Generating lower ethanol yields in fermentations by *Saccharomyces cerevisiae* via diversion of carbon flux towards the production of fructo-oligosaccharides

by

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Thesis presented in partial fulfilment of the requirements for the degree of
Master of Science

at
Stellenbosch University
Institute for Wine Biotechnology, Faculty of AgriSciences

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March 2013

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Summary

There is a growing international consumer demand for the production of lower ethanol wines. This can be attributed to various qualitative, social, economic and health concerns that are associated with high ethanol wines (Kutyna *et al.,* 2010; Varela *et al.,* 2012). There is continuous development and research into methods and technologies to lower the ethanol concentration in wine. However, in addition to the added cost and complexity these technologies all have various shortcomings. The development of yeast strains with lower ethanol productivity, yet desirable organoleptic and fermentation capacity, therefore remains a highly sought after research and development target in the wine industry.

Biologically based approaches aim to generate yeast strains with the capacity to divert carbon from ethanol production towards targeted metabolic endpoints (Kutyna *et al.,* 2010). This should ultimately be achieved without the production of unwanted metabolites that can negatively affect wine characteristics. In the context of these challenges, this study aimed to investigate the use of fructans as carbon sinks during fermentation to divert fructose from glycolysis and ethanol production toward intracellular fructan production by generating levan producing strains. In addition, the impact of fructan production on metabolic carbon flux during fermentation by these strains was analyzed. This was the first attempt to analyze intracellular fructan production in *Saccharomyces cerevisiae* under fermentative conditions with fructans acting as carbon sinks.

Fructans are fructose polymers that act as storage molecules in certain plants and function as part of the extracellular matrix in microbial biofilms, and are intensively studied due to their economic interest. Here we undertook the heterologous expression of a levansucrase (LS) M1FT from *Leuconostoc mesenteroides*, an enzyme producing β(2-6) levan-type fructans, in the *S. cerevisiae* BY4742Δ*suc2* strains without invertase activity (encoded by *SUC2*). Levansucrases indeed utilize sucrose as both fructose donor and initial polymerization substrate, and the sucrose concentration is of import to maintain transfructosylation activity of enzyme. High intracellular sucrose accumulation was achieved by the heterologous expression of either a sucrose synthase (Susy; cloned from potato) or by growing strains expressing the spinach sucrose transporter (SUT) in sucrose containing media. Endogenous sucrose synthesis was of specific interest to the overall goal of the project, which was to reroute carbon flux away from glycolysis in grape must containing only hexoses as carbon source. In addition, this approach of combining intracellular sucrose production with intracellular levan production could be used in various applications to limit the need for sucrose in media as both carbon source and LS substrate.

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The extracellular LS M1FT was introduced into Susy and SUT strains as either the complete gene (M1FT) or 50bp truncation (M1FT∆sp) without the predicted signal peptide. The data show that intracellular levan accumulation occurred in aerobic, but not anaerobic conditions. The data also suggest that the production of levan did not impact negatively on general yeast physiology or metabolism in these conditions. However, no significant reduction in ethanol yields were observed, suggesting that further optimisation of the expression system is required. This is the first report of levan synthesis by *S. cerevisiae*, and contributes towards expanding the possibilities for further industrial applications of these compounds.

Opsomming

Daar is toenemende aanvraag deur wynverbruikers na laër alkohol wyne. Hierdie neiging kan toegeskryf word aan verskeie kwalitatiewe, gesondheids en sosio-ekonomiese redes wat geassosieer word met die verbruik van hoër alkohol wyne. Daar is ’n deurlopende navorsing dryf toegespits op metodes en tegnologieë om die alkohol konsentrasie van wyne te verlaag. Hierdie tegnologieë het egter, bykomstig tot koste en kompliksiteits toename, verkeie tekortkominge. Die ontwikkeling van gisrasse met verlaagde alcohol produksie, maar steeds wenslike organoleptiese en fermentasie eienskappe, bly ‘n baie gesogte navorsings en ontwikkeling teiken in die internasionale wyn industrie.

Biologiese benaderings streef om gisrasse te genereer met die vermoë om koolstof weg van etanol produksie te herlei na geteikende metabolise eindpunte. Hierdie doelwit moet ook uiteindelik bereik word sonder die produksie van ongewenste metaboliete wat die wyn negatief kan affekteer. In die konteks van hierdie uitdaging, het hierdie studie gestreef om die gebruik van fruktane as ’n koolstof poel tydens fermentasie, met die doel om fruktose te herlei vanaf glikolise en etanol produksie na intrasellulêre fruktane produksie. Om hierdie doelwit te bereik, is gisrasse ontwikkel wat levaan (’n spesifieke fruktaan) produseer. Die impak van fruktaan produksie op metaboliese koolstof vloei tydens fermentasie deur hierdie gisrasse is bykomsrig ontleed. Hierdie verslag beskryf die eerste poging om intraselullêre fruktaan produksie in *Saccharomyces cerevisiae* te bewerkstellig, met die doel om fruktaan as ’n koolstof poel te gebruik.

Fruktane is fruktose polimere wat as bergings molekules optree in sekere plante en ook funksioneer as deel van die ekstrasellulêre matriks in mikrobiese biofilms. Hierdie polimere word tans internasionaal intensief bestudeer weens hul ekonomiese belang. Hierdie studie beskryf die uitdrukking van die levaansukrase (LS) M1FT van *Leuconostoc mesenteroides*, wat β(2-6) levaan-tipe fruktane produseer, in *S. cerevisiae* BY4742Δ*suc2* rasse, sonder invertase (gekodeer deur *SUC2*). Levaansukrases gebruik inderdaad sukrose as beide ’n fruktose donor en ook as ’n aanvanklike polimeriserings substraat. Die fruktose konsentrasie is belangrik om transfruktosilerings aktiwiteit van die ensiem te handhaaf. Hoë intrasellulêre sukrose akkumulasie was bereik deur die heteroloë uitdrukking van ’n sukrose sintase (Susy; gekloneer van aartappel) of die spinasie sukrose transporter (SUT) in media bevattende sukrose. Endogene sukrose sintese was van spesifieke belang tot die algehele doelwit om koolstof te herlei, weg van glikolise tydens fermentase van druiwe sap. Die benadering om intraselullêre sukrose produksie met levaan produksie te koppel, kan ook gebruik word in verskeie toepassings om die afhanklikheid op sukrose in die media, as substraat vir LS, te verminder.

Die ekstraselullêre LS, M1FT, was as vollengte geen (M1FT) of as ’n 50bp afkapping (M1FTΔsp), sonder seinpeptied, in die Susy en SUT gisrasse uitgedruk. Die data dui aan dat die produksie van levaan nie ’n negatiewe impak het op gis fisiologie of metabolisme in die toets kondisies nie. Daar was egter geen waarbeenbare afname in etanol opbrengs nie, wat aandui dat verdere optimisering van ekspressie sisiteem benodig word. Hierdie is die eerste verslag van levaan sintese in *S. cerevisiae* en dra by tot die uitbreiding van moontlikhede vir indutriële toepassings van die die verbindings.

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This thesis is dedicated to

My parents, Herman and Wilhelmina Brandt
For their continued love and support

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Biographical sketch

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Acknowledgements

I wish to express my sincere gratitude and appreciation to the following persons and institutions:

- **Prof Florian Bauer**, Institute for Wine Biotechnology, Stellenbosch University who was my supervisor for this project, for his invaluable input and advice;

- **Dr Jaco Franken**, Institute for Wine Biotechnology, Stellenbosch University for his continual input, technical advice and helpful discussions and laughs when the going got tough;

- **Dr Gavin George**, Institute for Plant Biotechnology, Stellenbosch University for kindly providing the levansucrase gene template used in this study;

- My friends at the Institute for Wine Biotechnology for their support especially **Thulile Ndlovu**, for her words of encouragement and support;

- The **IWBT** and **NRF** for their financial contributions;

- **My Friends** for their continual encouragement and willingness to put up with endless hours of thesis talk, especially **Warren Gillion** and **Charne Petzer** for their love and support;

- Last but not least, **my parents** and **siblings** for their love, support and encouragement.

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Preface

This thesis is presented as a compilation of 4 chapters. Each chapter is introduced separately.

Chapter 1 **General Introduction and project aims**

Chapter 2 **Literature review**
Diversion of carbon flux towards the production of fructo-oligosaccharides in *Saccharomyces cerevisiae* as a possible means of lowering ethanol production

Chapter 3 **Research results**
Generating lower ethanol yields in fermentations by *Saccharomyces cerevisiae* via diversion of carbon flux towards the production of fructo-oligosaccharides

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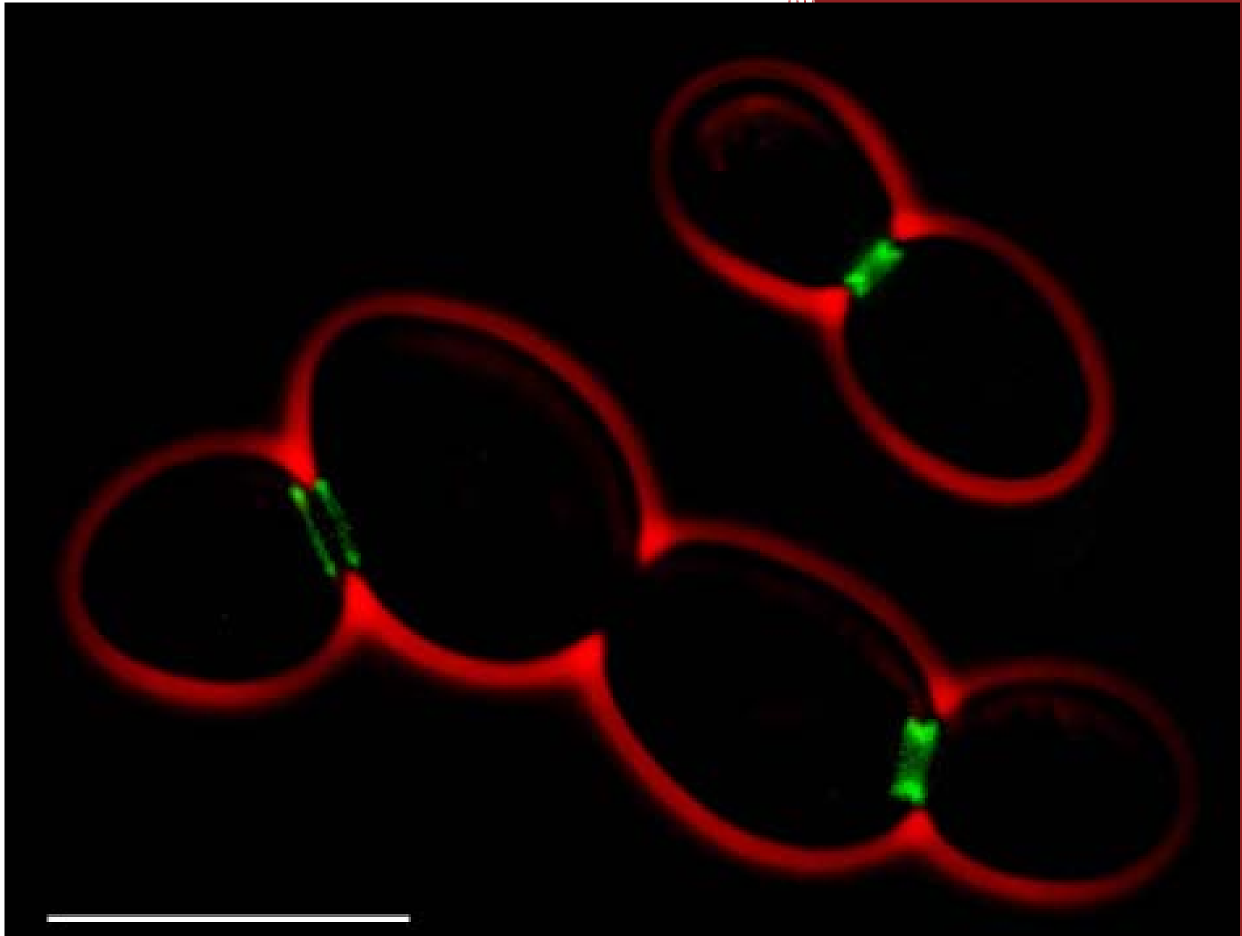
Chapter 1

Introduction and project aims



1. INTRODUCTION AND PROJECT AIMS

1.1 INTRODUCTION

Yeast fermentative capacity forms the basis for the production of a wide range of alcoholic beverages (Varela *et al.,* 2012). The commercial development of yeast starter cultures has specifically focused on improving yeast fermentation capacity measured in terms of ethanol productivity or yield, stress tolerance and early initiation of fermentation (Pretorius, 2000; Rainieri and Pretorius, 2000). Furthermore, the production of other yeast metabolites is also of importance, particularly in the alcoholic beverage industry context, as these molecules shape the organoleptic properties of beer and wines (Varela *et al.,* 2012).

Currently, the wine industry is under increasing consumer pressure for the production of easy to drink wines with moderate ethanol levels (Pickering, 2000). This is based on a combination of social, qualitative, economic and health issues associated with alcohol consumption in general. High ethanol content in wine can compromise product quality, increase perception of hotness and viscosity, and to a lesser extent, negatively impact sweetness, acidity, aroma, flavour intensity and textural properties of wine (Gawel *et al.,* 2007a; Gawel *et al.,* 2007b; Guth and Sies, 2001; Varela *et al.,* 2012). There has been significant interest in the development of technologies to produce lower ethanol wines that retain balance, flavour profile and other sensory and organoleptic characteristics (Kutyna *et al.,* 2010).

