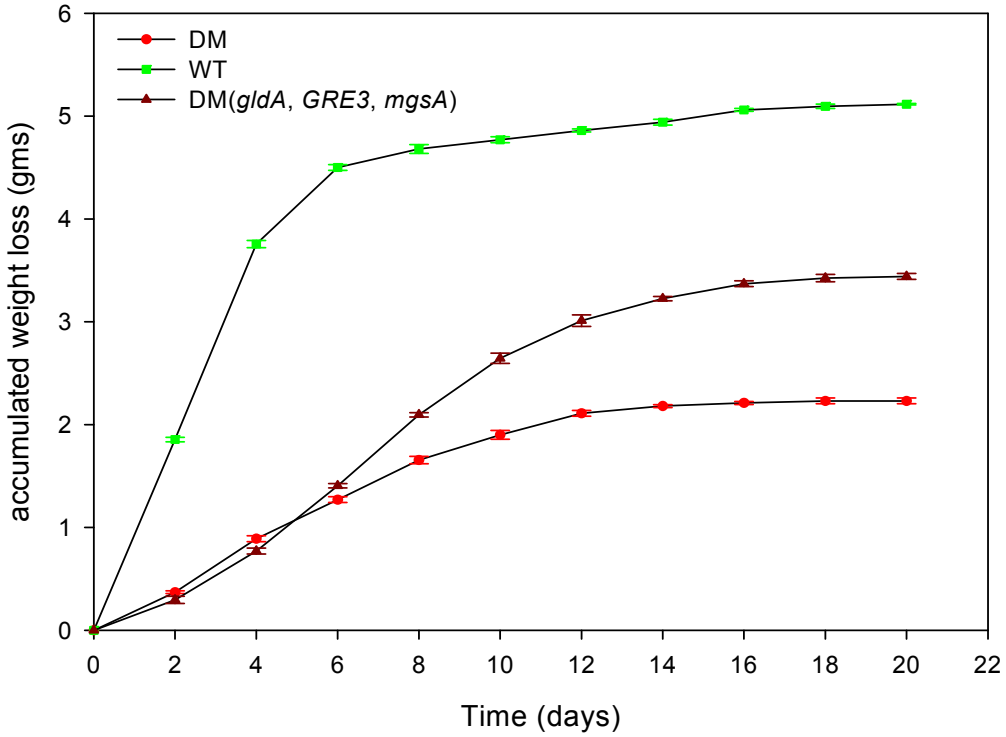

FIG. 4.3 Release of CO_2 by *S. cerevisiae* WT, DM and DM(*gldA*, *GRE3*, *mgsA*) when cultivated in 5% glucose-5% fructose and 6.7 g/l YNB carried out in four replicates. Error bars indicated are the standard deviation of the four replicates for each strain. Weight loss is assumed to only originate from CO_2.

Table 4.1 Effect of overexpression of *gldA*, *GRE3* and *mgsA* genes in *S. cerevisiae* *gpd1*Δ*gpd2*Δ strain on sugar consumption[a] cultivated in 5% glucose-5% fructose and 6.7 g/l YNB over 20 days.

Strains	Glucose used (g/l)	Fructose used (g/l)	Total sugar used (g/l)
WT	50 ± 0	49.89 ± 0.04	99.89 ± 0.04
DM	30.38 ± 1.58	16.8 ± 1.46	47.17 ± 1.59
DM(*gldA*, *GRE3*, *mgsA*)	44.9 ± 0.51	23.5 ± 1.06	68.4 ± 1.28
[a]Values represent the average and standard deviation of four independent cultivations.			

TABLE 4.2 Effect of overexpression of *gldA*, *GRE3* and *mgsA* genes in *S. cerevisiae* *gpd1*Δ*gpd2*Δ strain on product yield (moles/mole sugar utilized)[a] cultivated in 5% glucose-5% fructose and 6.7 g/l YNB during an oxygen limited fermentation. The values in brackets represent the metabolite or dry weight (for the biomass) concentrations (g/l)

	Strains		
Metabolite	**WT**	**DM**	**DM(*gldA*, *GRE3*, *mgsA*)**
CO_2	1.76 (43.00)	1.89 (21.79)	1.81 (30.22)
ethanol	1.76 (45.17)	1.89 (22.88)	1.81 (31.78)
biomass[b]	0.07 (0.98)	0.065 (0.42)	0.062 (0.58)
glycerol	0.080 (4.08)	0	0
propane-1,2-diol	0	0	0.047 (1.68)
acetate	0.021 (0.67)	0.83[c] (0.013)	0.45[c] (0.01)
succinate	0.011 (0.73)	0.009 (0.27)	0.015 (0.71)
malate	0.005 (0.42)	0.015 (0.50)	0.007 (0.33)
pyruvate	0.004 (0.161)	0.008 (0.182)	0.020 (0.73)

[a]Yield was calculated from the 20th day sample of four independent cultivations
[b]Molecular formula of *Saccharomyces cerevisiae* is taken as **$CH_{1.8}O_{0.5}N_{0.2}$**
[c]mmoles/mole sugar utilized
Coefficient of variation (CV) for concentration and yield of metabolites was less than 10% for all the strains.

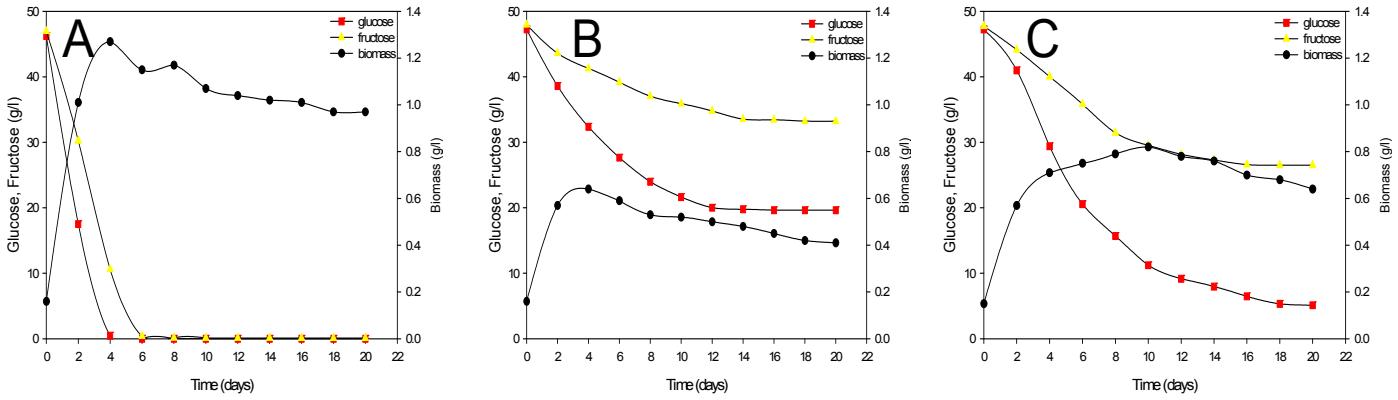

FIG. 4.4 Glucose and fructose consumption and growth (biomass: dry weight) by strains of *S. cerevisiae* WT (A), DM (B) and DM(*gldA*, *GRE3*, *mgsA*) (C) when cultivated in 5% glucose-5% fructose and 6.7 g/l YNB in a representative experiment.

4.3.4 Ethanol, glycerol and propane-1,2-diol production

As expected the DM and DM(*gldA*, *GRE3*, *mgsA*) strains did not produce glycerol due to the deletion of the *GPD1* and *GPD2* genes. The WT strain produced glycerol up to the 10th day and the concentration was constant thereafter (Fig. 4.5). DM(*gldA*, *GRE3*, *mgsA*) produced 1,2-propanediol (Fig. 4.5). Ethanol formation varied greatly among the strains with WT producing the highest concentration and DM the least. However, the highest molar ethanol yield was observed in DM whereas WT had the lowest ethanol yield (Table 4.2). This indicates that some glucose has been directed to glycerol and 1,2-propanediol formation instead of ethanol in the WT and DM(*gldA*, *GRE3*, *mgsA*) strains respectively whereas DM produced no glycerol due to the *gpd1*Δ*gpd2*Δ deletion. The available hydrogen in each metabolite was calculated from each of the products (Table 4.3). Approximately 100% of the hydrogen moles from the sugars were accounted for in the products for all the strains (Table 4.3).

TABLE 4.3 Moles of available hydrogen present[a] in each metabolite formed by strains cultivated 5% glucose-5% fructose and 6.7 g/l YNB under oxygen limited fermentation. The available hydrogen in one mole each of glucose and fructose is 24 thereby giving a total available 48 moles hydrogen.

	Strains		
Metabolite	**WT**	**DM**	**DM(*gldA*, *GRE3*, *mgsA*)**
CO_2	0	0	0
ethanol	42.24	45.36	43.44
biomass[b]	0.672	0.624	0.595
glycerol	2.24	0	0
propane-1,2-diol	0	0	1.504
acetate	0.336	13.28[c]	7.2[c]
succinate	0.308	0.252	0.42
malate	0.12	0.36	0.168
pyruvate	0.08	0.16	0.4
Total	45.99	46.76	46.53
%Hydrogen recovered	**95.81**	**97.42**	**96.94**

[a]moles of available hydrogen present in each metabolite is calculated from the final day (20th day) molar yield of each metabolite.
[b]Molecular formula of *S. cerevisiae* is taken as $\mathbf{CH_{1.8}O_{0.5}N_{0.2}}$
[c]mmoles of H formed/48 moles of H consumed from glucose and fructose
Coefficient of variation for available hydrogen moles was less than 13% for all the strains.

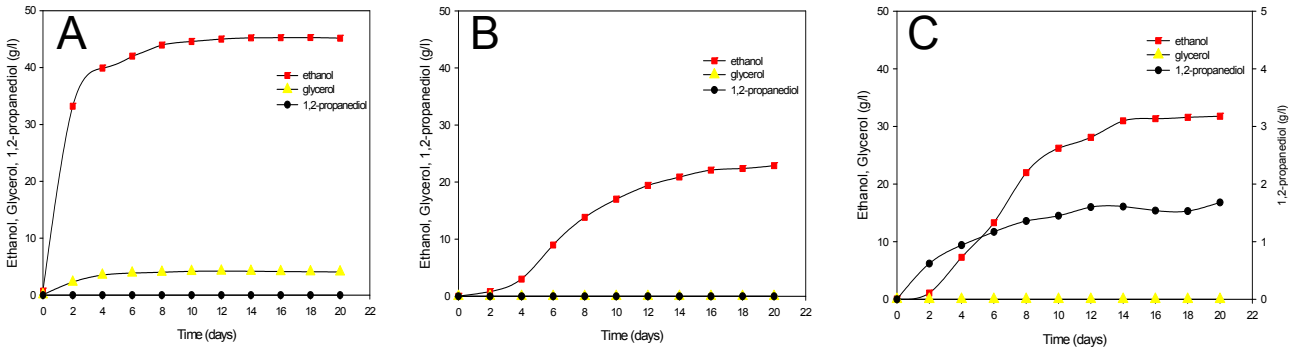

FIG. 4.5 Ethanol, glycerol and propane-1,2-diol production by strains of *S. cerevisiae* WT (A), DM (B) and DM(*gldA*, *GRE3*, *mgsA*) (C) when cultivated in 5% glucose-5% fructose and 6.7 g/l YNB in a representative experiment.

4.3.5 Cytosolic and mitochondrial acid production

WT produced much more acetate than DM and DM(*gldA*, *GRE3*, *mgsA*) (Fig. 4.6). Interestingly, the highest concentration of malate was produced by DM as compared to WT and DM(*gldA*, *GRE3*, *mgsA*). The increase in malate concentration in DM is linear even though there is no more growth. WT and DM(*gldA*, *GRE3*, *mgsA*) strains formed the highest concentration of succinate. The highest amount of pyruvate were found in DM(*gldA*, *GRE3*, *mgsA*) followed by WT and DM (Fig. 4.6). The molar yields of the metabolites are shown in Table 4.2.

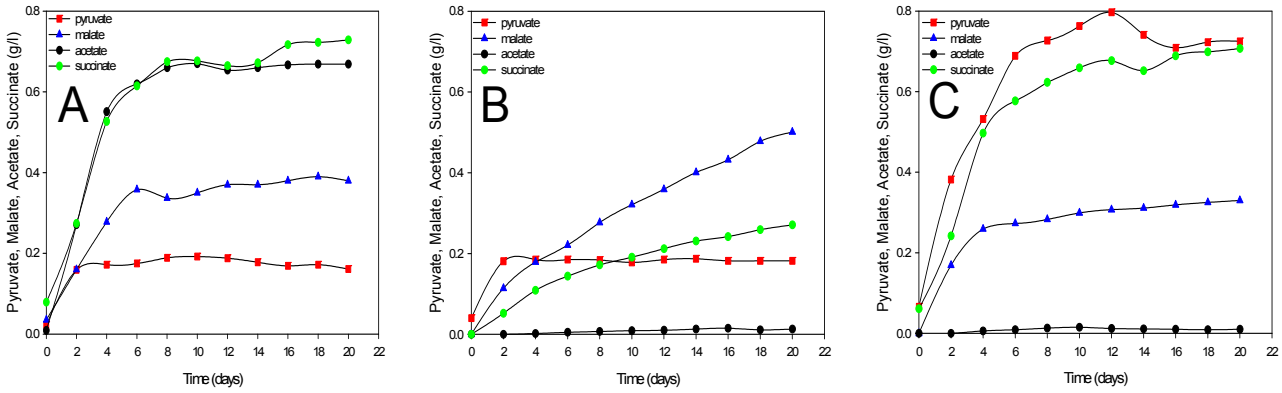

FIG. 4.6 Pyruvate, malate, acetate and succinate production by strains of *S. cerevisiae* WT (A), DM (B) and DM(*gldA*, *GRE3*, *mgsA*) (C) when cultivated in 5% glucose-5% fructose and 6.7 g/l YNB in a representative experiment.

4.3.6 Higher alcohols, minor acids and esters production

The highest concentrations of isobutanol were formed by DM(*gldA*, *GRE3*, *mgsA*) followed by DM and WT (Fig. 4.7). This shows that overexpression of *gldA*, *GRE3* and *mgsA* genes in DM stimulated isobutanol production. Similar concentrations of isoamyl alcohol and 2-phenyl ethanol were found in all the strains. None of the strains formed propanol or butanol (Fig. 4.7).

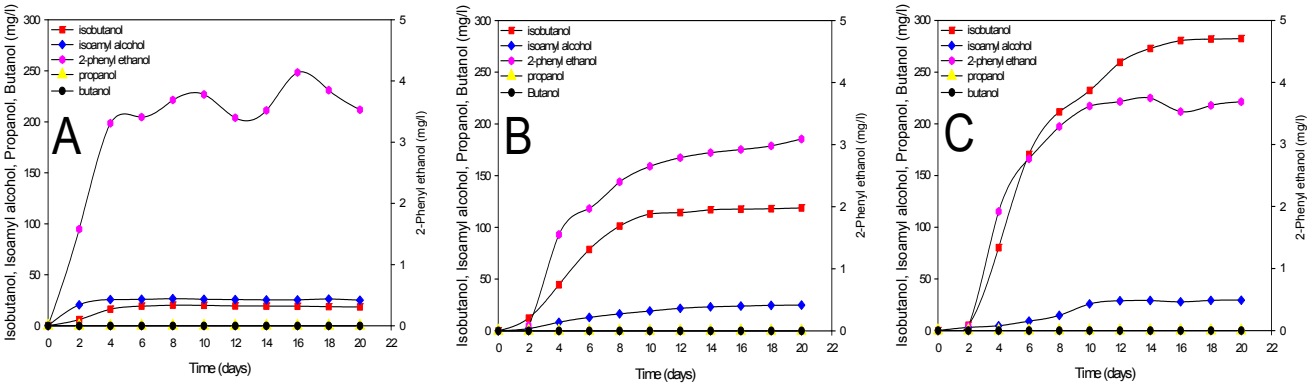

FIG. 4.7 Higher alcohol production by strains of *S. cerevisiae* WT (A), DM (B) and DM(*gldA*, *GRE3*, *mgsA*) (C) when cultivated in 5% glucose-5% fructose and 6.7 g/l YNB in a representative experiment.

None of the strains produced isoamyl acetate (Fig. 4.8). The *gpd1*Δ*gpd2*Δ deletion resulted in much lower ethyl acetate concentrations being produced compared to WT and overexpression of *gldA*, *GRE3* and *mgsA* genes in DM failed to restore levels back to those of WT (Fig. 4.8). All strains failed to produce butyric acid whereas DM(*gldA*, *GRE3*, *mgsA*) formed the highest concentration of isobutyric acid followed by DM and WT. The concentration and molar yields of higher alcohols, minor acids and esters are shown in Table 4.4.

Table 4.4 Effect of the overexpression of *gldA*, *GRE3*, *mgsA* genes in *S. cerevisiae* *gpd1*Δ*gpd2*Δ strain cultivated in 5% glucose-5% fructose and 6.7 g/l YNB on the concentration (mg/l) and the yield (mg/g sugar utilized; values in brackets) of higher alcohols, esters and minor acids during oxygen limited fermentation after 20 days.

	Strains		
Metabolite (mg/l)	**WT**	**DM**	**DM(*gldA*, *GRE3*, *mgsA*)**
Isobutanol	18.53 (0.18)	118.75 (2.52)	282.53 (4.13)
Isoamyl alcohol	25.09 (0.25)	24.89 (0.53)	29.38 (0.43)
Propanol	0	0	0
Butanol	0	0	0
2-phenyl ethanol	3.53 (0.04)	3.09 (0.066)	3.69 (0.054)
Ethyl acetate	26.51 (0.26)	4.63 (0.098)	5.72 (0.084)
Isoamyl acetate	0	0	0
Butyric acid	0	0	0
Isobutyric acid	0.91 (0.009)	2.14 (0.045)	6.11 (0.089)
Coefficient of variation for concentration and yield of metabolites was less than 12% for all the strains.			

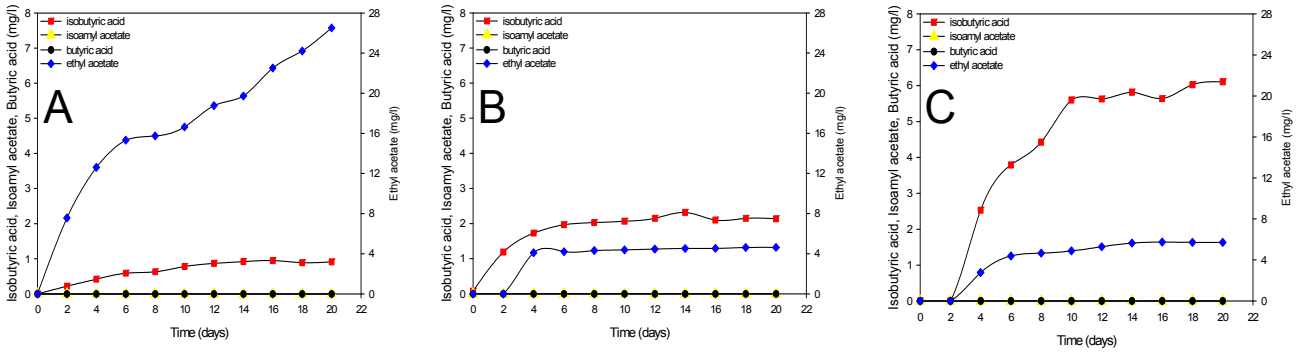

FIG. 4.8 Esters and minor acids production by strains of *S. cerevisiae* WT (A), DM (B) and DM(*gldA*, *GRE3*, *mgsA*) (C) when cultivated in 5% glucose-5% fructose and 6.7 g/l YNB in a representative experiment.

4.4 Discussion

This study has shown that the deletion of glycerol-producing genes in *S. cerevisiae* can be partially complemented by overexpressing propane-1,2-diol producing genes in liquid medium although the complementing effect was not visible on solid medium. Moreover, this genetic manipulation has marked impact on the concentration of metabolites formed.

