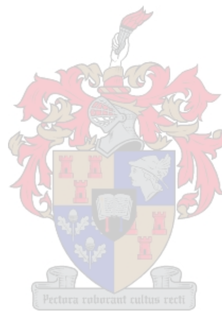
Channelling metabolic flux away from ethanol production by modification of gene expression under wine fermentation conditions

By

Eva Hutton Heyns



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This thesis is dedicated to my family and friends for their continuous support

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Biographical sketch

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Summary

There is a global demand for technologies to reduce ethanol levels in wine without compromising wine quality. While several chemical and physical methods have been developed to reduce ethanol in finished wine, the target of an industrially applicable biological solution has thus far not been met. Most attempted biological strategies have focused on developing new strains of the main fermentative organism, the yeast *Saccharomyces cerevisiae*. Gene modification approaches have primarily focused on partially redirecting yeast carbon metabolism away from ethanol production towards glycerol production. These techniques have met with some moderate success, thus the focus of the current study was to re-direct carbon flux towards trehalose production by moderate over-expression of the *TPS1* gene. This gene encodes trehalose-6-phosphate synthase, which converts glucose 6-phosphate and UDP-glucose to α,α-trehalose 6-phosphate. Previous data have shown that the overproduction of trehalose restricts hexokinase activity reducing the amount of glucose that enters glycolysis. Nevertheless, preliminary *TPS1* over-expression studies using multiple copy plasmids have shown some promise, but also indicated significant negative impact on the general fermentation behaviour of strains. In order to reduce such negative impacts of excessive trehalose production, a new strategy consisting in increasing the expression of *TPS1* only during specific growth phases and by a relatively minor degree was investigated. Our study employed a low-copy number episomal vector to drive moderate over-expression of the *TPS1* gene in the widely used industrial strain VIN13 at different stages during fermentation. The fermentations were performed in synthetic must with sugar levels representative of those found in real grape must. This, as well as the use of an industrial yeast strain, makes it easier to relate our results to real winemaking conditions. A reduction in fermentation capacity was observed for all transformed strains and controls. Expression profiles suggest that the *DUT1* promoter certainly results in increased *TPS1* expression (up to 40%) during early exponential growth phase compared to the wild type strain (VIN13). *TPS1* expression under the control of the *GIP2* promoter region showed increased expression levels during early stationary phase (up to 60%). Chemical analysis of the yeast and the must at the end after fermentation showed an increase in trehalose production =in line with the expression data of TPS1. Importantly, glycerol production was also slightly increased, but without affecting acetic acid levels for the transformed strains. Although ethanol yield is not significantly lower in the DUT1-TPDS1 strain, s statistically significantly lower ethanol yield is observed for over-expression under the *GIP2* promotor. Increasing trehalose production during stationary phase appears therefore to be a more promising approach at lowering ethanol yield and redirecting flux away from ethanol production. This controlled, growth phase specific over expression suggests a unique approach of lowering ethanol yield while not impacting on the redox balance.

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Opsomming

Wêreldwyd is daar ‘n aanvraag na tegnologie wat die etanol vlakke in wyn kan verminder sonder om wyngehalte te benadeel. Terwyl verskeie chemiese en fisiese metodes ontwikkel is om etanol in die finale wynproduk te verminder, is die soeke na 'n industrieel gebaseerde biologiese oplossing tot dusver nie gevind nie. Meeste biologiese strategieë fokus op die ontwikkeling van nuwe rasse van die primêre fermentatiewe organisme, naamlik *Saccharomyces cerevisiae*. Geen modifikasie benaderings het hoofsaaklik gefokus op die gedeeltelike kanalisering van koolstof metabolisme weg van etanol produksie na gliserol produksie. Hierdie benadering is net matiglik suksesvol, dus is ons huidige fokus om koolstof te kanaliseer na trehalose produksie deur gematigde oor-uitdrukking van die *TPS1* geen. Hierdie geen kodeer vir trehalose-6-fosfaat sintase, wat glukose-6-fosfaat en UDP-glukose omskakel na α, α-trehalose-6-fosfaat. Vorige data het getoon dat die oorproduksie van trehalose hexokinase aktiwiteit beperk en die hoeveelheid glukose wat glikolise binne gaan. Voorlopige *TPS1* oor-uitdrukking studies met behulp van multi-kopie plasmiede toon matige sukses, maar het ook ‘n negatiewe impak op die algemene fermentasie kapasiteit van die gis. Ten einde so 'n negatiewe impak van oormatige trehalose produksie te oorkom, is 'n nuwe strategie gevolg wat bestaan uit die verhoogde uitdrukking van die *TPS1* geen slegs gedurende spesifieke groei fases met baie lae vlakke van oor-uitdrukking. Ons studie gebruik 'n lae-kopie episomale vektor met matige oor-uitdrukking van die *TPS1* geen in die industriële ras VIN13 op verskillende stadiums tydens fermentasie. Die fermentasie is uitgevoer in sintetiese mos met suiker vlakke verteenwoordigend van dié van werklike wyn mos. Hierdie, sowel as die gebruik van 'n industriële gisras, maak dit makliker om ons resultate te vergelyk met regte wyn fermentasie kondisies. Verlaagde fermentasie kapasiteit is waargeneem vir alle getransformeerde stamme en hul kontroles. Geen uitdrukkings profiele dui op verhoogde *TPS1* uitdrukking (tot 40%) onder beheer van die *DUT1* promotor gedurende die vroeë eksponensiële groeifase wanneer vergelyk word met die wilde tiepe (VIN13). *TPS1* uitdrukking onder die beheer van die GIP2 promotor het verhoogde uitdrukking van tot 60% gedurende die vroeë stasionêre fase. Chemiese analise van die gis aan die einde van fermentasie dui op ‘n toename in trehalose produksie wat korreleer met die uitdrukking profiele van *TPS1*. Gliserol produksie is ook effens verhoog, maar sonder ‘n toename in asynsuur vlakke vir die getransformeerde rasse. Alhoewel etanol opbrengs nie aansienlik laer vir die DUT1-TPS1 ras is nie, is etanol opbrengs vir die oor-uitdrukking onder beheer van die *GIP2* promotor wel laer. Toenemende trehalose produksie gedurende stasionêre fase blyk dus 'n meer belowende benadering op die verlaging van etanol opbrengs en her-kanaliseering weg van etanol produksie. Hierdie benadering met die fokus op groeifase spesifieke oor-uitdrukking dui op 'n unieke strategie vir die verlaging van etanol opbrengs sonder om die redoks balans te beinvloed.

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Preface

This thesis is presented as a compilation of four chapters. Each chapter is introduced separately and is written according to the style of the journal Applied Microbiology and Biotechnology.

Chapter 1 **General Introduction and project aims**

Chapter 2 **Literature review**
Approaches to lowering ethanol in wine

Chapter 3 **Research results**
Construction of a recombinant industrial *Saccharomyces cerevisiae* strain for low ethanol fermentation

Chapter 4 **General discussion and conclusions**

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Chapter 1

Introduction and project aims

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1.1. INTRODUCTION

Over the past few decades, winemaking has changed dramatically and has had to keep up with the competitive nature of the global economy. There is a constant need for improving viticultural and oenological practices. Vine growing and wine making are biological processes, and the main contributors are grape vine and microbial organisms, in particular yeast. Many studies have focused on the improvement of wine making process and of wine quality by studying these biological systems (Pretorius, 2000). The traditional approach to wine making, and which continues to be used by some smaller and boutique wineries, was for the wine fermentation process to be carried out by the naturally occurring microbes in the vineyard and in the winery (Henschke, 1997). Today's competitive industry demands a more controlled, reliable and predictable production of wines on a larger industrial scale. This is the reason for the addition of pure yeast inocula that was introduced by Müller Thurgau in 1890 . In most instances *Saccharomyces cerevisiae* strains are inoculated into the grape must at the start of fermentation (Henschke, 1997; Pretorius, et al., 2003). *S. cerevisiae* not only converts fermentable sugars into ethanol but also plays a role in producing many flavour and aroma compounds in wine. These flavour compounds formed by yeast metabolism include esters, fatty acids and higher alcohols (Scudamore-Smith and Moran 1997; Pickering et al. 1998)

One of the more recent consumer and industry demands has been to lower the ethanol content of wines. One of the reasons for this is that high ethanol content can compromise the quality of wine, by creating a perception of increased hotness and viscosity and by masking other aromatic compounds (Gawel et al., 2007). Other reasons include the health risks involved in excessive alcohol consumption, and the cost to consumer as taxes are levied according to the alcohol content of beverages (de Barros, 2000; Kutyna et al., 2010). Comparative studies have shown that averarge ethanol concentrations of commercial wines have risen over the past two decades. This rise in ethanol content may be due to a number of factors including rising temperatures due to global warming (Catarino et al., 2011), as well as changes to viticultural practices aiming at increased ripeness of berries to improve flavour characteristics (Godden, 2000).

The different approaches for dealing with excessive ethanol can be divided into three groups, namely viticultural, mechanical or biological. Viticultural methods could include berry picking times and vine canopy control measures which influence the exposure of grapes to light and temperature. Physical methods may include removal of alcohol at the end of fermentation by reverse osmosis, dilution or distillation (Bui et al. 1986; Pickering et al.

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1999a; Mermelstein 2000). Fermentation management methods rely on regulation of fermentation conditions by temperature control, nutrient regulation or osmotic stress management (Attfield, 1997; d'Amore et al., 1987; Hinchcliffe et al., 1985).

Biological approaches focusing on the genetic modification of yeast also have the potential to address the ethanol problem, and have met with relative success in recent years (Kutyna et al., 2010).

These biological approaches target various genes that impact on central carbon metabolism, with the aim to redirect carbon flux. Most focus on genes involved in redirecting flux toward glycerol production. These include *GPD1* and *GPD2* encoding isozymes of glycerol 3-phosphate dehydrogenase (de Barros Lopes et al., 2000; Cambon et al., 2006; Eglington et al., 2002; Michnick et al., 1997; Nevoight et al., 1996; Remize et al., 2001; Remize et al., 1999), alcohol dehydrogenase (ADH) mutants (Drewke et al., in 1990), *PDC2* Pyruvate decarboxylase mutants (Nevoigt & Stahl, 1996; Schmitt & Zimmermann, 1982). Other approaches focused on the heterologous expression of genes that remove glucose from the system in order to lower ethanol, such as expression of the *GOX* gene from *Aspergillus niger*, encoding an enzyme converting glucose to gluconic acid (Pickering et al., 1999a). . Finally attempts have been made to modify the hexose transporters that facilitate the transport of glucose.

The approach described in this work is based on redirecting metabolic carbon flux towards the stress and reserve carbohydrates trehalose.The *TPS1* gene encodes trehalose-6-phosphate synthase, a key enzyme in the trehalose biosynthesis pathway (Francois et al., 2001). Trehalose is synthesized in two steps: First glucose 6-phosphate and UDP-glucose is converted to α,α-trehalose 6-phosphate by trehalose-6-phosphate synthase encoded by the *TPS1* gene. In the second step α,α-trehalose 6-phosphate and water are converted to trehalose and phosphate by trehalose-6-phosphate phosphatase (encoded by *TPS2* gene; Francois et al., 2001)(Fig1).

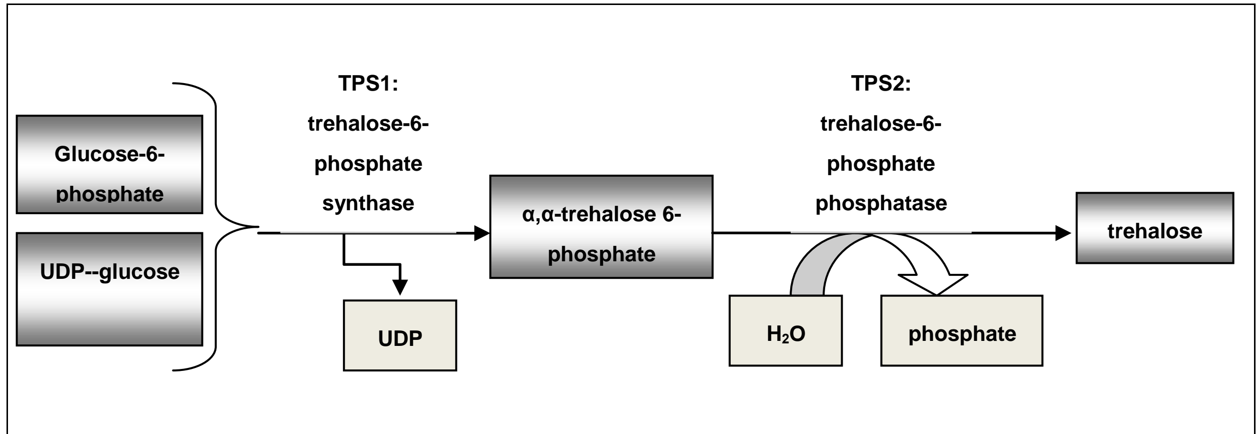

Figure 1: Trehalose synthesis from glucose 6-phophate and UDP-glucose

Trehalose-6-phosphate inhibits hexokinase activity. The overproduction of trehalose may therefore restrict the amount of glucose that enters glycolysis, in turn lowering the ethanol output, but also fermentative efficiency (Hohmann *et al.*, 1996). Preliminary studies on *TPS1* deletion and overexpression mutants in our laboratory (unpublished data) have shown that both over expression and deletion of the *TPS1* gene in the lab strain S288C leads to a decrease in ethanol yield, but also an overall reduction in fermentation rate (unpublished data). Both deletion and overexpression mutants produced less ethanol but had higher residual sugars at the end of fermentation (unpublished data). Glycolytic flux was impaired in the over expression strain thus accounting for the reduced fermentation efficiency and higher residual sugars. However, studies thus far have tended to use strong overexpression systems such as multiple copy plasmids and strong promoters combined to the TPS1 ORF. These excessively high expression levels may have been responsible for generating an excessive metabolic burden to the yeast, leading to the secondary effects that negatively impact on fermentation kinetics and a broad redirection of metabolic flux. Furthermore, in these studies, laboratory strains were employed for overexpression, and the fermentation conditions (low sugar levels) were not representative of real winemaking conditions.

Our study therefore focuses on improving the widely used industrial *Saccharomyces cerevisiae* strain - VIN13 to produce less ethanol in a controlled over expression study. The aim was to increase expression of the *TPS1* gene only during specific phases of growth and by a minor degree using two different promoters: The promoters of the *DUT1* gene to express the gene during the exponential growth phase and of the *GIP2* gene to activate gene expression during stationary phase. The aim therefore is to increase *TPS1* gene expression and hopefully enzyme activity without imposing additional stress on the yeast cell and without impacting on the redox balance. Maintaining redox balance is very problematic

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in most over-expression mutants as the production of ethanol regenerates reducing equivalents needed for the continuation of glycolysis.

1.2. PROJECT AIMS

The following aims were set for this project:

The first aim was to construct two *TPS1* over-expression strains under control of different promoters. These constructs and their controls (containing only promoter sequences) were transformed into the industrial VIN13 strain of *Saccharomyces cerevisiae*.

The second aim was to evaluate these two strains and their controls in synthetic wine, to establish the variations in ethanol yield, sugar consumption and trehalose production.

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Chapter 2

Literature review

Approaches to lowering ethanol in wine

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2.1. INTRODUCTION

Over past ten years there has been an increased demand for lower alcohol wines and de-alcoholised wines (Scudamore-Smith et al., 1997; Pickering et al., 1998; Schobinger et al., 1983; Anon et al., 1988; Heess et al., 1990; Hoffmann et al., 1990; Simpson et al., 1990; Howley et al., 1992). The demand for these wines mostly stems from health issues associated with excessive alcohol consumption and restrictions placed on the ethanol content in wines, such as taxes levied according to ethanol content in certain countries such asthe United States (Table 1) (de Barros et al., 2003; Scudamore-Smith et al., 1997; Pickering et al., 1998; Gladstones et al., 1999; Gladstones et al., 2000). In South Africa, the tax on unfortified wines is of R2.35/L, and R4.50 on fortified wines, as stated in the 2012 budget (http://www.treasury.gov.za/documents).