Maintaining the balance of ethanol in relation to wine flavour compounds is crucial when attempting to adjust ethanol concentration in wines. Ethanol is the most abundant volatile organic component in wine and is of particular importance as it has been shown to moderate the sensory impact of aroma compounds (Voilley and Lubbers, 1999; Williams, 1977). Given the complex interactions between ethanol and the various organoleptic aroma and taste components, careful consideration must be given to selecting techniques for lower ethanol wine production. Physical wine processing techniques aim to either decrease the sugar concentration in the grape must or reduce ethanol concentration post-fermentation. This, however, adds costs and complexity to the wine making process. Furthermore, post-fermentation wine processing can lead to loss of volatile aroma compounds and decrease other sensory characteristics of wine. These combined disadvantages have spurred various studies to investigate the generation of wine yeast strains with decreased ethanol productivity, yet maintained organoleptic and sugar utilization properties.

The screening of industrial wine yeast strains for lower ethanol production and developing methods with selective pressures toward lower ethanol production is ongoing.

Furthermore, several genetic modification (GM) strategies are available to divert yeast metabolism away from ethanol production towards alternative metabolic end-points (Kutyna *et al.,* 2010; Pretorius *et al.,* 2012; Varela *et al.,* 2012). In these strategies, metabolic end-points are selected to either complement the wine, e.g. glycerol, or be inert in the wine environment, thus minimising effect on wine bouquet. Glycerol is mainly formed in wine as a by-product of glycolysis by fermenting wine yeasts. It is thought to improve the overall balance between alcoholic strength, acidity, astringency and sweetness and is therefore considered to confer a degree of roundness and smoothness on the palate (Hickinbotham and Ryan, 1948; Nieuwoudt *et al.,* 2002). It is considered an ideal metabolic end-point to complement wine bouquet. Therefore several studies have endeavoured to generate yeast strains able to partially redirect carbon towards glycerol production, thus decreasing ethanol yield. There are several genetic modification approaches which can be used, such as overexpression of *GPD1* and/or *GDP2* genes which encode the glycerol-3-phosphate dehydrogenase isozymes (Cambon *et al.,* 2006; de Barros Lopes *et al.,* 2000; Nevoight and Stahl, 1996; Michnick *et al.,* 1997; Remize *et al.,* 1999; Varela *et al.,* 2012), disrupting or impairing alcohol dehydrogenase (*ADH*) expression and activity (Drewke *et al.,* 1990; Johansson and Sjostrom., 1984) or deleting pyruvate decarboxylase (*PDC)* genes (Nevoight and Stahl, 1996). These approaches have been successful in lowering ethanol yield. However, increased production of other metabolites that negatively impact wine quality such as acetic acid and acetoin (rancid butter aroma) was reported (Varela *et al.,* 2012). Therefore, additional genetic modifications are required to circumvent production of unwanted metabolites that can negatively affect the wine. Strategies such as diverting carbon from ethanol production towards storage carbohydrates or toward the synthesis of organic acids such as gluconic acid remain to be tested in wine environments.

The production of unwanted metabolites is frequently linked to the maintenance of the redox cycle during fermentation. Therefore, when modifications can be targeted to minimally impact on glycolysis, secondary unwanted metabolite production is expected to be minimal. The same holds true when considering storage carbohydrates to act as carbon sinks. The carbon is redirected from glycolysis in such a way as to not interfere with the redox cycle. The aim of these approaches is to decrease the carbon available for ethanol production, and thus a heterologously produced neutral polymer for which no native catabolic activity is present would be ideally suited for this purpose. With no active mechanism present to export, the storage carbohydrates should accumulate inside the cells, thus sequestering the synthesized polymers after fermentation and eliminating contact with the wine medium. The natural ability of *Saccharomyces cerevisiae* to produce storage carbohydrates such as glycogen and trehalose (Panek, 1991; O'Connor-Cox *et al.,* 1996; Pretorius, 2000) further illustrates the viability of polymers as carbon receptors. Thus, storage carbohydrates as carbon sinks provide a potential genetic modification approach to produce yeast strains with lower ethanolic capacity, yet maintained fermentative and organoleptic productivity.

This project specifically considers the use of heterologously produced fructans as potential carbon sinks with the aim of diverting carbon flux away from glycolysis and therefore, ethanol production. Fructans are sucrose-derived sugar polymers consisting of two up to more than a hundred thousand fructose units and are produced as part of the extracellular matrix in a broad range of micro-organisms and in a limited number of plant species as non-structural storage carbohydrates (Banguela *et al.,* 2011). The synthesis of distinct fructans, classified according to the type of bond formed, is catalysed by fructosyltransferase (FTF) enzymes. Levansucrases produce levan type fructans characterized by β(2-6) linkages between fructose monomers, whereas inulosucrases produce inulin type polymers with β(2-1) linkages between fructose monomers (Waterhouse and Chatterton, 1993). Fructans are not naturally produced by *S. cerevisiae*, thus theoretically, there should be no native fructan degradation activity in yeast cells. Furthermore, utilizing fructans as soluble storage carbohydrates has additional advantages, which include it being inert in wine environment and being osmotically less active than its sugar constituents, which would facilitate storage at higher concentrations (Altenbach and Ritsema, 2007). Sucrose is required by FTF enzymes as both fructose donor and acceptor molecules, yet *S. cerevisiae* does not naturally accumulate intracellular sucrose. Therefore, two distinct strains were developed, one which utilizes a sucrose transporter gene (SUT gene from spinach) and another which utilizes a sucrose synthase gene (Susy gene from potato) to yield intracellular sucrose as FTF substrate.

1.2 PROJECT AIMS

This study aims to investigate the use of fructans as carbon sinks during fermentation to divert fructose from glycolysis and ethanol production toward intracellular fructan production. Specifically, the production of intracellular levan by *S. cerevisiae* and the effect on carbon flux during fermentation was analyzed. This is the first study to investigate levan production in *S. cerevisiae*. Furthermore, incorporating intracellular sucrose production with intracellular fructan production is a new approach to fructan production. The cloning and expression of an active levansucrase with intracellular levan producing capacity in *S. cerevisiae* would allow for the validation of heterologous storage polymers as carbon sinks.

With these considerations in mind, the following broad aims were set out in the project:

i.) The generation of yeast strains that is able to accumulate intracellular sucrose, which will function as the substrate for levan production.

ii.) Heterologous expression of the *Leuconostoc mesenteroides* fructosyltransferase (M1FT) in the generated sucrose accumulation strains.

iii.) Assessing the generated strains for sucrose accumulation and also levan production.

iv.) Assessing the generated levan producing strains in terms of performance and impact on alcoholic fermentation.

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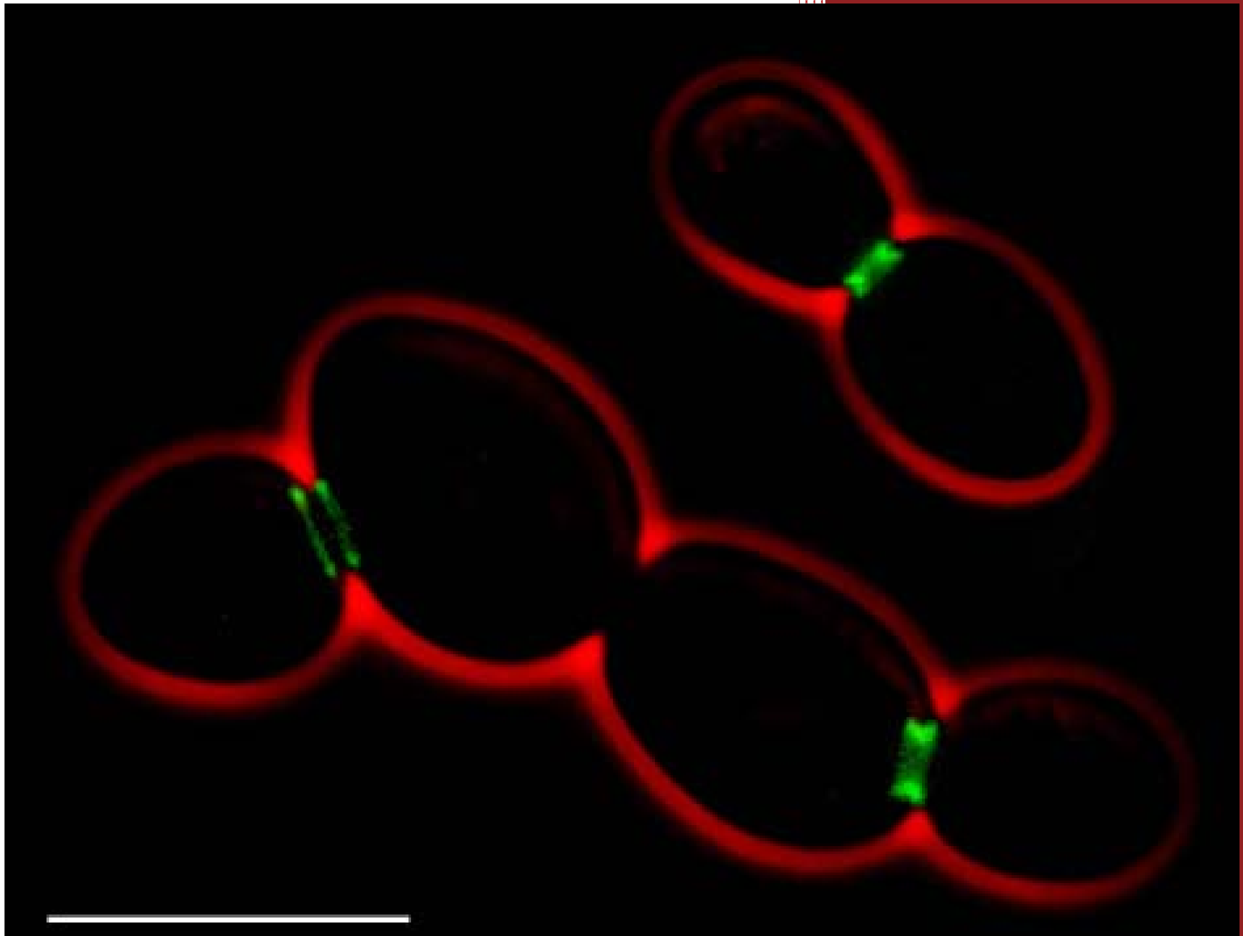
Chapter 2

Literature review:

Diversion of carbon flux towards the production of fructo-oligosaccharides in *Saccharomyces cerevisiae* a possible means of lowering ethanol production



2. LITERATURE REVIEW

2.1 INTRODUCTION

Fermentation-based processes have been used over thousands of years to prepare foods and beverages (Kutyna *et al*., 2010). Wine-making in particular has been dated through archaeological evidence in the Middle East to around 4000 B.C. (Poo, 1995). Traditional methods rely on microflora inocula that are present on the grapes, in the vineyard or in the "winery" for fermentation. The relative unpredictability and unreliability of these practises gave the wine-makers limited control over final wine quality. In recent times, with the advent of commercially available, pure *Saccharomyces cerevisiae* inocula with known properties, wine production on a larger, industrial scale with greater process predictability was made possible. With our improving understanding of yeast biology and fermentation processes, modern wineries can produce more predictable and reliable wines with established quality criteria (Henschke, 1997; Pretorius *et al.,* 2003).

The ethanol concentration of wine is primarily determined by the initial sugar concentration in the grapes and juice and the completeness and efficiency of the alcoholic fermentation (Yu and Pickering, 2008). The commercial development of *Saccharomyces cerevisiae* cultures for wine fermentations has focused mainly on the early initiation of fermentation, improving stress tolerance, and increasing fermentation efficiency (Pretorius, 2000; Rainieri and Pretorius, 2000). Currently, there is growing consumer demand for lower ethanol wines due to various economic, health and social reasons. Given that 90-95% of the sugar in the grape must is converted to ethanol, higher sugar musts, when fermented to dryness (< 5g/L sugar), can result in higher ethanol wines. This is especially a problem in regions with dry and warmer climates. In these climates, fruit deterioration is minimised, which allow winemakers greater flexibility in choosing when to harvest. The extension of time before harvests allows grapes to achieve phenolic ripeness which enhances the preferred flavour profile of wines and reduces unsavoury green characters. This extension however can also lead to higher sugar concentrations in the grapes and in turn the must. Therefore, extensive research is being done on methods to decrease ethanol produced during fermentations without compromising wine quality and flavour.

Early inventions and innovations in grape and wine production were based on little or no knowledge of the biology of grapevines or the microbes that drive fermentation (Chambers and Pretorius, 2010). Scientific advances in fermentation knowledge and techniques for the analyses of wine components has allowed for greater understanding of the dynamics of carbon flux during fermentations in the yeast cell, and how this relates to sugar utilisation and ethanol

production (Figure 2.1). When adjusting alcohol in wine, there are many factors that must be considered, ranging from consumer demand to the balance of alcohol and aroma compounds. The demand for lower ethanol wine has driven the wine industry to develop both physical and biotechnological approaches that tackle this problem. There are innovative processes designed to de-alcoholise, or lower/ reduce ethanol in wine, via viticultural or physical wine processing methods. There are also biotechnological approaches designed to redirect carbon flux from ethanol production towards molecules such as glycerol and organic acids such as gluconic acid and acids involved in the tricarboxylic acid (TCA) cycle. These molecules are selected to complement wine flavour or to be inert and not affect wine quality.