4.4.1 Novel pathways for NAD^+ regeneration alter growth

The results presented here suggest that the overexpression of *gldA*, *GRE3* and *mgsA* genes in DM lead to regeneration of NAD^+ thereby improving growth and metabolic activity of DM in liquid medium (Fig. 4.1). The inability of DM(*gldA*) and DM(*gldA*, *GRE3*) to even partially complement for *GPD1* and *GPD2* deletion could be due to lower availability of precursors (methylglyoxal and acetol) which might regenerate lower NAD^+ as compared to DM(*gldA*, *GRE3*, *mgsA*) strain. To my knowledge, overexpression of these genes to restore the redox balance in a *gpd1*Δ*gpd2*Δ DM has not been reported before but similar genetic manipulations have been carried out in *S. cerevisiae* WT to produce propane-1,2-diol (Cameron *et al.*, 1998; Jung *et al.*, 2008; Lee & Dasilva, 2006). However, it has been shown that overexpression of other native or heterologous genes can partially complement the double mutation (third chapter of this thesis). For example overexpression of genes such as *SOR1* and *srlD* were shown in the third chapter to partially complement DM. Several other studies have been carried out earlier where heterologous genes were overexpressed in DM. However, none of the alternative NADH-oxidizing systems such as the aldose reductase gene (*XYL1*) from *Pichia stipitis* (Liden *et al.*, 1996) and mannitol-1-phosphate dehydrogenase gene (*mtlD*) from *E. coli* (Costenoble *et al.*, 2003) can completely restore the growth defect phenotype of the double deletion of the glycerol synthesizing genes and none are as efficient as the glycerol synthesis pathway in maintaining the redox balance in *S. cerevisiae* under strictly anaerobic conditions.

The growth of WT and DM on 2% glucose and 5% glucose-5% fructose agar medium under aerobic and anaerobic conditions was same as shown in chapter 3. The different growth phenotype of DM(*gldA*, *GRE3*, *mgsA*) strain on solid medium under anaerobic conditions as compared to liquid medium could possibly be explained by the toxic effect

of methylglyoxal (Aguilera & Prieto, 2001; Ferguson *et al.*, 1998; Gomes *et al.*, 2005; Gomes *et al.*, 2006; Gomes *et al.*, 2008; Inoue *et al.*, 1985; Ispolnov *et al.*, 2008; Maeta *et al.*, 2005; Martins *et al.*, 2001; Murata *et al.*, 1986). Methylglyoxal formed by methylglyoxal synthase and also formed as an intermediate during the conversion of dihydroxyacetone phosphate (DHAP) to propane-1,2-diol might not diffuse away from the cell on solid medium and be more toxic. The toxic effect of methylglyoxal might also hold true for no growth phenotype of the DM(*gldA*) and DM(*gldA*, *GRE3*) strains under anaerobic conditions solid medium. The slightly better growth of DM(*gldA*, *GRE3*, *mgsA*) strain on 5% glucose-5% fructose agar medium under aerobic conditions might be because of comparatively less formation of methylglyoxal due to presence of oxygen. Experimental evidence of the greater toxic effect of methylglyoxal on solid medium than in liquid medium is required. However, this could be related to the degree of anaerobiosis found in the solid and liquid medium. Anaerobic conditions in the liquid medium are generated by the metabolic activity of the culture in flasks whereas in the solid medium, anaerobic conditions were developed by removing all oxygen from the incubation environment and would likely to be more stringent than in flasks. Presence of oxygen initially in the flasks increase sterol biosynthesis and membrane permeability (Principles and practices of winemaking by Roger B. Boulton, Vernon L. Singleton, Linda F. Bisson, Page 171) which might help the cells in diffusing more methlyglyoxal out in the liquid medium than in the solid medium.

4.4.2 Effect of genetic manipulation on sugar consumption, biomass formation and metabolite production

Transformation of DM with *gldA*, *GRE3* and *mgsA* improved sugar consumption. This is likely to be due to the higher regeneration of NAD^+ through propane-1,2-diol formation. The molar yield of biomass for DM(*gldA*, *GRE3*, *mgsA*) was lower compared to WT and DM. This might be due to the toxic effect of methylglyoxal on cell proliferation. The lower biomass yield for DM(*gldA*, *GRE3*, *mgsA*) strain than WT points to a lower amount of excess NADH being generated through biomass formation in DM(*gldA*, *GRE3*, *mgsA*) strain as compared to WT. Indeed, the propane-1,2-diol molar yield by DM(*gldA*, *GRE3*, *mgsA*) is lower than the glycerol molar yield by WT. Moreover, the higher ethanol molar yield by the DM(*gldA*, *GRE3*, *mgsA*) and the DM as compared to the WT is probably related to the higher need of regeneration of NAD^+. This reorientation of metabolic flux will also reduce biomass formation since more of the potential biomass precursors will

be directed towards ethanol production. One possible cause for the reduced efficiency of the heterologous system might be because of the greater affinity for NADH of Gpd1/2p in WT (Cronwright *et al.*, 2002) compared to the lower affinity of the glycerol dehydrogenase gene product in the manipulated DM(*gldA*, *GRE3*, *mgsA*) strain. As a result, DM(*gldA*, *GRE3*, *mgsA*) strain might be forced to regenerate NAD^+ through ethanol formation. Kinetic and flux studies of the glycerol forming pathway in *S. cerevisiae* revealed that the flux towards glycerol can be drastically increased by simultaneously increasing the NADH/NAD^+ ratio and Gpd1/2p activity (Cronwright *et al.*, 2002).

DM(*gldA*, *GRE3*, *mgsA*) produced the highest molar yield as well as concentration of pyruvate as compared to WT and DM. This might be related to the 2-oxoaldehyde dehydrogenase activity on methylglyoxal which might catalyze the conversion of this toxic compound to pyruvate (Murata *et al.*, 1986). However, 2-oxoaldehyde dehydrogenase activity has not yet been detected in *S. cerevisiae* (Murata *et al.*, 1986). In *S. cerevisiae*, natural formation of methylglyoxal occurs through triose phosphates (glyceraldehyde-3-phosphate and dihydroxyacetone phosphate) (Richard, 1993). Triose phosphate isomerase, *TPI1*, catalyzes the aldo-ketose isomerization of these triose phosphates. However, triose phosphates are very unstable molecules and β-elimination reaction of the phosphoryl group from the common 1,2-enediolate of both trioses may occur, leading to methylglyoxal formation (Richard, 1993) (Fig. 4.1). This is a non-enzymatic reaction and therefore methylglyoxal occurrence is an unavoidable consequence of glycolytic metabolism (Martins *et al.*, 2001). *S. cerevisiae* has mainly two different metabolic routes to eliminate methylglyoxal (Fig. 4.9). Both of these metabolic pathways, through a series of reactions convert methylglyoxal to lactate. The first is through a glyoxalase system consisting of glyoxalase I and glyoxalase II (Fig. 4.9). The second route to lactate is by a reduction/oxidation pathway where methylglyoxal is first reduced to lactaldehyde by NADPH-dependent methylglyoxal reductase and then oxidized to lactate by NAD^+-dependent lactaldehyde dehydrogenase (Fig. 4.9). Apart from these two systems, methylglyoxal has also been shown to be converted to pyruvate using 2-oxoaldehyde dehydrogenase in a bacterial system (Kalapos, 1999). In the DM(*gldA*, *GRE3*, *mgsA*) strain, methylglyoxal is not only the product of the non-enzymatic reaction but also through the enzymatic conversion of dihydroxyacetone phosphate to methylglyoxal. To handle the excess intracellular concentrations of this toxic compound, cells might choose another metabolic route such

as through methylglyoxal to pyruvate conversion. However, it is not known whether the 2-oxoaldehyde dehydrogenase enzyme is present in *S. cerevisiae*. Moreover, specificity of aldehyde dehydrogenases (*ALD*) present in *S. cerevisiae* towards 2-keto aldehydes (such as methylglyoxal) must also be investigated and cannot be ignored.

The lower formation of acetate in the genetically manipulated strains as compared to WT might again be due to lower availability of NAD^+ which is required for acetate formation. Similar formation of succinate by the WT and DM(*gldA*, *GRE3*, *mgsA*) could be due to functioning of glycerol-3-phosphate shuttle (Fig. 3.11) in WT and proposed acetol shuttle (Fig. 6.8) in the DM(*gldA*, *GRE3*, *mgsA*) strain. Functioning of these shuttles completes the reductive pathway of the TCA cycle thereby leading to reduction of fumarate to succinate which might not take place in DM as explained earlier. In the DM strain, succinate, if any, might only be formed through the degradation of glutamate as explained in chapter 3. The highest yield of malate in the DM indicates the urgency of this strain for NAD^+ regeneration through malate formation. Malate might then be scereted due to an incomplete reductive pathway of the TCA cycle.

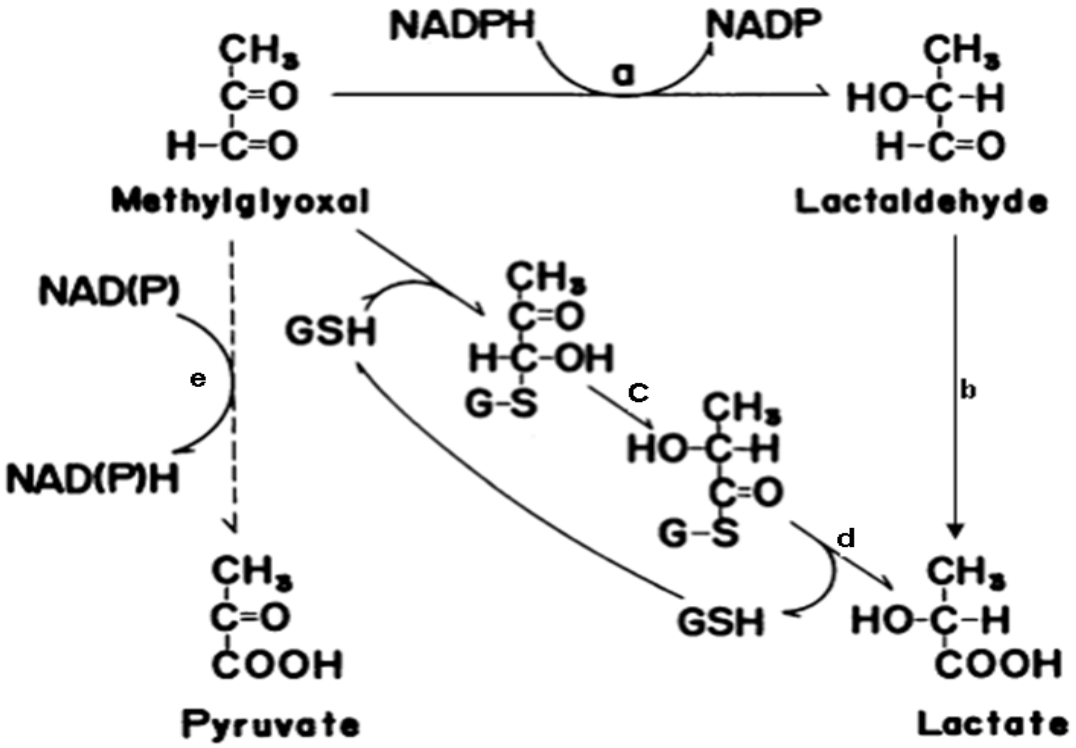

FIG. 4.9 Methylglyoxal metabolism by *S. cerevisiae*. The pathway, e, shown by dashed line has not been detected in *S. cerevisiae*. a, methylglyoxal reductase; b, lactaldehyde dehydrogenase; c, glyoxalase I; d, glyoxalase II; GSH, reduced glutathione (Murata *et al.*, 1985).

The higher alcohol, isobutanol was significantly affected as the highest concentration was produced by DM(*gldA*, *GRE3*, *mgsA*) followed by DM. In DM this could be related to regeneration of extra NAD^+ through isobutanol in the absence of NAD^+ regeneration through glycerol formation. The higher isobutanol concentration produced by DM(*gldA*, *GRE3*, *mgsA*) might be due to availability of more pyruvate (possibly by the oxidation of methylglyoxal) which acts as the principle precursor for isobutanol (Fig. 2.11).

4.4.3 Propane-1,2-diol production by the DM(*gldA*, *GRE3*, *mgsA*) strain

Our study has shown that the production of propane-1,2-diol can be further increased to 1.68 g/l which is more than the concentration achieved in other studies (Lee & Dasilva, 2006; Jung *et al*., 2008). The deletion of *GPD1* and *GPD2* genes from *S. cerevisiae* together with overexpression of *gldA*, *GRE3* and *mgsA* genes could force the metabolic flux towards propane-1,2-diol production in order to maintain a redox balance. In the earlier studies to produce propane-1,2-diol in *S. cerevisiae*, Cameron *et al*. (1998) and Lee and Dasilva (2006) overexpressed only *gldA* and *mgsA* genes and relied on *S. cerevisiae*, native *GRE3* gene to produce propane-1,2-diol. Although they found some propane-1,2-diol being formed by this genetically manipulated *S. cerevisiae*, the lower concentration as compared to the present study suggests that overexpression of native aldose reductase (*GRE3*) might also be necessary to further increase propane-1,2-diol concentration. Moreover, unlike the medium and cultivation conditions used by other authors (2% glucose with aeration), higher sugar medium (10% total sugar under fermentative conditions) was used in this study. High sugar medium and fermentative growth conditions have shown to lead to higher glycerol yields (Wang *et al.*, 2001). The same effect might occur in the genetically manipulated strain since propane-1,2-diol synthesis, like glycerol might also act as the principle redox balancing route under conditions in the *S. cerevisiae gpd1*Δ*gpd2*Δ strain.

There might be potential to increase the concentration of propane-1,2-diol even further by replacing the yeast aldose reductase (*GRE3*) used in this study which uses NADPH as the cofactor by the *Pichia stipitis* aldose reductase which has specificity for both NADH or NADPH as cofactor (Bengtsson *et al.*, 2009; Jeffries & Jin, 2004; Kang *et al.*, 2003; Traff-Bjerre *et al.*, 2004; Verduyn *et al.*, 1985; Wang *et al.*, 2007). Alteration of the cofactor preference of the *S. cerevisiae* aldose reductase from NADPH to NADH by site directed mutation could also result in an improved enzyme for propane-1,2-diol

production. Recently, structure site-directed mutagenesis guided by X-ray crystallography structural studies was used to change the coenzyme preference of *Candida tenuis* aldose reductase about 170-fold from NADPH in the wild-type to NADH while the enzyme still maintained its catalytic efficiency (Petschacher *et al.*, 2005; Petschacher & Nidetzky, 2005; Petschacher & Nidetzky, 2008).

4.5 Potential implications for the wine and chemical industry

This study has shown that the genetic manipulations of the pathways regulating the redox balance can affect the growth and the overall metabolic flux in *S. cerevisiae*. The implications are related to understanding how redox impacts on aroma compounds. Furthermore, the usefulness of this strain in the chemical industries where overproduction of propane-1,2-diol can be beneficial as mentioned previously cannot be ignored.

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Chapter 5

RESEARCH RESULTS III

PDC1 overexpression and *ALD6* deletion in a *Saccharomyces cerevisiae* glycerol synthesis mutant improves ethanol yield and reduces by-product formation

ABSTRACT

Ethanol and glycerol are the major by-products of alcoholic fermentation by *Saccharomyces cerevisiae* and typically 3-4% (0.06-0.08 moles/mole sugar) and 40-45% (1.6-1.8 moles/mole sugar) of the sugar fermented ends up in glycerol and ethanol respectively. Conversion of acetaldehyde to ethanol re-oxidizes the NADH formed during the glycolysis whereas glycerol formation accounts for the re-oxidation of excess NADH formed when the intermediates of glycolysis are withdrawn as precursors of biomass and the other metabolic reactions. *S. cerevisiae* synthesizes glycerol using NADH-dependent glycerol-3-phosphate dehydrogenases (Gpd1p and Gpd2p). The deletion of the corresponding genes renders the cells incapable of maintaining fermentative activity due to the accumulation of NADH. The possibility of eliminating glycerol from the alcoholic fermentation and re-oxidizing the excess NADH through additional ethanol formation in the *gpd1*Δ*gpd2*Δ double mutant (DM) was investigated. Transforming the DM with the pyruvate decarboxylase (*PDC1*) gene and deleting the aldehyde dehydrogenase (*ALD6*) gene from its genome yielded a strain that produced 32 ± 1.6 g/l ethanol (1.92 ± 0.06 moles/mole sugar consumed) as compared to 23 ± 1.8 g/l (1.89 ± 0.02 moles/mole sugar consumed) produced by DM in addition to improving the fermentative ability of DM by consuming more sugar. The ethanol concentration was less than the WT strain (45.17 ± 2.71 g/l) but the molar yield (1.76 ± 0.08) was greater. Furthermore, in a separate experiment, the effect of addition of pyruvate during the log phase on the fermentative ability of the DM was investigated. Addition of pyruvate to the final concentration of 1, 2 and 3 g/l on the 8^{th} day of growth of DM improved CO_2 release and sugar consumption by 23%, 29% and 35% respectively. The results show that increasing the flux from pyruvate to ethanol by either genetic manipulation or by externally adding pyruvate can help in re-oxidizing excess NADH.

5.1 Introduction

Ethanol and glycerol are quantitatively the most important by-products of alcoholic fermentation both for their biotechnological and metabolic importance (da Silva *et al.*, 2009; Wang *et al.*, 2001). During the growth of *S. cerevisiae* on glucose and fructose as the carbon and energy source, the pyridine nucleotides NAD^+ is converted to NADH in several biological anabolic processes (Albers *et al.*, 1996; Bakker *et al.*, 2001; van Dijken & Scheffers, 1986). Regulation of NAD^+ is required as only a catalytic amount is present inside the cell (Bakker *et al.*, 2001). NAD^+ used during glycolysis is regenerated through ethanol formation (Bakker *et al.*, 2001; van Dijken & Scheffers, 1986). However, when the intermediates of glycolysis are withdrawn as the precursors of biomass in addition to formation of acids such as acetate, excess NADH is generated (van Dijken & Scheffers, 1986). Furthermore, NADH is also formed in mitochondria converting 2-ketoglutarate to succinyl-CoA and is thought to be re-oxidized by means of an acetaldehyde-ethanol shuttle (Bakker *et al.*, 2000). This process also adds to NADH production in the cytosol. Formation of glycerol accounts for the re-oxidation of excess NADH under fermentative conditions.

The conversion of dihydroxyacetone phosphate to glycerol-3-phosphate and subsequent dephosphorylation leads to formation of glycerol in *S. cerevisiae* (Gancedo *et al.*, 1968a; Gancedo *et al.*, 1968b). The first reaction is catalyzed by two cytosolic NADH-dependent glycerol-3-phosphate dehydrogenases (Gpd1/2p) (Albertyn *et al.*, 1994; Eriksson *et al.*, 1995). A *gpd1*Δ*gpd2*Δ DM is unable to grow under strict anaerobic conditions because both cytosolic and mitochondrial excess NADH cannot be re-oxidized through glycerol production ultimately leading to intracellular accumulation of NADH (Ansell *et al.*, 1997). Therefore, in principle, this excess NADH can be used to drive other NADH dependent reduction reactions. The introduction of heterologous or overexpression of native proteins which catalyze NADH-dependent reactions could allow the DM to restore its NADH balance and permit anaerobic growth.