Table 1: Taxes levied on wine as per the Alcohol and tobacco tax and trade bureau US department of Treasury (http://www.ttb.gov/tax_audit/atftaxes.shtml) last reviewed **09/04/2012**

PRODUCT	TAX	TAX PER PACKAGE (usually to nearest cent)
Wine	**Wine Gallon**	**750ml bottle**
14% Alcohol or Less	$1.07[1]	$0.21
Over 14 to 21%	$1.57[1]	$0.31
Over 21 to 24%	$3.15[1]	$0.62

Another concern is that higher alcohol concentrations also compromise wine quality and can mask the sensorial characteristics of wines. High alcohol levels can also lead to sluggish or stuck fermentations (Guth & Sies, 2002).

However, in the same period, average alcohol content of wine has increased in many regions. There are a number of possible reasons for increased ethanol in modern day wines. One of the reasons can be linked to changes in viticulture such as vine canopy management techniques and/or berries that are left to mature for a longer period. A warmer climate may affect berry ripeness and sugar content. While many of these influences produce full, rich, complex and fruity properties, they also lead to higher sugar levels that in turn will lead to higher ethanol production, with many wines today reaching 15% ethanol (v/v) and above (Godden et al., 2000).

Several approaches have been used to reduce the ethanol content of wine. These include viticultural, physical and biological strategies.

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2.2. VITICULTURAL AND PHYSICAL APPROACHES

There are a few methods that can be used to lower ethanol in wine and these include viticultural methods like picking berries earlier to prevent over-ripening. However, this will have an influence on the sensory properties and complexity of the wines (Pickering et al., 2000). Other methods are used post fermentation and range from very basic procedures such as dilution and evaporation, to vacuum distillation and membrane filtration to more costly and complex techniques such as spinning cone technology and reverse osmosis (Schobinger et al., 1986).

2.2.1 REVERSE OSMOSIS

The most widely used method for reducing or removing ethanol from wine is reverse osmosis (Pickering et al., 2000). During reverse osmosis the larger molecules such as the flavour compounds of wine (organic acids and phenolics) are separated from the smaller water and alcohol molecules by a selective membrane (Fig I). This process involves wine being pumped through a membrane at a pressure greater than the osmotic pressure so as not to allow natural flow of the solvent (to equalise the concentrations of solutions at opposite sides of the membrane). This causes ethanol and water with smaller molecular weights to diffuse selectively through the membrane, leaving the concentrated organic acids and phenolic compounds behind. This is followed by perstraction (when a solution is permeated through a membrane and subsequently extracted with solvent) technology that separates the water and the alcohol, and the water is then added back into the wine. The removal of alcohol thus reduces the volume of the final product. Reverse osmosis relies on two types of a membranes, an ethanol-permeable and a selective ethanol-retention membrane. The permeate-exchange unit controls the water and ethanol balance of the system. The end product is still classified as wine based on its composition (Bui et al., 1986). This method is more advantageous than some other techniques, since there is no heating involved and the wine therefore retains its natural flavour. Besides the high cost of this process, an additional disadvantage of this method is wine volume loss due to the removal of the alcohol.

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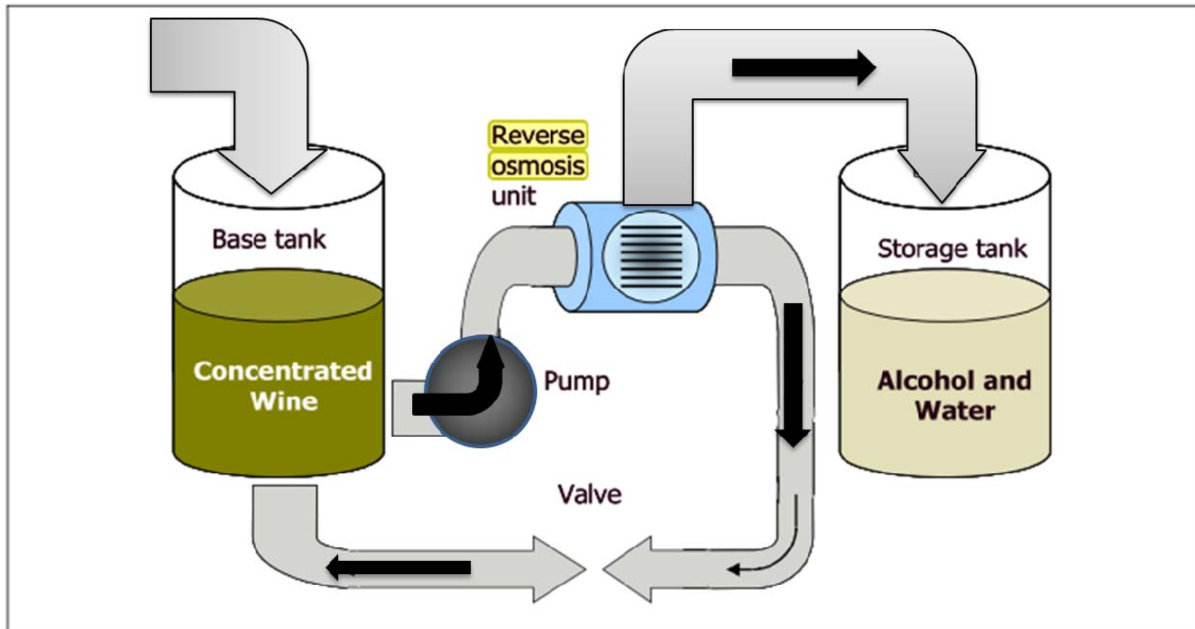

Figure 1. Reverse osmosis process of wine (adapted from Mermelstein, 2000)

2.2.2 SPINNING CONE COLUMN (SCC)

The technique was first developed in the USA in the 1930s and has since been modernised to a multi-stage strip column in Australia. This technology is currently marketed world-wide by the Californian Company ConeTech Inc. (Theron et al., 2006). With the SCC technique it is possible to reduce the level of alcohol to below that achieved by reverse osmosis. Both methods inevitably reduce the volume of the wine by the removal of the alcohol (Hay, 2001).The SCC is a gas-liquid contact device comprising a vertical counter-current flow system that includes a series of alternate rotating and stationary metal cones. The upper surfaces are moistened by a thin film of wine (Pickering et al., 2000) (see Fig II). A gravity and vacuum pump pulls the wine that is fed into the top of the column down through the first stationary cone and into the first rotating cone. The wine is spun into a fine liquid film, moving it up and over the lip of the cone into the next stationary cone, thus starting the process all over. About half of the wine volume is converted into an inert stripping gas called 'cold steam', which is just above room temperature (Hay, 2001). The vaporised cold steam feeds back into the bottom of the column and moves upward over the thin film of wine running downwards. Underneath each rotating cone is a fin that mobilises the rising stream into a turbulent state. The fins mobilising of the vapour combined with the spinning motion of the wine travelling downward removes the volatile flavour and aroma compounds and captures them in a liquid form.

There are three stages to this process: Firstly, when the wine passes through the cone it is stripped of its flavour and aroma compounds. In the second stage the wine runs back down the column where the cold steam vaporises the alcohol from the wine. During the third stage the flavour and aroma compounds are added back into solution (see Fig II).

The cost of this treatment is high but varies according to the volume of wine being treated (Theron et al., 2006).The main disadvantage is that the process requires heating of the wine (Pickering et al., 2000).This technique has its advantages as it preserves essential flavours and aromas. Other advantages include high efficiency, minimal thermal damage and the ability to handle highly viscous juice (Sykes et al., 1992; Gray et al., 1993; Pyle et al., 1994).

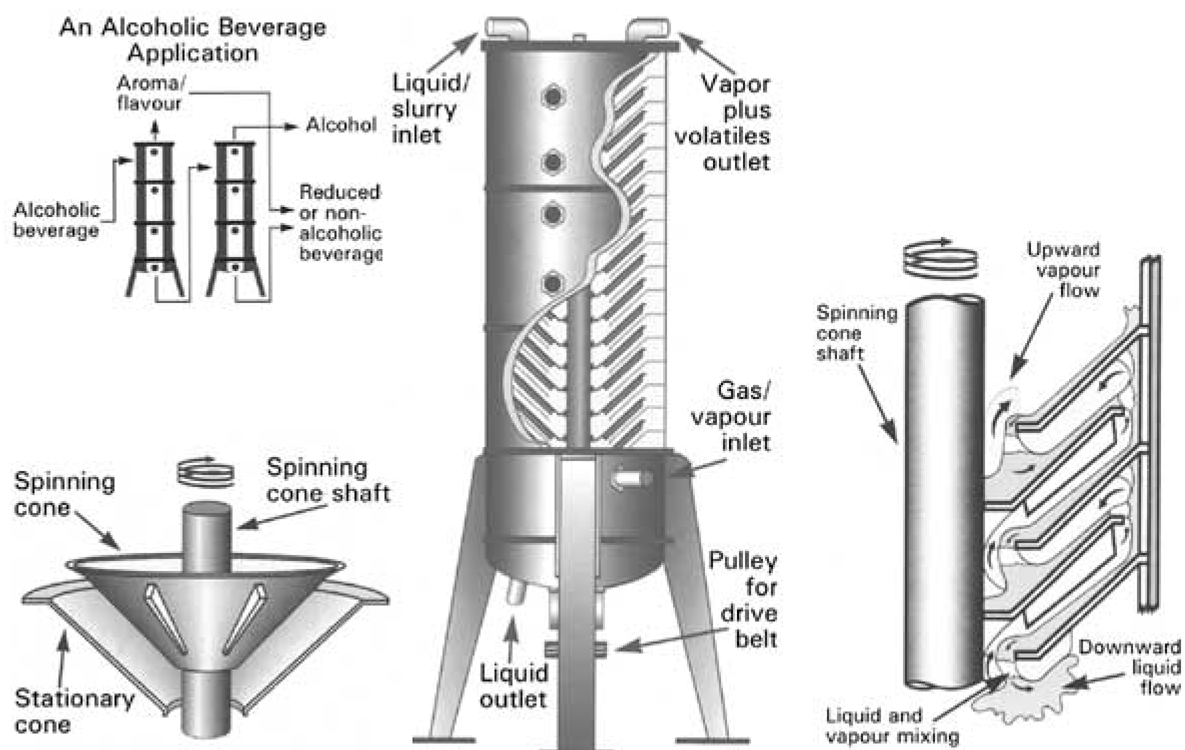

Figure 2: Spinning cone column (SCC) technique for lowering alcohol in wine (adapted from http://www.winebussiness.com)

The problem with these physical techniques is that they tend to change wine character and are very costly. The heating process that some of these post fermentation physical removal techniques include will have a direct effect on the aroma composition of wines. The problem of cost may not only arise from the process in itself, but also from the additional cost of equipment transport and hire since not all wineries can afford the equipment to perform these techniques (Bui et al., 1986; Pickering et al., 1999a; Mermelstein et al., 2000).

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2.3. NON-GMO BASED BIOLOGICAL APPROACHES

The public perception of genetically modified organisms is largely negative and in most countries the sale of wine either containing GMOs or having been manufactured with GMOs is problematic (Pretorius et al., 2000; Pretorius et al., 2005). Although GM studies improve our knowledge of how carbon flux is affected during alcoholic fermentation, they are not yet widely accepted and classical methods to improve wine yeast are therefore employed. Redirecting carbon flux in *S. cerevisiae* has proven difficult as the selection pressure for this species has maximised ethanol production capacity under aerobic and anaerobic conditions as the production of ethanol balances cellular redox and allows glycolysis to continue producing the energy needed for yeast cell growth and replication (Field et al., 2009; Piskur et al., 2006).

Several strategies have been attempted to generate lower ethanol-yielding wine yeast strains. A major target in many of these cases has been to redirect carbon flux towards glycerol instead of ethanol. The production of glycerol is considered favorable as glycerol can make positive contributions to the mouth feel and viscosity of wine, creating a perception of smoothness and sweetness (Gawel et al., 2007). Some attempts to enhance glycerol production by non-GM methodolgies such as breeding and directed evolution have been proposed in the past. Some other approaches include classical strain-selection and -modification methods, such as variant selection, mutagenesis, hybridization and spheroplast fusion (Pretorius et al., 2000). Yeast hybrids can be created from *S. cerevisiae* crossed with some of the senso stricto yeasts (including *Saccharomyces kudriavzevii*, *Saccharomyces cariocanus*, *Saccharomyces mikatae*, *Saccharomyces bayanus* and *Saccharomyces paradoxus*). A natural hybrid of *S. cerevisiae* and *Saccharomyces kudriavzevii* does show an increased production of glycerol but this seems to have no effect on ethanol yield (Combina et al., 2012). Directed evolution is the application of controlled selection pressures to growing cells to encourage adaptation and acquisition of a desired trait. To enhance glycerol production, conditions with high levels of sulphite at an alkaline pH were used. In these conditions, sulphite binds to acetaldehyde, reducing its availability for ethanol production and oxidation of NADH, and therefore channelling carbon flux towards glycerol biosynthesis. The adapted strain produced 41% more glycerol than the wild type and had enhanced tolerance to sulphite. The increase in glycerol production also led to a decrease in ethanol concentration in anaerobic conditions, decreasing from 47.6±0.1g/L to 46.5±0.4g/L with an increase in acetic acid (Chambers et al., 2012).

Another focus of such research has been based on observations that spontaneously fermented wine sometimes shows high levels of glycerol and a decrease in ethanol

production. This glycerol increase indicates a possible contribution of non-*Saccharomyces* yeast (Romano et al., 1997; Henick-Kling et al., 1998). *Candida stellata* has been known to produce increased glycerol concentrations of between 10 and 14 g/L (Ciani et al., 1995; Ciani et al., 1998), whereas *S. cerevisiae* usually produces only between 4 and 10 g/L (Radler et al., 1982; Ciani et al., 1998; Prior et al., 2000). As for *S. cerevisiae*, the increased acetic acid production that is coupled to increases in glycerol yield is problematic as it affects wine quality (Prior et al., 2000).

Other apiculate yeasts such as *Kloeckera apiculata* and *Hanseniaspora guilliermondii* also produce higher levels glycerol although acetic acid levels are also increased on these species (Ciani et al., 1995). However, there have been reports that not all strains of *Kloeckera* spp. form high levels of acetic acid (Romano et al., 1992). *K. apiculata* produces high-glycerol and low-ethanol ratios during fermentation. These results still need to be verified in real wine must (Romano et al., 1997). Although these approaches have not resulted in an effective lowering of ethanol yield without compromising wine quality, it indicates that adaptive evolution could possibly result in a lower ethanol producing yeast strain without the use of genetic modifications.

However, all the approaches described above are based on random processes in which genomic regions or entire genomes are recombined or reorganised. These methods are not controlled enough for modifying wine yeast in a specific manner. While any of the approaches may result in strains able to improve some desired aspects, they may simultaneously compromise other desired traits. These methods do have their advantages, as they do not involve GM. However up to date there seems to be no yeast strain that would have been generated through such approaches and produce significantly less ethanol. A particular challenge in this regard is the absence of selection conditions that would support the preferential survival of yeast strains with reduced ethanol production. If such conditions could be established, an approach based on directed evolution might prove successful.

2.4. GMO-BASED APPROACHES

As post fermentation processes to lowering alcohol are costly and influence wine quality and classical breeding strategies are unspecific and unreliable (Pretorius et al., 2000; Schobinger et al., 1986), perhaps the most straightforward and cost effective strategy is to look at genetically modified wine yeast strains. Most of the studies that are addressing ethanol reduction have focused on redirecting glycolytic flux away from ethanol, in particular towards increased glycerol production. Such strategies include modifying the expression of genes involved in central carbon and glycerol metabolism or transport such as *GPD1, GPD2,*

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FPS1 and *TPI1 (*as indicated by circles in Fig III below). Other studies have incorporated genes from bacterial or fungal species into *S. cerevisiae*. Examples of approaches based on heterologoes gene expression are the use of the glucose oxidase gene *GOX1* from *Apergillus niger*. The transformants reduce ethanol production by breaking down glucose into gluconic acid, making it unavailable for glycolysis (Malherbe et al., 2003). In a second example, the bacterial gene *noxE* (NADH oxidase) derived from *Lactococcus lactis* was incorporated into *S. cerevisiae* to reduce the intracellular NADH and reduce ethanol yield. Oxygen is required for the enzyme to be effective (Heux et al., 2006).