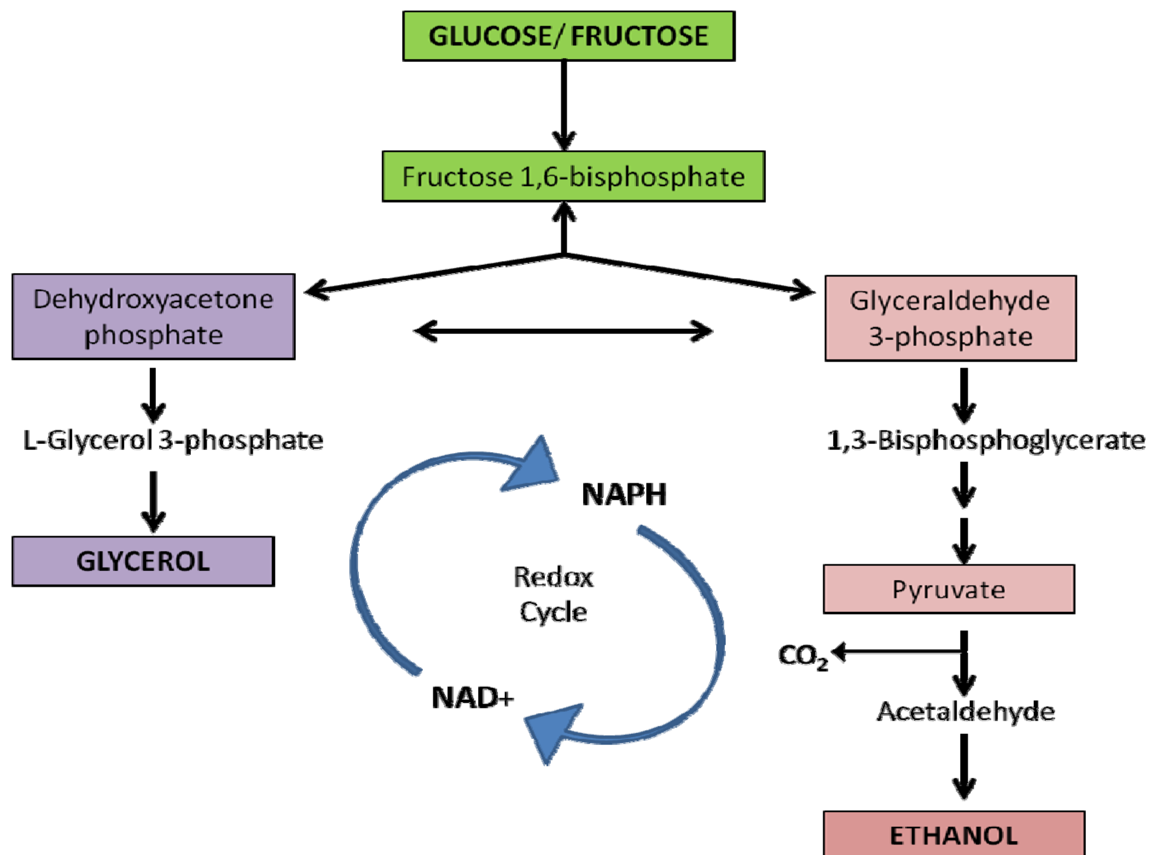


Figure 2.1: Simplified view of glucose/fructose metabolism during fermentation by wine yeast. Ethanol and CO_2 are major products formed during fermentation and to a lesser extent, glycerol.

Flavour is wine's most important distinguishing characteristic. The endless variety of flavours stem from a complex non-linear system of interactions among many hundreds of compounds, which then results in the overall impression of both aroma and taste components. These compounds include organic acids, alcohols, phenolics, sugars, glycerol, various esters, aldehydes, ketones, terpenes and other volatile compounds (Berg *et al.,* 1955; Rapp and Mandery, 1986). Of these, ethanol is the most abundant volatile organic component and is particularly important given its varied role in influencing the aroma and flavour of wine (Yu and Pickering, 2008). Ethanol has been shown to moderate the sensory impact of aromatic

compounds in wine by affecting their solubility, volatility and ability to bind with proteins (Voilley and Lubbers, 1999) and through a masking effect (Williams, 1977). Thus, given the complex nature of the interactions between ethanol and other wine components, understanding ethanol production and how aroma compounds interact with it, is of considerable import to wine makers. Methods that have minimal or no impact on aroma compounds but reduce ethanol are highly sought after.

The consumer demands for lower alcohol wines as well as the various other economic and health reasons have created a niche for lower alcohol wines in the market. Given the complex interactions between ethanol and aroma compounds, the methods used to generate lower ethanol wines are carefully selected by the wine makers to complement their wine style. This review describes current methods used to reduce ethanol in wine, with particular emphasis on biotechnological approaches designed to redirect carbon flux away from ethanol production. Furthermore, this review considers using carbon storage molecules as carbon receptors, and suggests a novel approach by proposing fructans as unique carbon storage molecules to act as carbon reservoirs, thereby redirecting carbon flux during fermentations.

2.2 THE QUEST FOR LOWER ETHANOL WINE

What is the optimal ethanol level required for a full bodied, high quality wine? This question has been debated by wine makers and consumers globally and formed the basis of many consumer panel based studies. Furthermore, the link between alcohol and consumer preference varies across consumer groups. It is thus important for wineries to consider market demands and their market segment when adjusting alcohol levels in wines.

2.2.1 The importance of lower alcohol wines in the global wine industry

Wine alcohol content is of growing importance to the wine industry (Varela *et al*., 2008). Over the past twenty years, alcohol levels in wines have increased significantly. This trend, observed in many producing areas, is linked to various factors, including global warming, the selection of grapes with a high sugar yield and the evolution of winemaking practices which favour the harvest of very mature grapes (Ehsani *et al.,* 2007). With the growing consumer demand for lower alcohol wines, wine makers are currently expected to optimize wine alcohol adjustments. It is therefore pertinent to establish how much of a change in ethanol in wine is required before it can be detected sensorially, which is known as the “difference threshold” (Yu and Pickering,

2008). The balance between the wine flavour compounds in relation to alcohol is crucial when adjusting/lowering alcohol in wine to maintain wine style and quality. Of equal importance is defining the term "lower ethanol wines", as it can be ambiguous and understanding the definition simplifies the approaches directed towards generating these specific wines.

When dealing with lower alcohol wines, it is important to remember that the term refers to a percentage decrease in the wine ethanol content via any of the various methods available. Many winemakers are seeking methods to slightly decrease the alcohol content of their wine, often by 1 or 2%, without lowering the concentration of other compounds involved with wine quality, especially aromatic compounds (Heux *et al.,* 2006). Hot wine growing climates such as in California, Spain, and Australia, where grapes may be harvested at very high sugar levels, often results in wines of high ethanol (e.g. 14 to 16% v/v). Many of these wines are considered out of balance and dominated by ethanol-associated attributes (Yu and Pickering, 2008). Thus alcohol is adjusted in the wine to the accepted alcohol levels of its particular style, thereby balancing the wine bouquet.

There are various reasons to why lower alcohol wines are in demand apart from high ethanol concentrations that affect the sensory properties of wines. Today's market, in line with the consumers' health concerns and prevention policies, focuses more on easy-to-drink wines with moderate alcohol levels. Social benefits may include improved productivity and function after activities involving alcohol (e.g. business lunches), lower risk of prosecution or accident while driving and more acceptable social behaviour in general. Health advantages may include reduced calorie intake, decreased risk from alcohol-related illness and disease (Pickering, 2000). Moreover, excessive ethanol content leads to higher costs in some countries which impose taxes on the alcohol degree (Ehsani *et al.,* 2007). This additional tax imposed on wines with elevated ethanol can tax the wine out of the competitive wine market.

Wines with reduced alcohol (ethanol) content have been commercially available for over two decades. Several technologies are used to produce de-alcoholised, low- and reduced-alcohol wine, while consideration is also given to the key quality, sensory, economic and marketing issues associated with wine of reduced alcohol content (Pickering, 2000).

2.2.2 Methods of generating lower ethanol wine

There are a number of techniques that can be used to reduce the alcohol content in wine. These fit broadly into one of three main groups; namely viticultural, physical and biological techniques (Kutyna *et al.,* 2010). Viticultural approaches are based on grape berry development and grapevine management. Physical methods to achieve lower ethanol in wines aim to reduce either sugar in the grape must or ethanol from the wine. Biological approaches include the possible use of genetically engineered yeast to divert carbon from ethanol towards various other

molecules, as well as selection of lower ethanol producing industrial wine yeast strains (Figure 2.2). Each of these techniques has its unique advantages and disadvantages. Wine makers thus have to carefully consider which process or combination of processes would be best suited to their needs and particular wine style.

Viticultural methods aim to reduce the amount of sugar that forms in the grape berry resulting in lower sugar content in grape must. This is made difficult due to the fact that fruity characters and reduced “green characters” develop as berries mature, and this maturation unavoidably produces fruit with a higher sugar content, which translates to higher ethanol concentrations in the wine (Chambers and Pretorius, 2010). The grape growers thus have to decide on the balance between phenolic ripeness and sugar content.

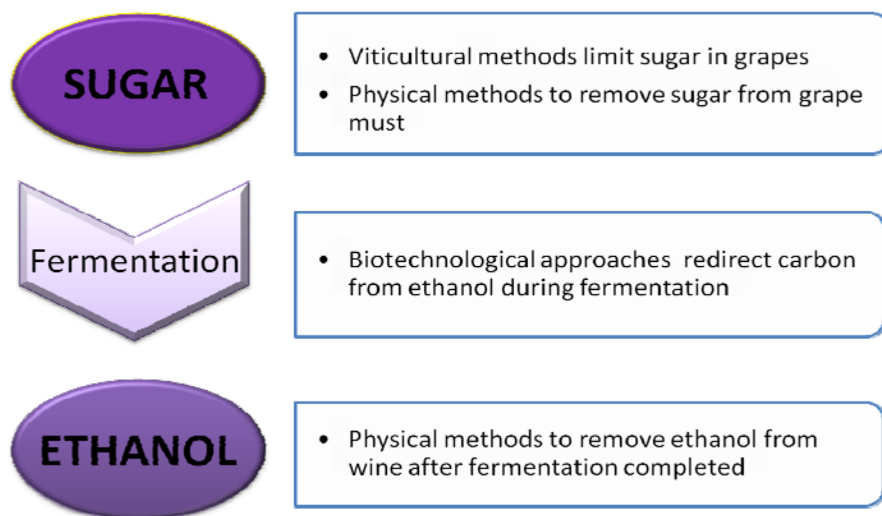


Figure 2.2: Methods to decrease ethanol levels in wine**.** The sugar content of the grape must equate to the concentration of ethanol in wine, when must is fermented. The three main phases of carbon flow to ethanol and the main ethanol reduction methods are shown.

One method of reducing sugar concentration in the berries is to shorten maturation period of berries. This however can lead to increased “green”, unripe characters and higher acid concentrations in wine (Varela *et al.,* 2008). The method therefore requires careful balance between wine flavour profile and maturity of grapes. Increases in pre-harvest irrigation can be also be used, but this does not appear to have any significant effect on sugar content of grapes. This technique also has the adverse effect of delaying ripening in high crop yields and prolonged maturation periods that might extend beyond onset of autumn-winter rains in some regions. Another method used by grape-growers is the adjusting of the leaf area to fruit weight ratio (LA/FW). This method requires lowering the LA/FW ratio after fruit onset, which then translates to a more balanced ratio between sugar and phenolic compounds. The drawback however is that ripening may be delayed or excessive bunch exposure may occur (Coulter, 2012).

There are various physical methods designed to reduce the ethanol content in wine. These methods at their simplest involve dilution (blending wine; water addition), heating and reverse osmosis (for review see Pickering, 2000). The volatile nature of ethanol allows for lower ethanol wines simply by fermenting at higher temperature. This however can lead to loss of volatile aroma compounds and increased production of unsavoury aroma compounds. The disadvantages of physical methods apart from loss of aroma, is that post fermentation wine processing to remove ethanol adds considerable cost to wine, while possibly lowering the quality of the wine produced.

Perhaps the simplest and most economical way to produce wine with lower ethanol concentrations would be the development of yeast strains with means of partially redirecting carbon metabolism away from ethanol production during fermentation (Kutyna *et al.,* 2010).

2.2.3 Biological approaches for carbon re-routing

Targeted changes can be made to the yeast genome that lead to a redirection of metabolic flux away from ethanol fermentation toward other end points, recognising that the choice of end-points is however constrained by likely incompatibility with wine composition and flavour (Kutyna *et al*., 2010). Metabolic end-points are often selected to either complement wine composition or to be completely inert in the wine environment thus minimising impact on yeast metabolism and wine bouquet. Various expression studies have been done to either delete or over-express key enzymes involved in the carbon metabolism of yeast during fermentation in an effort to redirect carbon away from ethanol production as seen in Figure 2.3. Wine complementary molecules (e.g. glycerol) are often selected as carbon receptors, as are molecules that yeast cannot metabolise, such as gluconic acid.

The complexity of carbon metabolism and also the need to maintain the NAD^+/NADH redox balance during fermentation complicates the selection of targets enzymes. The redox balance of yeast grown on high sugar concentrations is firmly linked to the production of metabolic end-products, such as ethanol, glycerol, and acetic acid. The need by yeast to maintain a redox balance has been used in recent years to design controlled and predictable metabolic rerouting systems that redirect carbon flux towards desired end points, e.g. glycerol overproduction (Kutyna *et al.,* 2010).

Expression studies often target enzymes catalyzing reactions in the glycerol production pathway. Glycerol is a polyol with a colourless, odourless and highly viscous character and is mainly formed in wine as a by-product of glycolysis by fermenting wine yeasts. It tastes slightly sweet, and has an oily and heavy mouth-feel. In addition to contributing to sweetness when present in quantities above its threshold taste level of 5.2g/L in wine (Hinreimer *et al.,* 1955), glycerol has been implicated in mouth-feel sensations by conferring “fullness” (also referred to

as "viscosity" or "weight") to wine. Glycerol is also thought to improve the overall balance between alcoholic strength, acidity, astringency and sweetness and hence is considered to confer a degree of roundness and smoothness on the palate (Hickinbotham and Ryan, 1948; Nieuwoudt *et al.,* 2002). Thus, given the positive attributes glycerol can contribute to wine, it is a choice molecule for diverting carbon from ethanol production.