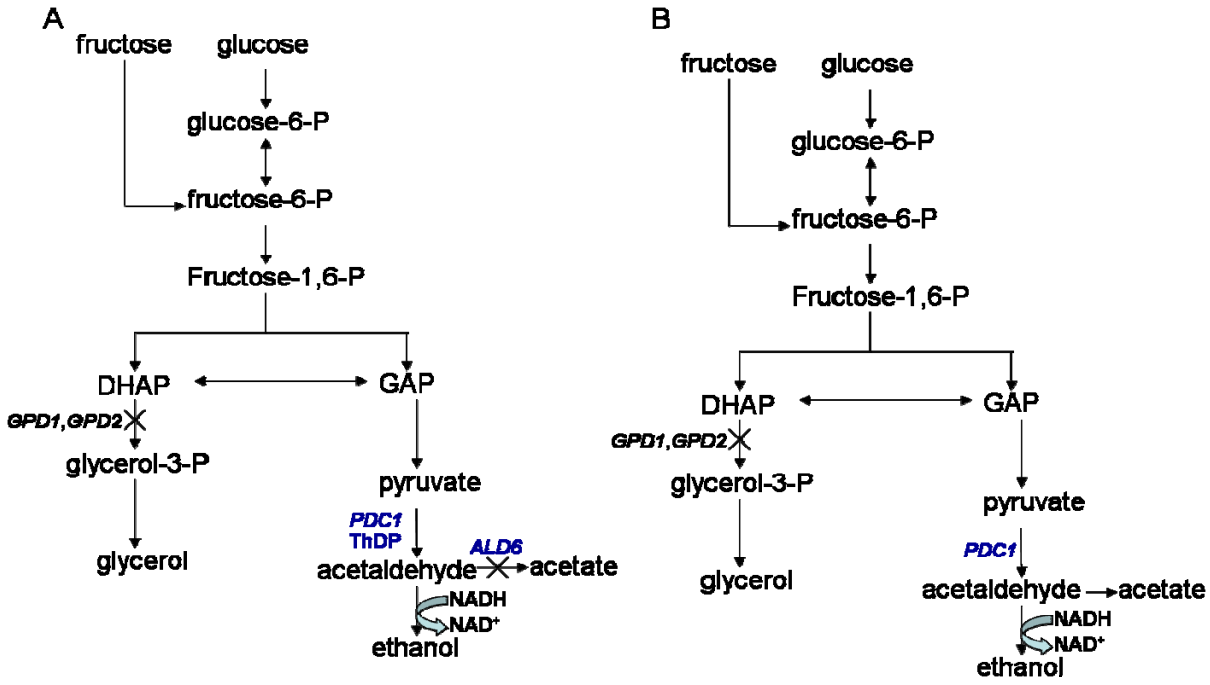

FIG. 5.1 Pathway showing the formation of "extra" ethanol in DM(*ald6*Δ, *PDC1*) strain and regeneration of NAD^+ during the conversion of acetaldehyde to ethanol due to overexpression of *PDC1* and deletion of *ALD6* gene (A) and formation of ethanol in DM (B). Formation of "extra" ethanol in DM(*ald6*Δ, *PDC1*) strain leads to regeneration of extra NAD^+ as compared to DM thereby increasing rate of glycolysis and sugar consumption in DM(*ald6*Δ, *PDC1*) as compared to DM. Thiamine diphosphate (2.5 mg/l) is added in the growth medium of DM(*ald6*Δ, *PDC1*) which acts as coenzyme for Pdc1p and added in the growth medium of DM as a control.

The objective of this study was to investigate the possibility of manipulating a *gpd1*Δ*gpd2*Δ DM of *S. cerevisiae* to re-oxidize excess NADH through ethanol formation by overexpressing the pyruvate decarboxylase (*PDC1*) gene in DM and then deleting the aldehyde dehydrogenase (*ALD6*) gene from its genome (Fig. 5.1). The *PDC1* gene (EC 4.1.1.1) is one of the major of three pyruvate decarboxylase isozymes and a key enzyme in alcoholic fermentation (Kellermann *et al.*, 1986; Pronk *et al.*, 1996; Schmitt & Zimmermann, 1982). It involves in the decarboxylation of pyruvate to acetaldehyde (Pronk *et al.*, 1996). The *ALD6* gene (EC 1.2.1.3) is required for conversion of acetaldehyde to acetate (Saint-Prix *et al.*, 2004; Wang *et al.*, 1998). In the manipulated strain DM(*ald6*Δ, *PDC1*), overexpression of *PDC1* should maximize the conversion of pyruvate to acetaldehyde and deletion of *ALD6* should reduce the flux of acetaldehyde towards acetate thereby forming more ethanol as compared to DM and re-oxidizing

excess NADH through ethanol formation. The effect of pyruvate addition on the fermentative ability of DM was also investigated.

Moreover, conservation of carbon by forming more ethanol and less glycerol could be beneficial for the bioethanol indistry where the primary focus is on increasing the carbon flux towards ethanol and decreasing towards other major byproducts formation such as acetate and glycerol. Deleting *GPD1* and *GPD2* genes under anaerobic condition could be a beneficial tool since usually significant sugar carbon ends up in glycerol and acetate which can otherwise be diverted towards ethanol.

5.2 Materials and methods

5.2.1 Yeast strains and genotypes

The genotypes of the WT and the DM strains are as described previously in Chapter 3. DM(*ald6*Δ) (BY4742; *gpd1*::*KanMx*; *gpd2*::*URA3*; *ald6*:: *LYS2*) was created by deleting *ALD6* gene from the genome of DM. The genotype of the mutant DM(*ald6*Δ, *PDC1*) is DM(*ald6*:: *LYS2*, PGK_P-*PDC1*-PGK_T).

5.2.2 Primers and PCR templates

Set of primers and PCR template used to create DM are described previously in Chapter 3. Primers for amplifying the *LYS2* gene with a 50bp region homologous to the 5' and 3' parts of *ALD6* were designed. The forward primer was 5'-ATGACTAAGCTACACTTTGACACTGCTGAACCAGTCAAGATCACACTTCCCTGCAG GTCGACGGATCCGG-3' and reverse primer was 5'-TTACAACTTAATTCTGACAGCTTTTACTTCAGTGTATGCATGGTAGACTTTACGTAAT GCTCAACCTTAAGCTGCTGC-3' (underlined parts of the primers are homologous to the *ALD6* gene). These primes were used to amplify the *LYS2* gene from the YdpK vector containing the *LYS2* marker (Berben *et al.*, 1991). The PCR product was then integrated into the *gpd1*Δ*gpd2*Δ DM to obtain a DM(*ald6*Δ) triple mutant. The *PDC1* gene was amplified using the *S. cerevisiae* BY4742 genomic DNA. The primers used for the amplification of the *PDC1* gene were: forward 5'-ACTAGTATGTCTGAAATTACTTTGGGTAAATATTTGTTC-3' and reverse 5'-CTCGAGTTATTGCTTAGCGTTGGTAGCAGCA-3'.

5.2.3 DNA manipulations, construction of plasmids and yeast and bacterial transformation

The *PDC1* gene was cloned into pDMPM multicopy shuttle vector (PhD thesis, Malherbe, DF 2010, Stellenbosch University) having an ampicillin resistance marker (amp^r), a leucine auxotrophic marker and a modified multiple cloning site flanked by the constitutive phosphoglycerate-kinase-1 gene promoter (*PGK1P*) and terminator (*PGK1T*) derived from pHVX2 (Volschenk *et al.*, 1997). The pDMPM(*PDC1*) plasmid was then transformed into the DM(*ald6*Δ) triple mutant to yield DM(*ald6*Δ, *PDC1*) strain. The gene was under the control of constitutive *PGK* promoter and terminator (Gellissen & Hollenberg, 1997). Prior to transformation, the gene was sequenced at the Central Sequencing Facility of Stellenbosch University. The sequence of *PDC1* gene was 100% homologous to the sequence obtained from its GenBank accession number. The accession number for *PDC1* gene is P06169.

Subcloning in *E. coli* DH5α, yeast and bacterial transformations and isolation of genomic DNA from *E. coli* and *S. cerevisiae* was done using standard protocols (Gietz & Schiestl, 2007; Harju *et al.*, 2004).

5.2.4 Medium and fermentation conditions

The fermentations were conducted in four-fold replicates 250 ml Erlenmeyer flasks with 100 ml medium. The medium and growth conditions were as described in chapter 3 except for few changes such as the medium was supplemented with thiamine diphosphate (ThDP) (2.5 mg/l) and strain DM(*ald6*Δ, *PDC1*) fermentation was monitored for 24 days since it continued to release CO_2 beyond 20 days. ThDP acts as the coenzyme for Pdc1p hence its addition in the medium of DM(*ald6*Δ, *PDC1*) strain might help in the higher conversion of pyruvate to acetaldehyde.

For the pyruvate addition experiment, 1 ml of 100, 200 and 300 g/l sodium pyruvate was added anaerobically into each of three different 250 ml Erlenmeyer flasks with caps and side arms containing 100 ml medium and inoculated with DM to give final concentration of pyruvate as 1, 2 and 3 g/l respectively. The pyruvate was injected through the cap

using a syringe to restrict oxygen diffusion during the 8th day of incubation of DM. As a control, 1 ml of water was injected into three different flasks inoculated with DM.

5.2.5 Plate assays of strains under aerobic and anaerobic conditions

Plate assays were performed under aerobic and anaerobic conditions. The medium and growth conditions were as described in chapter 3 except that the medium was supplemented with ThDP (2.5 mg/l).

5.2.6 Chemical analyses

Substrates consumed (glucose, fructose) and metabolites formed (ethanol, glycerol, pyruvate, acetate, malate, succinate) were analyzed using a Waters HPLC system as described in chapter 3.

Higher alcohols (isobutanol, isoamyl alcohol, propanol, butanol, 2-phenyl ethanol), esters (ethyl acetate, isoamyl acetate) and minor acids (isobutyric and butyric acid) formed by yeasts were determined with a gas chromatograph equipped with a flame ionization detector and Nukol free fatty acid phase fused-silica capillary column (DB-FFAP) (Hewlett Packard 6890 Plus) as described in chapter 3.

5.3 Results

5.3.1 Growth of strains on agar plates under aerobic and anaerobic conditions

All strains grew equally well under aerobic conditions on 2% glucose agar plates. However, both DM and DM(*ald6*Δ, *PDC1*) strains grew poorly compared to WT on 5% glucose-5% fructose agar plates under aerobic conditions. Anaerobic conditions did not affect the growth of WT strain on both 2% glucose and 5% glucose-5% fructose agar plates as reported in previous chapters. The growth phenotype of DM(*PDC*1) strain was analyzed under anaerobic conditions on 2% glucose agar plates. The hypothesis was to check if overexpression of *PDC1* could restore the growth defect of the DM. Nevertheless, no growth was observed for DM(*PDC1*) strain under these conditions (data not shown). Subsequently, *ALD6* gene was deleted in the DM(*PDC1*) background so that flux towards ethanol can be further improved which might then improve the

growth phenotype of DM(*PDC1*) strain. Contrary to the hypothesis, on both 2% glucose and 5% glucose-5% fructose agar plates under anaerobic conditions, growth of both DM(*ald6*Δ, *PDC1*) and DM was much poorer than their growth on the same plates under aerobic conditions. In fact, no growth by DM and DM(*ald6*Δ, *PDC1*) was visible on 5% glucose-5% fructose agar plates under anaerobic conditions. This shows that overexpression of *PDC1* and even further deletion of *ALD6* genes in DM failed to restore the growth defect on 5% glucose-5% fructose agar plates under aerobic conditions as well as both 2% glucose and 5% glucose-5% fructose agar plates under anaerobic conditions (Fig. 5.2).

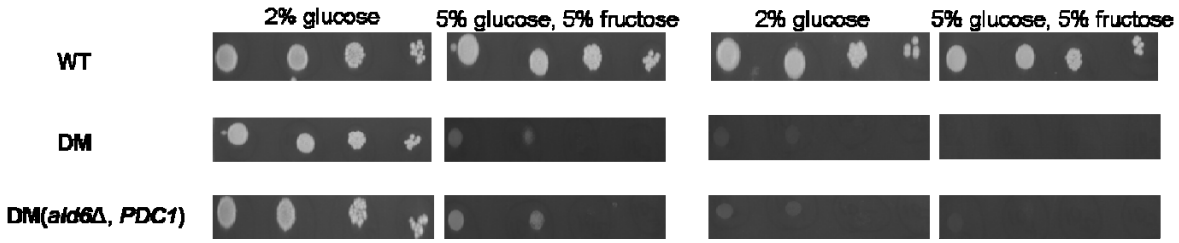

FIG. 5.2 Plate assays for the strains under aerobic and anaerobic conditions cultivated in 2% glucose and 5% glucose-5% fructose, 6.7 g/l YNB and thiamine diphosphate (2.5 mg/l). Ten µl of ten-fold dilution series were spotted for each row, beginning with a culture of an OD_{600nm} of one (corresponding to 2.7 X 10^5 cells / 10 µl).

5.3.2 Shift from respirofermentative to strictly anaerobic growth of the strains in liquid medium

The data are similar to those reported in the previous chapters. WT and DM behaved as reported previously, and DM(*ald6*Δ, *PDC1*) strain took a similar amount of time as DM to reach anaerobic conditions (Fig. 5.3).

5.3.3 Effect of *PDC1* overexpression and *ALD6* deletion on CO_2 release, total sugar consumption and biomass formation

The behaviour of WT and DM strains in terms of CO_2 release, total sugar consumption and biomass formation was similar to what observed in previous chapters (Fig. 5.3). The DM(*ald6*Δ, *PDC1*) strain released CO_2 slowly as compare to DM till 12^{th} day (Fig. 5.3). After 12^{th} day, CO_2 release by DM(*ald6*Δ, *PDC1*) overtook the CO_2 release by DM. However, the gas release was neither as rapid nor to the same amount as the WT.

Overexpression of just *PDC1* in DM failed to improve the sugar consumption phenotype of DM (data not shown). However, deletion of *ALD6* in the DM(*PDC1*) strain led to significant improvement in the sugar utilization albeit less than WT with DM(*ald6*Δ, *PDC1*) consuming 65% of the total sugar present in the medium (Table 5.1) as compared to DM which consumed 47% of the total sugar present in the medium. This shows that overexpression of *PDC1* and deletion of *ALD6* genes in DM certainly improved the growth in liquid medium unlike to growth on the solid medium. Biomass concentration for DM and DM(*ald6*Δ, *PDC1*) was similar, however, growth continued to increase until the 12th day in DM(*ald6*Δ, *PDC1*) whereas in DM growth ceased at ~2.5 days at which point strictly anaerobic conditions apparently commenced (Fig. 5.4). The molar yield of biomass was the highest for WT followed by DM and DM(*ald6*Δ, *PDC1*) strains (Table 5.2).

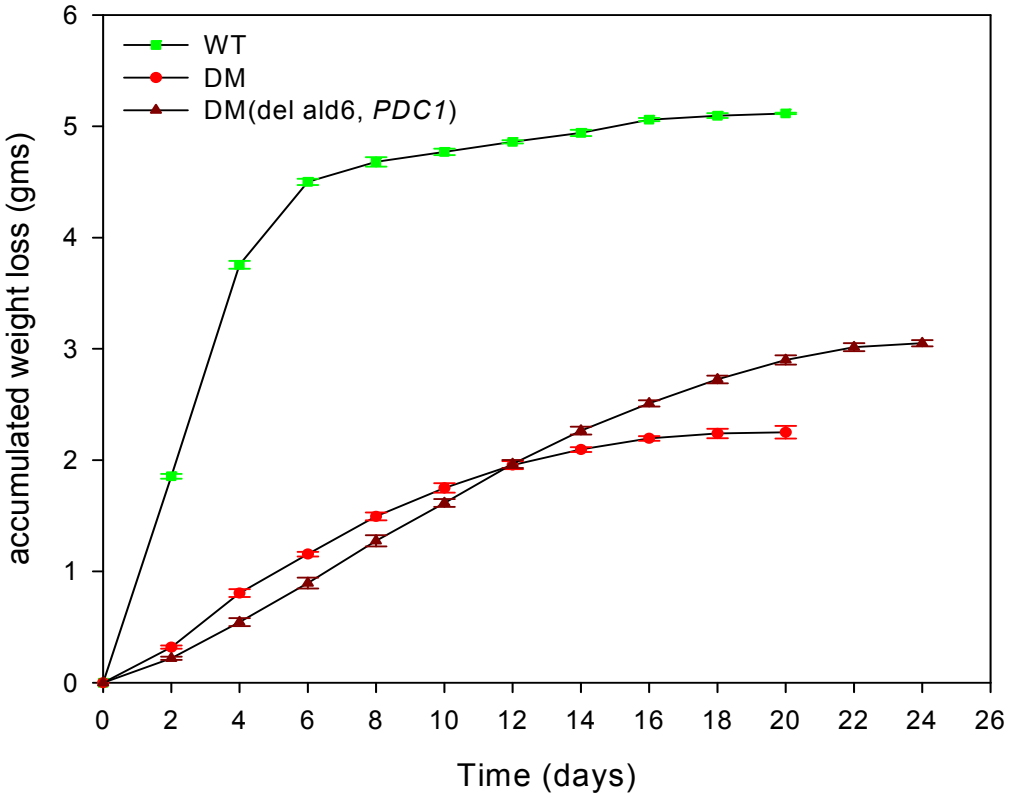

FIG. 5.3 Release of CO_2 by *S. cerevisiae* WT, DM and DM(*ald6*Δ, *PDC1*) when cultivated in 5% glucose-5% fructose, 6.7 g/l YNB and thiamine diphosphate (2.5 mg/l) carried out in four replicates. Error bars indicated are the standard deviation of the four replicates for each strain. Weight loss is assumed to only originate from CO_2.

Table 5.1 Effect of the overexpression of *PDC1* and deletion of *ALD6* genes in *S. cerevisiae gpd1*Δ*gpd2*Δ strain on sugar consumption[a] cultivated in 5% glucose-5% fructose, 6.7 g/l YNB and 2.5 mg/l thiamine diphosphate over maximum 24 days[b].

Strains	Glucose used (g/l)	Fructose used (g/l)	Total sugar used (g/l)
WT	50 ± 0	49.89 ± 0.04	99.89 ± 0.04
DM	30.38 ± 1.58	16.8 ± 1.46	47.17 ± 1.59
DM(*ald6*Δ, *PDC1*)	43.62 ± 0.67	21.98 ± 0.59	65.59 ± 0.99

[a]Values represent the average and standard deviation of four independent cultivations.
[b]DM(*ald6*Δ, *PDC1*) growth was monitored for 24 days whereas growth for WT and DM was monitored for 20 days.

TABLE 5.2 Effect of the overexpression of *PDC1* and deletion of *ALD6* genes in *S. cerevisiae gpd1*Δ*gpd2*Δ strain on product yield (moles/mole sugar utilized)[a] cultivated in 5% glucose-5% fructose, 6.7 g/l YNB and 2.5 mg/l thiamine diphosphate during an oxygen-limited fermentation. The values in brackets represent the metabolite or dry weight (for the biomass) concentrations (g/l).

	Strains		
Metabolite	**WT**	**DM**	**DM(*ald6*Δ, *PDC1*)**
CO_2	1.76 (43.00)	1.89 (21.79)	1.92 (30.58)
ethanol	1.76 (45.17)	1.89 (22.88)	1.92 (32.01)
biomass[b]	0.07 (0.98)	0.065 (0.42)	0.044 (0.39)
glycerol	0.080 (4.08)	0	0
acetate	0.021 (0.67)	0.83[c] (0.013)	0.18[c] (0.004)
succinate	0.011 (0.73)	0.009 (0.27)	0.006 (0.261)
malate	0.005 (0.42)	0.015 (0.50)	0.007 (0.326)
pyruvate	0.004 (0.161)	0.008 (0.182)	0.006 (0.196)

[a]Yield was calculated from the final day sample (20th for WT and DM and 24th for DM(*ald6*Δ, *PDC1*) of four independent cultivations
[b]Molecular formula of *Saccharomyces cerevisiae* is taken as $\mathbf{CH_{1.8}O_{0.5}N_{0.2}}$
[c]mmoles/mole sugar utilized
Coefficient of variation (CV) for concentration and yield of metabolites was less than 11% for all the strains.

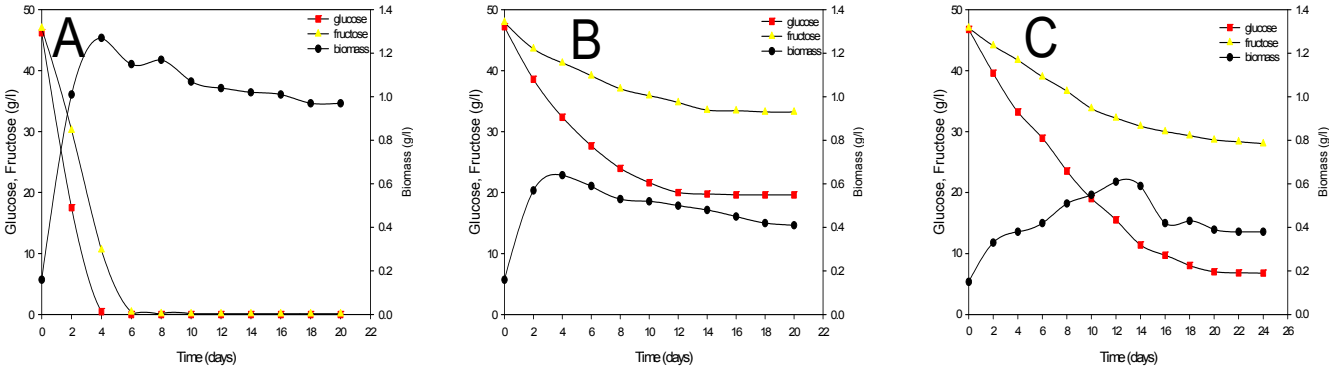

FIG. 5.4 Glucose, fructose consumption and growth (biomass: dry weight) by strains of *S. cerevisiae* WT (A), DM (B) and DM(*ald6*Δ, *PDC1*) (C) when cultivated in 5% glucose-5% fructose, 6.7 g/l YNB and thiamine diphosphate (2.5 mg/l) in a representative experiment.