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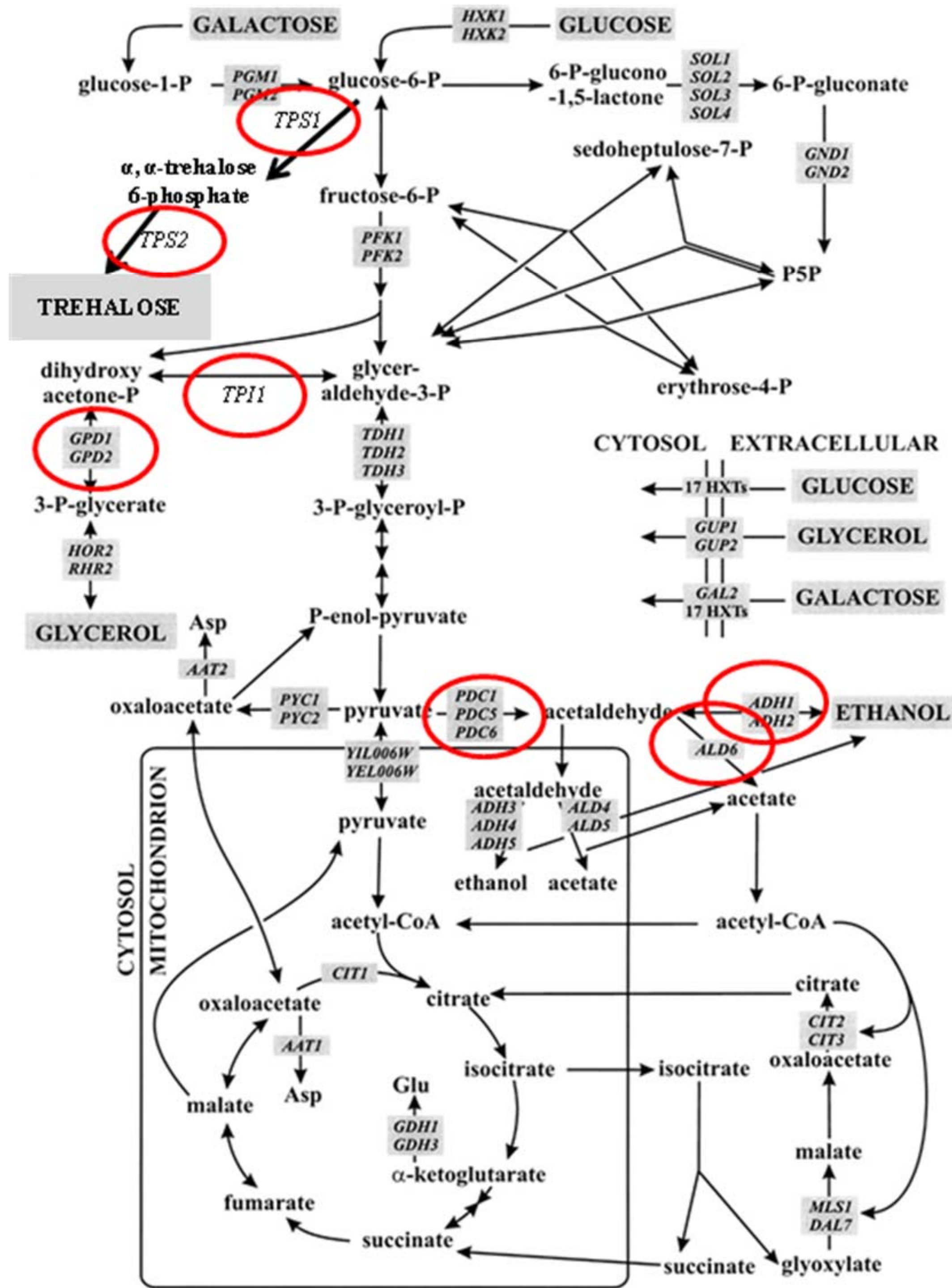

Figure 3: Central carbon metabolism and genes encoding the relevant enzymes. Genes circled in red indicate those that have been targeted to generate low ethanol strains (adapted from Kuepfer, 2005, Genome Res; 15:1421-1430).

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2.4.1 DELETION OF ALCOHOL DEHYDROGENASE (ADH) ENCODING GENES

In *S. cerevisiae* five isozymes of alcohol dehydrogenase (ADH) have been described. *ADH1* encodes one of the most important enzymes in alcoholic fermentation as it reduces acetaldehyde by converting it to ethanol (Leskovac et al., 2002; de Smidt et al., 2011; Lutstorf *et al*., 1968). This reaction regenerates NAD^+ from NADH and is essential for maintaining redox balance in the cytoplasm during fermentation as the oxidised co-factor is vital for glyceraldehydes-3-phosphate oxidation during glycolysis. The second isozyme is encoded by *ADH2,* which oxidises ethanol to form acetaldehyde (Cirlacy et al., 1975; Cirlacy et al., 1979; Denis et al., 1981). *ADH2* is involved in converting acetaldehyde into ethanol. This is observed during prolonged fermentation where the yeast cell is stressed (Millan et al., 1990). *ADH1* and *ADH2* are cytosolic isozymes whereas *ADH3* is a mitochondrial isozyme that under anaerobic conditions transports NADH to the cytosol for the production of NAD^+ by reduction of acetaldehyde (Bakker et al., 2000). The other two known isozymes are *ADH4* and *ADH5* but the function of these are yet unknown. Other than the classic isozymes ADH1-5 other enzymes that relate to ADH activity are *SFA1*, *ADH6* and *ADH7*. *SFA1* has both glutathione- dependent formaldehyde and alcohol dehydrogenase activity, and is involved in formaldehyde detoxification (Wehner et al., 2003). ADH6 and ADH7 gene products show a stringent specificity for NADPH and are described as cinnamyl ADHs (Gonzalez et al., 2000; Larroy et al., 2002). Although many studies have since been done on ADH, one of the first was that of Drewke et al. (1990). An adh0 strain of *S. cerevisiae* was created by deleting *ADH1*, *ADH3*, and *ADH4* and a point mutation was introduced in the gene *ADH2* coding for the glucose-repressible isozyme *ADH2*, thus completely removing our alcohol dehydrogenase (ADH) isozymes of the five that where at that time identified (ADH1-5).This point mutation inactivates ADH2 completely.. During glucose metabolism this strain (adh0) produced more glycerol and less ethanol but also high levels of acetaldehyde and acetate. Ethanol production in adh0 cells seemed to be dependent on mitochondrial electron transport. Fermentations using these deletion strains could not run to completion and were left with high residual sugars (Ciriacy, 1975; Johansson et al., 1984; Drewke et al., 1990). Although carbon flux is re-directed towards glycerol production lowering the ethanol yield, there are high levels of acetaldehyde and acetate produced formed that would compromise wine quality. Acetaldehyde can give wine a sour or metallic taste when concentrations are too high, where as acetic acid affects the volatile acidity, leading to a vinegary taste is present in too high amounts. A more recent study by de Smidt et al. (2011) aimed at establishing the role of alcohol dehydrogenase isozymes ADH1 to ADH5 in *S. cerevisiae* and to determine whether the enzymes are able to substitute functions *in vivo*. Quadruple deletion mutants were created, each mutant containing only one genomic *ADH* gene. During

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this study the Q1 mutant (quadruple deletion mutant containing only *ADH1* in genome) showed that *ADH1* is the only enzyme that efficiently performs the task of reducing acetaldehyde to ethanol and regenerating the NAD^+ from NADH that is necessary for carbohydrate metabolism. This Q1 mutant was also able to utilize ethanol as sole carbon source or during diauxic growth on glucose (de Smidt et al., 2011; Lutstorf and Megnet, 1968). The deletion of *ADH1* lead to an increase in glycerol production and in turn increased acetaldehyde levels. Strains expressing only *ADH2* (Q2) or *ADH3* (Q3) respectively yielded less ethanol than the Q1 strain, and were able to oxidise the additional ethanol added. The strains expressing only *ADH4* (Q4) and *ADH5* (Q5) were unable to utilise produced ethanol, and were unable to grow on media containing ethanol as carbon source. The study suggests that it is unlikely that ADH4 and *ADH5* are involved in ethanol production.

2.4.2 ALTERATIONS OF GLYCEROL METABOLISM

Genes involved in glycerol production and transport, namely *GPD1*, *GPD2* and *FPS1* have been major targets to achieve lower ethanol yields. Glycerol is produced by converting dihydroxyacetone to glycerol 3-phosphate by glycerol 3-phosphate dehydrogenase (GPDH) and then dephosphorylated by the glycerol 3-phosphatase enzyme (Gancedo *et al.,* 1968). Studies have been conducted on enhancing glycerol production by over-expressing *GPD1* or *GPD2* genes. By over-expression of the *GPD1* gene glycerol production is increased. The overproduction of glycerol through this pathway leads to an excess in NAD^+ production. The system tries to maintain redox balance and rectifies the NAD^+ over production by converting NAD^+ to NADH increasing acetaldehyde and acetic acid levels in the process. This is the reason why an increase in glycerol is usually associated with an increase in acetic acid (de Barros Lopes et al., 2000; Cambon et al., 2006; Eglington et al., 2002; Michnick et al., 1997; Nevoight et al., 1996; Remize et al., 2001; Remize et al., 1999).

Another approach to increase flux towards glycerol production is to target genes that regulate channeling proteins. The *FPS1* gene encodes Fps1p which is a member of the Major Intrinsic Protein (MIP) family of channeling proteins with the main function of regulating intracellular glycerol by glycerol export rather than uptake. It has been shown that the overexpression of *FPS1* increases glycerol production and suppresses the growth defect of the *TPS1* mutant on carbon sources such as glucose and fructose. *TPS1* over expression plays a role in the regulation of glycolysis, as its gene product restricts the influx of glucose into the pathway. The proposed reason for this is that trehalose 6-phophate inhibits hexokinase *in vitro*.(Blázquez et al., 1993 Teusink et al.,1998; Thevelein et al.,1995)

A mutated form of the *FPS1* gene leads to a constantly open form of the channelling protein, resulting in glycerol leakage from the cell which is compensated for by the production of more glycerol. Unfortunately, this mutant also affects biomass production and yeast growth on glucose (Luyten et al., 1995; Tamás et al., 1999; Van Aelst et al., 1991).

Cordier et al. (2007) attempted to combine some approaches in a single strain in the hope of decreasing ethanol and increasing glycerol production. The genes that they selected for this study can be placed into groups: *GPD1* and *FPS1 (*involved in glycerol transport and production), *TPI1* (involved in the glycolytic branch point conversion of DHAP to GAP), and *ADH1* and *ALD3* (involved in the production of ethanol and acetic acid from acetaldehyde).

Firstly *GPD1* encoding glycerol phosphate dehydrogenase was introduced into a tpi1Δ mutant defective in triose phosphate isomerase. This reduced the dihydroxyacetone phosphate and glycerol-3-phospate which in turn inhibit myo-inositol synthase that catalyzes the formation of inositol-6-phosphate from glucose-6-phosphate. The *ADH1* gene that encodes major NAD+ alcoholic dehydrogenase enzyme was then deleted. *ALD3* which encodes cytosolic NAD+ dependent aldehyde dehydrogenase was over-expressed to ascertain whether the increase in acetaldehyde formation could be reduced in favour of NADH for glycerol production. This newly combined mutant was able to produce 0.46 g glycerol/g glucose) at a production rate of 3.1mmol /(g biomass h).The flux control coefficient was shifted to glycerol efflux due to intracellular accumulation of glycerol that can be overcome by the overproduction of glycerol exporter encoded by the *FPS1* gene.

The overexpression of glyceraldehyde-3-phosphate dehydrogenase gene, *GPD1* under control of the *ADH1* promoter, is currently seen as most effective method of lowering ethanol yield by up to 35% and increasing glycerol production, but the problem is that the decrease in ethanol yield does not restore redox balance and results in higher acetate yields. These expression strains also produced elevated concentrations of acetaldehyde, acetoin and 2,3-butanediol and succinate.

Excessive acetic acid production can be prevented by deletion of *ALD* genes in *GPD* overexpression strains. The *ALD6* gene encodes a cytosolic aldehyde dehydrogenase, and converts acetaldehyde to acetate, it is activated by Mg2+ and utilizes NADP+ as the preferred coenzyme (Saint-Prix et al*.,* 2004; Navarro-Avino et al**.**,1999) In yeasts cells lacking glucose-6-phosphate dehydrogenase activity the aldehyde dehydrogenase ALD6 gene is essential in providing NADPH (Grabowska et al., 2003). Deletion of the ALD6 gene results in lowered acetate yield (Remize et al., 2000). This deletion was applied to wine-derived laboratory *GPD1* overexpression strains but these strains cannot be compared to

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industrial strains as lab strains usually don't perform well under industrial wine making conditions, and is thus not representative of industrial strains (Dequin, 2001; Eglington et al., 2002; Remize et al., 1999).

A study by Cabon et al (2006) reported on *GPD1* over-expression combined with deletion of *ALD6* in a wine yeast strains. These strains had lowered acetate production and glycerol production was increased with the ethanol production being 15 to 20% lower compared to the control. The wine quality is still influenced because inefficient reduction of 2, 3-butanediol leads to acetoin accumulation. The acetaldehyde branch point needs to be investigated to optimally adjust metabolite formation (Cambon et al., 2006; Eglington et al., 2002)

In order to rectify the problem of increased acetoin overproduction in mutants over-expressing *GPD1* with *ALD6* deletions *BDH1* was over-expressed. *BDH1* encodes 2, 3-butanediol dehydrogenase that converts acetoin to innocuous 2, 3- butanediol. Over-expression of the BDH1 gene enables 85-90% of the overproduced acetoin to be converted into 2, 3-butanediol, a compound that does not affect the sensory attributes of the wine (Ehsani et al*.,* 2009).A study by Varela et al., 2012 showed a decrease in acetoin levels by converting it to 2, 3-butanediol and also showed a decrease in acetaldehyde levels. With all strains the acetoin levels were below the sensory threshold although the acetaldehyde levels were still above the acceptable sensory threshold.

2.4.3 INTRODUCTION OF GLUCOSE OXIDASE (GOX) INTO *S. CEREVISIAE* TO REDUCE GLUCOSE AVAILABILITY

The *GOX* gene encodes the glucose oxidase enzyme (GOx) that catalysis the breakdown of glucose into D-glucono-δ-lactone and hydrogen peroxide. A transgenic strain of *S. cerevisiae* was generated by incorporating the *GOX* gene from *Aspergillus niger* under transcriptional control of the yeast *PGK1* promoter into the yeast genome. The secretion of Gox by the transgenic strain into the must lowers the glucose content of the must by converting it to D-glucono-δ-lactone and gluconic acid (Fig IV) thus reducing the ethanol content. The problem is that large amounts of gluconic acid are produced leaving the wine with a high titratable acidity (Pickering et al*.,* 1999a). The transgenic strains reduced the ethanol content by up to 1.8 %(v/v).This method may be unsuitable for industrial wine fermentations as the Gox enzyme activity requires high levels of oxygenation (Malherbe et al., 2003).