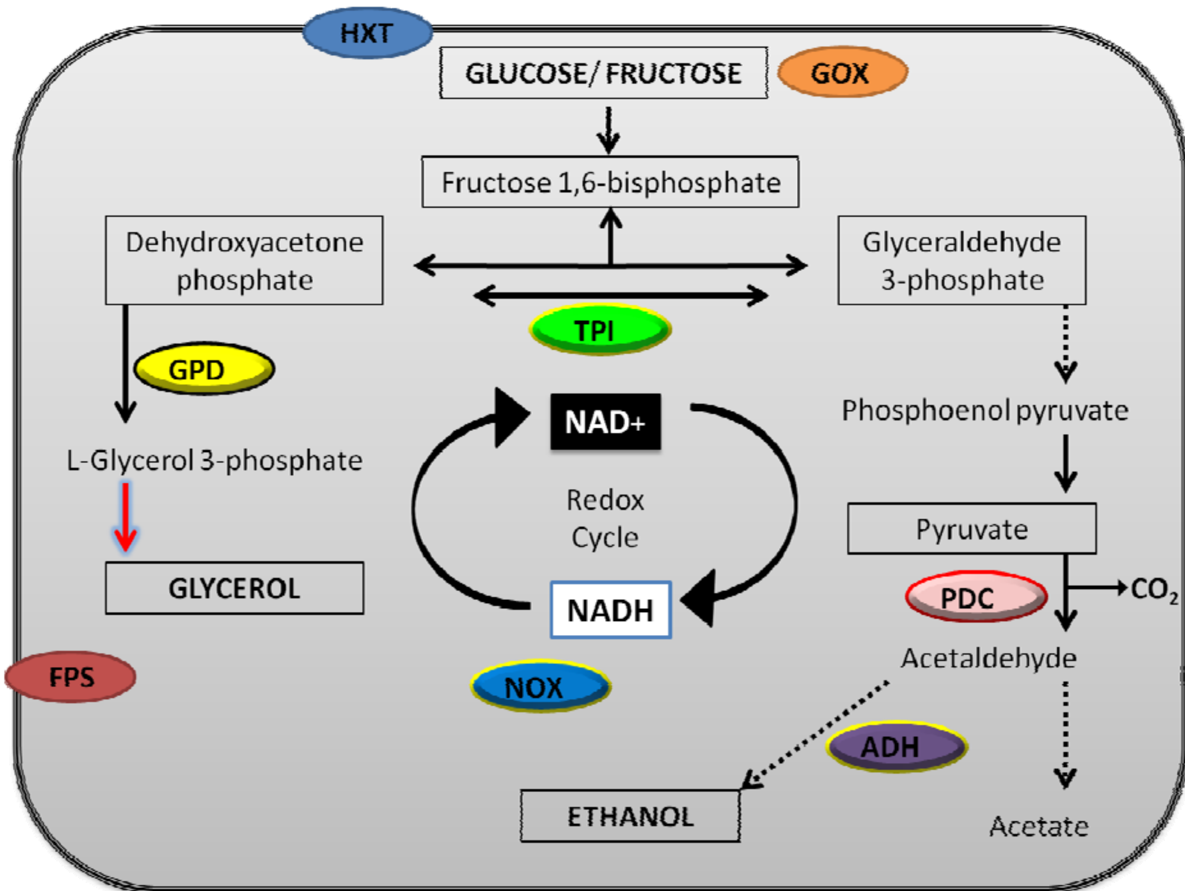


Figure 2.3: Examples of the targeted enzymes in various expression studies which were either modified, over-expressed or deleted in an effort to re-direct carbon away from ethanol production. These enzymes all function within carbon metabolism during fermentation by wine yeast. The red arrow indicated NAH^+ producing reaction whereas the dotted arrows indicate NADH formation. GPD- Glycerol 3-phosphate dehydrogenase; PDC- Pyruvate decarboxylase; ADH-Alcohol dehydrogenase; TPI-Triose phosphate isomerise; NOX-NADH oxidase; FPS-Glycerol transporter; GOX-Glucose oxidase; HXT-Hexose transporter.

One approach used to enhance glycerol production is the over-expression of *GPD1* or *GPD2* genes (Nevoigt and Stahl, 1996; Michnick *et al.,* 1997; Remize *et al.,* 1999; de Barros Lopes *et al.,* 2000; Remize *et al.,* 2001; Eglinton *et al.,* 2002; Cambon *et al.,* 2006; Kutyna *et al.,* 2010). Gpd1p and Gpd2p are isozymes that reductively convert dihydroxy- acetone phosphate (DHAP) to glycerol 3-phosphate (G-3-P), which is subsequently dephosphorylated to glycerol by glycerol-3-phosphatase (Figure 2.3). Over-expression of *GPD1* or *GPD2* has been shown to increase glycerol yield by up to 548%, depending on the yeast strain, medium and fermentation

conditions (Kutyna *et al.,* 2010). The ethanol yield observed showed reduction of up to 35%. However, increased glycerol production results in a shift in the redox balance, through excessive NAD^+ regeneration. In response to this imbalance, acetate is produced by the yeast to regenerate NADH. In addition, several other redox-dependent metabolic pathways will show modified flux resulting in other, mostly unwanted metabolites such as succinate, acetaldehyde, acetoin and 2,3-butanediol also being produced in higher quantities (Cambon *et al.,* 2006; Eglinton *et al.,* 2002; Michnick *et al.,* 1997; Remize *et al.,* 1999). These metabolites have an undesirable impact on wine quality. Further genetic modifications of *GPD* yeast mutants are therefore required to avoid producing excessive amounts of these metabolites (Kutyna *et al.,* 2010). These modifications include *GDP* overexpression in combination with *ALD6* (aldehyde dehydrogenase) which reduces acetic acid concentrations. However, this resulted in increased acetoin production (Cambon *et al.,* 2006).

Alternatively, molecules that are inert in wine can be used as carbon receptors to minimise genetic modification and preferably have little to no impact on the redox balance during fermentation. Current research is being done to identify such molecules (e.g. fatty acids in TCA) and modulate expression of said molecules in wine yeast to ascertain the impact on fermentation and ethanol production. In addition, ongoing efforts are underway to identify agents for selective pressure that favours redirection of carbon in yeast during fermentation.

2.3 FRUCTANS AS STORAGE MOLECULES

The natural production of intra-cellular polysaccharides shows the capacity of yeast cells to accumulate carbon, such as glycogen and trehalose for storage as survival mechanism. Such sugar polymers can potentially be used as carbon receptors to partially redirect carbon from ethanol production toward polymer production. Targeted approaches aim to produce inert intracellular molecules that cannot be metabolised by yeast to act as metabolically neutral, non-lethal carbon receptors. The remainder of this review will evaluate the potential of sugar polymer molecules as storage carbohydrates and their potential usage as carbon sinks when aiming to divert carbon flux away from ethanol formation.

2.3.1 Storage molecules as carbon receptors

Many microorganisms, including yeast and bacteria, accumulate carbon energy reserves as a means to cope with starvation conditions frequently encountered in the environment. The

biosynthesis of glycogen is a conserved and widely utilised strategy for such metabolic storage and a variety of sensing and signalling mechanisms have evolved in evolutionarily distant species to ensure the production of this homopolysaccharide (Wilson *et al.,* 2010). Glycogen and trehalose are the main storage carbohydrates in yeast cells (Panek, 1991) and it has been clearly illustrated how important these carbohydrates are for the viability, vitality and physiological activity of yeasts (O'Connor-Cox *et al.,* 1996; Pretorius, 2000). The example of glycogen production in yeast can be used to illustrate how natural storage molecules act as carbon receptors or reservoirs for later utilization by cells.

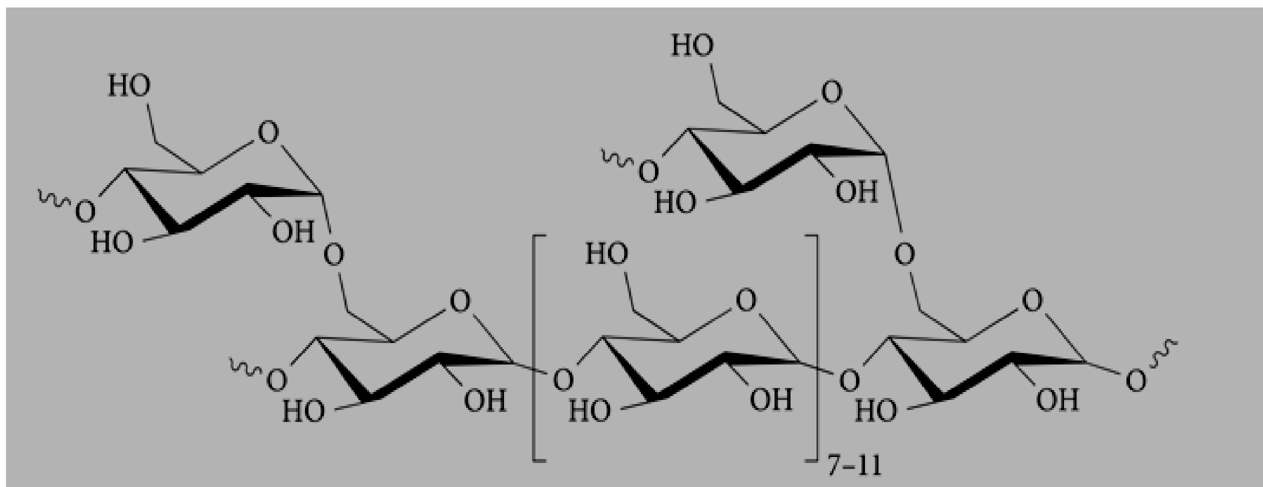


Figure 2.4: The chemical structure of glycogen. The linear α-1,4-glycosidic linkages can be seen as well as the α-1,6-branch points (Rapp, 2012).

Glycogen is a major intracellular reserve polymer consisting of α-1,4-linked glucose subunits with α-1,6-linked glucose at the branching points (Figure 2.4) (Wilson *et al.,* 2010). The structure of yeast glycogen is similar to that of other glycogens, with a chain length of 11–12 glucose residues (Northcote, 1953) and a particle diameter of around 20nm (Mundkur, 1960). The synthesis of glycogen requires the activities of glycogenin and a self-glucosylating initiator, glycogen synthase, *GSY1/GSY2* (Farkas *et al.,* 1991; Cheng *et al*., 1995), which catalyzes bulk synthesis. In addition, it requires the activity of the branching enzyme (*GLC3*), which introduces the branches characteristic of the mature polysaccharide (Figure 2.5) (Rowen *et al.,* 1992).

Glycogen is formed upon limitation of carbon, nitrogen, phosphorous or sulfur (Lillie and Pringle, 1980). The one outstanding advantage in using glycogen as a reserve compound is that this macromolecule has little effect on the internal osmotic pressure of the cell (Wilson *et al.,* 2010). Glycogen provides a readily mobilizable carbon and energy source that can be accessed while the yeast adapt to a new growth medium (Pretorius, 2000). Glycogen breakdown is also accompanied by sterol formation, which is essential for yeast vitality and

successful fermentation (Francois *et al.,* 1997). In yeast, the importance of glycogen reserves in survival during long-term nutrient deprivation has been demonstrated clearly (Sillje *et al.,* 1999).

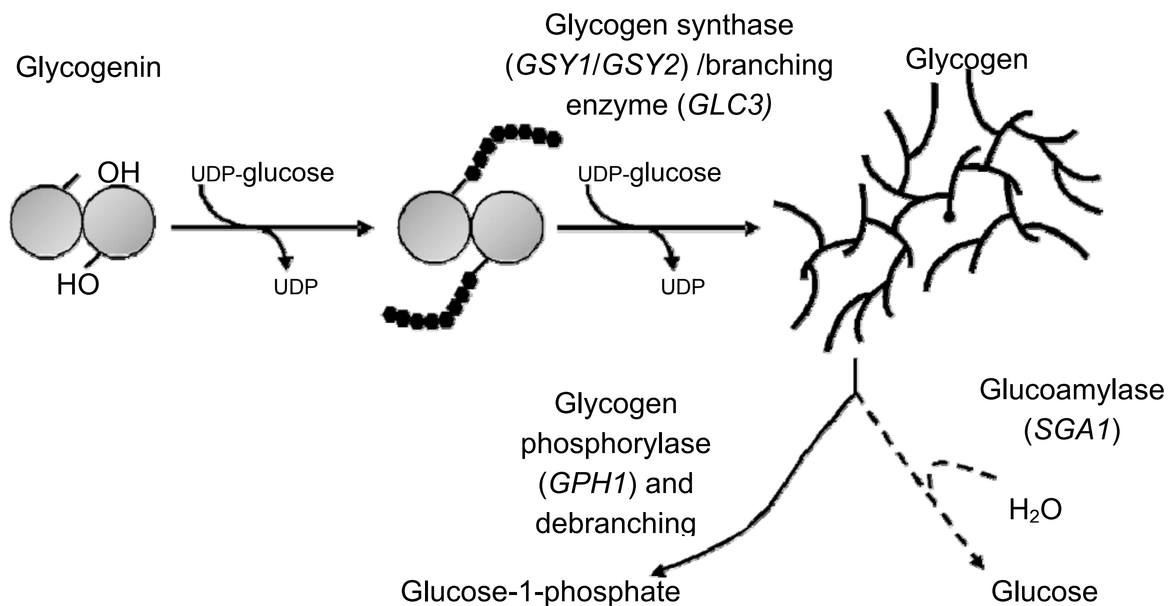


Figure 2.5: Schematic representation of the pathways of glycogen synthesis and degradation in yeast.The initiator protein, glycogenin, attaches a glucose residue from UDPG to a tyrosine residue within its own sequence. Glycogenin then adds additional glucose residues, in α-1,4-glycosidic linkage, forming a short oligosaccharide. This oligosaccharide serves as a primer for glycogen synthase (*GSY1/GSY2*), which catalyzes bulk glycogen synthesis by processively adding additional glucose residues in a-1,4-glycosidic linkage. The branching enzyme (*GLC3*) introduces the α-1,6-branch points characteristic of glycogen. Degradation occurs via the concerted action of glycogen phosphorylase (*GPH1*), which releases glucose as glucose-1-phosphate from linear α-1,4-linked glucose chains, and the debranching enzyme (*GDB1*), which eliminates the α-1,6-branch points. Alternatively, glycogen can be hydrolyzed in the vacuole by a glucoamylase (*SGA1*) activity, generating free glucose (Wilson *et al.,* 2010)

As an example of a carbon reservoir, glycogen shows that (i) polysaccharides can act as carbon reservoirs without harming cells, and (ii) can only be broken down by specific native enzymes in cells when carbon is required. This allows for the possible expression of heterologous polysaccharide genes that can act as carbon reservoirs. The advantage of heterologous expression is that when potential targets for expression are chosen thoughtfully, the transgenic cells should not have any native enzymes with which to degrade heterologous polysaccharides or storage molecules. Therefore, once carbon is captured in these molecules, it will effectively remain unavailable for utilisation by cells. In principle, yeast expressing carbon polymer synthesis genes during alcoholic fermentation, will thus decrease the amount of carbon available to produce ethanol.