5.3.4 Ethanol and glycerol production

Deletion of the *GPD1* and *GPD2* genes resulted in no glycerol formation in DM(*ald6*Δ, *PDC1*) strain as also observed in DM strain. Glycerol production in WT strain followed similar pattern as observed in previous chapters (Fig. 5.5). WT produced the highest concentration of ethanol and DM the least. However, the highest molar ethanol yield was observed in DM(*ald6*Δ, *PDC1*) whereas WT had the lowest ethanol yield (Table 5.2). This indicates the carbon conservation through glycerol formation in WT and through ethanol formation in DM(*ald6*Δ, *PDC1*) strain. Furthermore, the higher molar yield of ethanol in DM(*ald6*Δ, *PDC1*) as compared to DM indicates that overexpression of *PDC1* and deletion of *ALD6* genes in DM directed more sugar towards ethanol formation in DM(*ald6*Δ, *PDC1*) as compared to DM (Table 5.2). Approximately 100% of the hydrogen moles from the sugars were accounted for in the products for all the strains (Table 5.3).

TABLE 5.3 Moles of available hydrogen present[a] in each metabolite formed by strains cultivated 5% glucose-5% fructose, 6.7 g/l YNB and 2.5 mg/l thiamine diphosphate under oxygen limited fermentation. The available hydrogen in one mole each of glucose and fructose is 24 thereby giving a total available 48 moles hydrogen.

	Strains		
Metabolite	**WT**	**DM**	**DM(*ald6*Δ, *PDC1*)**
CO_2	0	0	0
ethanol	42.24	45.36	46.08
biomass[b]	0.672	0.624	0.42
glycerol	2.24	0	0
acetate	0.336	13.28[c]	2.88[c]
succinate	0.308	0.252	0.168
malate	0.12	0.36	0.17
pyruvate	0.08	0.16	0.12
Total	45.99	46.76	46.96
% Hydrogen recovered	**95.81**	**97.42**	**97.83**

[a]moles of available hydrogen present in each metabolite is calculated from the final day (20th for WT and DM and 24th for DM(*ald6*Δ, *PDC1*) molar yield of each metabolite.
[b]Molecular formula of *S. cerevisiae* is taken as $\mathbf{CH_{1.8}O_{0.5}N_{0.2}}$
[c]mmoles of H formed/48 moles of H consumed from glucose and fructose
Coefficient of variation for available hydrogen moles was less than 10% for all the strains.

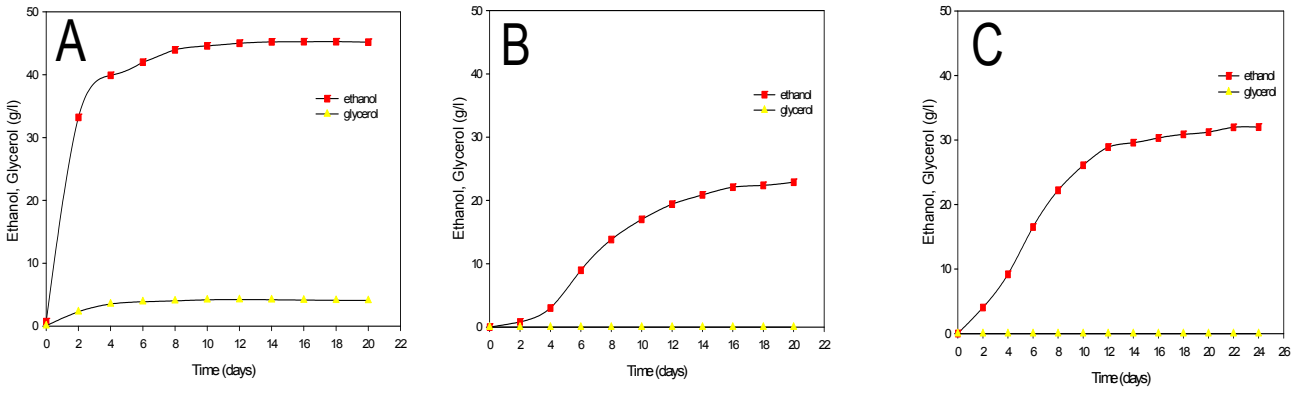

FIG. 5.5 Ethanol and glycerol by strains of *S. cerevisiae* WT (A), DM (B) and DM(*ald6*Δ, *PDC1*) (C) when cultivated in 5% glucose-5% fructose, 6.7 g/l yeast nitrogen base and thiamine diphosphate (2.5 mg/l) in a representative experiment.

5.3.5 Cytosolic and mitochondrial acid production

Acetate production was the highest in WT and the least by DM(*ald6*Δ, *PDC1*). The highest concentration of malate was produced by DM (Fig. 5.6) followed by WT and DM(*ald6*Δ, *PDC1*). WT formed the highest concentration of succinate whereas DM and DM(*ald6*Δ, *PDC1*) formed similar amount of this metabolite. Pyruvate concentration did not vary much among the strains (Fig. 5.6). The molar yields of the metabolites are shown in Table 5.2.

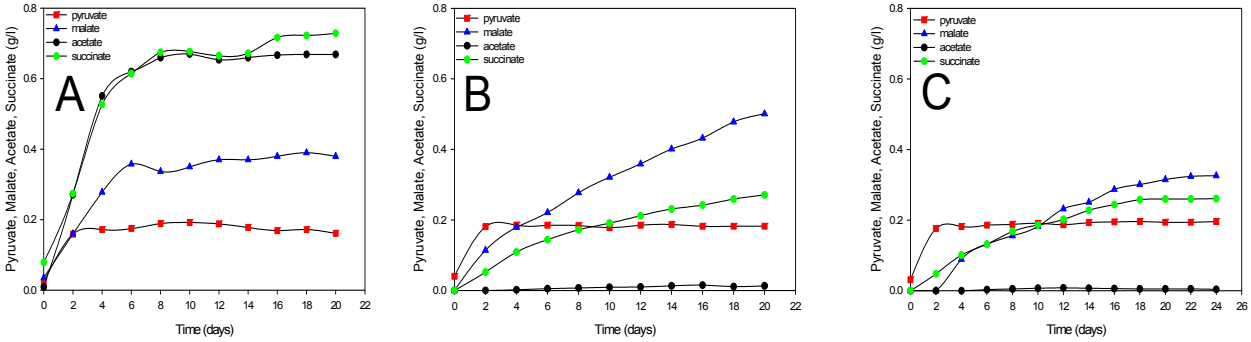

FIG. 5.6 Pyruvate, malate, acetate and succinate production by strains of *S. cerevisiae* WT (A), DM (B) and DM(*ald6*Δ, *PDC1*) (C) when cultivated in 5% glucose-5% fructose, 6.7 g/l YNB and thiamine diphosphate (2.5 mg/l) in a representative experiment.

5.3.6 Higher alcohols, minor acids and esters production

The highest concentration of isobutanol was formed by DM followed by DM(*ald6*Δ, *PDC1*) and WT strains (Fig. 5.7). Similar concentrations of isoamyl alcohol and 2-phenyl ethanol were formed in all the strains. None of the strains formed propanol or butanol (Fig. 5.7).

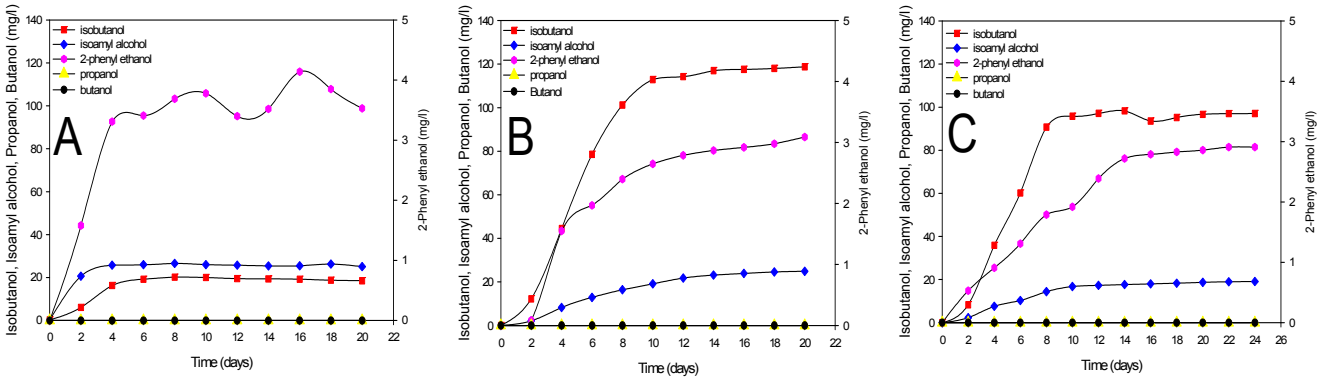

FIG. 5.7 Higher alcohol production by strains of *S. cerevisiae* WT (A), DM (B) and DM(*ald6*Δ, *PDC1*) (C) when cultivated in 5% glucose-5% fructose, 6.7 g/l YNB and thiamine diphosphate (2.5 mg/l) in a representative experiment.

None of the strains produced isoamyl acetate (Fig. 5.8). The highest concentration of ethyl acetate was formed by WT followed by DM and DM(*ald6*Δ, *PDC1*) strains (Fig. 5.8). This shows that overexpression of *PDC1* and deletion of *ALD6* genes in DM led to further decrease in ethyl acetate concentration. All strains failed to produce butyric acid whereas DM formed the highest concentration of isobutyric acid followed by DM(*ald6*Δ, *PDC1*) and WT (Fig. 5.8). The concentration and molar yields of higher alcohols, minor acids and esters are shown in Table 5.4.

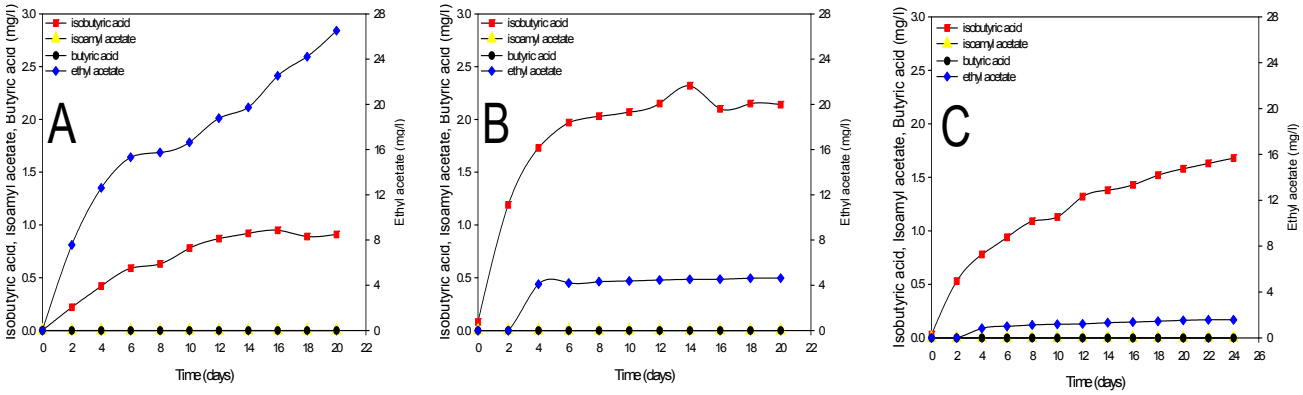

FIG. 5.8 Esters and minor acids production by strains of *S. cerevisiae* WT (A), DM (B) and DM(*ald6*Δ, *PDC1*) (C) when cultivated in 5% glucose-5% fructose, 6.7 g/l YNB and thiamine diphosphate (2.5 mg/l) in a representative experiment.

Table 5.4 Effect of the overexpression of *PDC1* and deletion of *ALD6* genes in *S. cerevisiae gpd1*Δ*gpd2*Δ strain cultivated in 5% glucose-5% fructose, 6.7 g/l YNB and 2.5 mg/l thiamine diphosphate on the concentration (mg/l) and the yield (mg/g sugar utilized; values in brackets) of higher alcohols, esters and minor acids during oxygen limited fermentation over a maximum of 24 days[a].

	Strains		
Metabolite (mg/l)	**WT**	**DM**	**DM(*ald6*Δ, *PDC1*)**
Isobutanol	18.53 (0.18)	118.75 (2.52)	97.07 (1.49)
Isoamyl alcohol	25.09 (0.25)	24.89 (0.53)	19.09 (0.29)
Propanol	0	0	0
Butanol	0	0	0
2-phenyl ethanol	3.53 (0.04)	3.09 (0.066)	2.91 (0.045)
Ethyl acetate	26.51 (0.26)	4.63 (0.098)	1.58 (0.024)
Isoamyl acetate	0	0	0
Butyric acid	0	0	0
Isobutyric acid	0.91 (0.009)	2.14 (0.045)	1.68 (0.023)

Coefficient of variation for concentration and yield of metabolites was less than 12% for all the strains.

[a]DM(*ald6*Δ, *PDC1*) growth was monitored for 24 days whereas growth for WT and DM was monitored for 20 days.

5.3.7 Effect of pyruvate addition during the growth of DM on sugar consumption, biomass formation, CO_2 release and ethanol formation

Addition of pyruvate on the 8th day of growth of DM increased CO_2 release (Fig. 5.9) and sugar consumption was increased with increasing pyruvate concentration of 1, 2 and 3 g/l by 23%, 29% and 35% respectively as compared to DM without any pyruvate addition (Table 5.6). Morever, pyruvate addition had very little impact on the biomass formation as compared to DM (Table 5.6). Furthermore, ethanol formation increased by 25%, 30% and 36% when pyruvate was added to the final concentration of 1 g/l, 2 g/l and 3 g/l respectively as compared to DM (Table 5.6).

Table 5.5 Effect of the addition of pyruvate after the 8th day of growth of *S. cerevisiae gpd1*Δ*gpd2*Δ strain on sugar consumption[a] and ethanol formation when cultivated in 5% glucose-5% fructose and 6.7 g/l YNB.

Strains	Glucose used (g/l)	Fructose used (g/l)	Total sugar used (g/l)	Ethanol formed (g/l)	Biomass formed (g/l)
DM(1 ml water)	31.8 ± 0.91	15.7 ± 0.85	47.5 ± 1.72	23.1 ± 1.45	0.41 ± 0.02
DM(1 g/l pyruvate)	37.78 ± 0.21	20.8 ± 0.66	58.6 ± 0.79	28.8 ± 2.20	0.45 ± 0.002
DM(2 g/l pyruvate)	39.2 ± 0.70	21.98 ± 1.72	61.2 ± 1.99	30.02 ± 2.22	0.47 ± 0.01
DM(3 g/l pyruvate)	40.9 ± 1.77	23.1 ± 0.98	63.98 ± 2.75	31.54 ± 0.49	0.47 ± 0.01

[a]Values represent the average and standard deviation of three independent cultivations.

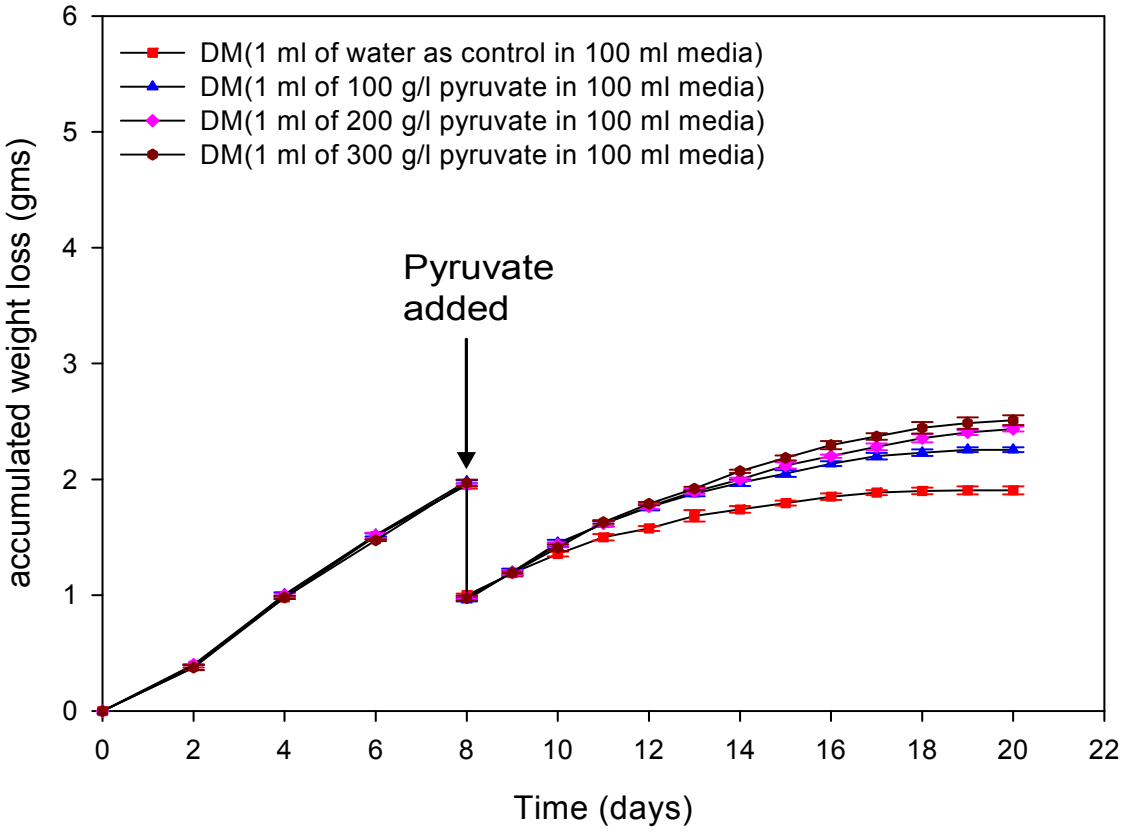

FIG. 5.9 Release of CO_2 by *S. cerevisiae gpd1*Δ*gpd2*Δ DM (DM 1 ml water), DM (1 g/l pyruvate), DM (2 g/l pyruvate) and DM (3 g/l pyruvate) when cultivated in 5% glucose-5% fructose and 6.7 g/l YNB carried out in triplicates. Error bars indicated are the standard deviation of the triplicates for each strain. Weight loss is assumed to only originate from CO_2.

5.4 Discussion

It was shown in this study that the overexpression of *PDC1* and deletion of *ALD*6 genes in DM did not appear to stimulate growth significantly on solid medium. However, a clear improvement was seen in liquid medium. This could be related to the strict anaerobic conditions established for growth on solid medium whereas in the liquid medium, conditions were semi-anaerobic. The genetic manipulations improved growth, sugar utilization and ethanol production compared to DM although these parameters were lower than those found in a fermentation by WT. Addition of pyruvate during the growth of DM also increased sugar consumption as well as ethanol formation. Moreover, these genetic manipulations had a marked impact on the concentration of metabolites formed.

5.4.1 Overexpression and deletion of native genes of *S. cerevisiae* in *gpd1*Δ*gpd2*Δ DM alter growth

The results presented here suggest that the overexpression of *PDC1* along with the deletion of *ALD6* genes (native genes of *S. cerevisiae*) in DM lead to higher regeneration of NAD^+ thereby improving growth and metabolic activity of DM in liquid medium. To my knowledge, this kind of genetic manipulation which leads to overproduction of ethanol by *S. cerevisiae* and improves the growth of DM has not been reported before. However, it has been shown that production of sorbitol (third chapter of this thesis) and propane,1-2-diol (fourth chapter of this thesis) by overexpressing native or heterologous genes in DM can partially complement the double mutation and improve growth of DM. Several other studies have been carried out earlier where heterologous genes were overexpressed in DM. However, none of the alternative NADH oxidizing systems reported could completely complement the double deletion of glycerol synthesizing genes and none are as efficient as the glycerol synthesis pathway in maintaining the redox balance in *S. cerevisiae* under strictly anaerobic conditions (Costenoble *et al.*, 2003; Liden *et al.*, 1996).