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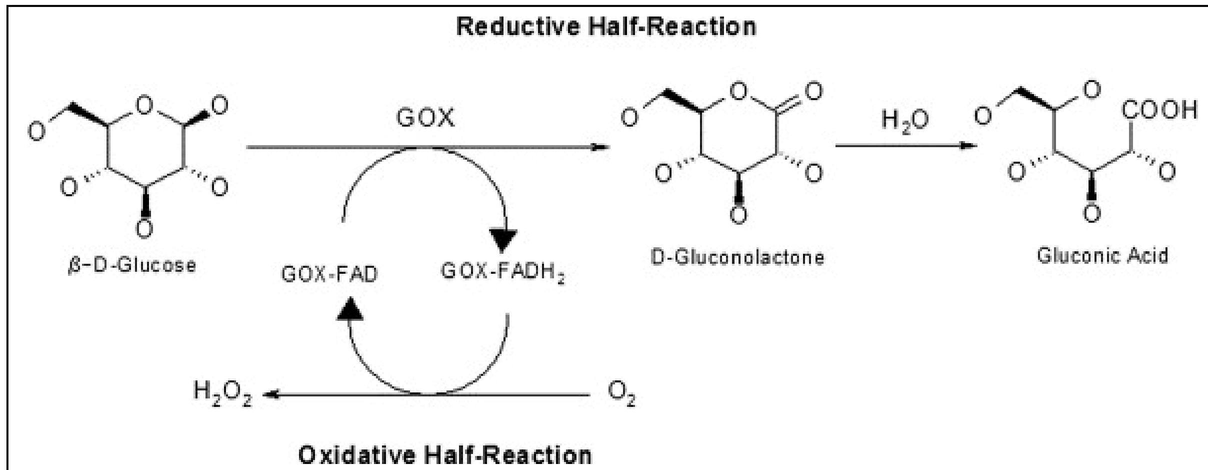

Figure 4: Glucose oxidase (GOX) pathway (Simpson et al., 2007)

2.4.4 NADH OXIDASES (NOX) OVER-EXPRESSION TO REDUCE INTRACELLULAR NADH

Another approach based on co-factor engineering was used by Heux et al. (2006) by over-expressing the *Lactococcus lactis* gene *noxE* (which codes an H_2O-forming NADH oxidase).The focus was to develop a yeast strain producing NADH oxidase to reduce ethanol yield. This enzyme specifically utilises NADH in the presence of oxygen (Heux et al., 2006)., thus anaerobic conditions are necessary. The approach was to direct carbon flux towards multiple metabolites rather than something specific which could lead to the accumulation of a compounds which negatively affect wine quality. This led to a reduction in ethanol of up to 15% but the mutants showed impaired growth and fermentation performance reducing sugar consumption by 50% and increasing acetaldehyde, acetate and acetoin production.

2.4.5 DIMINISHED PYRUVATE DECARBOXYLASE (PDC) ACTIVITY TO INCREASE GLYCEROL PRODUCTION

Pyruvate decarboxylase is the enzyme that catalyses the decarboxylation of pyruvic acid to acetaldehyde and carbon dioxide during fermentation. Previous deletion studies have been done on the pyruvate decarboxylase (PDC) mutants, but the deletion of all three genes *PDC1*, *PDC5* and *PDC6* rendered *S. cerevisiae* incapable of growing in medium containing only glucose as carbon source with excess NADH inhibiting glycolytic flux. However, deletion of only the regulatory *PDC2* gene led to diminished transcription of the *PDC1* structural gene that in turn resulted in diminished PDC activity. Diminished transcription of *PDC1* yielded 4.7

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times more glycerol than that of the wild type in a strain producing only 19% of its normal PDC activity. Overexpression of *GPD1* resulted in a 20-fold increase in GPD activity with a 5.6 times increase in glycerol production. When both the deletion of *PDC2* and the overexpression of *GPD1* were combined in one mutant strain, the glycerol increase was 8.1 times that of the wild type. All these mutants resulted in decreased ethanol production and increased glycerol production although there is an increase in acetate yield (Nevoigt & Stahl, 1996; Schmitt and Zimmermann, 1982).

2.4.6 DELETION OF TRIOSE PHOSPHATE ISOMERASE (TPI) TO INCREASE GLYCEROL PRODUCTION

During glycolysis triose phosphate isomerase (TPI) plays an important role in efficient energy production and is of interest as it is an important branch point in the glycolytic pathway(Fig III), as it catalysis the conversion between dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (GAP). The TPI gene deletion causes an accumulation of dihydroxyacetone phosphate which can no longer be channelled into the glycolytic pathway, leading to an increased glycerol of as high as 80-90% with an yield of 1 mol of glycerol per mol of glucoseand a decrease in ethanol production (Compagno et al., 1996; Ciriacy et al., 1979; Cordier et al., 2007). These deletion strains are not able to grow on media with glucose as sole carbon source due to lack of NADH supply (Compagno et al., 2001; Overkamp et al., 2002). Although the total elimination of the *TPI1* gene is therefore unsuitable for biotechnological purposes, a partial or controlled regulation of the expression of this gene might yield desirable results. Deletions in *REB1*, *RAP1* or *GCR1* binding sites of the *TPI1* promoter region reduce Tpi1p activity. However, the deletion of *RAP1*- and *GCR1*-binding sites has been shown to have no impact on glycerol and ethanol production (Scott et al., 1993; Clifton et al., 1981; Uemura., 1990; Uemura., 1999).

2.4.7 DELETION AND OVER EXPRESSION OF TREHALOSE-6-PHOSPHATE SYNTHASE (TPS) TO SHIFT CARBON FLUX TOWARD TREHALOSE PRODUCTION

TPS1 encodes the synthase subunit of trehalose-6-phosphate synthase/phosphatase complex, which synthesizes the storage carbohydrate trehalose. TPS expression is induced by a stress response and repressed by the Ras-cAMP pathway (Winderickx et al., 1996; Bell et al., 1992; Bell et al., 1998). Trehalose is synthesized in two steps: First glucose 6-phosphate plus UDP-glucose is converted to α,α-trehalose 6-phosphate by trehalose-6-phosphate synthase encoded by the *TPS1* gene. In the second step α,α-trehalose 6-phosphate and water are converted to trehalose and phosphate by trehalose-6-phosphate phosphatase encoded for by the *TPS2* gene (Francois et al., 2001). Trehalose-6-phosphate

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inhibits hexokinase activity (Hohmann et al., 1996), which can affect glycolysis by restricting the amount of glucose that enters glycolysis during the switch to fermentative metabolism (Hohmann et al., 1996).

In a study done by Bosch et al. (unpublished data) deletion mutants were screened for altered ethanol yields. The strain with a deletion of the *TPS1* gene in a laboratory strain (selected from the EUROSCARF deletion library) showed an accelerated fermentation rate, lower ethanol yield and significantly higher glycerol yield (3.6±0.4) than the wild type (2.3±0.2)). During the same study the *TPS1* gene was over-expressed in a laboratory strain under control of the PGK1 promoter. Fermentations for over-expression strains showed lower ethanol and glycerol yield and a reduced fermentation capacity with higher residual sugars (unpublished data).The reduced fermentation capacity of this over-expression strain can be due to partial inhibition of glycolytic flux. Although trehalose was not measured during this study it is hypothesised that trehalose levels might be increased, not only inhibiting hexokinase mediated glucose flux trough glycolysis but also the distribution of carbohydrates. This hypothesis was supported by the reduced levels of glycerol and ethanol produced.

2.4.8 COMBINED APPROACHES

The most recent study of these combined approaches was reported by Varela et al in 2012. This study used previously studied gene modifications that influenced ethanol production and combined them in one study using the same genetic background. The Strain that was used for all gene modification was AWRI1631, a stable haploid with a deletion of the HO locus (Borneman et al., 2008). As indicated in Table 2 (significant changes in ethanol highlighted in red) some of the gene modifications led to significantly lower ethanol levels, the most significant being those involving over-expression of *GPD1* with a reduction of up to 35% when compared to the parental strain. Additional modifications had to be implemented to avoid production of unwanted metabolites such as acetate, which could be improved by the deletion of *ALD6* in *GPD1* over-expression strains (Remize et al., 2000)

Table 2: Genetic modification of constructed strains and ethanol production compared to parental strain AWRI1631 strain

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Strain	Modified gene(s)	Genetic modification	Ethanol (%)[a]	Significance[b]
AWRI1631	None	None (parental strain)	100 ± 1.4	NS
AWRI1631 ΔACO1	*ACO1*	ORF deletion	99 ± 0.6	NS
AWRI1631 ΔACO2	*ACO2*	ORF deletion	99 ± 0.2	NS
AWRI1631 ΔADH1	*ADH1*	ORF deletion	100 ± 0.6	NS
AWRI1631 ΔADH3	*ADH3*	ORF deletion	99 ± 0.4	NS
AWRI1631 ΔADH1ΔADH3	*ADH1, ADH3*	ORF deletion	99 ± 0.6	NS
AWRI1631 ΔFRDS1	*FRD1*	ORF deletion	100 ± 0.9	NS
AWRI1631 ΔGPH1	*GPH1*	ORF deletion	99 ± 0.3	NS
AWRI1631 ΔGRR1	*GRR1*	ORF deletion	100 ± 1.5	NS
AWRI1631 ΔHXK2	*HXK2*	ORF deletion	97 ± 0.4	S
AWRI1631 ΔIDH1	*IDH1*	ORF deletion	99 ± 0.4	NS
AWRI1631 ΔIDP2	*IDP2*	ORF deletion	99 ± 0.6	NS
AWRI1631 ΔKGD1	*KGD1*	ORF deletion	100 ± 0.1	NS
AWRI1631 ΔMDH1	*MDH1*	ORF deletion	99 ± 1.4	NS
AWRI1631 ΔMIG1	*MIG1*	ORF deletion	97 ± 0.8	S
AWRI1631 ΔMIG2	*MIG2*	ORF deletion	99 ± 0.4	NS
AWRI1631 ΔOSM1	*OSM1*	ORF deletion	99 ± 0.4	NS
AWRI1631 ΔPDC1	*PDC1*	ORF deletion	100 ± 2.1	NS
AWRI1631 ΔPDC5	*PDC5*	ORF deletion	97 ± 0.3	S
AWRI1631 ΔPYC1	*PYC1*	ORF deletion	99 ± 0.8	NS
AWRI1631 ΔPYC2	*PYC2*	ORF deletion	99 ± 1.7	NS
AWRI1631 ΔTPI1	*TPI1*	ORF deletion	Stuck ferment[c]	NA
AWRI1631 FPS1Δ11	*FPS1*	Truncated Fps1p	92 ± 0.4	S
AWRI1631 gcrTPI1	*TPI1*	Point mutation in TPI1 promoter at GCR1 binding site	98 ± 0.9	S
AWRI1631 rapTPI1	*TPI1*	Point mutation in TPI1 promoter at RAP1 binding site	100 ± 0.7	NS
AWRI1631 aspTPI1	*TPI1*	Point mutation in TPI1 to change Glu_{165} to Asp in Tpi1p active site	94 ± 0.3	S
AWRI1631 GOX	*GOX*	Expression of glucose oxidase from *Aspergillus niger*	95 ± 2.1	S
AWRI1631 PYC1	*PYC1*	Promoter replacement	99 ± 0.4	NS
AWRI1631 MDH2	*MDH2*	Promoter replacement	98 ± 0.3	S
AWRI1631 FUM1	*FUM1*	Promoter replacement	100 ± 0.5	NS
AWRI1631 FRDS1	*FRD1*	Promoter replacement	98 ± 0.5	NS
AWRI1631 ICL1 MLS1	*ICL1, MLS1*	Promoter replacement	98 ± 0.7	NS
AWRI1631 ADH2	*ADH2*	Promoter replacement	100 ± 0.6	NS
AWRI1631 ZWF1	*ZWF1*	Promoter replacement	99 ± 0.8	NS
AWRI1631 GND1	*GND1*	Promoter replacement	[illegible]	NS
AWRI1631 GPD1	*GPD1*	Promoter replacement	89 ± 0.3	S
AWRI1631 2GPD1	*GPD1*	Two copies of the *FBA1p-GPD1* cassette	81 ± 0.4	S
AWRI1631 3GPD1	*GPD1*	Three copies of the *FBA1p-GPD1* cassette	71 ± 0.3	S
AWRI1631 GPD1 FPS1Δ11	*FPS1, GPD1*	*GPD1* promoter replacement and truncated Fps1p	87 ± 0.5	S
AWRI1631 GPD1 ΔTPI1	*GPD1, TPI1*	*GPD1* promoter replacement and *TPI1* deletion	Stuck ferment	NA
AWRI1631 2GPD1 ACS1	*GPD1, ACS1*	Two copies of the *FBA1p-GPD1* cassette, and *ACS1* promoter replacement	84 ± 0.9	S
AWRI 2531	*GPD1, ALD6*	Two copies of the *FBA1p-GPD1* cassette and *ALD6* deletion	71 ± 0.4	S
AWRI 2532	*GPD1, ALD6*	Three copies of the *FBA1p-GPD1* cassette and *ALD6* deletion	65 ± 1.2	S

[a] Values are averages and standard deviations from nine replicates for the parental strain and three replicates for all other strains.
[b] S, $P < 0.05$ (strain produced a significantly lower ethanol yield); NS, not significantly different; NA, not applicable.
[c] Unfinished fermentation with high residual sugar.

2.5. CONCLUSION

The examination of all strategies to achieve lower alcohol wines, including viticultural approaches, post fermentative removal of alcohol and GM and non GM approaches clearly shows that all current solutions are either inapplicable in industry or have significant cost and /or quality implications. A biological approach appears the most suitable strategy for ethanol reduction, as a yeast strain producing less ethanol may be more cost effective and have less of an influence on wine quality (Pretorius, 2000; Schobinger et al., 1986). The majority of biological approaches focus on shifting flux away from ethanol towards metabolites such as glycerol. The over expression of the glyceraldehyde-3-phosphate dehydrogenase gene, *GPD1* was the most efficient strategy to lower ethanol concentrations by increasing glycerol production, although additional modifications were necessary to remove unwanted metabolites. Very little research has been done on diverting carbon to the formation of reserve carbohydrates such as trehalose. The shift towards trehalose production could reduce the amount of glucose entering glycolysis as formation of trehalose is believed to inhibit hexokinase mediated glucose flux trough glycolysis (Hohmann et al., 1996). Although previous studies on gene modifications have given us a good understanding of which genes

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to target for lowering ethanol yield, how these modifications and their regulation by different promotors influence the regulatory networks is still unclear.

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Chapter 3

Research results

Construction of a recombinant industrial *Saccharomyces cerevisiae* strain for low ethanol fermentation

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3.1 INTRODUCTION

The demand for lower alcohol wines has increased over the last decade (Howley et al., 1992). Problems relating to the sensorial quality of wine, as well as economic and health issues are associated with this increased demand. The quality of wine is indeed compromised as high alcohol levels change the mouthfeel (viscosity) of the wine and may create the perception of 'hotness' on the palate (Guth et al., 2002). Economic interest in low alcohol wines is driven by the tax penalties that are levied according to alcohol content of wines in some countries (de Barros, 2000; Kutyna et al., 2010). High alcohol consumption is also associated with numerous health risks. Stricter drink and drive policies also contribute to a consumer demand for lower alcohol beverages.

Several reasons have been proposed to explain the observed increase in average ethanol content of wines in recent times. A global increase in temperatures due to global warming might be a contributing factor (Catarino et al., 2011). However, it is more likely that the demand for more full bodied and fruity wines has led to delayed harvesting to ensure fully matured (and sometimes overripe) berries. Due to the higher sugar levels of these berries, the alcohol content of the fermented wine is inevitably higher (Godden, 2000).

Existing procedures for removal or lowering of ethanol post-fermentation include spinning cone columns and reverse osmosis, both of which are costly (due to loss of volume, transport costs and expensive equipment) and may have a negative impact on wine quality. Another approach is to pick berries earlier to reduce the sugar levels of the must, but this frequently runs counter the desired flavour and aroma profile of the wine (Bui et al., 1986; Pickering et al., 1999a; Mermelstein, 2000). An appropriate biological alternative would be to develop yeast strains producing lower levels of ethanol during fermentation.

The genetic modification of yeast has the potential to address the high ethanol problem, and has met with relative success in recent years (Kutyna et al., 2010). Even though GM yeasts are not yet widely accepted and therefore not used in the wine industry, GM studies have significantly improved our knowledge of carbon flux and its regulation during alcoholic fermentation. Most gene modification strategies have been focusing on shifting carbon flux towards glycerol production rather than ethanol. The deletion of the genes encoding alcohol dehydrogenase (ADH) isozymes, *ADH1*, *ADH3*, and *ADH4* combined with a point mutation in the gene *ADH2* serves as a good example of this approach (Drewke et al., in 1990). Fermentation with this mutant strain lowers ethanol yield and leads to higher production of

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glycerol. However, this mutant strain struggles to complete fermentation and results in high levels of acetaldehyde and acetate (Drewke et al., 1990).

The most successful strategy to date involved the over expression of the *GPD1* gene (Michnick et al., 1997). *GPD1* encodes the glycerol 3-phosphate dehydrogenase which converts dihydroxyacetone to glycerol 3-phosphate. As with the previous approach, the strain also produces higher levels of acetaldehyde and acetic acid, a consequence of the excessive regeneration of NAD^+. This shortcoming has since been relatively successfully mitigated through additional deletion and over-expression of genes that block the synthesis of these unwanted by-products (Grabowska et al., 2003; Remize et al., 2000).