2.3.2 Introducing fructo-oligosaccharides (FOSs) as alternate carbon receptors in yeast

Fructo-oligosaccharides (FOSs), or fructans, are sucrose-derived sugars consisting of two to up to more than a hundred thousand fructose units. In nature, fructan synthesis occurs in a broad range of micro-organisms and a limited number of plant species as non-structural storage carbohydrates (Banguela *et al.,* 2011). Within eukaryotic plants, the storing of fructans instead of sucrose as soluble reserve carbohydrate has several advantages, which includes the fact that as soluble polysaccharides, fructans are osmotically less active than sucrose and can therefore be stored in much higher concentrations (Altenbach and Ritsema, 2007). In prokaryotic microbes however, fructans function within the extracellular matrix. Thus, intracellular fructans in eukaryotic yeast are expected to theoretically have advantages similar to fructan utilization in plants and glycogen in yeast. Fructans of distinct origin can differ by their degree of polymerization (DP), the presence of branches, the type of linkage connecting the fructose units, and the position of the glucose residues (Figure 2.6) (Waterhouse and Chatterton, 1993). For the purpose of this review, the focus will be primarily on microbial fructans, with a brief overview of plant fructans to give a collective view of characterised fructans.

Fructans are composed entirely of fructose monomers. Fructans are classified as inulins, levans, mixed levans (gramminans in plants) and the so-called neo-series (neo-inulin and neo-levan, in plants), according to the type of bond that the extended β-D fructosyl chain forms with sucrose (Figure 2.6; Velazquez-Hernandez *et al.,* 2009). Microbial fructans differ from plant fructans in several key functions and structures. Inulin polymers from plants have a DP of 30–150 fructosyl residues, while microbial inulins have a DP of 20–10,000 (Van Hijum *et al.*, 2006). Levans of plant origin (fleins) have a DP < 100 fructosyl residues, while microbial levans usually have a DP > 100 (Velazquez-Hernandez *et al.,* 2009).

In plants, fructans occur in many prominent orders such as the *Asterales*, the *Liliales*, and the *Poales*, among which are representatives of economic importance (e.g. wheat, barley, onion) (Pollock and Cairns, 1991; Alenbach and Ritsema, 2007). Fructans are not only a carbon source for storage but also play an important role as anti-stress agents in many plants species (Xiang *et al.,* 2010). Several reviews have been published on plant fructans metabolism and their physiological roles (Pontis, 1990; Pollock and Cairns, 1991; Vijn and Smeekens, 1999), beneficial roles as prebiotics in human and animal feeding (Roberfroid and Delzenne, 1998; Delzenne *et al.,* 2005; Roberfroid, 2005; Verdonk *et al.,* 2005), industrial applications (Han, 1990) and biosynthesis in transgenic plants (Cairns, 2003; Ritsema and Smeekens, 2003; Banguela and Hernandez, 2006).

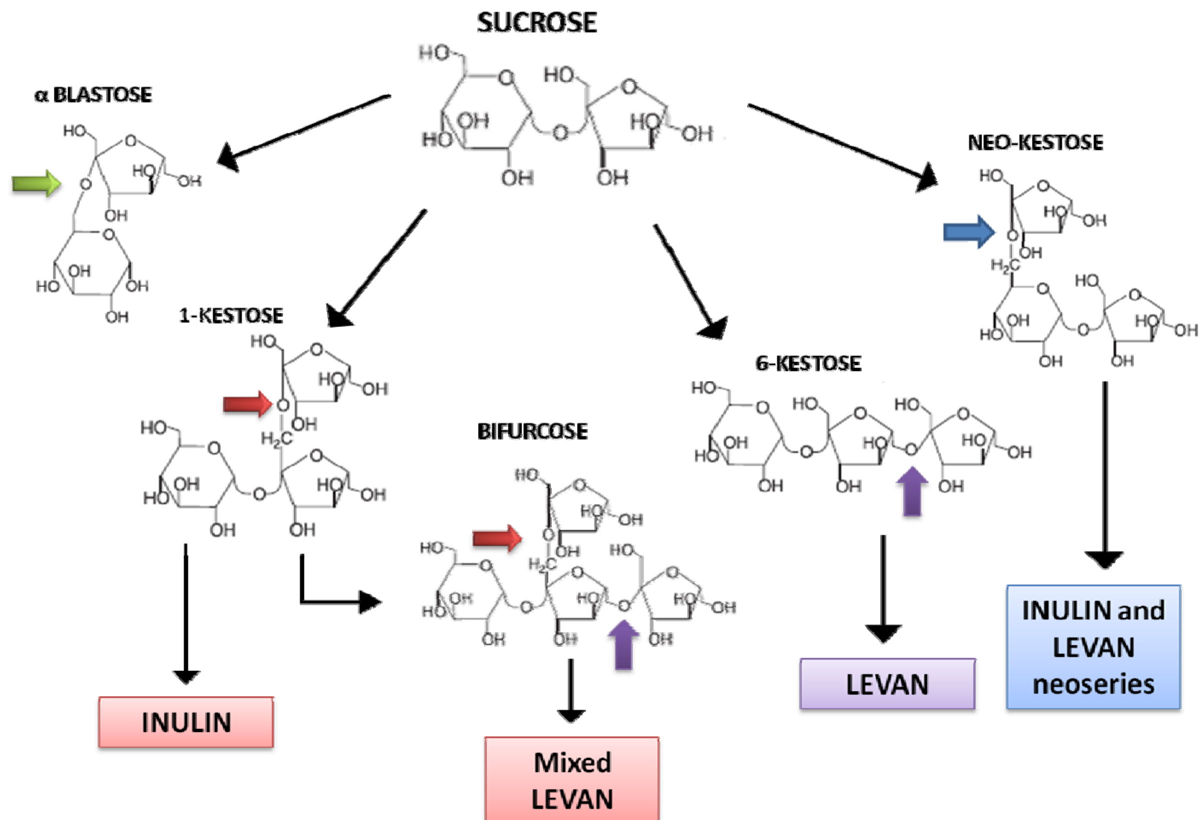


Figure 2.6: Fructan structures. Arrows indicate the type of bond with which the fructosyl moieties are bound to sucrose molecule. Inulins are linear polymers of fructose with β(2-1) bonds (red arrows). Addition of fructosyl residues in a β(2-1) bond to sucrose results in the formation of 1-kestose (inulin precursor). Levans are linear polymers of fructose, with β(2-6) bonds (purple arrows). The addition of a fructosyl residue to sucrose with β(2-6) bond results in the formation of 6-kestose (levan precursor). Mixed levans have both β(2-1)and β(2-6) linked fructosyl residues. In the neo-series, the β-D-fructosyl units are linked by a β(2-1) bond (inulin) or β(2-6) bond (levan) but the fructosyl chains are attached either to C1 or C6 of the glucose moiety of sucrose (blue arrows). (Adapted from Velazquez-Hernandez *et al.,* 2009)

Microbial fructans have been isolated from both Gram-positive and Gram-negative bacteria, as well as fungi from the genera *Aspergillus* and *Rhodotorula*. Microbial fructans are involved in the extracellular matrix by conferring resistance to environmental stress such as water deprivation, nutrient assimilation, biofilm formation, and as virulence factors in colonization (Velazquez-Hernandez *et al.,* 2009). Levan and inulin are the predominate forms of microbial fructans.

Bacterial levan, due to its higher DP and better solubility in water, is preferred over plant inulin as an emulsifier or encapsulating agent in a wide range of industrial products, including bio-degradable plastics, cosmetics, glues, textile coatings, and detergents (Banguela *et al.,* 2011). In the food industry, levan is more relevant as a prebiotic ingredient, but it is also a preferred substrate for the production of High Fructose Syrup because of the very low glucose content. For medical application, levan is attractive as a blood plasma volume extender. Despite all this potential application, levan is not yet commercialized at a significant scale since its industrial production from sucrose is costly and low-yielding (Kang *et al.,* 2009).The biological

and industrial importance of fructans has been the subject of extensive research, conducted to improve their production or to elucidate their biological role in nature. These molecules due to their storage capacity and industrial importance should therefore be considered as potential candidates for carbon reservoirs, with the aim of diverting carbon flux away from the dominant end-products of alcoholic fermentation. Since the genes involved in levan synthesis have been cloned and characterised from several organisms, a range of potential targets for heterologous expression already exists.

2.3.3 Fructosyltransferases as possible targets for genetic manipulation in yeast

Microbial fructosyltransferases (FTFs) are polymerases that are involved in microbial fructan (levan, inulin and fructo-oligosaccharide) biosynthesis. These enzymes polymerize the fructose moiety of sucrose into levan or inulin fructans, with β(2-6) and β(2-1) linkages respectively (Anwar *et al.,* 2010). Microbial FTFs are classified according to (i) the type of linkage between β-D-fructosyl units in the polymer that they synthesize and (ii) their enzymatic properties (Velazquez-Hernandez *et al.,* 2009). These enzymes have been extensively studied due to the industrial demand for the fructans they produce (Velazquez-Hernandez *et al.,* 2009). Microbial FTFs differ from their plant counterparts; plants require 2 distinct FTFs to achieve the same outcome as single microbial FTFs.

According to the carbohydrate-active enzyme database (CAZy), FTFs belong to the glycoside hydrolase family 68 (GH68). GH68 is part of Clan-J, together with the family GH32, which includes yeast, plant and fungal FTFs. FTFs are β-retaining enzymes, employing a double-displacement mechanism that involves formation and subsequent hydrolysis of a covalent glycosyl–enzyme intermediate (a pingpong type of mechanism) (Chambert *et al.,* 1974; Hernandez *et al.,* 1995; Song and Jacques, 1999). Two distinct FTFs from Lactic Acid Bacteria, (LABs) showing high sequence similarities (>60% identity), have been characterized that produce either levan (made by levansucrase) with characteristic β(2-6) bonds, or inulin (made by inulosucrase) with β(2-1) bonds (Anwar *et al.,* 2010). These FTFs have been extensively characterized and will be used as examples to describe the characterization of microbial FTFs and their mechanism of function.

FTF enzymes are known to catalyse two different reactions: (i) trans-glycosylation, using the growing fructan chain (polymerization), sucrose, or gluco- and fructosaccharides (oligosaccharide synthesis) as the acceptor substrate; (ii) hydrolysis of sucrose, using water as the acceptor (Figure 2.7). Levansucrases and inulosucrases, though similar in the reactions they catalyze, differ markedly in their reaction and product specificities, i.e. in β(2-6) versus β(2-1) glycosidic bond specificity (resulting in levan and inulin synthesis, respectively), and in the ratio of hydrolysis versus trans-glycosylation activities (Ozimek *et al.,* 2006). Examples of the 3D

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structures of both levansucrase (SacB from *Bacillus subtilis*) and inulosucrase (InuJ from *Lactobacillus johnsonii*) shows that both enzyme types use the same fully conserved structural framework for the binding and cleavage of the donor substrate sucrose in the active site (Pijning *et al.,* 2011). These differences can be explained by differences in the catalytic mechanism of the enzymes, and differences in their product specificities. A model to explain these differences was proposed by Ozimek and co-workers (Ozimek *et al.,* 2006; illustrated in Figure 2.7).