The growth of DM and WT on high and low sugar plates both aerobically and anaerobically was same as observed in earlier chapters. Like DM, DM(*ald6*Δ, *PDC1*) also struggled to grow on 5% glucose-5% fructose agar medium under aerobic conditions as well as on 2% glucose and 5% glucose-5% fructose agar medium under anaerobic conditions. This observation was surprising because the same genetic manipulation in DM improved the growth and fermentative ability in liquid medium. One possible explanation for this phenotype of DM(*ald6*Δ, *PDC1*) strain on solid medium could be because of the higher ethanol molar yield and less biomass molar yield by this strain (Table 5.2). Higher ethanol yield could be because of the higher regeneration of NAD^+ through this metabolite formation due to overexpression and deletion of *PDC1* and *ALD6* genes in DM respectively. Furthermore, lower biomass yield by this strain as compared to DM shows that less NAD^+ might be used for biomass formation and available NAD^+ is used to promote glycolysis and regenerated through ethanol formation. Overexpression of *PDC1* gene might increase the flux of pyruvate towards acetaldehyde. Furthermore, deletion of *ALD6* gene stops the acetaldehyde being channeled towards acetate and most of the acetaldehyde formed might go for the ethanol formation (Fig. 5.5).

TABLE 5.6 NADH formed and NAD^+ regenerated for WT, DM and DM(*ald6*Δ, *PDC1*) strains through metabolite formation. NAD^+ regenerated in WT through ethanol formation is taken as zero by assuming that its formation is the result of a redox neutral process. In other strains, NAD^+ regenerated through ethanol formation are "extra" moles of ethanol formed as compared to moles of ethanol formed by WT strain.

	Strains					
	WT		**DM**		**DM(*ald6*Δ, *PDC1*)**	
Metabolite	NADH formed	NAD^+ regenerated	NADH formed	NAD^+ regenerated	NADH formed	NAD^+ regenerated
Biomass	0.07	0	0.065	0	0.044	0
Acetate	0.021	0	0.001	0	0.0002	0
Glycerol	0	0.08	0	0	0	0
Succinate	0	0.011	0	0.009	0	0.006
Malate	0	0.005	0	0.015	0	0.007
Total*	**0.091**	**0.096**	**0.066**	**0.024**	**0.044**	**0.013**
Ethanol	0	1.76-1.76 = **0**	0	1.89-1.76=**0.13**	0	1.92-1.76=**0.16**
Total	**0.091**	**0.096**	**0.066**	**0.154**	**0.044**	**0.173**

*Without taking into account the NAD^+ regenerated through ethanol production

Coefficient of variation for NADH formed and NAD^+ regenerated was less than 12% and 10% respectively for all the strains.

5.4.2 Effect of genetic manipulation on sugar consumption, biomass formation and metabolite production

Overexpression and deletion of DM with *PDC1* and *ALD6* genes respectively improved sugar consumption. As the molar yield of biomass is lower for DM(*ald6*Δ, *PDC1*) as compared to DM means that less of the glycolytic intermediates were used for the biomass formation and therefore available more for ethanol formation. This could be due to overexpression and deletion of *PDC1* and *ALD6* genes respectively which might force more pyruvate flux towards ethanol and less towards biomass formation. Indeed, around 96% of the available hydrogen present in sugar ended up in ethanol in DM(*ald6*Δ, *PDC1*) strain as compared to 94% of the available hydrogen present in the sugar which ended up in ethanol formation in DM strain (Table 5.3).

Deletion of the *ALD6* gene (Cambon *et al.*, 2006) which is major aldehyde dehydrogenase catalyzing the conversion of acetaldehyde to acetate led to a further

decrease in the acetate formation as compared to DM. Moreover, similar succinate concentration and yield (g/g biomass formed) (Table 6.3 and 6.4) by DM and DM(*ald6*Δ, *PDC1*) strains should lead to similar biomass formation which the data also confirmed. Decreased availability of pyruvate for formation of products other than ethanol in DM(*ald6*Δ, *PDC1*) strain due to genetic manipulation also lead to reduced concentration of malate as compared to DM.

The higher alcohol, isobutanol was significantly affected as the highest concentration was produced by DM followed by DM(*ald6*Δ, *PDC1*) and WT strain. In DM this could be related to regeneration of extra NAD^+ through isobutanol in the absence of NAD^+ regeneration through glycerol formation. However generation of more NAD^+ through ethanol formation might result in lower formation of isobutanol in strain DM(*ald6*Δ, *PDC1*) as compared to DM. Moreover, lower availability of pyruvate for products other than ethanol formation might again play role in the lower formation of higher alcohols in DM(*ald6*Δ, *PDC1*) strain as compared to DM. Decrease in the ethyl acetate concentration in DM(*ald6*Δ, *PDC1*) strain was not surprising and could be due to lower availability of acetate required for ester formation.

5.4.3 Addition of “extra” pyruvate during the growth increases CO_2 release, sugar consumption and ethanol production by DM

According to the results presented earlier in this chapter, it was found that by channeling more pyruvate towards ethanol formation, the fermentative ability of the DM can be improved as the strain DM(*ald6*Δ, *PDC1*) consumed more sugar as well as released more CO_2 as compared to DM. To check if the same improvement in DM can be carried out without overexpressing *PDC1* and deleting *ALD6* but just by adding pyruvate, most of which might end up in ethanol thereby reoxidizing NADH, the data show that by adding pyruvate in different concentrations in DM improved CO_2 release (Fig. 5.9) as well as sugar consumption by DM (Table 5.5). Increase in sugar consumption because of the addition of pyruvate is likely due to the higher ethanol formation which regenerates NAD^+. However, the only very slight increase in the biomass concentration following the addition of pyruvate might be because of the higher production of ethanol as compared to DM. This shows that most of the pyruvate added ended up in ethanol rather than biomass. To our knowledge, injection of a glycolytic intermediate (pyruvate) has never been carried out previously to increase the rate of

glycolysis. However, research has been carried out in previous studies where acetoin or acetaldehyde has been added as an electron acceptor thereby improving the re-oxidation of NADH and improving growth of *S. cerevisiae* (Roustan & Sablayrolles, 2002). Addition of acetoin or acetaldehyde regenerated NAD^+ through the formation of 2,3-butanediol (Gonzalez *et al.*, 2000; Gonzalez *et al.*, 2001). Our result suggest that pyruvate addition might also help in NAD^+ regeneration through ethanol formation.

5.5 Potential implications for the ethanol production industry

This study has shown that the genetic manipulations of the pathways regulating the redox balance can affect the growth and the overall metabolic flux of *S. cerevisiae*. Increased formation of ethanol in wine might not be useful due to its bad effects on the drinker. However, the genetic manipulations might be used to produce more ethanol in the chemical industry. Ethanol-producing industries try to maximize the ethanol yield by genetically manipulating strains that produce less glycerol and more ethanol. Our result shows that ethanol yield can be significantly increased from the WT strain by genetic manipulations. However, this has an impact on the rate of ethanol formation and sugar utilization. Microarray analysis of such strains could be carried out in future which might pinpoint the genetic modifications that may lead to improved strains.

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Chapter 6

RESEARCH RESULTS IV

Relationships between sugar consumption, growth and metabolite production by wild type *S. cerevisiae* and genetically manipulated strains

ABSTRACT

Saccharomyces cerevisiae undergoes alcoholic fermentation in high sugar medium and forms ethanol and carbon dioxide as the major metabolites via glycolysis. *S. cerevisiae* also forms other metabolites such as glycerol, organic acids, higher alcohols, esters and fatty acids, many of which are aroma compounds. The aim of this study was to compare the impact of the manipulation of redox balancing pathways or introduction of new pathways to restore the redox balance on growth, sugar consumption and the production of organic acids and secondary aroma compounds. The data show that modification of the redox balancing ability of a strain significantly impacts on the production of many metabolites. In particular, significant differences were observed in the concentration of acids, higher alcohols, esters and fatty acids. Principal component analysis (PCA) of the concentrations and yields of biomass and primary and secondary metabolites and sugar consumed revealed that the cluster groupings formed by strains where not only dependent upon the NAD^+ equivalents generated during fermentation but also other unknown biological properties. The data provide an initial assessment of the impact of redox balance on the production of many aromatic compounds of significant biotechnological interest. Indeed, the production of these secondary metabolites plays an essential role in the character of wine as they impart flavour and aroma in wine.

6.1 Introduction

Aroma in wine originates either from wine grapes or from yeasts which ferment the grape juice and synthesize aroma compounds (Herrero *et al.*, 2008; Lambrechts *et al*., 2000). During fermentation, *S. cerevisiae* produces a broad range of aroma-active substances, which greatly affect the complex flavour of fermented alcoholic beverages such as wine and beer (Polaskova *et al.*, 2008). Although these secondary metabolites are often formed only in trace amounts, their concentrations determine the distinct aroma of these beverages because their perception threshold is very low. The main fermentation-derived secondary metabolites which contribute to the wine aroma are aldehydes, higher alcohols, esters and fatty acids (Lilly *et al.*, 2006a; Vilanova & Sieiro, 2006). The individual secondary metabolites and their impact on perception are listed in Table 6.1.

Table 6.1 Secondary metabolites and the aroma imparted by them in wine (Lambrechts *et al*., 2000)

Compounds	Aroma
Isobutanol	Alcohol, nailpolish
Isoamyl alcohol	Banana
Amyl alcohol	Banana
2-phenyl ethanol	Rose, honey
propanol	Alcohol, ripe fruit
butanol	Medicinal, wine like
Isoamyl acetate	Solvent, marzipan, malt
Ethyl acetate	Apple, pineapple, fruity, solvent, balsamic
Isobutyric acid	Rancid butter, cheese
Butyric acid	Rancid cheese, sweet

The biochemical pathways leading to the production of these compounds in yeast have been reasonably well mapped and documented (Hazelwood *et al.*, 2008). Such pathways include the Ehrlich pathway describing the conversion of amino acids to aldehydes and higher alcohols and the production of esters through the combination of activated acyls with such alcohols (Hazelwood *et al*., 2008; Cordente *et al*., 2007). These pathways form a complex network of shared intermediates and reversible reactions (Rossouw *et al*. 2008) (Fig. 2.11, 2.12 and 2.13). However, while well mapped, many questions regarding the physiological role and the genetic and metabolic regulation of this network remain to be addressed.

Several reactions within this network use NAD^+ and NADH as co-factors. The redox balance is therefore expected to significantly impact on the yields of many volatile compounds. *Vice versa*, the production of higher alcohols, aldehydes and associated acids has been suggested to directly contribute to the maintenance of redox balance during fermentation. However, little direct evidence for such a role has been provided, and the impact of redox balance on the production of individual volatile aroma compounds remains unknown.

Within this network, specific amino acid precursors can be transformed into a number of intermediates and final aroma compounds. For example, propanol can be formed from the degradation of threonine (Fig. 2.11). Threonine is dehydrated to α-ketobutyrate catalyzed by threonine dehydratase *CHA1* and then decarboxylated to propanal using unspecific decarboxylases. Propanal is ultimately reduced to propanol using unspecific reductases. Esters are formed intracellularly in an enzyme-catalyzed reaction between a higher alcohol and an activated acyl-CoA molecule (Fig. 2.13) (Cordente *et al.*, 2007). The most characterized enzymes involved in ester synthesis are the alcohol acetyltransferases namely *ATF1* and *ATF2* (Lilly *et al.*, 2000; Verstrepen *et al.*, 2003). These enzymes catalyze the production of acetate esters from an alcohol and acetyl-CoA. Fatty acids are usually formed by the oxidation of corresponding aldehydes using NAD^+ as the cofactor (Fig. 2.11). The major higher alcohols, esters and fatty acids and the aroma they impart to wine are listed in Table 6.1.

The objective of this chapter is to compare modifications on the metabolism and growth of yeast as well as on the production of aroma compounds by all strains described previously (Chapters 3-5). In particular, it is of interest to investigate whether the variable redox balancing capability of the strains can be linked to the modification in metabolic fluxes. For this purpose, linkage patterns in the production of the compounds were analyzed by principal component analysis (PCA). We measured most of the aroma compounds which might influence the regeneration or consumption of NAD^+ except amyl alcohol since this compound was not calibrated on the gas chromatograph instrument with a flame ionization detector used in this study. The data showed a marked increase in isobutanol concentration in most of the genetically manipulated strains. However, the production of other higher alcohols did not increased in some strains revealing that NAD^+ might not be the only driving factor on their production. A

significant correlation was observed between yields of acetate and ethyl acetate and isobutanol and isobutyric acid as expected.

6.2 Materials and methods

6.2.1 Yeast strains and plasmids

All the yeast strains with their genotypes are listed in Table 6.2.

TABLE 6.2 Yeast strains used in this study

Strains	Genotype	Reference
S. cerevisiae, BY4742	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	Brachmann *et al.*,1998
DM	BY4742; *gpd1*::*KanMx*; *gpd2*::*URA3*	This study
DM(*srlD*)	DM[PGK_P-*srlD*-PGK_T]	This study
DM(*SOR1*)	DM[PGK_P-*SOR1*-PGK_T]	This study
DM(*gldA*, *GRE3*, *mgsA*)	DM[PGK_P-*gldA*-PGK_T, PGK_P-*GRE3*-PGK_T, PGK_P-*mgsA*-PGK_T]	This study
DM(*ald6*Δ, *PDC1*)	DM(*ald6*:: *LYS2*, PGK_P-*PDC1*-PGK_T)	This study

6.2.2 Medium and fermentation conditions

Four replicates of each fermentation was conducted for all strains. The medium and growth conditions were as described in Chapters 3, 4 and 5.

6.2.3 Analytical methods

Glucose, fructose, ethanol, sorbitol, glycerol, pyruvate, acetate, succinate and malate concentrations were measured by HPLC as described in Chapter 3. A GC-FID as described in Chapter 3 was set up to detect and measure the concentrations of the following compounds: isobutanol, isoamyl alcohol, propanol, butanol, 2-phenyl ethanol, ethyl acetate, isobutyric acid, methanol, hexanol, ethyl butyrate, ethyl hexanoate, hexyl acetate, acetoin, ethyl lactate, ethyl caprylate, propionic acid, ethyl caprate, iso-valeric acid, diethyl succinate, valeric acid, 2-phenylethyl acetate, isoamyl acetate, hexanoic

acid, octanoic acid, decanoic acid and butyric acid. Biomass concentration was determined as described in Chapter 3.

6.2.4 Standardized biplot

Standardized biplot based on PCA (Syms 2008) was used to graphically display relationships between variables (metabolites concentrations, metabolites yields on the basis of gram per gram biomass formed and metabolites yields on the basis of gram per gram sugar consumed) as well as clustering of samples from different strains.

In this bi-plot, variables are plotted on two perpendicular imaginary axes (principle components t1 and t2). On this two-dimensional graph, each variable is represented as an axis in a specific direction determined by the bi-plot technique. A perpendicular line drawn from the clustering of the samples on the axis assigned to a particular variable gives the rough estimate of the value of that variable. The goodness of the estimate depends upon the R-squared value for that variable in that particular biplot, *i.e*, closer the R-squared value to 1, the better the estimate. Univariate correlations between variables were calculated using Spearman correlations (Syms 2008). Spearman was selected due to possible outliers present in the data. Each variable is correlated to another variable in terms of their Spearman value (r) and Sperman p-value (p). A significant positive correlation between two variables is likely if the “r” is in the range of $0.70 \leq r \leq 1$ and $p<0.05$ and a significant negative correlation is likely if the “r” is in the range of $-1 \leq r \leq -0.70$ and $p<0.05$. This implies that it does not matter how close or far the variables are on the bi-plot, the correlation between them is determined by the “r” and p-value.

6.3 Results

6.3.1 Comparison of sugar utilisation and biomass and ethanol production

General fermentation parameters such as biomass, ethanol, glycerol, sorbitol and propane-1,2-diol were measured in response to sugar consumption by different strains (Fig. 6.1). WT rapidly consumed the highest amount of sugar of all the strains. Among the genetically manipulated strains, DM consumed the least whereas DM(*SOR1*) strain showed the highest sugar consumption and the other strains showed similar sugar utilization (Fig. 6.1A). WT produced the highest amount of ethanol and DM the least. Among strains created to improve NAD^+ regeneration, DM(*SOR1*) showed the highest capability to form ethanol (Fig. 6.1B). Biomass production was the highest in the WT strain. Compared to DM, genetic manipulation improved biomass production but not to the extent of the WT strain (Fig. 6.1C). In all the strains, biomass concentration decreased or remained the same after the 4^{th} day except for DM(*ald6*Δ, *PDC1*) strain where it continued to increase till the 12^{th} day (Fig. 6.1C). Absorbance measurement (A_{600}) confirmed the patterns of growth determined by dry mass measurement (data not shown). No metabolite replaced glycerol in significant concentrations in cultures of DM and DM(*ald6*Δ, *PDC1*) strains. The DM(*srlD*) and DM(*gldA*, *GRE3*, *mgsA*) strains expressing heterologous enzymes respectively produced similar molar concentrations of sorbitol and propane-1,2-diol whereas DM(*SOR1*) produced a lower concentration of sorbitol. No strain produced a metabolite concentration similar to the glycerol concentration produced by WT indicating the NAD^+ capacity of these strains were lower. (Fig. 6.1D).

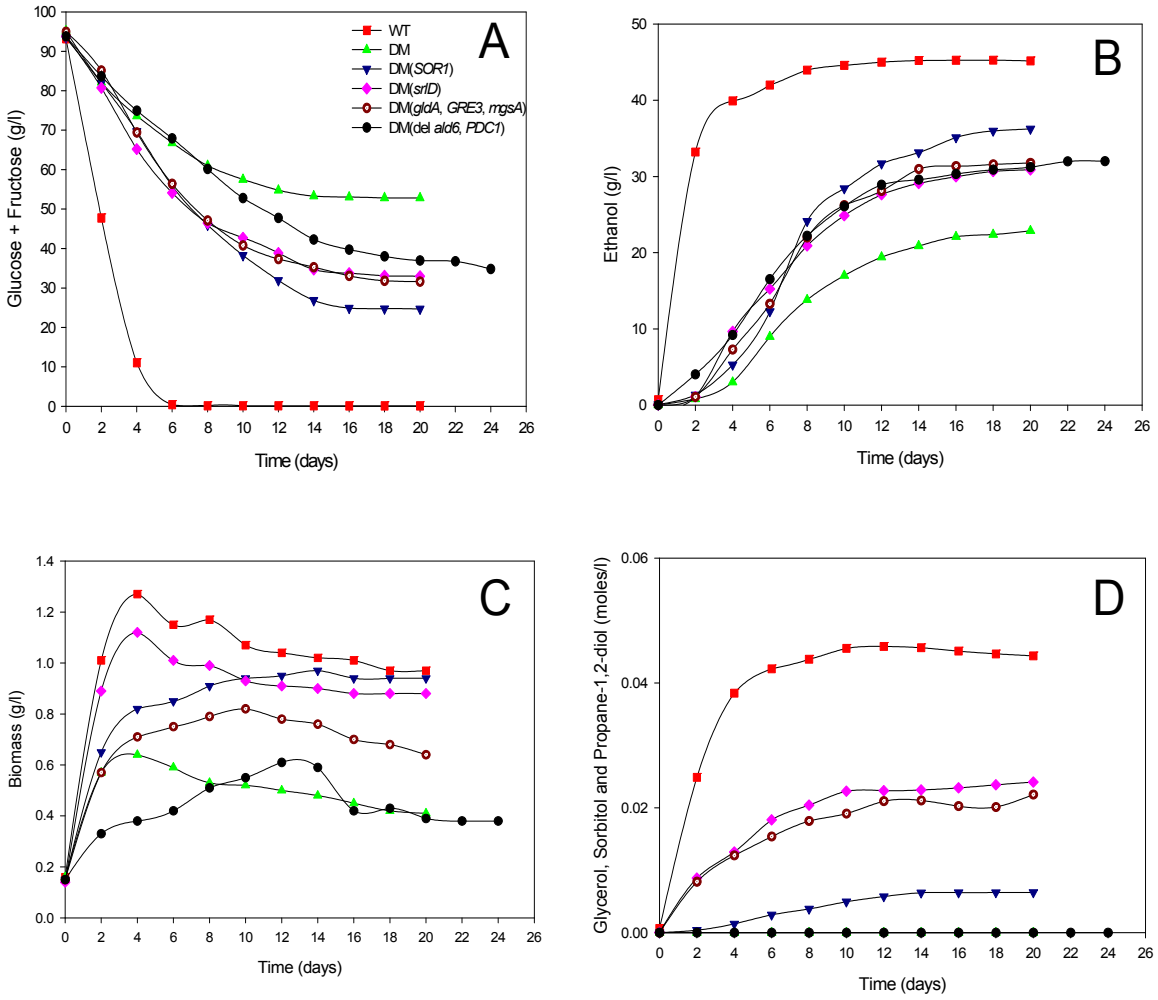

FIG. 6.1 Sugar consumption (A), ethanol production (B), biomass (dry weight) (C) and metabolite (glycerol, sorbitol and propane-1,2-diol) production (D) by the WT and genetically manipulated strains in a representative experiment. WT formed glycerol, DM(*SOR1*) and DM(*srlD*) formed sorbitol and DM(*gldA*, *GRE3*, *mgsA*) formed propane-1,2-diol as metabolite whereas DM and DM(*ald6*Δ, *PDC1*) strains formed no metabolite in significant concentrations to replace glycerol.