Very few alternatives to redirecting carbon flux towards other carbon sinks have been explored. Redirecting carbon flux towards reserve carbohydrates for example might have the potential for lowering ethanol by directing glucose towards the production of metabolites such as trehalose.

The key enzyme in the trehalose biosynthesis pathway is trehalose-6-phosphate synthase, encoded by the *TPS1* gene (Francois et al., 2001). This enzyme converts glucose-6-phosphate and UDP-glucose to α,α-trehalose-6-phosphate. In the second step α,α-trehalose 6-phosphate and water are converted to trehalose and phosphate by trehalose-6-phosphate phosphatase encoded by the *TPS2* gene (Francois et al., 2001). The overproduction of trehalose through *TPS1* overexpression however also restricts hexokinase activity, reducing the amount of glucose entering glycolysis and slowing glycolytic flux (Hohmann et al., 1996). In our laboratory, preliminary studies with strains over-expressing or carrying a deletion of the *TPS1* gene in the laboratory strain S288C showed a decrease in ethanol yield for both scenarios, which was accompanied by an overall reduction in fermentation rate (unpublished data).

As the TPS mutants used in this initial study were generated in laboratory strains which perform poorly in industrial fermentation conditions, the results may not be fully indicative of the effect of such modifications in a real wine yeast strain. Furthermore, these studies employed strategies such as expression from multiple copy plasmids or the use of strong promoters leading to a very high level of *TPS1* expression, possibly resulting in an excessively high metabolic burden on the yeast, with the lowering of the fermentation rate as an indirect side-effect.

The chromosomal *TPS1* gene is expressed during late exponential and early stationary growth phase, resulting in trehalose levels peaking during early stationary phase. (Rautio et al., 2007, Rossouw et al., 2009). This specific increase in trehalose at early stationary phase is also linked to the response of *S. cerevisiae* to the increase in ethanol, as trehalose is produced as a stress

protectant by stabilising the cell membrane (Alexandre et al., 2001; Gasch et al., 2000; Thevelein., 1984; Van Laere, 1989; Wiemken,. 1990).The consequent lowering in trehalose towards the end of fermentation can be linked to the increase in expression of trehalose degrading enzymes (Thevelein *et al.*,1982). To further assess whether carbon flux can be redirected to trehalose without impacting general fermentative behaviour, we assessed the impact of changing the expression of the *TPS1* gene during alcoholic fermentation by using two different promoters derived from the *DUT1* and *GIP2* genes. These promoters were chosen due to their intermediate strength and their growth phase specific expression pattern during fermentative growth in industrial wine yeast strains (Rossouw at al. 2008). The promoter of the *DUT1* gene is mainly active during exponential growth, while the promoter of the *GIP2* gene is linked to gene expression during stationary phase. These stage specific promotors were chosen to ascertain whether over-expression of the *TPS1* gene is more effective during exponential growth phase or stationary phase. The controlled over-expression study was carried out in the genetic background of an industrial *Saccharomyces cerevisiae* strain, VIN13. The resulting strains therefore continue to express native *TPS1*, but will in addition express the gene in either exponential phase (when the native gene is repressed), or show moderately increased expression during stationary phase. The final goal is to increase trehalose accumulation at the expense of ethanol, without affecting general fermentation parameters. The data indicate that both test strains produce higher levels of trehalose and lower levels of ethanol, with expression under control of the GIP2 promotor being more effective The accumulation of trehalose coincides with the stage specific expression of the *TPS1* gene, with trehalose levels being higher for the DUT-TPS strain during exponential phase and higher for the GIP-TPS strain during early and mid stationary phase.

3.2 MATERIALS AND METHODS

3.2.1 STRAINS AND CULTURE CONDITIONS

The strains generated during this study are shown in Table 1 and have all been derived from VIN13, a yeast strain commonly used in industrial wine fermentation.

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Table 1: Strains described in this chapter

Strain	*Description*
Vin13	Industrial wine yeast strain (Anchor yeast)
DUT-TPS	VIN 13 with inserted plasmid containing Ycplac22 centromeric fragment, *DUT1* promoter sequence and *TPS1* Gene
GIP-TPS	VIN 13 with inserted plasmid containing Ycplac22 centromeric fragment, *GIP2* promoter sequence and *TPS1* Gene
DUT CONTROL	VIN 13 with inserted plasmid containing Ycplac22 centromeric fragment, *DUT1* promoter sequence without the *TPS1* gene
GIP CONTROL	VIN 13 with inserted plasmid containing Ycplac22 centromeric fragment, *GIP2* promoter sequence without the *TPS1* gene

To verify the presence of the correct episomal plasmid in the yeast cell, plasmid isolation was performed using the Zymoprep^{TM} Yeast Plasmid Miniprep II kit (Inqaba Biotech, Johannesburg, South Africa). The isolated plasmid from the various transformed strains were used in a back transformation and plated on zeocin selective media. Restriction digests of isolated plasmids were also performed to confirm the identity of plasmids isolated from the yeast.

All strains were maintained on YPD plates containing 1 g/L zeocin for industrial strains and 0.6 g/L zeocin for the lab strains. Small scale fermentations were carried out in 250 ml Erlenmeyer flask containing the 200 ml synthetic wine must MS300 (Bely et al., 1990) and sealed with rubber bung and S-bend airlocks. Glucose and fructose was added in equal amounts at 100 g/L each for the lab strains and 125 g/L for the industrial strains. Pre-inoculated cultures were grown overnight at 30°C in YPD broth containing 1 g/L and 0.6 g/L zeocin for the genetically modified strains. These cultures were then used to inoculate small scale fermentations to a cell density (OD_{600}) of 0.1. All fermentations were carried out in triplicate. Fermentations were carried out at 30°C and the progress monitored by weight loss which is indicative of CO_2 production and sugar consumption. Cell growth and biomass production was determined by OD_{600} readings (Shimadzu UV-1601PC UV-Visible, Scanning Spectrophotometer, Japan).

3.2.2. DNA MANIPULATION AND PLASMID CONSTRUCTION

General DNA manipulation protocols were as described in Ausubel et al. (1994). The centromeric plasmids were constructed by inserting the centromere from the Ycplac22 plasmid into the pTEF/Zeo expression vector (Invitrogen). The pTEF/zeoYcplac22 plasmid was digested with BglII and SpeI and the pTEF/Zeo vector was digested with BglII and NheI. The Ycplac22 centromeric fragment was then cloned into the pTEF/Zeo vector, resulting in the construct pTEF-CEN.

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Following this, the two promoter regions (upstream of the *DUT1* and *GIP2 genes*) and the *TPS1* ORFwere cloned from the genomic DNA of the industrial *S. cerevisiae* strain, VIN13. The DNA fragments were amplified using the primers listed in Table 2. The two promotor regions were identified based on the expression of the corresponding genes (*DUT1* and *GIP2*) at different time points during fermentation in transcriptional studies conducted using five different wine yeast strains (Rossouw *et al*., 2008). A subset of genes were initially selected (genes showing expression during either the exponential growth phase or stationary phase) and subsequently narrowed down to only those genes showing overall moderate expression levels since high expression levels were undesirable in light of the goal of this study. From this final set of genes *DUT1* and *GIP2* were selected as fulfilling all criteria. The corresponding upstream promoter sequences of these two genes were identified (www.yeastgenome.org) and primers designed for amplification. The promoters are flanked by *Sph*I and *Pvu*II restriction sites, and the genes are flanked by *Pvu*II and *Nar*I digestion sites. Fragments were cloned into the pGEM-T Easy vector (Promega, Charbonnières, France) and transformed into the *E.coli* strain DH5α. Transformed cells were plated out on LB; Ampicillin; X-gal; IPTG blue/white selection plates (Ampicillin final concentration of 100 ug/ml; 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-gal) final concentration of 40 ug/ml (2 ul of Xgal stock solution per ml of media); Isopropyl β-D-1-thiogalactopyranoside (IPTG) final concentration of 0.1 mM (1 ul IPTG stock solution per ml of media)). White colonies were picked and screened by colony PCR. Gene and promoter sequences of positive transformants were confirmed by sequencing. The promoter fragments and *TPS1* ORF were excised and cloned into the pTEF-CEN plasmid, resulting in the two final constructs pTEF-CEN-DUT1-TPS1 and pTEF-CEN-GIP2-TPS1 (Fig 1)

Table 2: PCR primers used to amplify the gene, *TPS1* and the two promoter regions of the *DUT1* and *GIP2* genes

Name	***Sequence (5’-3’)***
TPS1 Forward	5’-GATC<u>CAGCTG</u>ATGACTACGGATAACGCTAAGG-3’
TPS1 Reverse	5’-GATC<u>GGCGCC</u>TAACAGCGCTACAGACAGGC -3’
DUT1 Forward	5’- GATC<u>GCATGC</u>ACTATGTACATACACACGCACC- 3’
DUT1 Reverse	5’- GATC<u>CAGCTG</u>TTGGTTATTTTTTGGCTCGCTGTA- 3
GIP2 Forward	5’- GATC<u>GCATGC</u>GCTGTCTAGAATGCATTTTTCCA - 3’
GIP2 Reverse	5’- GATC<u>CAGCTG</u>TGTTGCGTTGATGAAATCCTAA- 3’

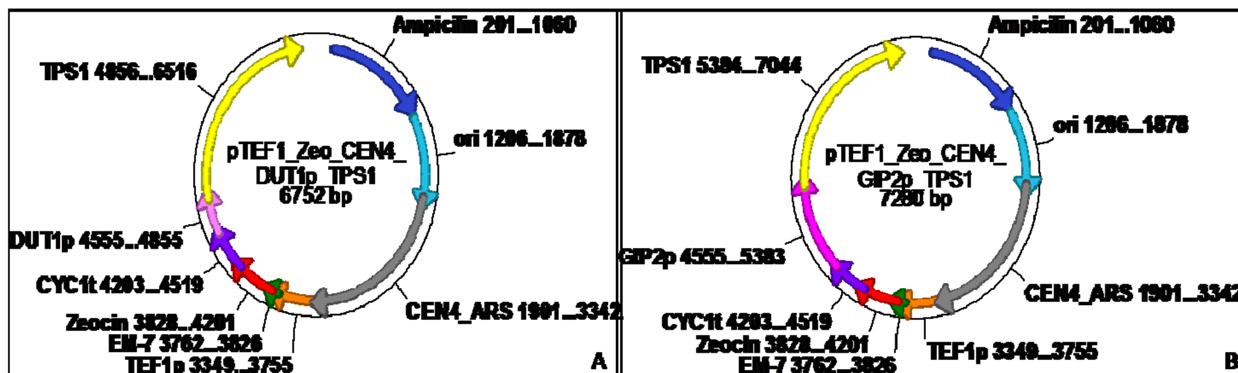

Figure 1: Complete constructs of pTEF plasmid with inserted Ycplac22 centromeric region and inserted *DUT1* (A) and *GIP2* (B) promoters in tandem with the *TPS1* gene.

3.2.3. YEAST TRANSFORMATION

An electroporation transformation technique was used to transform the constructs and empty vector controls into VIN13. The transformation was done by inoculating 10 ml overnight cultures of VIN13 colonies. The overnight cultures were added to 100 ml YPD, and incubated with shaking at 30 °C until an OD_{600} of between 0.8 and 1.5 was reached. Cells were harvested at 5000 rpm at 4 °C and re-suspended in 40 ml ddH_2O. Following the addition of 5 ml TE Buffer and 5 ml LiOAc, the cells in suspension were incubated for 45 minutes at 30 °C with gentle agitation. After addition of 1.25 ml 1M DTT another 15 minute incubation step was carried out at 30 °C. Eighty micro litres of ice cold ddH_2O was added and cells were centrifuged at 5000 rpm for 4 minutes. Cells were washed with 120 ml ddH_2O and harvested by centrifugation. The last wash step was performed with 1M Sorbitol and cells were harvested by centrifugation. The cells were re-suspended in 200 µl Sorbitol (1M). Eighty micro litres of the cell suspension was added to an eppendorf tube containing 10 µl of plasmid DNA (total of 40ug). The mixture of plasmid DNA and competent cells were transferred to a 0.2 cm gap electroporation cuvette and pulsed at 1.5 kV, 25 mF and 200 ohms for 5-5.7 milliseconds. Immediately after the pulse 1ml of ice cold YPD was added, the sample was transferred to sterile 1.5 ml eppendorf tubes and incubated at 30 °C overnight. Positive transformants were selected by plating on selective media containing 1 g/L zeocin (for the industrial strains) and 0.6 g/L zeocin (for the laboratory strains) (Wenzel et al., 1992; Lilly et al., 2006).

3.2.4. VERIFICATION OF GENE EXPRESSION BY QUANTITATIVE REAL-TIME PCR ANALYSIS (QRT-PCR)

RNA isolations were performed on samples taken at time points T2, T5, T 11 and T18 to cover the range of different growth phases of the yeast during fermentation. RNA was extracted using the hot phenol extraction protocol (Schmitt et al., 1990). RNA was quantified using the

Nanodrop® (ND-1000 Spectrophotometer, Thermo Fisher Scientific, Delaware, USA) and integrity assessed on denaturing and non-denaturing gels. The Improm-II™ Reverse Transcription System was used to synthesise cDNA using a random primer set.

Primers for target genes and primers for normalisation were designed using the Primer Express software v. 3 (Applied Biosystems) and a KAPA™ SYBR® FAST qPCR Kit was used to perform QRT-PCR analysis. Spectral data were captured by the 7500 cycler (Applied Biosystems by Life technologies, California, USA). Data analyses were conducted using Signal Detection Software (SDS) v. 1.3.1. (Applied Biosystems) to determine the corresponding Ct values and PCR efficiencies respectively for the samples analysed (Ramakers et al., 2003). The primer sequences used for QRT-PCR are described in Table 3 below.

Table 3: QRT-PCR primers used to amplify the *TPS1* gene and the *ACT1* gene

Name	***Sequence (5'-3')***
TPS1	5'- TTGCACGCCATGGAAGTG-3'
TPS1	5'- AACAACCTTGCCCCTCCATT - 3'
ACT1	5'- GCCGAAAGAATGCAAAAGGA - 3'
ACT2	5'- TCTGGAGGAGCAATGATCTTGAC - 3'

3.2.5 METABOLITE ANALYSIS

Samples were taken throughout fermentation to quantify key metabolites. Samples and standards were prepared in accordance with the method described by Eyéghé-Bikong et al. (2012). The media was analyzed by high performance liquid chromatography (HPLC) on an AMINEX HPX-87H ion exchange column (Bio-Rad, California, USA) using 5 mM H_2SO_4 as the mobile phase. Peak detection and quantification was performed by Agilent RID and UV detectors in tandem. Analysis was carried out using the HPChemstation software package.

Trehalose extraction (Yoshikawa et al., 1994) and quantification was performed as follows: Trehalose sampling was performed at selected time points during fermentation by harvesting 5mls of fermentation culture. The cells were dried and weighed. For the extraction 500µl of 0.25M NA_2CO_3 was added for every 25mg of cells followed by addition of acid washed glass beads. The buffer/cell mixture was vortexed and incubated at 95°C for twenty minutes, followed

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by centrifugation at to pellet cell resides. Supernatants were analysed for trehalose using the Megazyme Trehalose assay kit (Megazyme International, Ireland) according to specifications.

3.2.6 PROTEIN EXTRACTION AND QUANTIFICATION

Protein extraction and quantification was performed as follows: Protein sampling was performed at selected time points during fermentation by harvesting 5mls of fermentation culture. The cells were dried and weighed. A protein extraction buffer (pH 6.8) was prepared as follows: 120mM Tris-HCl, 20mM EDTA, 4% SDS, 6M urea. For the extraction 500µl extraction buffer was added for every 25mg of cells. Acid washed glass beads were added and vortexed vigorously followed by an incubation step at 65°C for twenty minutes. Samples were then centrifuged and supernatants transferred to clean tubes.

Quantification was done using the BCA Protein Assay Reagent Kit (Pierce, Rockford, USA). OD readings were taken at 562nm for quantification relative to the standard curve.