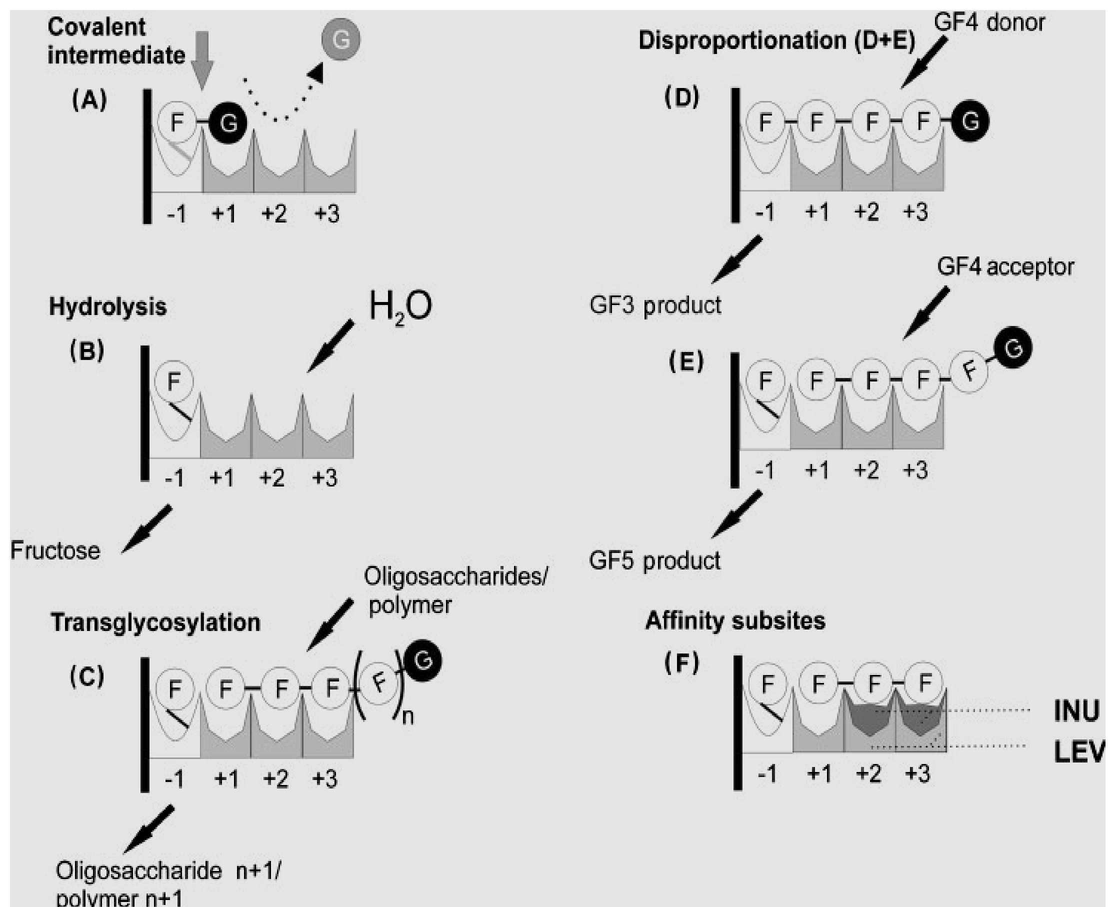

Figure 2.7: Schematic representation of the reaction sequences occurring in the active site of FTF enzymes. The donor and acceptor subsites of FTF enzymes are mapped out based on the available three-dimensional structural information (Martinez-Fleites *et al.,* 2005; Meng and Futterer, 2003), and data obtained in the Ozimek *et al.,* 2006 study. (A) Binding of sucrose to subsites-1 and +1 results in cleavage of the glycosidic bond (glucose released, shown in grey), and formation of a (putative) covalent intermediate at subsite-1 (indicated by a grey line). Depending on the acceptor substrate used, hydrolysis (with water) (B) or trans-glycosylation (C) reactions may occur [with oligosaccharides or the growing polymer chain, resulting in FOS synthesis (n+1) or polymer synthesis (n+1), respectively]. *Lb. reuteri* 121 FTF enzymes also catalyse a disproportionation (D, E) reaction with inulin-type oligosaccharides. Kestopentaose (GF4), for instance, is converted into GF3 and GF5 (D, E). (F) The differences in affinity between Inu and Lev at the +2 and +3 subsites are shown by a shallow cleft (dark grey; low affinity), and a deep cleft (light grey; high affinity), respectively. Sugar-binding subsites are shown either in white ("1 subsite), reflecting specific and constant affinity for binding of fructosyl residues only, or in light/dark grey (+1, +2 and +3 subsites), reflecting their ability to bind fructosyl, glucosyl (with GFn substrate) or galactosyl (with raffinose) residues. The vertical grey arrow indicates the position where glycosidic bond cleavage/formation occurs. The vertical black bar indicates the salt bridge in FTF enzymes (E342 and R246 in SacB from *B. subtilis*) (Martinez-Fleites *et al.,* 2005; Meng and Futterer, 2003) that possibly blocks further donor sugar-binding subsites. F, fructose; G, glucose (Ozimek *et al.,* 2006).

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The ratio of hydrolysis to trans-glycosylation in levansucrases and in inulosucrase can thus be explained by their acceptor binding sub-sites having a stronger or weaker affinity for large polymers (DP 5 and larger). The industrial applications of both enzymes thus vary. Levansucrase enzymes can be used for the production of larger levan polymers, whereas inulosucrases allow for the production of shorter chain FOSs. The storage potential for the larger levan polymers is more pronounced as a larger amount of fructose is utilized, and thus levansucrases are ideal candidates for heterologous gene expression in yeast *Saccharomyces cerevisiae* to divert carbon via the polymerization of available fructose.

2.4 LEVANSUCRASE EXPRESSION STUDIES

2.4.1 Characterisation of Levansucrases (LSs)

Levansucrases (LSs) described so far differ widely with respect to their kinetic and biochemical properties. There is still no clear understanding of which structural elements of LSs determine the poly/oligomerization ratio and the outcome of the transfructosylation reaction (Tian *et al.,* 2011). Only a few LSs have been fully characterised with respect to their transfructosylation product spectra and their acceptor/donor specificity. LSs can be used to synthesize novel β-(2-6)-FOSs and levan from various acceptors, not just sucrose. This, however, is hampered by the fact that the levansucrases that have been characterized all incidentally have low stability, providing limited information on the lesser common LSs of higher stability. To address this, current research aims to characterize LSs with improved properties from selected microbial sources of biotechnological interest (Tian *et al.,* 2011).

The tri-dimensional structures of LSs from *Bacillus subtilis* (Meng and Futterer, 2003) and *Gluconacetobacter diazotrophicus* (Figure 2.8; Martinez-Fleites *et al.,* 2005) are available. This had led to greater understanding of how the conserved catalytic site interacts with substrates and acceptor specificities. Detailed acceptor and donor substrate studies of LS from *B. subtilis* were coupled with a structural model of the substrate enzyme complex in order to investigate, in detail, the roles of the amino acids (Asp86, Glu342, Asp247 in conserved active site Asp-Glu-Asp) in the catalytic action of the enzymes and the scope and limitations of substrates (Seibel *et al.,* 2006). The most energy efficient binding was surprisingly with D-glycopyranoside (D-Gal-Fru) rather than sucrose (Figure 2.9).

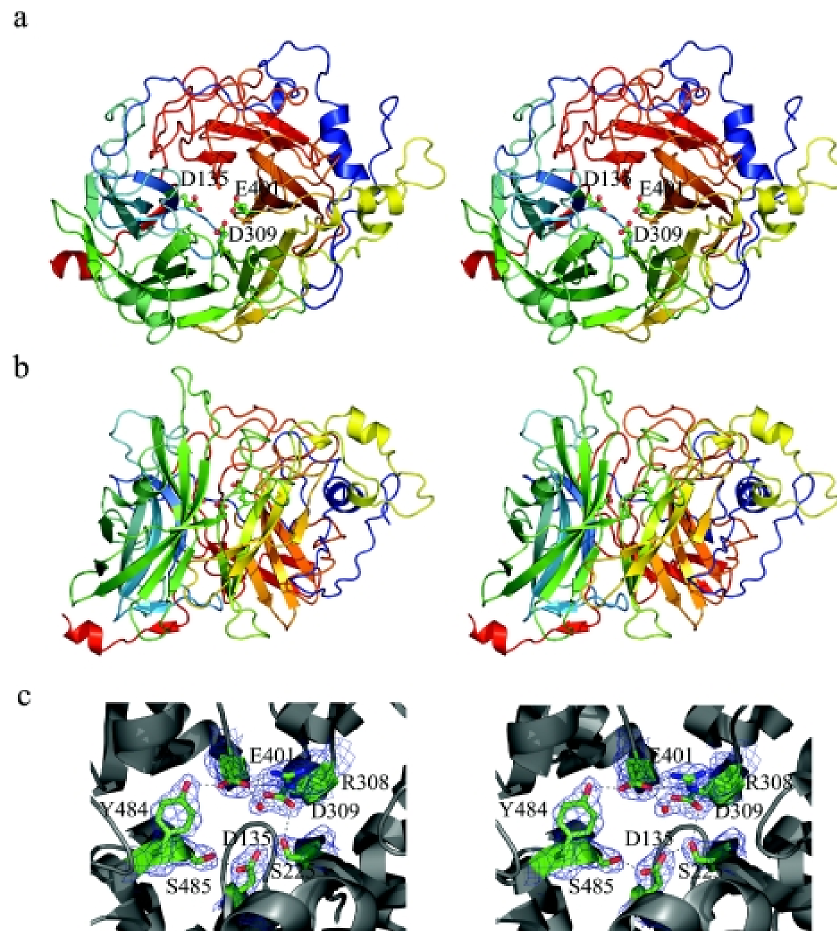

Figure 2.8: Three-dimensional structure of LsdA from *G. diazotrophicus*. Superior (a) and lateral (b) stereo views of the five-bladed β-propeller fold. The colour is 'ramped' from N- (blue) to C- (red) terminus. Catalytic residues Asp^{135}, Asp^{309} and Glu^{401} are shown in ball-and-stick representation. (c) Stereo view of the electron density map (contoured at 1σ level) 'carved' around catalytic residues and other residues involved in the hydrogen-bond (broken lines) network at the active site. These Figures were prepared with PYMOL. (Martinez-Fleites *et al.,* 2005)

The production of novel β-(2-6)-FOSs and levan from various acceptors is thus shown to be possible, with varying degrees of efficiency. The acceptor affinity for the single binding site seems to be an important factor with regards to FOSs/polymer formation. As the acceptors determine to a degree the ratio of polymerisation (fructose donors) to hydrolysis (H_2O as acceptor), it is important to understand which motifs they interact with to specifically determine the role of acceptors. A separate study undertook the characterization of *Bacillus megaterium* levansucrase SacB mutagenesis variants, Y247A, Y247W, N252A, D257A, and K373A (Strube *et al.,* 2011). This study revealed novel surface motifs remote from the sucrose binding site with distinct influence on the polysaccharide product spectrum. The structures of the SacB variants reveal clearly distinguishable subsites for polysaccharide synthesis as well as an intact, active site architecture. Amino acids outside the active site of enzyme have a well-defined and rationally explainable effect on the polymer formation activity (Figure 2.10).

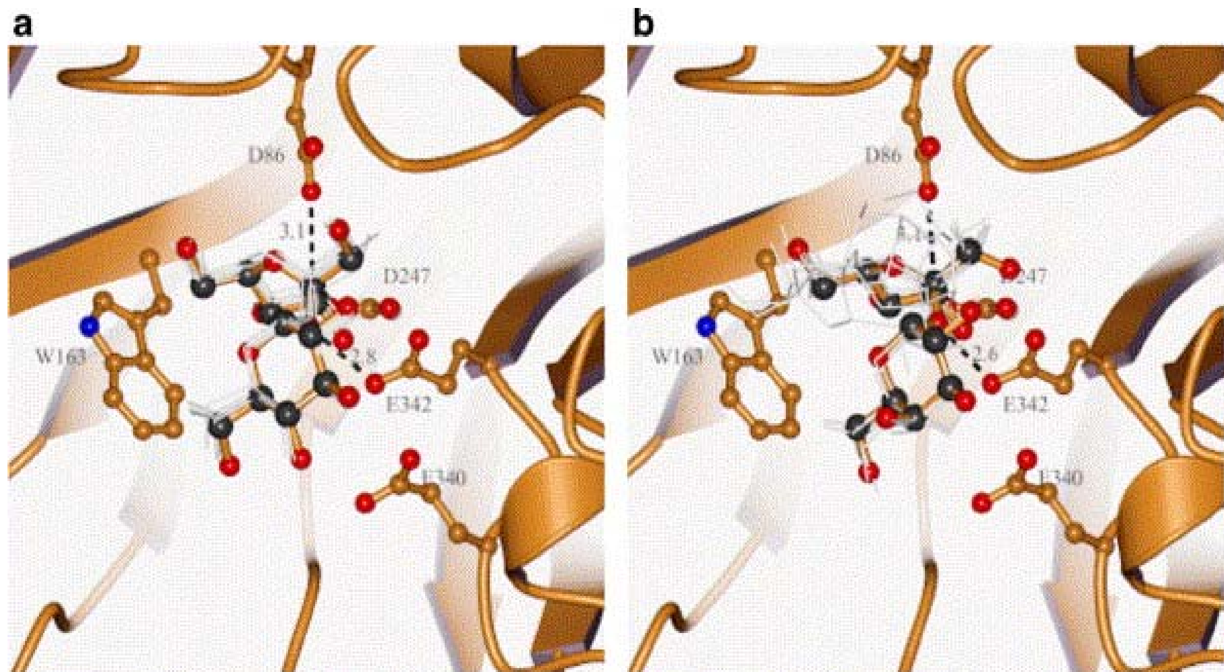

Figure 2.9: Lowest energy dockings of the substrates sucrose (left) and D-Gal-Fru (right) with FTF show identical orientation in the active site of the enzyme. Further conformations of D-Gal-Fru docking experiments are also superimposed (grey) (Seibel *et al.,* 2006).

Olvera and co-workers (2012) described the design of chimeric levansucrases with improved trans-glycosylation activity. LSs, as mentioned previously, have both trans-fructosylation activity and hydrolytic activity, which may account for as much as 70 to 80% of substrate conversion, depending on reaction conditions. In this study, it was attempted to shift enzyme specificity towards trans-fructosylation. It was found that in some cases the hydrolytic activity was reduced to less than 10% of substrate conversion. However, all of the constructs were as stable as SacB. Specific kinetic analysis revealed that this change in specificity of the SacB chimeric constructs was derived from a 5-fold increase in the transfructosylation activity and not from a reduction of the hydrolytic activity, which remained constant.

There are various factors that influence the enzymatic production of fructans. Characterisations of various microbial LSs show that the substrate/donor interactions with both the active site and subsites on enzyme surface play a pivotal role in polymer production. These enzymes, however, still have low availability and stability. The study by Olvera *et al.* (2012) may address this problem, with the construction of chimeric LS enzymes as a rational strategy to modify single domain fructansucrases or mutants to increase the efficiency and reduce substrate loss by hydrolysis, without affecting the enzyme stability.