6.3.2 Comparison of organic acids production

Concentrations of organic acids such as pyruvate, malate, acetate and succinate produced by the strains were affected by the genetic manipulations (Fig. 6.2) showing that the redox balance inside the cell influences their production (Fig. 2.8, 2.9). Pyruvate production by WT, DM and DM(*ald6*Δ, *PDC1*) showed a similar trend with a steady increase until approximately the 2nd day and the concentration stayed constant thereafter (Fig 6.2A). However, the other strains DM(*srlD*), DM(*SOR1*) and DM(*gldA*, *GRE3*, *mgsA*)) continued to produce pyruvate beyond the 2nd day and the final values were considerably higher than the WT, DM, DM(*ald6*Δ, *PDC1*) strains. Malate production was the highest in DM whereas the other strains showed a similar trend in

malate production (Fig 6.2B). Acetate production was significantly reduced in the genetically manipulated strains compared to the WT strain and only the DM(*srlD*) strain showed acetate production above 0.2 g/l. Compared to the WT, succinate production was reduced in the genetically manipulated strains except DM(*gldA*, *GRE3*, *mgsA*) where the production pattern was similar to the WT strain. Final concentrations of organic acids are shown in Table 6.3.

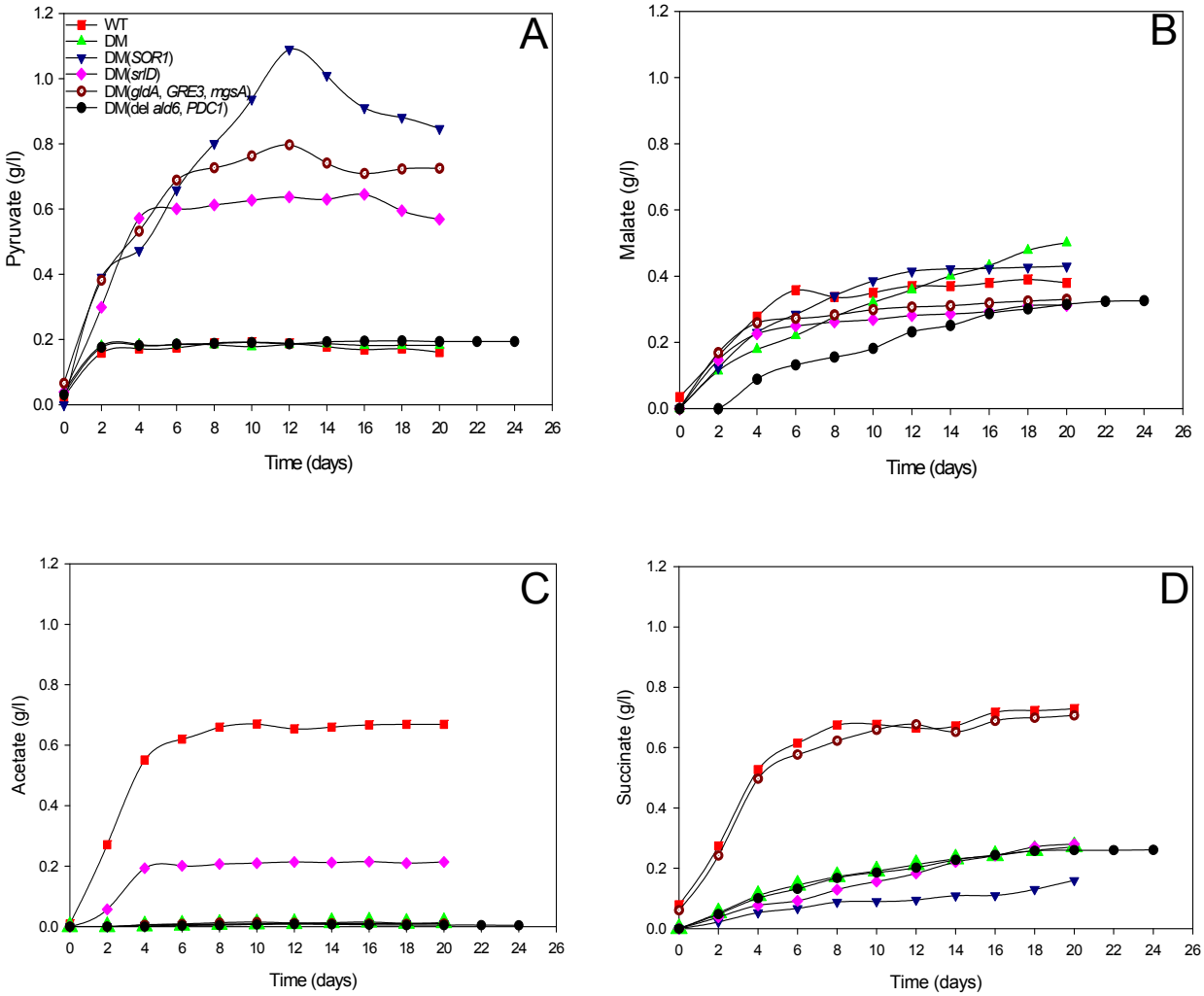

FIG. 6.2 Pyruvate (A), malate (B), acetate (C) and succinate (D) production by the WT and genetically manipulated strains in a representative experiment.

6.3.3 Comparison of secondary metabolites production

Production of higher alcohols may be driven by the necessity to regenerate NAD^+ in the genetically manipulated strains. This hypothesis was apparently valid for isobutanol production since all the genetically manipulated strains produced more isobutanol than the WT strain although the concentration produced by DM(*srlD*) was only slightly higher

(Fig. 6.3A). However, the comparative analysis of other higher alcohols revealed that NAD^+ regeneration might not be the only factor that drives their production. For example isoamyl alcohol production decreased in DM(*ald6*Δ, *PDC1*) as compared to WT strain whereas the metabolite production in DM(*SOR1*) and DM(*srlD*) strains was considerably greater (Fig. 6.3B). The pattern of isoamyl alcohol production varied greatly between strains. 2-phenyl ethanol production followed similar trend in WT and genetically manipulated strains with only slight variations (Fig. 6.3C). Concentrations of secondary metabolites at the end of fermentation are shown in Table 6.3.

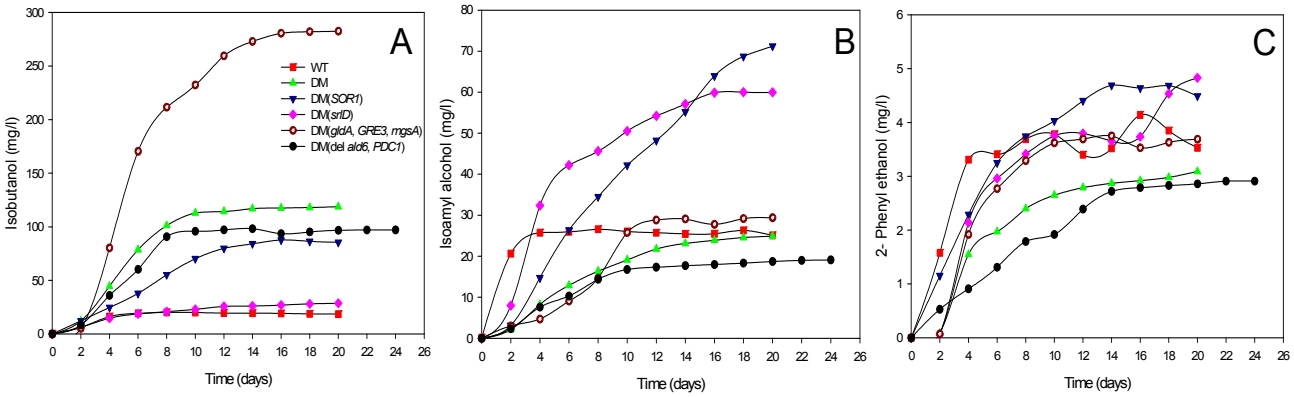

FIG. 6.3 Isobutanol (A), isoamyl alcohol (B) and 2-phenyl ethanol production (C) by the WT and genetically manipulated strains in a representative experiment.

The ethyl acetate concentration produced by the WT strain was higher than all the other strains indicating that these genetic manipulations negatively impacted on the ability of the strains to produce this metabolite (Fig 6.4A). On the other hand, isobutyric acid concentrations increased significantly in cultures inoculated with genetically manipulated strains as compared to the WT strain albeit to a different extent (Fig. 6.4B).

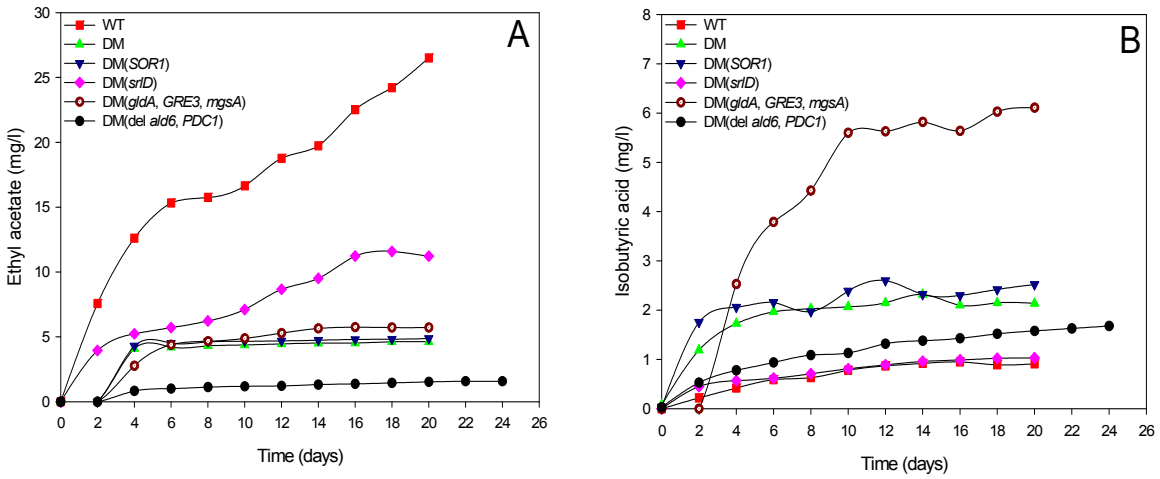

FIG. 6.4 Ethyl acetate (A) and isobutyric acid (B) production by the WT and genetically manipulated strains in a representative experiment.

6.3.4 Comparison of yields of primary and secondary metabolites

Yields (g/g biomass and g/g sugar consumed) for the metabolites were calculated using the concentrations formed by different strains at the end of fermentation. DM(*ald6*Δ, *PDC1*) strain had the highest ethanol yield (g/g biomass) followed by DM, DM(*gldA*, *GRE3*, *mgsA*), WT, DM(*SOR1*) and DM(*srlD*) strains (Table 6.4). WT showed the lowest ethanol yield (g/g sugar consumed) whereas all genetic manipulations lead to an increase with DM(*ald6*Δ, *PDC1*) revealing the highest yield followed by DM and DM(*SOR1*) with the same yield and DM(*srlD*) and DM(*gldA*, *GRE3*, *mgsA*) giving a slightly lower yield (Table 6.5). WT and DM(*gldA*, *GRE3*, *mgsA*) strains had a similar molar yield of glycerol and propane-1,2-diol (0.04 mole/gram biomass) followed by a sorbitol yield of DM(*srlD*) (0.03 mole/gram biomass) and DM(*SOR1*) (0.007 mole/gram biomass).

Pyruvate and malate yields (g/g biomass and g/g sugar consumed) increased in the genetically manipulated strains as compared to WT strain (Table 6.4 and 6.5). Moreover, among genetically manipulated strains, its yields showed huge variation with DM(*SOR1*) and DM(*gldA*, *GRE3*, *mgsA*) strains revealing very high yields of this metabolite (Table 6.4 and 6.5). Acetate yields (g/g biomass and g/g sugar consumed) decreased greatly in the other strains as compared to WT strain and among genetically manipulated strains, DM(*srlD*) showed the highest acetate yield (Table 6.4 and 6.5). DM(*gldA*, *GRE3*, *mgsA*) showed the highest succinate yield (g/g biomass and g/g sugar consumed).

Yields (g/g biomass and g/g sugar consumed) of higher alcohols and isobutyric acid increased in the genetically manipulated strains as compared to WT strain (Table 6.4 and 6.5). However, some genetically manipulated strains showed greater increase than other strains. This shows that other factors than just NAD^+ regeneration might also be responsible for their production. Yields (g/g biomass and g/g sugar consumed) of ethyl acetate decreased due to genetic manipulation.

TABLE 6.3 Concentration[a] of sugar consumed, biomass and primary (g/l) and secondary (mg/l) metabolites formed by WT and genetically manipulated strains at the end of fermentation[b] cultivated in 5% glucose-5% fructose and 6.7 g/l YNB under oxygen limited fermentation.

Primary metabolites	**WT**	**DM**	**DM(*SOR1*)**	**DM(*srlD*)**	**DM(*gldA*, *mgsA*, *GRE3*)**	**DM(*ald6*Δ, *PDC1*)**
sugar consumed	99.85±0.07	47.41±1.95	75.31±1.58	67.01±1.21	68.39±1.28	65.19±0.68
biomass	0.97±0.008	0.41±0.03	0.94±0.02	0.88±0.03	0.58±0.02	0.38±0.006
ethanol	45.17±2.56	22.88±1.68	36.31±2.47	30.89±2.34	31.78±1.23	32.01±1.68
glycerol	4.08±0.09	0	0	0	0	0
sorbitol	0	0	1.17±0.13	4.39±0.14	0	0
propane-1,2-diol	0	0	0	0	1.68±0.02	0
pyruvate	0.16±0.01	0.18±0.004	0.85±0.01	0.57±0.01	0.72±0.02	0.19±0.004
malate	0.38±0.01	0.50±0.06	0.43±0.03	0.31±0.04	0.33±0.04	0.33±0.01
acetate	0.67±0.03	0.01±0.004	0.01±0.001	0.21±0.03	0.01±0.002	0.004±0.0002
succinate	0.73±0.04	0.27±0.01	0.16±0.02	0.28±0.01	0.71±0.01	0.26±0.02
Secondary metabolites						
isobutanol	18.53±0.55	118.75±3.02	85.52±3.36	28.63±1.46	282.53±6.03	97.07±1.72
isoamyl alcohol	25.09±2.49	24.89±2.07	71.23±4.33	59.95±1.06	29.38±0.75	19.09±2.92
2-phenyl ethanol	3.53±0.44	3.09±0.10	4.49±0.14	4.83±0.14	3.69±0.35	2.91±0.09
isobutyric acid	0.91±0.12	2.14±0.49	2.52±0.37	1.03±0.28	6.11±0.15	1.68±0.12
ethyl acetate	26.51±1.72	4.63±0.33	4.87±0.09	11.22±1.58	5.72±0.30	1.58±0.12

[a]Mean ± standard deviation of four independent cultivations.
[b]Fermentation was completed on 20th day for WT, DM, DM(*SOR1*), DM(*srlD*) and DM(*gldA*, *mgsA*, *GRE3*) and on 24th for DM(*ald6*Δ, *PDC1*).

TABLE 6.4 Yields[a] of primary (g/g biomass) and secondary metabolites (mg/g biomass) formed by WT and genetically manipulated strains at the end of fermentation[b] cultivated in 5% glucose-5% fructose and 6.7 g/l YNB under oxygen-limited fermentation.

Primary metabolites	**WT**	**DM**	**DM(*SOR1*)**	**DM(*srlD*)**	**DM(*gldA*, *mgsA*, *GRE3*)**	**DM(*ald6*Δ, *PDC1*)**
ethanol	46.55±2.32	55.96±4.73	38.62±2.26	35.11±2.56	54.86±2.71	84.24±4.5
glycerol	4.2±0.07	0	0	0	0	0
sorbitol	0	0	1.24±0.12	4.99±0.23	0	0
propane-1,2-diol	0	0	0	0	2.90±0.15	0
pyruvate	0.17±0.01	0.44±0.03	0.90±0.03	0.65±0.04	1.25±0.04	0.51±0.008
malate	0.39±0.01	1.22±0.08	0.46±0.04	0.35±0.05	0.57±0.07	0.86±0.03
acetate	0.69±0.03	0.03±0.01	0.01±0.001	0.24±0.03	0.02±0.003	0.01±0.0005
succinate	0.75±0.04	0.66±0.04	0.17±0.02	0.32±0.01	1.22±0.05	0.69±0.04
Secondary metabolites						
isobutanol	19.10±0.55	290.60±17.60	90.97±2.2	32.6±2.52	488.13±31.04	255.47±4.86
isoamyl alcohol	25.87±2.66	60.69±1.03	75.75±3.41	68.18±2.18	50.71±2.19	50.20±7.37
2-phenyl ethanol	3.64±0.44	7.56±0.44	4.78±0.22	5.49±0.26	6.35±0.38	7.66±0.29
isobutyric acid	0.94±0.12	5.17±0.80	2.68±0.39	1.17±0.32	10.55±0.49	4.42±0.30
ethyl acetate	27.33±1.81	11.33±1.08	5.18±0.18	12.73±1.48	9.87±0.45	4.16±0.34

[a]Mean ± standard deviation of four independent cultivations.
[b]Fermentation was completed on 20th day for WT, DM, DM(*SOR1*), DM(*srlD*) and DM(*gldA*, *mgsA*, *GRE3*) and on 24th for DM(*ald6*Δ, *PDC1*).

TABLE 6.5 Yields[a] of primary (g/g sugar consumed) and secondary (mg/g sugar consumed) metabolites formed by WT and genetically manipulated strains at the end of fermentation[b] cultivated in 5% glucose-5% fructose and 6.7 g/l YNB under oxygen-limited fermentation.

Primary metabolites	**WT**	**DM**	**DM(*SOR1*)**	**DM(*srlD*)**	**DM(*gldA*, *mgsA*, *GRE3*)**	**DM(*ald6*Δ, *PDC1*)**
biomass	0.009±0.0001	0.008±0.00001	0.012±0.000003	0.013±0.00001	0.007±0.000002	0.006±0.000001
ethanol	0.45±0.03	0.48±0.001	0.48±0.0003	0.46±0.0004	0.46±0.0002	0.49±0.0003
glycerol	0.04±0.001	0	0.00	0.00	0.00	0.00
sorbitol	0.00	0	0.016±0.00002	0.065±0.00002	0.00	0.00
propane-1,2-diol	0.00	0	0.00	0.00	0.02±0.00001	0.00
pyruvate	0.002±0.0001	0.004±0.000001	0.011±0.000004	0.009±0.000001	0.01±0.000002	0.003±0.000001
malate	0.004±0.0002	0.011±0.00001	0.006±0.000003	0.005±0.00001	0.005±0.00001	0.005±0.000001
acetate	0.007±0.0003	0.0003±0.000001	0.0001±0.0000001	0.003±0.00001	0.0001±0.0000003	0.0001±0.0000001
succinate	0.007±0.0004	0.0057±0.000004	0.002±0.000003	0.004±0.000001	0.01±0.000002	0.004±0.000003
Seconday metabolites						
isobutanol	0.19±0.006	2.508±0.001	1.14±0.0004	0.45±0.0002	4.13±0.002	1.49±0.0004
isoamyl alcohol	0.25±0.03	0.525±0.0004	0.95±0.0005	0.89±0.0002	0.43±0.0001	0.29±0.0005
2-phenyl ethanol	0.04±0.004	0.065±0.00002	0.06±0.00002	0.07±0.00001	0.05±0.00004	0.04±0.00001
isobutyric acid	0.009±0.001	0.0451±0.0001	0.033±0.00004	0.02±0.00004	0.09±0.00003	0.03±0.00002
ethyl acetate	0.27±0.02	0.098±0.00004	0.065±0.00001	0.17±0.0003	0.08±0.00003	0.02±0.00002

[a]Mean ± standard deviation of four independent cultivations.
[b]Fermentation was completed on 20th day for WT, DM, DM(*SOR1*), DM(*srlD*) and DM(*gldA*, *mgsA*, *GRE3*) and on 24th for DM(*ald6*Δ, *PDC1*).