3.3. RESULTS

3.3.1 MONITORING FERMENTATION PERFORMANCE AND BIOMASS FORMATION

Untransformed wild type VIN13 and all transformed strains were inoculated into synthetic must, and fermentation performance was evaluated. The fermentation performance was monitored by measuring weight-loss through CO_2 release and OD_{600} reading indicative of sugar consumption and biomass formation, respectively. The fermentation performance of the strains containing plasmid constructs when compared to wild type VIN13 was slightly reduced with regard to both biomass formation and weight loss. However, all transformed strains with the exception of the DUT control strain showed similar fermentation performance, suggesting that the slightly reduced performance was due to the presence of the plasmid, and not linked to the increased expression of the *TPS1* gene. Importantly, all strains completed fermentation within a broadly similar time frame (Fig 2 and 3).

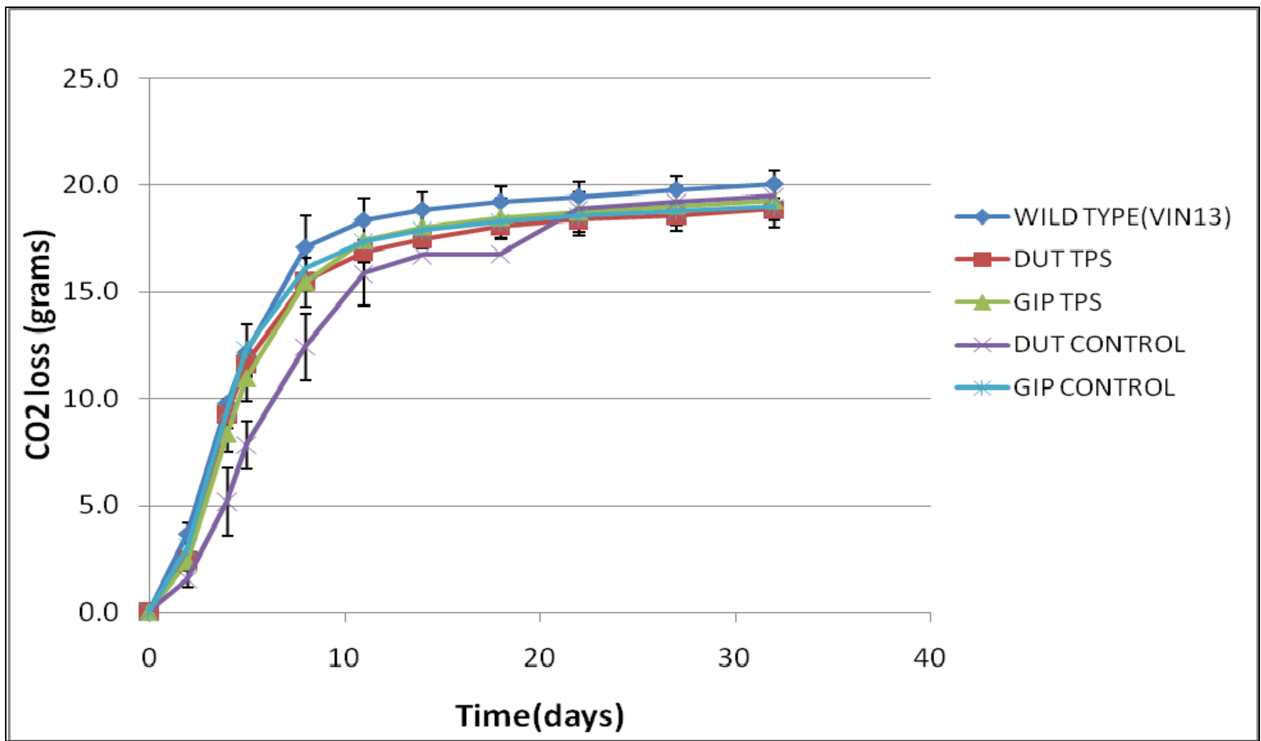

Figure 2: Fermentation performance by monitoring the accumulated CO_2 loss for all test strains and their controls. Values are the average of three biological repeats ± standard deviation.

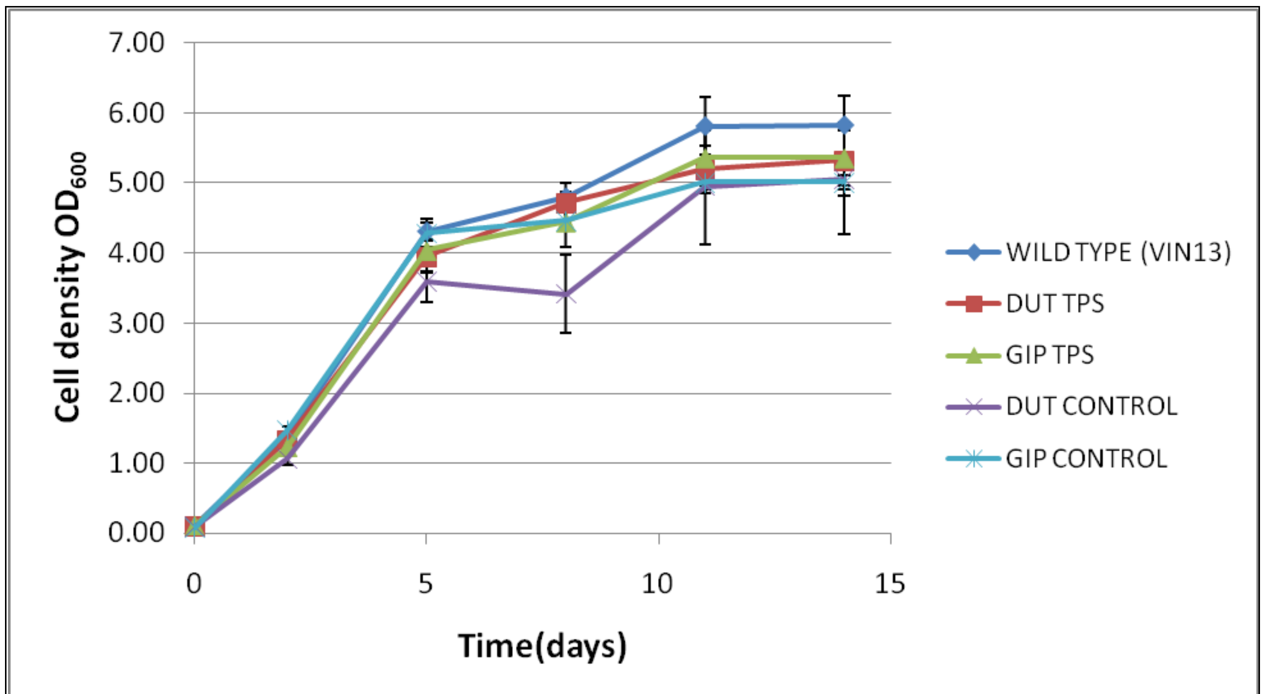

Figure 3: Fermentation performance by monitoring the OD_{600} readings for all test strains and their controls. Values are the average of three biological repeats ± standard deviation.

3.3.2 EXPRESSION OF THE *TPS1* GENE

To determine the expression levels of the *TPS1* gene under the control of the *DUT1* and the *GIP2* promoter, RNA extraction was performed at time points T2, T5, T11 and T14 for all industrial strains. The expression data for the qRT-PCR was normalized relative to the expression of the housekeeping *ACT1* gene (Fig 4). The first time point T2 is representative of exponential growth phase. There is a significant (> 2-fold) over-expression of the *TPS1* gene under the control of the *DUT1* promoter during the exponential growth phase compared to the wild type (VIN13) and DUT control (plasmid containing promoter only). T-tests were performed to confirm the significant change in *TPS1* expression during early exponential growth phase. The second time point T5 is representative of early stationary phase and a slight but statistically significant over-expression of the *TPS1* gene under the control of the *GIP2* promoter is observed (Fig 4). There also appears to be an additional increase in *TPS1* expression at days 11 and 14 for the DUT-TPS1 strain (representative of late stationary phase).

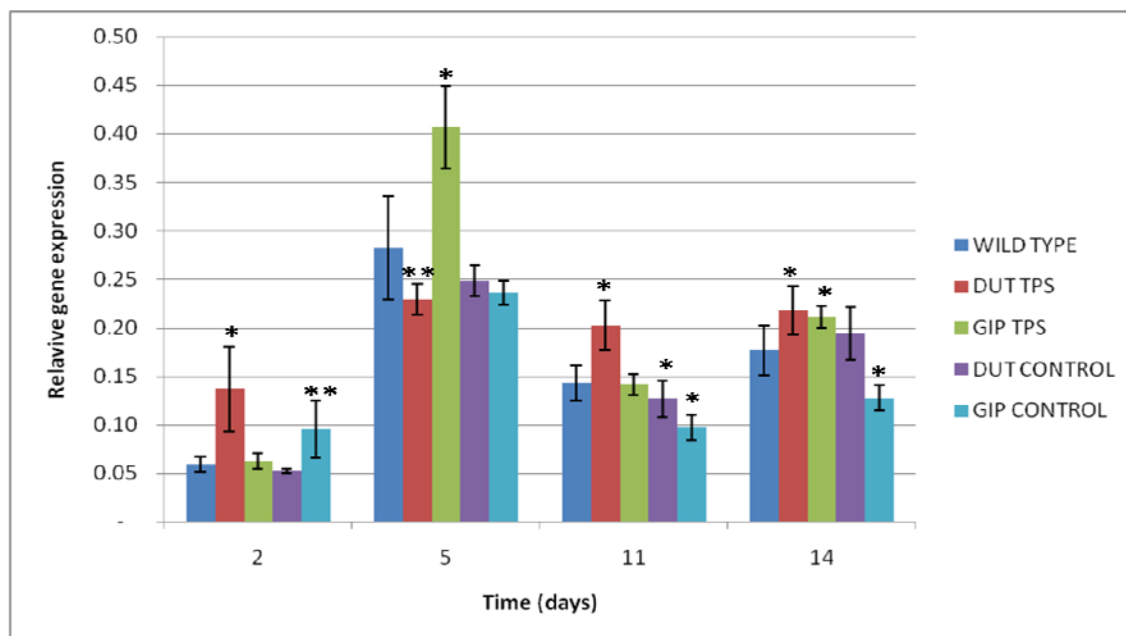

Figure 4: Relative gene expression levels at different stages of fermentation. Values are the average of three biological repeats ± standard deviation.(* denotes $p<0.05$ (95% confidence)**denotes $p<0.1$ (90% confidence))

3.3.3 CHEMICAL ANALYSIS

The main products and by-products of alcoholic fermentation, ethanol, glycerol and aetic acid, were measured by HPLC analysis at the relevant time points and ethanol yields (g of ethanol produced / g of sugar consumed were calculated (Fig 5C, 5D, 6, 7 (A-D)). Fig 5,6 and 7A show that total ethanol production was lower in all the transformants when compared to the wild type

VIN13 strain. However, when comparing ethanol yields (Fig 7B), only the two strains expressing *TPS1* show a statistically significant reduction. The glucose consumption rates of all strains appear similar (Fig 5A). However, the DUT-TPS strain and the promoter controls display residual fructose levels that are significantly higher than the wild type and the GIP-TPS strain (Fig 5B). The strain carrying the GIP-TPS construct showed similar total sugar consumption to that of the wild type, and was clearly the most efficient in terms of reducing ethanol yields. The DUT control strain showed significant standard deviations, and as the fermentation kinetics already suggested, appears to have experienced fermentation problems which are not directly related to the presence of the plasmid. This is also reflected in the significantly higher amount of acetic acid produced by this strain, suggesting that this strain experienced some redox- balance related fermentation stress. Surprisingly, all transformed strains produced higher levels of glycerol than the wild type. This increased level of glycerol however did not translate in higher levels of acetic acid but for the strain DUT Control as discussed above (Fig 7C and 7D).

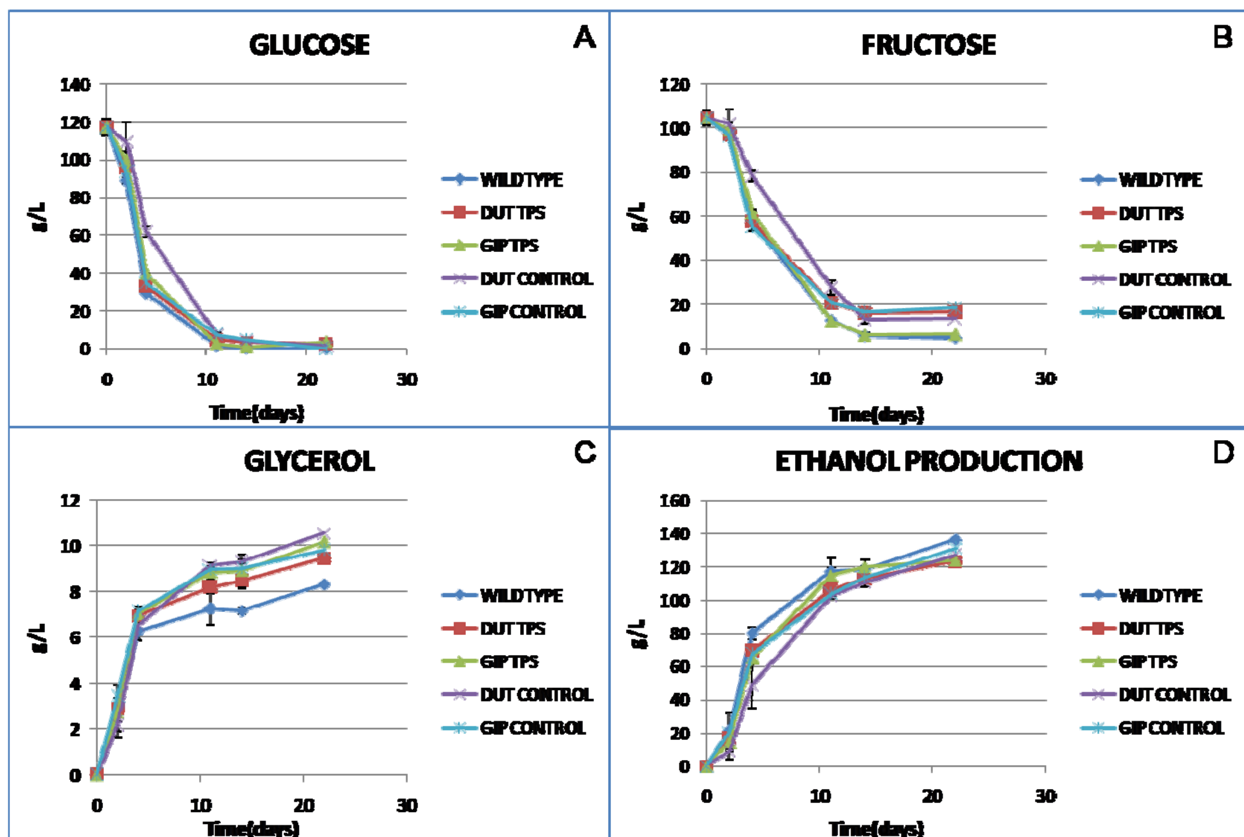

Figure 5: Metabolite distribution for all test strains and their controls throughout fermentation. Values are the average of three biological repeats ± standard deviation.