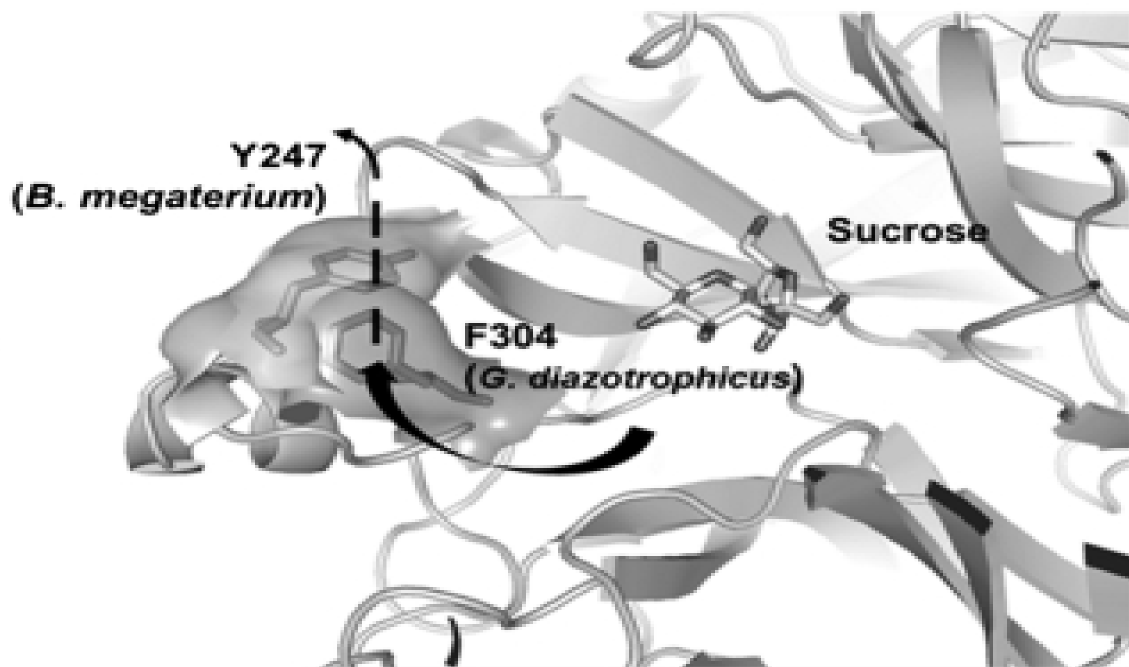

Figure 2.10: Superimposition of the levansucrases SacB from *B. megaterium* and LsdA from *G. diazotrophicus***.** The differential surface motif Tyr^{247} of the levansucrase SacB from *B. megaterium* and LsdA from *G. diazotrophicus* leads to the synthesis of polysaccharide and oligosaccharides, respectively. The structural alignment of the levansucrases SacB and LsdA shows a conformational difference in the surface motif Tyr^{247} essential for polysaccharide synthesis in SacB (*C*). In LsdA, the orientation of this motif might block the polysaccharide chain, thus leading to the synthesis of short oligosaccharides of 3–5 carbohydrate units (Strube *et al.,* 2011).

2.4.2 Levansucrases expression studies

Fructans produced by levansucrases thus depend not only on the source of the enzyme, and the hydrolysis to transfructosylation ratio of the enzyme, but also on the substrates that act as both donors and acceptors of fructosyl moieties. This all has to be taken into account when investigating possible LSs for heterologous expression. Another factor to consider is the heterologous host. To date, LSs have been expressed in both prokaryotic (*E. coli*) and eukaryotic (yeast and plant species) hosts of biotechnological interests.

The industrial applications of LSs apply to the production of high molecular weight levan, as well as novel β-(2-6)-FOSs and levan from various acceptors. Fructan formation strongly depends on the specific enzyme catalyzing its production; therefore current research focuses on identifying novel LSs of native levan producing species. However, the production of levan using these species is usually not cost effective (Rairakhwada *et al.,* 2010). The example of *G. diazotrophicus* LsdA LS can be used to illustrate this point. The low expression levels of the lsdA gene, the limited cell number in bioreactors, and the technological constraints derived from the polysaccharides causing high density culture supernatants make the native bacterium inadequate for industrial scale, cost effective production. Currently, there is no commercial

technology for levan production by either natural or recombinant means (Kang *et al.,* 2009 Banguela *et al.,* 2011).

Riarakhwada *et al. (2010)* cloned and expressed levansucrase gene of *B. amyloliquefaciens* type 1 in *E. coli* and in *Bacillus megaterium* and enhanced the LS production in the recombinant *B. megaterium,* through optimisation of fermentation conditions using response-surface methodology (RSM) (Riarakhwada *et al.,* 2010). *B. megaterium* was selected as alternative host for recombinant protein production due to its intrinsic lack of alkaline proteases as well as high stability in replication and maintenance of recombinant plasmids it hosts. Also, *Bacillus* strains can produce and secrete large quantities (20–25 g/l) of extracellular enzymes which have resulted in them becoming part of the most important industrial enzyme producers, producing about 60% of the commercially available enzymes. In the study, the authors were able to induce a 62-fold increase in levansucrase production when compared to the wild type strain. They proposed a model that shows the individual and interactive effects that media components (donors/acceptors) have on production of levansucrase.

The expression of microbial LSs in prokaryotic systems has the advantage that the expression and secretion systems are comparable to that of the wild-type producer strains. However, the possibility exists of heterologous expression of microbial LS in eukaryotic systems. The post translational as well as secretory processing of enzyme by the host must be taken into account, however, as it might alter activity or decrease stability of the enzyme. Recent studies have investigated the use of eukaryotic systems as a possible alternative, which will be considered in the following sections.

2.4.3 Levansucrase expression studies in eukaryotic models

There are various studies that undertook the expression of microbial LS in eukaryotic hosts of biotechnological interest, such as *S. cerevisiae, Pichia pastoris* and *Nicotiana tabacum*. Expression in eukaryotic hosts may however be complicated due to the many eukaryotic post-translational modifications such as glycosylation and proteolytic processing. In an effort to obtain a large quantity of recombinant levansucrase for the enzymatic production of levan, the secretory expression of levansucrases has been examined in yeast heterologous hosts (Kang *et al.,* 2011).

In a study by Scotti *et al.* (1996), the ability of signal sequences of various *Bacillus* spp. or yeast secreted proteins to direct *B. subtilis* SacB LS into the secretion pathway of *S. cerevisiae* were compared (Scotti *et al.,* 1996). This was based on previous work by this group, which reported the extracellular production of *B.* subtilis SacB in yeast. However, SacB accumulated intracellularly in an unprocessed form that remained weakly associated with the

inner face of the cytoplasmic membrane. This precursor was unable to enter the secretion pathway of yeast (Scotti *et al.,* 1994). The 1996 study found that the efficiency of the signal sequences correlated with the overall hydrophobicity of their h-domain and was independent of their origin. Furthermore, the net-charge of the proximal protein sequence downstream from the signal sequence contributed to the competence of the heterologous proteins to be excreted by the yeast. Modification of the net charge allowed the protein to be translocated under the control of the yeast invertase signal sequence. Moreover, the glycosylation of LS did not modify significantly the transfructosylation polymerase activity. These studies showed that heterologous expression was possible, though various factors must be taken into account.

In a study by Trujillo *and co-workers* (2004), the influence of N-glycosylation on the kinetic and catalytic properties of *G. diazotrophicus* LsdA LS produced in *P. pastoris* was reported. The use of *P. pastoris* offers many advantages, including its ease of usage relative to other eukaryotic expression systems. They found that the glycosylated enzyme behaved similarly to non-glycosylated LsdA when substrate specificity, fructooligosaccharide (FOS) production, sucrose hydrolysis or levan formation reactions were carried out under different experimental conditions. These results indicated that the presence of N-linked oligosaccharides did not alter the catalytic properties of LsdA. This study showed for the first time, the possibility of modulating LsdA catalysis, including FOS production, by manipulating the concentration of organic solvents (Acetone; acetonitrile; dimethyl sulfoxide) and temperature in the reaction media. This study gave deeper insight into the catalytical and kinetic properties of the *P. pastoris* produced LsdA, confirming that this recombinant glycosylated enzyme represents a promising candidate for mass production of FOS from sucrose in both aqueous or water restricted environments. However, the recombinant LsdA produced levan of low molecular weight.

A more recent study by Kang and colleagues, the LS M1FT from *Leuconostoc mesenteroides* was cloned and expressed in *P. pastoris (*Kang *et al.,* 2011). Previous work done by this group showed that when M1FT was expressed in *E. coli* for large scale production of levan *in vitro*, significant amounts of recombinant protein were expressed as cytoplasmic inclusion bodies during fermentation (Kang *et al.,* 2005). The 2011 study revealed that M1FT was glycosylated at its 2 potential N-glycosylation sites. While this did not alter enzyme optimal conditions, it did increase acceptor specificity. The results showed that recombinant *L. mesenteroides* M1FT was highly expressed and secreted in *P. pastoris.* It also showed that the recombinant M1FT efficiently catalysed transfructosylation to polymerize high molecular weight levan from sucrose. This study reaffirms the observation that *P. pastoris* is a suitable heterologous host for recombinant LS expression. Thus, *Pichia* can be used for both production of levan FOS (Trujillo *et al.,* 2004) and high molecular weight levan (Kang *et al.,* 2011).

Microbial LSs has also been expressed in tobacco, as a novel alternative source of highly polymerized levan. In a study by Banguela and co-workers (2012), *P. pastoris* and *N. tabacum* were used as host for LsdA production and direct levan synthesis, respectively. A previous study by this group reported the constitutive expression of *G. diazotrophicus* LS (LsdA) fused to the vacuolar targeting pre-pro-peptide of onion sucrose:sucrose 1-fructosyltransferase (1-SST) in tobacco (Banguela *et al.,* 2011). The production of levan with a degree of polymerization above 10^4 fructosyl units was detected in leaves, stem, root, and flowers, but not in seeds. It was further illustrated that the constitutive expression of LsdA in tobacco allowed for the accumulation of highly polymerized levan in mature tobacco leaves where the polymer represented between 10-70% (w/w) of total dry weight (Banguela *et al.,* 2012). Also, polymer production remained stable in the plant progenies, making a possible biotechnological application feasible. The recombinant LsdA expressed in *P. pastoris* displayed a saccharolytic (sucrose cleavage) capacity and had a levan yield 9-fold increased relative to wild-type.

These studies all show the inherent difficulty of heterologous expression of prokaryotic LS in eukaryotic expression systems. The post-translational glycosylation, however, does not seem to affect the enzymatic mechanism. Further study is required for the expression of novel microbial LSs, and characterisation of the heterologous protein and products they produce. *P. pastoris* appears currently to be the most suited host to study heterologous expression levansucrases.

2.5 CONCLUSIONS

Wine ethanol content is of growing importance to the wine industry, due to various economic, social and health reasons. There are various methods available to reduce the ethanol content in wine, each with its unique advantages and disadvantages. The ideal method would allow for a percentage decrease in ethanol content with minimal impact on aroma compounds. Current studies often select for metabolic end points that either compliment wine composition (e.g. glycerol) or are completely inert in the wine environment. Furthermore, carbon storage molecules can function as carbon receptors to divert carbon from ethanol production. This review proposed a novel approach by suggesting fructans as receptors for carbon during fermentation, thereby, decreasing available carbon for ethanol production.

The natural ability of *S. cerevisiae* to produce and store high concentration of glycogen polymer, without excessive metabolic burden, indicates a potential for intracellular fructan polymer production. Furthermore, fructans function as non structural storage carbohydrates in

certain plants, and are thus expected to function in similar way as glycogen in yeast. However, as fructan is heterologously expressed, there should not be native degradation activity, allowing fructans to act as carbon sinks.

Heterologous expression of levansucrases in eukaryotic systems has been shown to be possible with *P. pastoris* and *T. tabacum* as hosts. The expression in *S. cerevisiae* as seen in chapter 3 offers a novel approach to expression of LS and production of levan. Levan was shown to function as carbon receptor in *S. cerevisiae* during aerobic growth. However, further study is required into the metabolic pressure diversion of carbon toward levan production may place upon yeast during fermentation. The potential of levan production during fermentation would allow wine maker to harvest both wine and levan containing cells. LS expression in yeast however requires future work into characterization of novel LSs with higher stability as well as characterization of the resulting products in heterologous expression system, which may yield a viable industrial system for levan production.