6.3.5 Comparative study of strains using standardized biplot analysis

Two different groups of standardized biplot were drawn. In the first group, the values of glycerol, sorbitol and propane-1,2-diol were entered separately whereas in the second group those metabolites were referred as NAD^+ equivalent (moles/l or moles/g biomass or moles/g sugar consumed). Both groups had three different types of biplot (i) according to concentration of metabolites and sugar consumed (ii) according to yield (g/g biomass) and (iii) according to yield (g/g sugar consumed). The different clusters of the strains in the biplots were characterized by certain metabolites which were either formed in very high amount or in very low amount. The strains did not cluster together in one area of the biplot indicating that they differ in terms of metabolite production (Fig 6.5). Cluster A is characterized by the higher production of acetate, ethyl acetate, NAD^+ equivalent, sugar consumption and ethanol and lower production of isobutyric acid as compared to other strains. Cluster B is characterized by the higher production of 2-phenyl ethanol, isoamyl alcohol and pyruvate as compared to other strains. Cluster C is characterized by the higher production of isobutanol and isobutyric acid and cluster D is characterized by the higher production of malate and lower production of biomass as compared to other strains. These observations could also be related to the amount of NAD^+ regeneration in different strains. DM(*SOR1*) and DM(*srlD*) strains clustered together and similarly DM together with DM(*ald6*Δ, *PDC1*). On the other hand, DM(*gldA*, *GRE3*, *mgsA*) and WT did not cluster with any other strain. Moreover, similar clustering of the strains was obtained in two different groups of biplots based on concentrations and yields (data not shown). This suggests that not only the production of glycerol, sorbitol or propane-1,2-diol and NAD^+ equivalents which impacted on the clustering pattern but also the production of several other metabolites also influenced the clustering pattern.

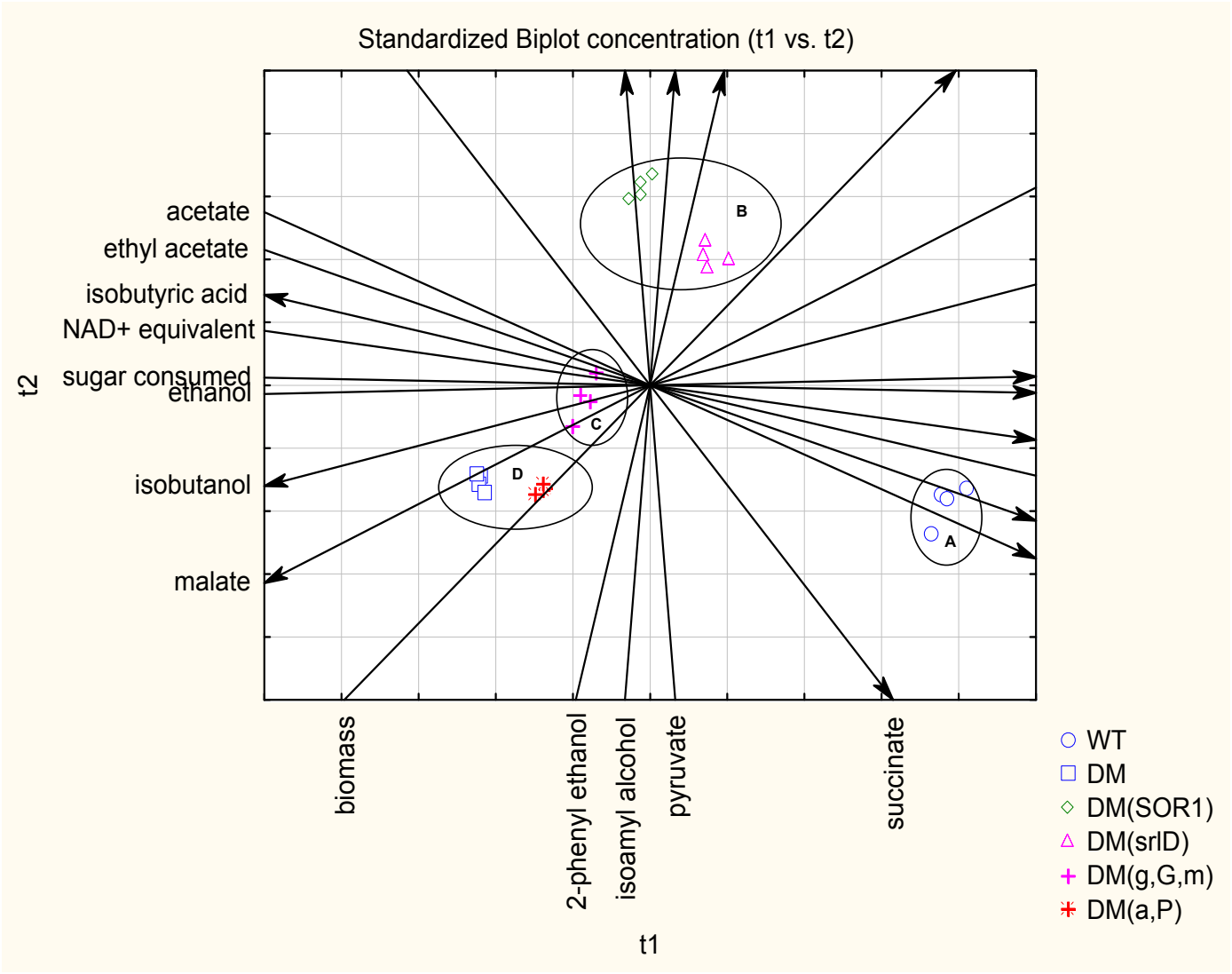

FIG. 6.5 Standardized biplot used to graphically display relationships between concentrations of metabolites as well as clusterings of samples (cluster A= WT, cluster B= DM(*SOR1*) and DM(*srlD*), cluster C= DM(*gldA*, *GRE3*, *mgsA*) and cluster D= DM and DM(*ald6*Δ, *PDC1*) strains).

6.3.6 Correlation analysis of the metabolites

A statistical study was carried to analyze positive or negative correlation between the concentration of metabolites and between yields (g/g biomass) and (g/g sugar consumed) (Table 6.6). This study showed both obvious and non-obvious correlations. For example as expected sugar consumed and ethanol production and sugar consumed and biomass production was significantly correlated. Furthermore, concentrations and yields (g/g biomass and g/g sugar consumed) of isobutanol/isobutyric acid, acetate/ethyl acetate were also significantly correlated. On the other hand, concentration and yield (g/g sugar consumed) of pyruvate/isoamyl alcohol and isoamyl alcohol/2-phenyl ethanol was also significantly correlated which is not apparent. This might be due to the involvement of some unknown pathways taking part in higher alcohols production.

However, the yield (g/g biomass) of pyruvate/isoamyl alcohol and isoamyl alcohol/2-phenyl ethanol was not correlated.

Table 6.6 Spearman values for relationships[a] between concentrations and yields of metabolites in PCA biplots (All p-values were less than 0.01).

concentration biplot		
variable 1	**variable 2**	**Spearman**
sugar consumed	biomass	0.85
sugar consumed	ethanol	0.77
sugar consumed	NAD^+ equivalent	0.74
biomass	ethanol	0.70
biomass	NAD^+ equivalent	0.8
biomass	acetate	0.72
biomass	ethyl acetate	0.75
NAD^+ equivalent	acetate	0.76
NAD^+ equivalent	ethyl acetate	0.94
pyruvate	isoamyl alcohol	0.73
acetate	ethyl acetate	0.85
isobutanol	isobutyric acid	0.81
isoamyl alcohol	2-phenyl acetate	0.82
yield(g/g biomass) biplot		
ethanol	malate	0.78
NAD^+ equivalent	malate	-0.71
NAD^+ equivalent	2-phenyl ethanol	-0.74
malate	isobutanol	0.76
malate	isobutyric acid	0.78
malate	2-phenyl ethanol	0.79
acetate	ethyl acetate	0.92
succinate	isoamyl alcohol	-0.81
isobutanol	isobutyric acid	0.95
yield(g/g sugar consumed) biplot		
biomass	ethanol	-0.94
NAD^+ equivalent	malate	-0.70
NAD^+ equivalent	acetate	0.73
NAD^+ equivalent	ethyl acetate	0.73
pyruvate	isoamyl alcohol	0.78
acetate	ethyl acetate	0.95
isobutanol	isobutyric acid	0.90
isoamyl alcohol	2-phenyl ethanol	0.81

[a]Correlation is assumed to be significant if $0.70 \leq r \leq 1$ (positive correlation) or $-1 \leq r \leq -0.70$ (negative correlation) and $p<0.05$ (Syms 2008).

6.4 Discussion

6.4.1 Impact of NAD^+ regeneration ability of strains on general fermentation parameters

WT and DM strains were two extremes in terms of both absolute and rate of sugar utilisation and ethanol and biomass production. This could be due to the strongest NAD^+ regeneration ability in WT and the weakest in DM. The other strains lay between these two extremes. Different factors such as sorbitol, propane-1,2-diol and ethanol production might determine individual performance. DM(*SOR1*) was the best strain in terms of sugar utilisation and biomass production in spite of forming the least concentration of metabolite to replace glycerol (sorbitol). This might be due to the highest production of ethanol by this strain which could also account for NAD^+ regeneration. DM(*gldA*, *GRE3*, *mgsA*) and DM(*srlD*) strains showed similar sorbitol and propane-1,2-diol production concomitant with similar sugar utilisation and ethanol production. However, lower biomass production in the former could be due to the toxic effect of methylglyoxal on biomass production as explained earlier in chapter 4. DM(*ald6*Δ, *PDC1*) strain did not form any metabolite to replace glycerol but still consumed similar amount of sugar and produced similar concentration of ethanol as compared to DM(*gldA*, *GRE3*, *mgsA*) and DM(*srlD*) strains. The higher ethanol yield (g/g sugar consumed) of DM(*ald6*Δ, *PDC1*) as compared to DM(*gldA*, *GRE3*, *mgsA*) and DM(*srlD*) strains might explain. However, slower growth and rate of sugar consumption by DM(*ald6*Δ, *PDC1*) as compared to DM(*gldA*, *GRE3*, *mgsA*) and DM(*srlD*) strains indicate that NAD^+ regeneration through ethanol production is not as efficient compared to NAD^+ regeneration through sorbitol or propane-1,2-diol production in other strains. Moreover, DM(*ald6*Δ, *PDC1*) produced much lower amount of biomass as compared to DM(*gldA*, *GRE3*, *mgsA*) and DM(*srlD*) strains. Several factors such as lower intracellular availability of NAD^+ or toxic effect of acetaldehyde might explain this observation. However, further experiments could support these findings.

WT had the lowest ethanol yields (g/g biomass and g/g sugar consumed) which could be due to the highest production of glycerol whereas DM(*ald6*Δ, *PDC1*) strain had the highest ethanol yields which could be due to the lower production of other by-products as compared to other strains. The higher yield of sorbitol by DM(*srlD*) as compared to DM(*SOR1*) could be due lower availability of free intracellular fructose in the latter strain as discussed in chapter 3.

6.4.2 Influence of redox balance on organic acid production

Under fermentative conditions, several factors such as NAD^+ or NADH accessibility or availability of precursors might determine the production of organic acids (Fig. 2.8, 2.9). For example pyruvate production depends on the availability of glycolytic precursors. Once formed, most of it is converted to ethanol to maintain redox balance. However, some pyruvate might be exported from the cell due to low activity of alcohol dehydrogenase (*ADH1*) at the start of fermentation. This hypothesis might be valid for DM, WT and DM(*ald6*Δ, *PDC1*) strains where its production increased until approximately 2nd day and the concentration remained constant thereafter. However, in other strains pyruvate export was much greater which could be due to inhibition of the *ADH1* activity by sorbitol or propane-1,2-diol. This could lead to intracellular accumulation of pyruvate and later export but requires experimental evidence to support this argument. Moreover, availability of non glycolytic precursor such as methylglyoxal in DM(*gldA*, *GRE3*, *mgsA*) as explained in Chapter 4 might also contribute to higher pyruvate production and export.

Malic acid concentration in the range of 0.3-0.5 g/l imparts tart (green and sour) taste to wine. The highest production as well as yields (g/g biomass and g/g sugar consumed) of this compound in DM as compared to other strains could be due to the highest need to regenerate NAD^+ in the absence of *GPD1* and *GPD2* genes.

Acetic acid is desirable in wine if its concentration is around 300 mg/l as it contributes to smell and taste (Cambon *et al*., 2006; Remize *et al*., 1999). Much lower concentrations and yields of acetate in genetically manipulated strains as compared to WT strain could be due to lower availability of NAD^+ (due to deletion of *GPD1* and *GPD2* genes) as compared to WT strain. Among genetically manipulated strains, DM(*srlD*) strain formed the highest concentration and yields of acetate which could be related to production of $NADP^+$ in the *srlD* catalyzed reaction which can then be used to produce acetate through $NADP^+$-dependent aldehyde dhydrogenase (*ALD6*). There is experimental support for the presence of NADPH dependent *srlD* in plants (Tao *et al*., 1995). However, further investigation is needed to show the presence of NADPH-dependent *srlD* in *Escherichia coli*.

Succinic acid confers wine a taste that is a combination of saltiness, bitterness and acidity. The normal level of succinic acid in wine is around 1 g/l and its concentrations between 0.6-1 g/l contribute positively to the taste of wine (Coulter *et al*., 2004). WT and DM(*gldA*, *GRE3*, *mgsA*) were the only strains which formed succinate in this range indicating that redox balance adversely affected the production of this metabolite. Succinate is formed through the reductive pathway of the TCA cycle (Camarasa *et al*., 2003). The highest production of this compound in WT and DM(*gldA*, *GRE3*, *mgsA*) could be due to functioning of glycerol-3-phosphate (Fig. 3.11) and proposed acetol shuttle (Fig. 6.8) respectively which completes the reductive pathway thereby completing the conversion of fumarate to succinate. The low but slight production of succinate in other strains could be through degradation of glutamate to succinate via the GABA pathway as explained in chapter 3.

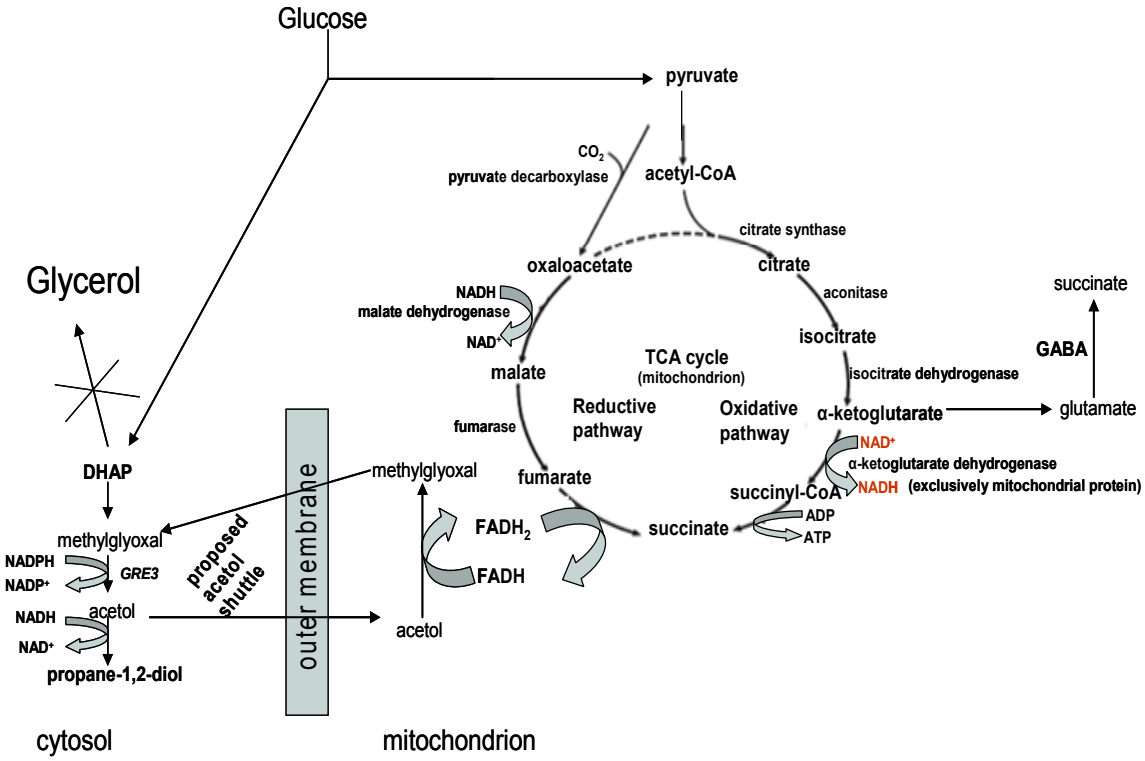

FIG. 6.8 Proposed acetol shuttle for the production of succinate through the reductive pathway in the DM(*gldA*, *GRE3*, *mgsA*) strain. Conversion of methylglyoxal to acetol is carried out in the cytosol using an aldose reductase (Gre3p). Acetol then crosses the outer membrane and enters the mitochondrion. In the mitochondrion, acetol could be converted to methylglyoxal using the mitochondrial *GPD* gene and reducing FADH to $FADH_2$ in this process. The $FADH_2$ formed could reduce fumarate to succinate. Adapted from Larsson *et al.* (1998).

6.4.3 Secondary metabolites as aroma compounds

Secondary metabolites such as higher alcohols, esters and fatty acids are considered as aroma compounds in the wine industry. These aroma compounds play major role in improving wine quality by influencing the flavour of wine. It was shown in this study that the genetic manipulation of the *gpd1*Δ*gpd2*Δ DM not only improved the fermentative ability but influenced the overall metabolic flux in terms of aroma compound production. Not all the aroma compounds usually found in wine were detected in this study. This could be due to unavailability of precursors in the medium (Prior *et al*., 2000) and also might be due to the use of laboratory yeast strains to conduct fermentation (Prior *et al*., 2000). Furthermore, production of certain aroma compounds might also require molecular oxygen (Bardi *et al*., 1999) which was depleted early (after 2.5 days) during the fermentation conducted in this study. On the other hand, during wine making process, the must is in contact with molecular oxygen for most part of the fermentation process.

6.4.3.1 Regulation in the production of higher alcohols

Availability of precursors such as pyruvate and amino acids determine the production. Moreover, production of higher alcohols under fermentative conditions has been associated with the need to regenerate NAD^+. Genetically manipulated strains continued to produce higher alcohols even when growth ceased whereas WT stopped their production after cessation of growth indicating a general need to regenerate NAD^+ in the genetically manipulated strains. Furthermore, the final concentration of isobutanol was higher in manipulated strains than the WT strain indicating that some NAD^+ regeneration is directly linked to higher alcohol production. However, the slight increase in higher alcohols production and yields such as isoamyl alcohol and 2-phenyl ethanol by genetically manipulated strains suggests that only certain higher alcohol synthesis pathways may be preferentially used in NAD^+ regeneration. Moreover, higher alcohol concentrations varied greatly among the genetically manipulated strains indicating the involvement of other factors in their production. For example, much higher production of isobutanol in DM(*gldA*, *GRE3*, *mgsA*) might reflect a mechanism by cells to detoxify toxic methylglyoxal by converting it to pyruvate and ultimately to isobutanol. High levels of isoamyl alcohol in DM(*SOR1*) and DM(*srlD*) strains is difficult to explain.

A closed NADH and NAD^+ balance (Tables 6.7 and 6.8) was observed for WT and DM(*srlD*) strains indicating that most of the NADH formed through biomass and metabolites (glycerol and sorbitol) production was regenerated through primary and secondary metabolites production. In the case of DM(*gldA*, *GRE3*, *mgsA*) strain, it is assumed that NAD^+ regenerated through propane-1,2-diol production might be used for the oxidation of methylglyoxal to pyruvate (Murata *et al.*, 1985) thereby giving rise to "extra" 0.047 moles of NADH formed through pyruvate production. However, this possibility needs to be experimentally confirmed and further investigation is required. If this assumption is valid, a closed redox balance will also be possible for DM(*gldA*, *GRE3*, *mgsA*) strain (Table 6.9). Surprisingly, DM, DM(*SOR1*) and DM(*ald6*Δ, *PDC1*) strains did not show a closed redox balance (Tables 6.7 and 6.9) with NAD^+ regenerated much higher than NADH formed by these strains. The possibility of oxygen regenerating NAD^+ in the early stage of fermentation in these strains cannot be ignored.