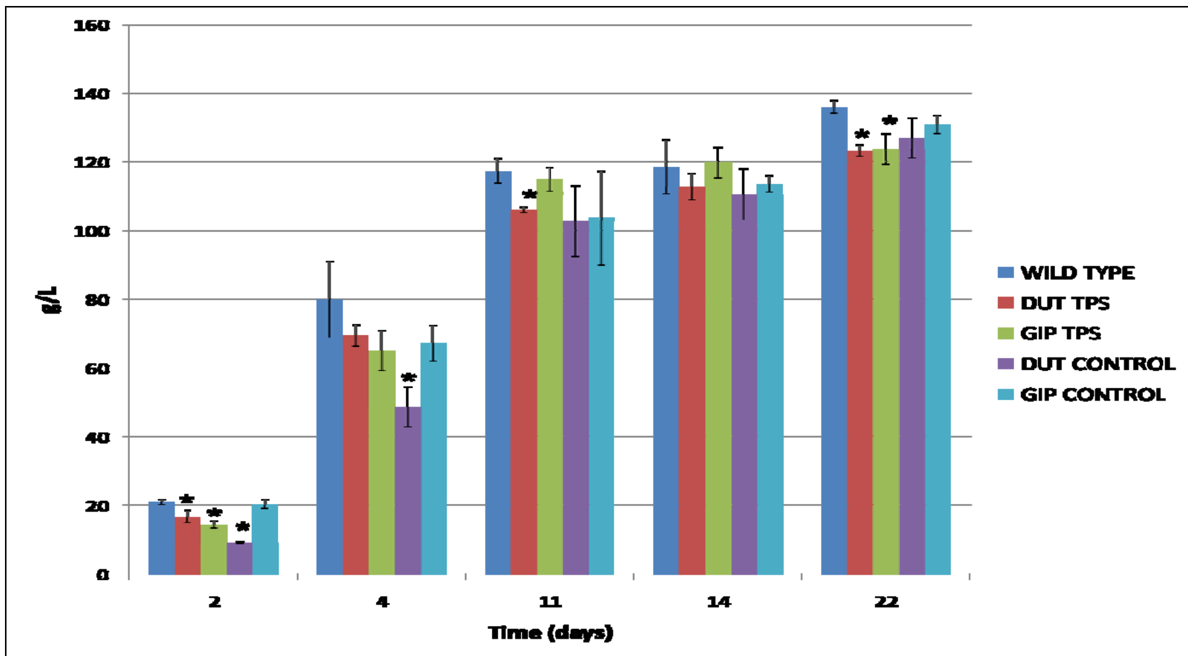

Figure 6: Ethanol production for all strains and controls throughout fermentation. Values are the average of three biological repeats ± standard deviation. (* denotes p<0.05(95% confidence)**denotes p<0.1 (90% confidence))

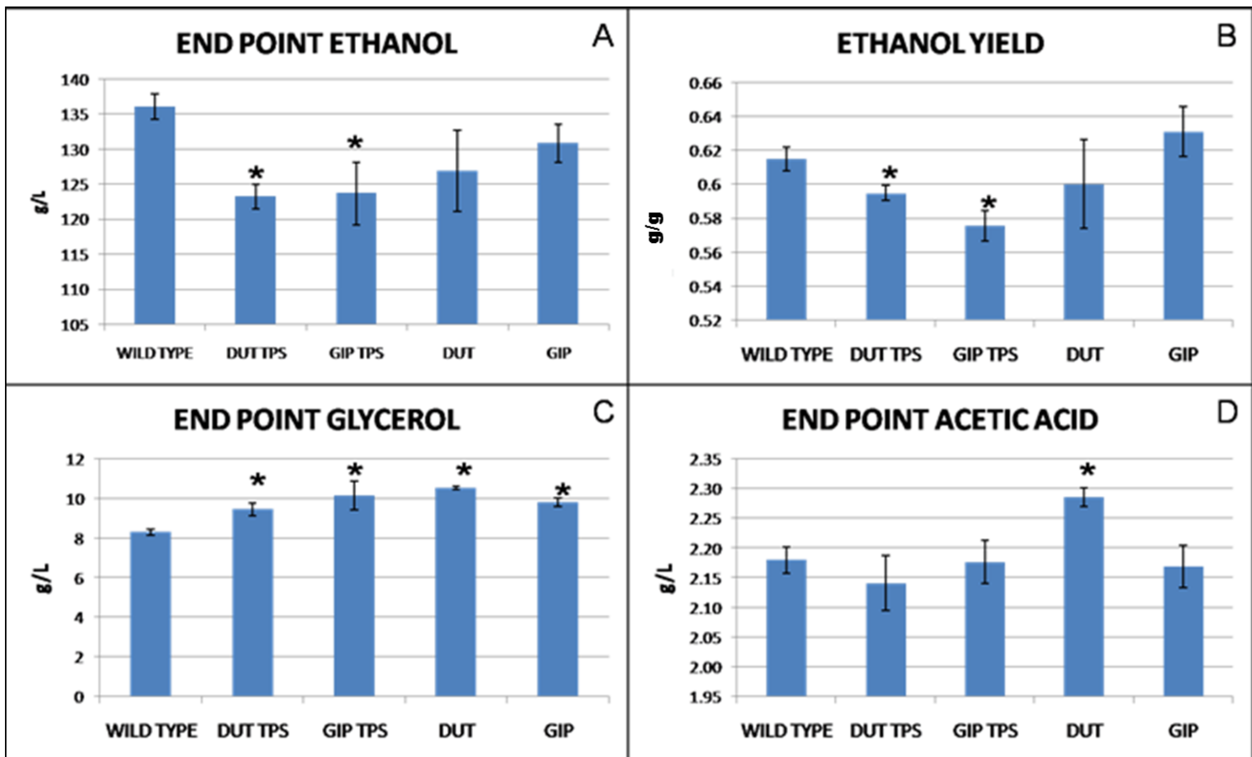

Figure 7: Ethanol (frame A) ,acetic acid (frame D) and glycerol levels (frame C) at the end of fermentation (T22). Ethanol yield as determined at the end of fermentation are depicted in frames B. Values are the average of three biological repeats ± standard deviation. * denotes p<0.05 (95% confidence) **denotes p<0.1 (90% confidence)

The sugar utilisation is less for the test strains and their controls indicating that the reduced fermentation capacity is not due to the over expression of the *TPS1* gene. Replica plating was performed for strains and controls at day eleven to assess the plasmid retention during fermentation (since no selection pressure was maintained during fermentation). The plasmid is retained in approximately 97% of the yeast cells.

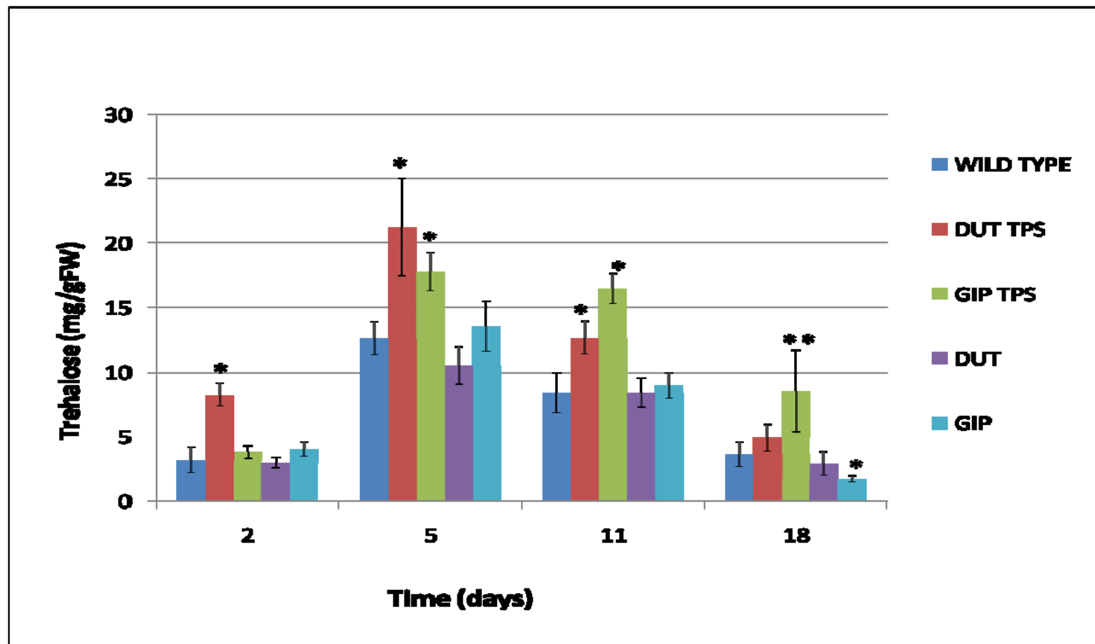

Figure 8: Trehalose production for all strains and controls throughout fermentation. Values are the average of three biological repeats ± standard deviation. * denotes $p<0.05$ (95% confidence) **denotes $p<0.1$ (90% confidence)

Trehalose extractions were performed for quantification of trehalose at different stages of fermentation. The trehalose levels for the DUT-TPS strain is significantly higher than its controls during early and late exponential growth phase, (Fig 8), while the trehalose levels for the GIP-TPS strain are significantly higher than the control during early and mid stationary phase . Trehalose levels therefore closely follow the *TPS1* expression data slight over-expression of the *TPS1* gene under control of the *GIP2* promotor (Fig 8). In line with the ethanol yield data, the GIP-TPS strain was the only strain that retained significantly higher levels of trehalose at the end of fermentation, in line with the ethanol yield data, and highlighting the importance of trehalose degradation during stationary phase. Indeed, the significant amount of trehalose produced by the DUT-TPS strain during exponential growth is almost entirely degraded to close to Wild Type levels at the end of fermentation (Fig 8).

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3.4 DISCUSSION

The CO_2 release curves for the strains that contain the episomal plasmid seem to suggest that these strains have a slower fermentation rate, which is also indicated by the slightly higher residual sugars in some of the strains. This may be due to pre-inoculation conditions, as the strains containing the plasmid were cultured under selection pressure in media containing the antibiotic zeocin. Another explanation could be that the episomal plasmid itself is causing the cell stress. An episomal plasmid requires energy from the host cell for maintenance and reproduction. This energy is taken from the host's cellular resources, and may represent a metabolic burden to the cell (Stouthamer and van Verseveld, 1987).

The expression profiles of the two TPS over-expressing strains compared to their plasmid-only controls and the wild type, suggests that the *DUT1* promoter drives increased *TPS1* expression during early exponential growth phase. The expression of *TPS1* under the control of the *GIP2* promoter region showed increased expression levels during early stationary phase. Expression of *TPS1* under control of the *DUT1* promoter was up to 40% higher than the wild type strain (VIN13) during early exponential growth, whereas the expression of *TPS1* under control of the *GIP2* promoter was up to 60% higher than the wild type during early stationary phase. The levels of expression indicate that the aim of achieving a growth-phase specific and moderate increase in TPS1 expression has been achieved. Preliminary studies where the *TPS1* gene was over-expressed at high levels indeed indicated strongly reduced fermentative activity as already described in the literature, The expression of *TPS1* both under control of the *DUT1* promoter during the early exponential growth phase as well as the *GIP2* promoter in early stationary phase have some metabolic impact in terms of decreasing the total ethanol yield, and in the case of GIP-TPS without impacting significantly on fermentative performance of the strain. Indeed, expression under control of the *GIP2* promoter seems not to reduce the fermentation capacity and the sugar utilization in a statistically significant manner (even though its expression levels are slightly higher than that of *TPS1* under control of *DUT1*). This might suggests that the expression of the *TPS1* gene during early stationary growth (regulated by the *GIP2* promoter) is more effective and causes less stress to the cell than expression during early exponential growth (*DUT1*). The main aim of this study was to redirect flux towards trehalose production, and the trehalose data indicates that there has indeed been a shift in flux towards trehalose production by the over-expression of the *TPS1* (trehalose 6 –phosphate synthase) gene. Trehalose production is increased substantially for both test strains. The DUT-TPS strain produces higher levels of trehalose during early exponential growth phase compared to its control strain, whereas the trehalose production of the GIP-TPS strain increased during early stationary phase, increasing to levels above those of the DUT-TPS strain.

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The ethanol yield for the DUT-TPS and the GIP-TPS test strains do seem to be lower than the wild type. Although when compared to their relevant controls it is not clear that there is a significant reduction for the DUT-TPS strain. The GIP-TPS shows a significant reduction in ethanol yield and appears to be the more effective strain.

Although there is no obvious link to the over-expression of the *TPS1* gene, there is a slight increase in glycerol production observed for all transformed strains. In *S. cerevisiae* glycerol production plays an important role in stress tolerance, maintaining intracellular phosphate levels and redox balance (NAD+/NADH levels) (Blomberg & Adler., 1992; Hohmann., 2002). In studies where glycerol production has been up regulated, such as the over-expression of *GPD1*(Michnick et al., 1997) it was found that acetic acid levels increase with an increase in glycerol production. An increase in acetic acid has been linked to excessive NAD regeneration (Michnick et al., 1997). In this study, although glycerol production has increased there is no increase in acetic acid levels for either of the test strains, which is a positive outcome but also expected as the trehalose production pathway should not influence the NADH/NAD+ balance as would a modification in gene expression linked to glycerol over-expression This suggests that the slight over-expression of the *TPS1* gene by these specific promoters has successfully shifted carbon flux toward trehalose production with minimal affect on redox balance.

As all test strains and their controls show an increase in glycerol production this cannot be linked to *TPS1* over-expression, although minimal plasmid loss at day eleven suggests that the plasmid is stable. This is indicative that the plasmid is not causing the cell intolerable strain. It may also be hypothesized that with less glucose entering glycolysis and downstream pathways (as some glucose is re-directed toward trehalose production), the increase in glycerol production is due to the cell's need to maintain redox balance. This is because less ethanol may be produced (due to less glucose flux through glycolysis), while the cells' metabolic needs for biomass production from the pyruvate branch point remain unchanged. Re-oxidation of NADH via glycerol production may then provide an alternative pathway to maintain redox balance.

Overall the GIP-TPS strain seems to outperform the DUT-TPS strain, with lower ethanol levels and more favourable residual sugar content at the end of fermentation as well as a lower ethanol yield suggesting that over-expression during stationary phase is a more effective approach. The flux towards trehalose away from ethanol production seems to be more effective than that of the DUT-TPS strain.

During this study the expression under phase specific promoters and the level at which genes are over expressed have been of more significance than perhaps the over-expression of the

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TPS1 gene in itself. The novel idea of slight overexpression using phase-specific promoters for expression of target genes may be a good approach for future research using other genes involved in reserve carbohydrate production as well as previously studied genes involved in glycerol over-expression such as *GPD1*.

With each biological approach aimed at re-directing carbon flux, we gain more insight into how different pathways affect each other and where compensation occurs to maintain redox balance. Putting all this information together might in future bring us closer to finding an effective way of reducing ethanol yield as well as improving other aspects of wine quality.

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Chapter 4

General discussion and conclusions

4.1. GENERAL DISCUSSION AND CONCLUSION

Worldwide there is a need to produce wines with reduced alcohol levels without affecting the quality and aroma composition of the wine (Scudamore-Smith et al., 1997; Pickering et al., 1998). There are different approaches to this problem, most of which are applied post fermentation to reduce or remove the ethanol. These procedures are costly and could affect wine quality. To address the consumer and industry demand to find avenues for reducing the ethanol content in wine, we have adopted a genetic modification approach. Such a strategy would eliminate the cost involved in post fermentation ethanol removal without adversely affecting the quality of the wine. Our study focused on directing carbon flux towards the production of the storage carbohydrate trehalose. This approach differs from previous studies where the focus has mainly been on redirecting carbon flux towards glycerol. Our aim of flux redirection was achieved by slightly over-expressing the *TPS1* gene, encoding the trehalose-6-phosphate synthase enzyme, under the control of two growth phase specific promoters of the *DUT1* and *GIP2* genes. The novelty of our particular over-expression strategy lies in the selection and use of these two promoter regions. These promoters were identified as follows: Large scale gene expression datasets from several wine yeast strains during time-course fermentation experiments were interrogated to identify promoters that drive growth-stage specific gene expression in these conditions. These genes where then split into two groups, those showing expression during exponential growth phase and those showing expression during stationary phase. These were then further narrowed down to those only showing moderate levels of expression. Of those genes, the upstream promoter regions where identified. Those that were selected for this study are the promoter regions of *DUT1* for the exponential growth phase expression and of GIP2 for the stationary phase specific expression.

A single copy episomal plasmid was then used to incorporate the promoter sequences and TPS ORF and subsequently transformed into a widely used industrial yeast strain. The growth stage –specific expression patterns of the *DUT1* and GIP2 promoters were confirmed by the expression profiling performed in this study.

TPS1 expression was slightly higher under the control of the *GIP2* promoter than the *DUT1* promoter. Over-expression under control of the stationary phase promoter *GIP2* seems to be more effective in terms of lowering ethanol yield as well as showing improved sugar utilisation than expression under the control of the exponential growth stage specific promoter *DUT1*. This strategy shows promise for targeting other genes of interest with phase specific promoters. The over-expression strains as well as their controls seem to have

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slightly reduced fermentation performance which show that it is not the over-expression of the *TPS1* gene alone that is responsible for the slightly reduced fermentative capacity of transformed strains. The increase in trehalose production has an effect on lowering ethanol yield, indicating an altered flux away from ethanol towards trehalose production. The increase in glycerol for all test strains and controls again indicates that it is not the over-expression of the *TPS1* gene causing the stress. The other positive outcome of our approach is that the increase in glycerol production was not accompanied by an increase in acetic acid production, as was the case for several other genetic modification studies (de Barros Lopes et al., 2000; Cambon et al., 2006; Eglington et al., 2002). The trehalose data indicate that the over-expression of the *TPS1* gene has been successful in shifting carbon flux towards trehalose production. The over-expression of the *TPS1* gene under control of the *DUT1* promotor causes a significant increase in trehalose production in early and late exponential growth phase which is expected as the *TPS1* expression is increased by about 40% during this phase. The 60% over-expression of the *TPS1* gene under control of the *GIP2* promoter causes an increase in trehalose production during early, mid and late stationary phase that is linked to the phase specificity of the *GIP2* promotor.

When comparing the two test strains it seems that the GIP-TPS strain is definitely the more efficient of the two, with slightly less ethanol yield, better sugar consumption and slightly higher production of trehalose when compared to DUT-TPS.

The novel approach used in this study has given us the insight over-expression of trehalose biosynthesis under the *TPS1* gene during early to late stationary phase yields better results than over-expression during exponential growth phase. Although this may not be exactly the same for other genes, growth phase specific promoters could positively impact on the effectiveness of over-expression. Another important factor is the level of over-expression. During this study we have proven that by only slightly over-expressing the target gene we are able to minimize unwanted stress and undesirable impacts on cellular redox balance.

There are some aspects that need further investigation and that might have been helpful to have included in this study. Other genes involved in trehalose biosynthesis can be targeted together with different phase specific primers identified in the preliminary studies. Different plasmid vectors can also be investigated as to ascertain the impact of the specific plasmid vector. The effectiveness of this approach can also be checked in different real wine must, as it may differ from results found in synthetic must. As we observe a decrease of trehalose towards the end of fermentation, targeting genes involved in trehalose degradation could also reduce the amount of sugar that re-enters glycolysis. An approach similar to the current

study with phase specific promoters with only moderate over-expression would be preferable as not to impact on the redox balance.

For commercial purposes even a 0.5% reduction in ethanol yield would be considered significant as this can mean the difference between tax penalties and wine being suitable for export. During this study we get very close to that 0.5% which could perhaps be further optimised by looking at other promoters as to find the perfect balance between alcohol reduction and fermentation performance. For the approach to be commercially viable the episomal plasmid will have to be replaced by either an insertion cassette into the yeast genome itself or the endogenous trehalose promoter need to be replaced, as the episomal plasmid is not stable enough for commercial use. These approaches are still considered genetically modified strategies that are currently not acceptable in South Africa as well as most other countries.

The knowledge gained during this study in terms of phase specific promoters as well as the choice of promotor with regards to the level of expression can aid future work. Expressing previously studied genes such as *GPD1* that has in the past been one of the most successful targets can be re investigated with the use of phase specific promotors that also lessen the amount of over-expression. Although during our study an increase in acetic acid was not expected as NAD+/NADH ratios should not be disturbed by overproduction of trehalose, the choice of these low-strength phase-specific promoters might address redox balance challenges such as acetic acid over-production that has been a problematic feature of *GPD1* over-expression (de Barros Lopes et al., 2000; Cambon et al., 2006; Eglington et al., 2002).Adding a deletion such as *ALD6* might then not be necessary. The same approach using different target genes for channelling carbon to other reserve carbohydrates could be a possible alternative for lowering ethanol yield.

With each gene modification study we gain more knowledge as to how carbon flux and redox balance is regulated, enabling us to develop more effective strategies to lower ethanol yield during fermentation. This study has given us the novel insight that gene regulatory systems such as promoters are just as important as the genes themselves with regards to selecting an effective gene modification approach.

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ADDENDUM A

METHOD VALIDATION DATA

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SEQUENCE DATA FOR GENES AND PROMOTORS

TPS1 GENE SEQUENCE

The *TPS1* gene sequence is the full coding sequence from 10bp upstream (allowed for cloning (enzyme digests as described in cloning strategy in chapter 3)) from start codon ATG to 181bps downstream from the stop codon as to include terminator sequence of the *TPS1* gene. The VIN13 *TPS1* gene had 3 base pair changes, changing the position 210 amino acid from a cysteine to arginine, the 329 amino acid from an alinine to a threonine and the position 531 from a histidine to a leucine.. The *GIP2* promoter sequence derived from the 314bp fragment upstream of the *GIP2* gene and the *DUT1* promoter sequence 444bp upstream from the *DUT2* gene.

TPS1 GENE SEQUENCE

5’-

GATCCAGCTG<u>ATG</u>ACTACGGATAACGCTAAGGCGCAACTGACCTCGTCTTCAGGGG
GTAACATTATTGTGGTGTCCAACAGGCTTCCCGTGACAATCACTAAAAACAGCAGTA
CGGGACAGTACGAGTACGCAATGTCGTCCGGAGGGCTGGTCACGGCGTTGGAAGG
GTTGAAGAAGACGTACACTTTCAAGTGGTTCGGATGGCCTGGGCTAGAGATTCCTG
ACGATGAGAAGGATCAGGTGAGGAAGGACTTGCTGGAAAAGTTTAATGCCGTACCC
ATCTTCCTGAGCGATGAAATCGCAGACTTACACTACAACGGGTTCAGTAATTCTATT
CTATGGCCGTTATTCCATTACCATCCTGGTGAGATCAATTTCGACGAGAATGCGTGG
TTGGCATACAACGAGGCAAACCAGACGTTCACCAACGAGATTGCTAAGACTATGAA
CCATAACGATTTAATCTGGGTGCATGATTACCATTTGATGTTGGTTCCGGAAATGTT
GAGAGTCAAGATTCACGAGAAGCAACTGCAAAACGTTAAGGTCGGGTGGTTCCTGC
ACACACCATTCCCTTCGAGTGAAATTTACAGAATCTTACCTGTCAGACAAGAGATTTT
GAAGGGTGTTTTGAGTTGTGATTTAGTCGGGTTCCACACATACGATTATGCAAGACA
TTTCTTGTCTTCCGTGCAAAGAGTGCTTAACGTGAACACATTGCCTAATGGGGTGGA
ATACCAGGGCAGATTCGTTAACGTAGGGGCCTTCCCTATCGGTATCGACGTGGACA
AGTTCACCGATGGGTTGAAAAAGGAATCCGTACAAAAGAGAATCCAACAATTGAAG
GAAACTTTCAAGGGCTGCAAGATCATAGTTGGTGTCGACAGGCTGGATTACATCAA
AGGTGTGCCTCAGAAGTTGCACGCCATGGAAGTGTTTCTGAACGAGCATCCAGAAT
GGAGGGGCAAGGTTGTTCTGGTACAGGTTGCAGTGCCAAGTCGTGGAGATGTGGA
AGAGTACCAATATTTAAGATCTGTGGTCAATGAGTTGGTCGGTAGAATCAACGGTCA
GTTCGGTACTGTGGAATTCGTCCCCATCCATTTCATGCACAAGTCTATACCATTTGA
AGAGCTGATTTCGTTATATGCTGTGAGCGATGTTTGTTTGGTCTCGTCCACCCGTGA
TGGTATGAACTTGGTTTCCTACGAATATATTGCTTGCCAAGAAGAAAAGAAAGGTTC

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CTTAATCCTGAGTGAGTTCACAGGTGCCGCACAATCCTTGAATGGTGCTATTATTGT
AAATCCTTGGAACACCGATGATCTTTCTGATGCCATCAACGAGGCCTTGACTTTGCC
CGATGTAAAGAAAGAAGTTAACTGGGAAAAACTTTACAAATACATCTCTAAATACACT
TCTGCCTTCTGGGGTGAAAATTTCGTCCATGAATTATACAGTACATCATCAAGCTCA
ACAAGCTCCTCTGCCACCAAAAACTGATGAACCCGATGCAAATGAGACGATCGTCT
ATTCCTGGTCCGGTTTTCTCTGCCCTCTCTTCTATTCACTTTTTTTATACTTTATATAA
AATTATATAAATGACATAACTGAAACGCCACACGTCCTCTCCTATTCGTTAACGCCT
GTCTGTAGCGCTGTTAGGCGCCGATC-3’

DUT1 PROMOTER SEQUENCE

5’-
GATCGCATGCGCTGTCTAGAATGCATTTTTCCATGCTACGTCGATTTTTTGCCCGGA
AGAGGCTGACGTAGCGCTGGAAGGTACCGACAACTATGCCTATTGTGGAGATGGG
CGGCAAACTCCTGTCGCAGAGTGGGGGCGGGGAAAGGTTCTTTTTGCCGTGGAAT
GAAGCGCATAAAAGAAAAACAGTATGCCATATTAAGTCTTTTTAAGGGGAAAGGGG
CTGCTACCATGAGGTCTTTTACCAAAAATGTGTATCAGCTACGCTTCTCAATGAAGG
GGCCAAGAAGTTCGTTCTATCCAACAGGAAATATTTCGATATTGCAACGGTCTTTCT
ATATCTTGCATATTCTTCTTCAGGTTGAGTTCCTTTATATATACTGCATTGCGTAATAA
GCAACACAGGGCCCATCCGTTCAACAGAAGAAAACTAAAGCTTTTCAAACATAGCCT
TTCCAGTTCAGTGCATATTTATAGAAGGTAAACCTGCATACAATACGGTTAAAACAAT
AGGAACGAAAAGAAAGCTAGACGGGAAAGCAGTTACTAAAGGCAGGGTTGACGGG
ACGTTTAAAGAGAGAAGTCTGGTTTATAAAACCTTTTAGTAGTAAAAAAGGAAAAGA
AACAAGGAAGGTTGGCATTCTGTTTGATTGAGGAGAAAGGAACCACATTGGACTTTT
TTTTTTCTTTCTGTAAGGTATAGTTTTTAGTAGGCTGAACATCAAAAAGATCTCCGTT
GTACTCAGAAACAAGTTTAATAGGAACGGGTACAGATATCTTGTCAAAATTTTATTCT
TACAGCCCAAGTTTATTAGGATTTCATCAACGCAACACAGCTGGATC-3’

GIP2 PROMOTER SEQUENCE

5’-
GATCGCATGCACTATGTACATACACACGCACCATTATCTCTCGTTTTACATAAGTAAA
TACAGCAATAATAATACCTGTAAATATCTCAACATACTCAATCAAATGAGCTGATAAG
CATATTCAATTTTTCTTCATAGCAATATTTTTTTCTGTCCAAGTTTTATTTTTTTTCCAT
AATTTCTGATTTACTACCATTGAAAATTATAAAAGGAAAAATATTACGCGCTACCATT
TAATAAGTAGAAACAACTAAGTTCTCGAATGAGATGTTTGCACTGATACAGCGAGCC
AAAAAATAACCAACAGCTGGATC-3’

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VERIFICATION OF SINGLE GENE AMPLIFACATION PRODUCT (RT-PCR PRIMERS –ACT1) FROM cDNA

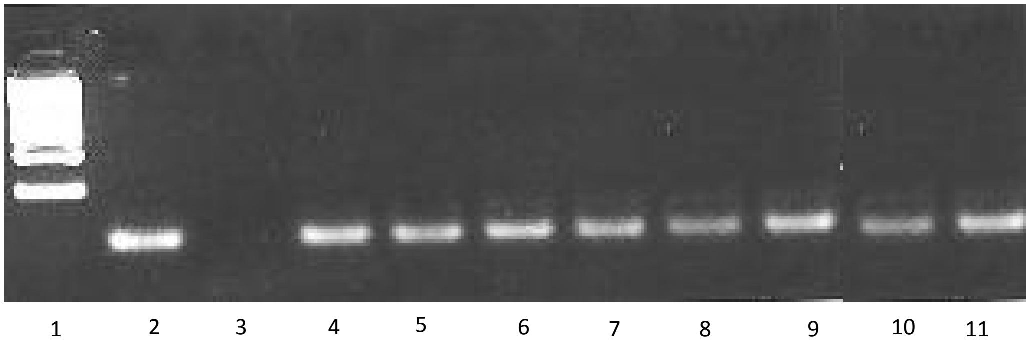

Fig 1 Single PCR product using ACT 1 housekeeping gene. lane 1 100bp ladder; lane 2: positive control; lane3:negative control;lane 4: 5x cDNA dilution T2; lane 5: 10x cDNA dilution T2; lane 6: 5x cDNA dilution T5; lane 7: 10x cDNA dilution T5; lane 8: 5x cDNA dilution T11; lane 9: 10x cDNA dilition T11; lane 10: 5x cDNA dilution; lane 11: 10x cDNA dilution.

REALTIME PCR SPECTRAL DATA SETS

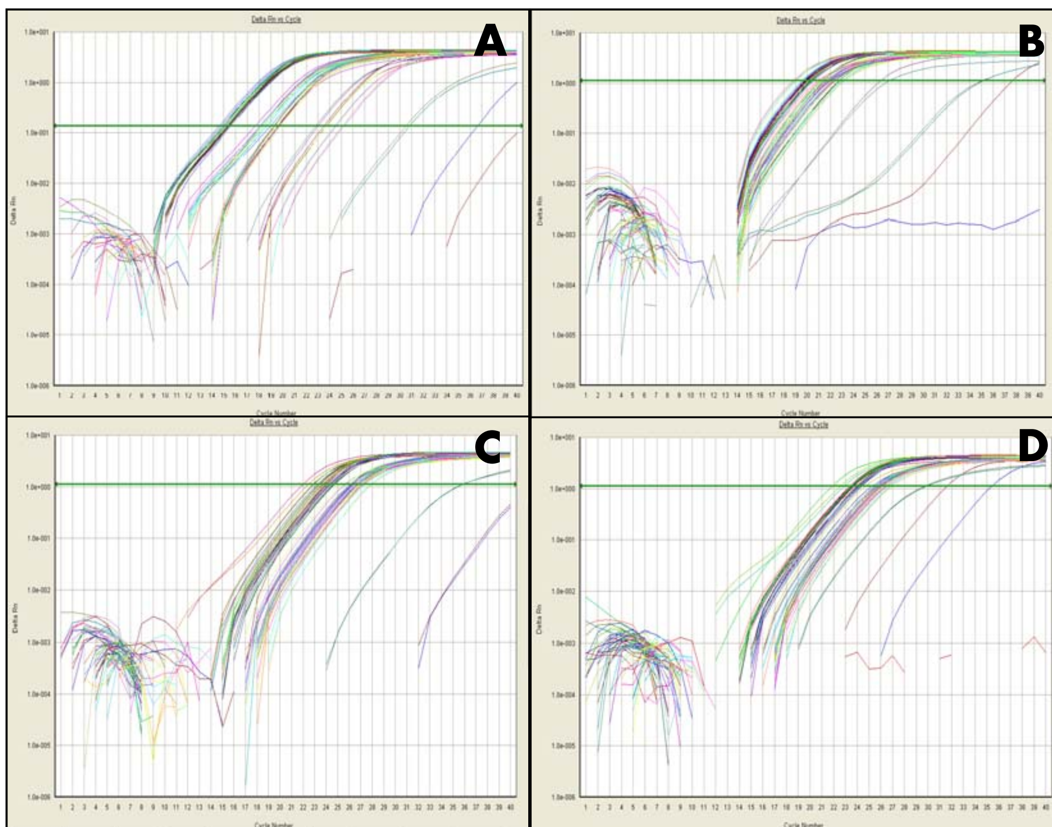

Fig 2 Amplification plots for RT-PCR Data. Graphical representation of real-time PCR data. A: Time point T2; B: Time point T5; C: Time point T11; D: Time point T14. Rn is the fluorescence of the reporter dye divided by the fluorescence of a passive reference dye. Rn is plotted against PCR cycle number. ΔRn is Rn minus the baseline; ΔRn is plotted against PCR cycle number. The amplification plot shows the variation of log (ΔRn) with PCR cycle number.