6.4.3.2 Regulation of esters and fatty acids production

Production of esters is regulated differently as compared to production of higher alcohols in terms of requirement of cofactor. Production of higher alcohols regenerates NAD^+ whereas production of acetate (an intermediate in the production of esters) requires NAD^+ (Fig. 2.13). Therefore the higher concentration and yields of ethyl acetate in WT compared to the other strains reflects a greater availability of NAD^+ and acetate. The NAD^+ concentration would be lower in the mutant strains as compared to WT strain due to the deletion of *GPD1* and *GPD2* genes. This might explain the lower production of ethyl acetate by the genetically manipulated strains as compared to WT strain. Among genetically manipulated strains, DM(*srlD)* showed the highest production of ethyl acetate. The acetate needed for ethyl acetate production could be produced by the $NADP^+$ dependent oxidation of acetaldehyde to acetate. Measurement of the intracellular concentrations of NADH/NAD^+ and metabolites could help to resolve this issue.

Production of isobutyric acid was boosted in all the genetically manipulated strains as compared to WT. This might be related to the greater production of isobutanol which acts as the direct precursor of isobutyric acid (Fig. 2.11). None of the strains including the WT formed isoamyl acetate or butyric acid which was surprising because these compounds are usually formed by *S. cerevisiae* under fermentative conditions.

However, the laboratory yeast strains used in this study might produce low undetectable levels of these aroma compounds as compared to industrial yeast strains. Moreover, much higher concentration of sugar might be required (~ 200 g/l) than used in this study (100 g/l) to produce detectable levels of these compounds.

Table 6.7 NADH formed and NAD^+ regenerated for WT and DM through primary and secondary metabolites production[a]. NAD^+ regenerated in WT through ethanol production is taken as zero by assuming that its production is the result of a redox neutral process. In DM, NAD^+ regenerated through ethanol production is "extra" moles of ethanol formed as compared to moles of ethanol formed by WT strain.

	WT		**DM**	
Metabolite	NADH formed	NAD^+ regenerated	NADH formed	NAD^+ regenerated
biomass	0.0711	0.0000	0.0633	0.0000
glycerol	0.0000	0.0801	0.0000	0.0000
sorbitol	0.0000	0.0000	0.0000	0.0000
propane-1,2-diol	0.0000	0.0000	0.0000	0.0000
malate	0.0000	0.0051	0.0000	0.0142
acetate	0.0201	0.0000	0.0008	0.0000
succinate	0.0000	0.0112	0.0000	0.0087
isobutanol	0.0000	0.0005	0.0000	0.0061
isoamyl alcohol	0.0000	0.0005	0.0000	0.0011
isobutyric acid	0.0000	0.0000	0.0000	0.0001
2-phenyl ethanol	0.0000	0.0001	0.0000	0.0001
total[b]	**0.0912**	**0.0974**	**0.0641**	**0.0303**
ethanol	**0.0000**	**0.0000**	**0.0000**	**0.1182**
total	**0.0912**	**0.0974**	**0.0641**	**0.1485**

[a]Coefficients of variation for NADH formed and NAD^+ regenerated were less than 13% and 10% for both strains.
[b]Without taking into account the NAD^+ regenerated through ethanol production

Table 6.8 NADH formed and NAD^+ regenerated for DM(*SOR1*) and DM(*srlD*) through primary and secondary metabolites production[a]. NAD^+ regenerated through ethanol production is "extra" moles of ethanol formed as compared to moles of ethanol formed by WT strain.

	DM(*SOR1*)		**DM(*srlD*)**	
Metabolite	NADH formed	NAD^+ regenerated	NADH formed	NAD^+ regenerated
biomass	0.0914	0.0000	0.0961	0.0000
glycerol	0.0000	0.0000	0.0000	0.0000
sorbitol	0.0000	0.0154	0.0000	0.0649
propane-1,2-diol	0.0000	0.0000	0.0000	0.0000
malate	0.0000	0.0077	0.0000	0.0062
acetate	0.0004	0.0000	0.0096	0.0000
succinate	0.0000	0.0033	0.0000	0.0064
isobutanol	0.0000	0.0028	0.0000	0.0010
isoamyl alcohol	0.0000	0.0019	0.0000	0.0018
isobutyric acid	0.0000	0.0001	0.0000	0.0000
2-phenyl ethanol	0.0000	0.0001	0.0000	0.0001
total[b]	**0.0918**	**0.0312**	**0.1057**	**0.0805**
ethanol	**0.0000**	**0.1166**	**0.0000**	**0.0336**
total	**0.0918**	**0.1478**	**0.1057**	**0.1141**

[a]Coefficients of variation for NADH formed and NAD^+ regenerated were less than 11% and 10% for both strains.
[b]Without taking into account the NAD^+ regenerated through ethanol production

Table 6.9 NADH formed and NAD^+ regenerated for DM(*gldA*, *GRE3*, *mgsA*) and DM(*ald6*Δ, *PDC1*) through primary and seconday metabolites production[a]. NAD^+ regenerated through ethanol production is "extra" moles of ethanol formed as compared to moles of ethanol formed by WT strain.

	DM(*gldA*, *GRE3*, *mgsA*)		**DM(*ald6*Δ, *PDC1*)**	
Metabolite	NADH formed	NAD^+ regenerated	NADH formed	NAD^+ regenerated
biomass	0.0621	0.0000	0.0427	0.0000
glycerol	0.0000	0.0000	0.0000	0.0000
sorbitol	0.0000	0.0000	0.0000	0.0000
propane-1,2-diol	0.0000	0.0470	0.0000	0.0000
malate	0.0000	0.0065	0.0000	0.0067
acetate	0.0004	0.0000	0.0002	0.0000
succinate	0.0000	0.0158	0.0000	0.0061
isobutanol	0.0000	0.0100	0.0000	0.0036
isoamyl alcohol	0.0000	0.0009	0.0000	0.0006
isobutyric acid	0.0000	0.0002	0.0000	0.0001
2-phenyl ethanol	0.0000	0.0001	0.0000	0.0001
pyruvate	**0.047**	**0**		
total[b]	**0.1095**	**0.0805**	**0.0429**	**0.0172**
ethanol	**0.0000**	**0.0481**	**0.0000**	**0.1511**
total	**0.1095**	**0.1286**	**0.0429**	**0.1683**

[a]Coefficients of variation for NADH formed and NAD^+ regenerated were less than 10% for both strains.
[b]Without taking into account the NAD^+ regenerated through ethanol production

6.4.4 Relationships between metabolites produced using correlation analysis

As expected, sugar consumption was significantly positively correlated with the production of biomass and ethanol and NAD^+ regeneration capacity of strains. Isobutanol/isobutyric acid and acetate/ethyl acetate production were also significantly correlated. This might be due to the single step reaction needed for the conversion of isobutanol to isobutyric acid and acetate to ethyl acetate (Fig. 2.11 and 2.13). Therefore availability of the immediate precursor will effect the production. Biomass production was linked to acetate production which might be due to regeneration of $NADP^+$ through biomass production (van Dijken & Scheffers, 1986) which can then be used as a cofactor for the oxidation of acetaldehyde to acetate (Saint-Prix *et al.*, 2004). Some unexpected correlations were also observed such as between isoamyl alcohol and 2-phenyl ethanol production and yield (g/g sugar consumed). The data showed that DM(*SOR1*) and DM(*srlD*) strains which formed high levels of isoamyl alcohol also formed high level of 2-phenyl ethanol but no obvious link in the production of these metabolites is apparent. No significant correlation was observed in the production or

yields of organic acids implying that the factors which affected the production of these compounds were probably not directly related to the modified redox balancing potential of the strains.

6.5 Overall conclusion

This study has shown that the need to restore the redox balance in the cell plays an important but not exclusive role in the production of secondary metabolites. The introduction of different pathways for NAD^+ regeneration also influenced the production of aroma compounds in different manner. In the absence of ability to form glycerol and to regenerate NAD^+, the production of higher alcohols under fermentative conditions was expected in the genetically manipulated strains. However, isobutanol production was stimulated more than isoamyl alcohol production. This might be related to a requirement for acetate (acetyl-CoA) used in the production of isoamyl alcohol from pyruvate (Fig. 2.11). Among the group of fatty acids, none of the strains formed butyric acid (which could be related to the inability to produce butanol) but isobutyric acid production was boosted in all the genetically manipulated strains as compared to WT strain. Among esters, isoamyl acetate was not formed by any of the strains. This was especially surprising for the WT strain because isoamyl acetate is usually formed by yeast under fermentative conditions (Lambrechts *et al*., 2000). Ethyl acetate production was lower in the strains as compared to WT strain might again be due to of lower availability NAD^+.

This work was carried out on the laboratory yeast strains and whether the same effects would be observed in wine yeast strains need further investigation. Furthermore, as these higher alcohols are mostly formed by the degradation of amino acids, measurement of intracellular levels of amino acids (valine and leucine in this case) could reveal the relationship to higher alcohol production. The affect of introduction of a new pathway for NAD^+ regeneration in wine yeast strains and its influence on aroma compounds production might help us to design new strategies to improve the aroma profile of wine.

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Chapter 7

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

7.1 Concluding remarks

The importance of glycerol formation by the yeast *Saccharomyces cerevisiae* under fermentative conditions has been clearly defined (da Silva *et al*., 2009; Gancedo *et al*., 1968a; Gancedo *et al*., 1968b; Petrovska *et al*., 1999; Wang *et al*., 2001). The enzymes Gpd1p and Gpd2p involved in the formation of glycerol have been isolated (Eriksson *et al*., 1995) and it has been shown that deletion of these genes makes the yeast defective in glycerol formation. Such mutants are unable to grow under strict anaerobic conditions due to the subsequent inability of these strains to regenerate NAD^+ (Bjorkqvist *et al*., 1997; Nissen *et al*., 2000).

To address this growth defective phenotype, native and heterologous genes were overexpressed in the *gpd1*Δ*gpd2*Δ double mutant (DM) and later used to ferment high sugar medium. Interestingly, the anaerobic sugar consumption and carbon dioxide evolution phenotype of the DM observed in this study was different when compared to earlier studies where the sugar consumption and carbon dioxide evolution of the DM used to cease as soon as system becomes strictly anaerobic (Bjorkqvist *et al.*, 1997; Liden *et al.*, 1996; Costenoble *et al.*, 2003). Nevertheless, the growth phenotype of the DM observed in this study was similar to those studies since biomass formation ceased as soon as the system becomes completely anaerobic (Bjorkqvist *et al.*, 1997; Liden *et al.*, 1996; Costenoble *et al.*, 2003). The observation that sugar consumption and carbon dioxide evolution by the DM differed between this study and earlier studies could be either due to strain difference or to the different growth conditions, and in particular the high sugar concentrations, used. The bacterial mannitol-1-P dehydrogenase gene was earlier cloned and overexpressed in the DM (Costenoble *et al.*, 2003). Glucose consumption by the DM overexpressing mannitol-1-P dehydrogenase increased only slightly under strict anaerobic conditions which could be related to low amounts of mannitol being formed and consequently the insufficient regeneration of NAD^+ required for growth. Our study has shown that overexpression of *E. coli* sorbitol-6-P dehydrogenase (*srlD*) gene partially restores the growth defect of the DM in high sugar medium under fermentative conditions with a relatively higher amount of sorbitol produced as compared to mannitol production in the other study (Costenoble *et al.*, 2003). In another study, overexpression of transhydrogenase from bacteria in the DM did not result in growth under anaerobic conditions probably due to the localization of the enzyme in a membrane without an appropriate proton gradient (Nissen *et al*., 2001).

Our study has shown that overexpression of *gldA*, *GRE3* and *mgsA* genes simultaneously in the DM partially improved the growth defect. Furthermore, overexpression of native genes such as *SOR1* and *PDC1* (in combination with *ALD6* deletion) in the DM has not been described earlier. Interestingly, overexpression of *SOR1* led to the best complementation in terms of sugar utilization and growth compared with the other manipulated strains. This observation may be related to the presence of fructose as carbon source in the growth medium. *SOR1* is a native gene of *S. cerevisiae* strain and is induced when sorbitol is used as carbon source (Sarthy *et al.*, 1994; Toivari *et al.*, 2004). However, this is the first study to our knowledge where it was shown that *SOR1* might also catalyze the reverse reaction thereby converting fructose to sorbitol and regenerating NAD^+.

The expression of these genes in the DM improved the growth phenotype albeit not to the same extent as compared to the WT strain. This shows that glycerol formation and NAD^+ regeneration through Gpd1p and Gpd2p cannot be replaced to the same degree by insertion of the genes selected in this study. However, the degree of restoration was better than the strategies used for complementation in earlier studies (Costenoble *et al.*, 2003; Nissen *et al*., 2000; Liden *et al.*, 1996). Nevertheless, it must be noted that the growth system and medium composition used for fermentation in this study was different when compared to those studies (Costenoble *et al.*, 2003; Nissen *et al*., 2000; Liden *et al.*, 1996). Firstly, this study was not conducted in strictly anaerobic conditions unlike these earlier studies (Costenoble *et al.*, 2003; Nissen *et al*., 2000; Liden *et al.*, 1996) where nitrogen was continuously sparged in the bioreactor to maintain a strictly anaerobic environment. Furthermore fructose was added along with glucose while only glucose was provided as carbon and energy source in those studies. The poor growth on a solid medium under strict anaerobic conditions for some of the genetically manipulated strains (Fig. 3.3, 4.2, Fig. 5.2) suggest that the strains will behave differently in such conditions. Moreover, growth of some of the genetically manipulated strains might also be affected negatively if fructose will be eliminated from the growth medium. Nevertheless, a goal of this study was to investigate fermentation properties of the manipulated strains in a high sugar medium as is found in grape juice. Fermentation of grape juice is not a strictly anaerobic process and both glucose and fructose are present in ample amounts in grape juice.

An investigation of the growth and metabolite analysis of a *gpd1*Δ*gpd2*Δ DM and a DM with heterologous or native genes overexpression under fermentative conditions in high sugar medium has not been conducted earlier to our knowledge. In all the genetically manipulated strains, ethanol formation is the main source for NAD^+ regeneration. However, no significant correlation was observed between formation of ethanol or biomass and other primary or secondary metabolites. The only significant correlation was between sugar consumption and ethanol or biomass formation. This indicates that the metabolic network is indeed very complex and it is difficult to pin point a single factor that can control the structure of the metabolome. Nevertheless, the interesting point which came out of this study was that by changing the mode of re-oxidation of NADH, a completely different metabolite profile can be obtained. The formation of much higher concentrations of isobutanol by DM(*gldA*, *GRE3*, *mgsA*) compared to WT is an indication that secondary metabolite synthesis can be increased. However, the fact that these strains were cultivated in semi-anaerobic environment cannot be ignored and.growth in strictly anaerobic environment might lead to a completely different metabolite profile.

7.2 Future prospects

This study confirms that alternative NAD^+ regeneration pathways can be inserted into *S. cerevisiae* and directly impact on the growth and flux of primary and secondary metabolites. However, the new pathways introduced in the DM were not as efficient in NADH re-oxidation as glycerol formation in WT. To further improve the growth and fermentative ability of the DM, availability of NAD^+ must be increased inside the cell. A possible approach to further increase NAD^+ availability could be achieved by overexpressing both *srlD* and *SOR1* genes simultaneously and deleting the *ALD6* gene so that NAD^+ would be regenerated through alternative routes. Furthermore, overexpression of *PDC1* might further enhance NAD^+ regeneration through “extra” ethanol formation. Moreover overexpression of both *srlD* and *SOR1* genes might also lead to increase in sorbitol yield. Sorbitol is an important polyol with applications in food, pharmaceutical and cosmetic industries (Nissen *et al.*, 2005; Liu *et al.*, 2009). Therefore, by eliminating glycerol from the ethanol fermentation process and possible replacement with sorbitol could have significant commercial advantages. The genetic manipulation used in this study might also be used in the WT strain to decrease ethanol production. By overexpressing *srlD* and *SOR1* genes in the WT strain, carbon flux from

ethanol could be directed towards sorbitol formation. Improvement in NADH re-oxidation in DM(*gldA*, *GRE3*, *mgsA*) strain might be carried out by using an aldose reductase from *Pichia stipitis* having affinity for both NADH or NADPH as cofactors (Verduyn *et al.*, 1985) instead of using the aldose reductase from *S. cerevisiae* (Gre3p) which can only recognize NADPH as cofactor for the reduction of methylglyoxal (Kuhn *et al.*, 1995). Overexpression of *P. stipitis* aldose reductase in DM(*gldA*, *GRE3*, *mgsA*) strain might also help in further increasing the yield of propane-1,2-diol. By overexpressing *P. stipitis* aldose reductase in DM(*gldA*, *GRE3*, *mgsA*) strain, re-oxidation of excess NADH can be carried out in two pathways (conversion of methylglyoxal to acetol and acetol to propane-1,2-diol) instead of only one (acetol to propane-1,2-diol) (Fig. 4.1). Propane-1,2-diol is a major commodity chemical with wide variety of uses in chemical industries (Cameron *et al.*, 1998; Jung *et al.*, 2008; Lee & Dasilva, 2006). Worldwide propane-1,2-diol (also known as propylene glycol or 1,2-propanediol) is mainly used for the production of unsaturated polyester resins (Jung *et al.*, 2008). It can also be used as an industrial solvent, antifreeze and in cleaning agents, paint and surfactants (Jung *et al.*, 2008). Moreover, propane-1,2-diol is also used as an approved additive in foods, in cosmetics and as a solvent in pharmaceutical preparations (Cameron *et al.*, 1998; Jung *et al.*, 2008; Lee & Dasilva, 2006). Both sorbitol and propane-1,2-diol could be more valuable co-products in ethanol fermentation than glycerol. Eliminating glycerol from alcoholic fermentation and increasing ethanol yield could be useful for the bioethanol industry. One of the candidates for this purpose could be DM(*ald6*Δ, *PDC1*) strain because no metabolite in significant concentrations was formed by this strain to replace glycerol. DM(*ald6*Δ, *PDC1*) had much higher ethanol yield as compared to WT strain. At present there is excess glycerol on the world market due to the production of glycerol as a byproduct of the biodiesel industry (da Silva *et al.*, 2009). However, ethanol productivity and concentration produced must improve in this strain before any such application could be considered. Further metabolic engineering of these pathways to restore their fermentation ability and NADH re-oxidation ability would be required.

A systems biology approach using transcriptrome analysis would be useful in establishing the relationships between overexpression of different genes and the growth and secondary metabolites formation (Brul *et al.*, 2008). This knowledge can then be used to target specific pathways or genes which might further improve the fermentative ability as well as aroma profile of the fermented medium. Earlier a systems biology approach was used to analyze the physiological and transcriptional response of *S.*

cerevisiae by deleting an important subunit of succinate dehydrogenase complex (*SDH3*) playing an important role in respiration (Cimini *et al.*, 2009). A genome-wide transcription analysis of this mutant linked mitochondrial respiration to several different parts of the metabolism, including fatty acid and sterol metabolism (Cimini *et al.*, 2009). Growth of strains in a nitrogen or sugar-limiting chemostat cultivation can open a new sphere where a different phenotypic analysis of the strains could be possible. Moreover, microarray analysis along with the knowledge of biochemical properties of enzymes involved in secondary metabolite biosynthesis might also be beneficial in that manipulation of a particular pathway can be carried out with specific objectives in mind. For example knowledge of the biochemical properties of enzymes involved in isoamyl alcohol and isobutanol formation might provide more information regarding different regulation in their formation in this study keeping in mind that they share common precursor (2-keto isovalerate) (Fig. 2.11) for their formation. To date no studies based on this approach have appeared.

Finally, addition of pyruvate as the precursor of ethanol formation in *S. cerevisiae* could potentially improve the fermentative ability of yeast and aroma properties of wine.

7.3 Final conclusion

In conclusion, this study has shown that genetic manipulation altering the NADH/NAD^+ level inside the cell has broad implications on the overall metabolic fluxes because of the high connectivity of these cofactors within the metabolic network. The insights generated in this study might benefit future biotechnological strategies to modify the aroma properties of wine. However, wine made from the genetically modified yeast strains are not accepted at present; therefore a non genetic way of improving strains such as breeding would be the only option. Furthermore, more genetic changes are necessary in the manipulated strains constructed in this study to improve the fermentative ability close to the level of WT strain.

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