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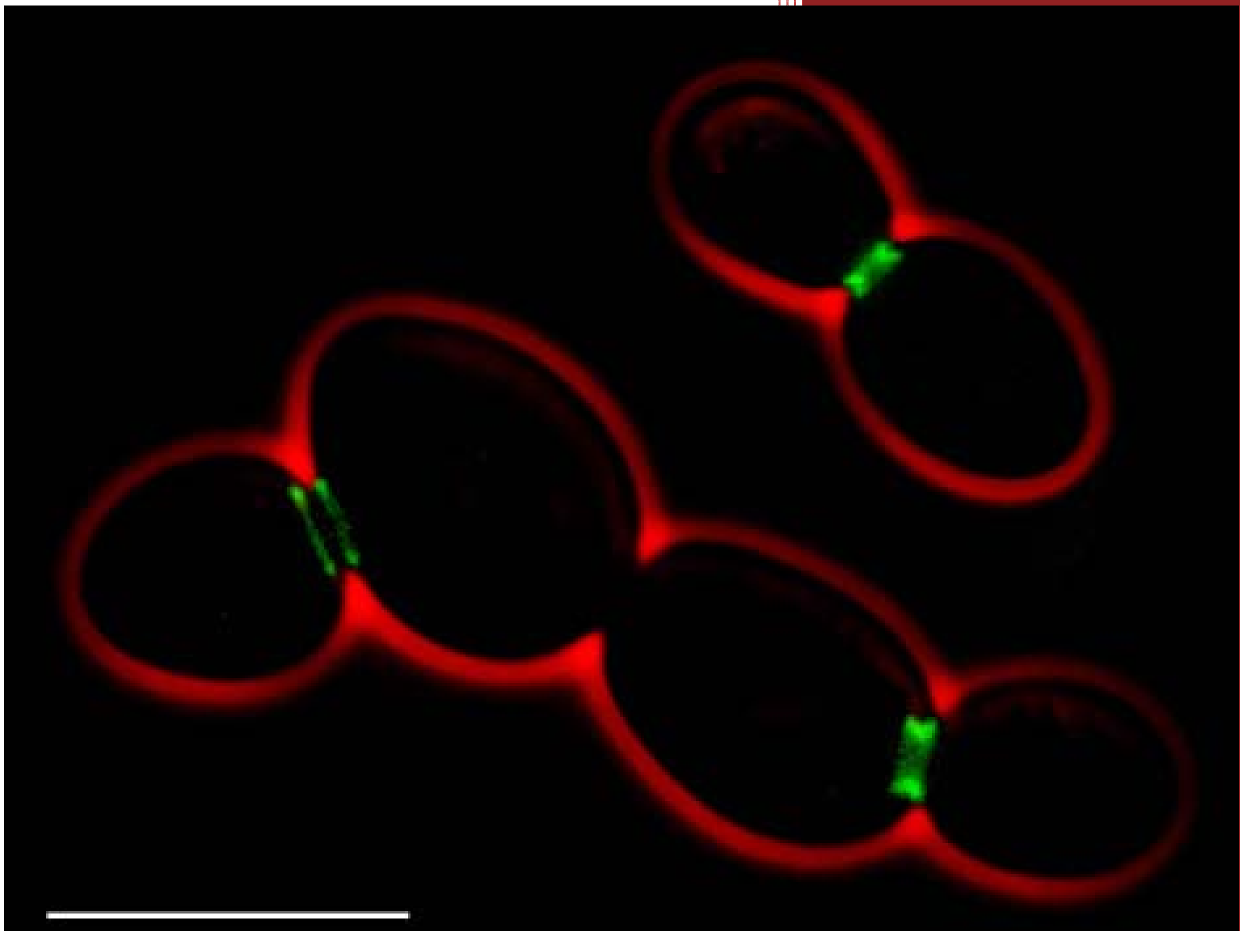
Chapter 3

Research results

Generating lower ethanol yields in fermentations by *Saccharomyces cerevisiae* via diversion of carbon flux towards the production of fructo-oligosaccharides



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3. RESULTS CHAPTER

3.1 INTRODUCTION

The increasing consumer demand for easy-to-drink wines with moderate alcohol levels due to a combination of economic, social, qualitative and health issues associated with higher ethanol wines, has spurred research into development of technologies to reduce/lower ethanol concentrations in wine without compromising wine sensory characteristics (Pickering, 2000; Kutyna *et al.,* 2010; Varela *et al.,* 2012). There are many strategies currently available to reduce alcohol concentrations in wine; however, all have been shown to have serious limitations (Shmidtke *et al.,* 2012; Varela *et al.,* 2012).

The strategies can be broadly classified into 3 groups: i) viticultural, ii) biological and iii) physical approaches (Kutyna *et al.,* 2012). Viticultural methods to reduce ethanol contrive to lower the sugar concentrations in the grape berry, resulting in decreased availability of sugar for ethanol production during fermentation. These practises however, can result in delayed ripening of berries, and excessive bunch exposure. Furthermore, it can also result in increased unsavoury, “green” characters and acidity in the resulting wine.

Physical wine processing methods aim to decrease either the sugar concentration in the grape must, or the ethanol concentration post fermentation. These methods however add cost and complexity to the wine making process. Furthermore, the loss of volatile aroma compounds and other sensory characteristics of wine make these approaches subject to cautious scrutiny by wine-makers.

Biological approaches aim to develop and screen for yeast strains with lower ethanol productivity, yet maintained organoleptic and fermentative capacity. It is widely believed that the biological approach to decreased ethanol productivity has the capacity to deliver the best outcome (Kutyna *et al*., 2010; Varela *et al.,* 2012). However, GMO based strategies remain subject to intense debate in wine sector and currently no adequate selection pressure can be applied in order to select specifically for lower alcohol production as a phenotypic outcome (Pretorius, 2000; Pretorius and Hoj, 2005).

The development of technologies to produce a balanced wine with lower ethanol levels that retain the flavour profile and other sensory characteristics is ongoing. The biological GMO strategies to reduce ethanol productivity in yeast aim to redirect carbon from ethanol production during fermentation towards selected alternate metabolic end points (Kutyna *et al.,* 2010). The experimental designs are such that the selected metabolic end points complement the wine bouquet or are completely inert in a wine environment, thus minimising the effect on wine

characteristics. Targeted changes can be made to the yeast genome by either deleting or over-expressing key enzymes involved in the carbon metabolism of yeast during fermentation in an effort to redirect carbon away from ethanol production (Kutyna *et al.,* 2010). However, maintaining the yeast fermentation capacity and wine quality in lower ethanol producing GM strains remains a major challenge (Varela *et al.,* 2012).

Selected metabolic end-points include glycerol, storage carbohydrates and organic acids such as gluconic acid and organic acids in the TCA cycle. There are various documented GM modifications which result in increased glycerol production (Nevoigt and Stahl, 1996; Michnick *et al.,* 1997; Remize *et al.,* 1999; de Barros Lopes *et al.,* 2000; Remize *et al.,* 2001; Eglinton *et al.,* 2002; Cambon *et al.,* 2006; Kutyna *et al.,* 2010). However these approaches impact on the redox cycle during fermentation, resulting in increased production of metabolites that negatively affect wine quality such as acetic acid (Cambon *et al.,* 2006; Eglinton *et al.,* 2002; Michnick *et al.,* 1997; Remize *et al.,* 1999). Therefore, multiple genetic modifications are required to circumvent the production of unsavoury metabolites. Alternatively, molecules that are inert in wine can be used as carbon receptors to minimise genetic modifications and preferably have little to no affect the redox balance during fermentation.

Several studies have shown that the deletion or overexpression of target genes involved in the TCA cycle affect ethanol production (Arikawa *et al.,* 1999; Peleg *et al.,* 1990; Selecky *et al.,* 2008). These studies use organic acids as carbon receptors to limit carbon available for ethanol production. However, increased production of malate, fumerate and citrate was reported. The production of unwanted metabolites may be circumvented by using storage carbohydrates as metabolic end points. The natural ability of *S. cerevisiae* to produce and store glycogen and trehalose (Panek, 1991) as storage molecules indicates the potential for intracellular polymer production without major metabolic burdens (O'Connor-Cox *et al.,* 1996; Pretorius, 2000). The advantage of this approach is that limited genetic modification is required and there should theoretically be no impact on the redox cycle during fermentation.

Fructans are fructose polymers consisting of multiple fructose units, which occur in a broad range of micro-organisms and a limited number of plant species as non-structural storage carbohydrates (Banguela *et al.,* 2012). These molecules have been increasingly used in production on functional foods and pharmaceutical formulations due to their pre-biotic properties and other health enhancing roles (Lafraya *et al.,* 2011). Fructan synthesis is catalyzed by a group of enzymes referred to as fructosyltransferases (FTFs). FTF enzymes are known to catalyse two different reactions: (i) trans-glycosylation, using the growing fructan chain (polymerization), sucrose, or gluco- and fructosaccharides (oligosaccharide synthesis) as the acceptor substrate; (ii) hydrolysis of sucrose, using water as the acceptor. This study aimed to use fructans, produced by a heterologously expressed FTF as carbon sinks to redirect carbon from ethanol production. As these carbohydrates are foreign to yeast, there should be no native

degradation agents in *S. cerevisiae*. Furthermore, this strategy should result in a limited metabolic burden as there is no theoretical impact on redox cycle. Fructan production should, in principle, act as carbon sink to decrease fructose available for ethanol production, thereby decreasing ethanol productivity. This should theoretically occur without the production of unwanted metabolites.

To obtain such a fructan producing yeast, a sucrose accumulating *S. cerevisiae* strain first had to be constructed as a base strain. This was achieved by introducing either a sucrose synthase (Susy; cloned from potato) or by growing strains expressing the spinach sucrose transporter (SUT) in sucrose containing media. Both the sucrose accumulating strains were transformed with the plasmids bearing M1FT, a levansucrase from *Leuconostoc mesenteroides* or a truncated version of the same gene, with its N-terminal secretion signal removed. The data indicate that strains bearing either of the fructose accumulation genes in combination with either of the M1FT constructs do indeed lead to a yeast strain that produces levan polymers that accumulate inside the cell. This is the first report of fructan-accumulating yeast strains. Such engineered yeast strains, however, fail to produce detectable levan during alcoholic fermentation.

3.2 MATERIALS AND METHODS

3.2.1 Strains, plasmids and culture conditions

Escherichia coli DH5α (GIBCO-BRL/Life Technologies) was used as host for the cloning and propagation of all plasmids. The yeast vector pYCplac33-PGK1$_{PT}$ (this laboratory) was used for over-expression purposes. Plasmid-carrying *E. coli* strains were grown at 37°C on Luria-Bertani medium, supplemented with 100μg ampicillin mL^{-1}. General procedures for cloning, DNA manipulations, transformations and agarose gel electrophoresis were performed as described by Sambrook *et al*., 1989. Restriction enzymes, T4 DNA-Ligase, and Expand Hi-Fidelity polymerase used in the enzymatic manipulation of DNA were obtained from Roche Diagnostics (Randburg, South Africa) and used according to the specifications of the supplier. All yeast strains used in this study are derived from the BY4742 (S288c) genetic background and are listed in Table 3.1. Yeast strains were grown either on rich YPD (Biolab, Merck) or on minimal SCD medium, containing 0.67% (w/v) yeast nitrogen base without amino acids (DIFCO) and 2% (w/v) glucose supplemented with amino acids according to the specific requirements of the respective strains. For the sucrose accumulation and levan production experiments, cultures were grown in SCD medium, containing 0.67% (w/v) yeast nitrogen base without amino acids

(DIFCO). Susy strains generate intracellular sucrose from glucose and fructose thus, the carbon for Susy strains were 4% (w/v) glucose and 4% (w/v) fructose. SUT strains import sucrose from the medium, and were thus grown with 5% (w/v) sucrose and 3% (w/v) glucose. The medium was supplemented with amino acids according to the specific requirements of the respective strains.

Table 3.1: Description of all yeast strains used in this study

Strain	Genotype	Reference
BY4742	*MATα Δhis3Δlys2Δleu2Δura3*	EUROSCARF Library
BY4742Δ*suc2*	*MATα Δleu2Δlys2Δhis3Δura3Δsuc2::LEU2*	This laboratory
BY4742Δ*suc2*-Susy	*MATα Δleu2Δlys2Δhis3Δura3Δsuc2::LEU2 PGK1*$_P$-Susy-*PGK1*$_T$*::HIS3*	This Study
BY4742Δ*suc2*-Susy-M1FT	*MATα Δleu2Δlys2Δhis3Δura3Δsuc2::LEU2 PGK1*$_P$-Susy-*PGK1*$_T$*::HIS3 YCplac33-URA3*-M1FT	This Study
BY4742Δ*suc2*-Susy-M1FTΔsp	*MATα Δleu2Δlys2Δhis3Δura3Δsuc2::LEU2 PGK1*$_P$-Susy-*PGK1*$_T$*::HIS3 YCplac33-URA3*-M1FTΔsp	This Study
BY4742Δ*suc2*-SUT	*MATα Δleu2Δlys2Δhis3Δura3Δsuc2::LEU2 PGK1*$_P$-SUT-*PGK1*$_T$*::HIS3*	This Study
BY4742Δ*suc2*-SUT-M1FT	*MATα Δleu2Δlys2Δhis3Δura3Δsuc2::LEU2 PGK1*$_P$-SUT-*PGK1*$_T$*::HIS3 YCplac33-URA3*-M1FT	This Study
BY4742Δ*suc2*-SUT-M1FTΔsp	*MATα Δleu2Δlys2Δhis3Δura3Δsuc2::LEU2 PGK1*$_P$-SUT-*PGK1*$_T$*::HIS3 YCplac33-URA3*-M1FTΔsp	This Study
BY4742Δ*suc2*-M1FTΔsp	*MATα Δleu2Δlys2Δhis3Δura3Δsuc2::LEU2 YCplac33-URA3*-M1FTΔsp	This Study
BY4742Δ*suc2*-M1FT	*MATα Δleu2Δlys2Δhis3Δura3Δsuc2::LEU2 YCplac33-URA3*-M1FTΔsp	This Study

3.2.2 Construction of SPR-HIS-SUT-SPR and SPR-HIS-SUSY-SPR integration cassettes

Work previously done in this laboratory to generate Susy and SUT strains sucrose accumulation strains. All primers and plasmids used in this study are indicated in tables 3.2 and 3.3, respectively. The integration cassette combined either Susy (sucrose synthase gene from potato) or SUT (sucrose transporter from spinach) in PGK promoter/terminator cassette with a HIS3 selection marker using fusion PCR. The Susy open reading frame (ORF) was amplified from pHVXII-SUSy plasmid using primers (PGK_LacZ_F and PGKt_SPR_R). DNA amplification was conducted with 30 cycles of denaturation (30 sec at 94°C), annealing (45 sec at 54°C), and elongation (4min 30 sec at 72°C). The Histidine auxotrophic marker was amplified from the pPVΔI plasmid using primers (SPR_HIS_F and HIS_PGKp_R) and amplified using a similar PCR protocol to what was used for Susy. PCR products were ligated into pGEM-T Easy vector (Promega, USA) and sequenced using an ABI PRISM^{TM} automated sequencer at the Central Analytical Facility (CAF), Stellenbosch University®. Positive Susy clones were digested using *Apa*I and *Sbf*I. Positive *HIS3* clones were digested with *Apa*I and *Sal*I. Susy and the *HIS3* auxotrophic marker were then ligated and the resulting product was used as template for fusion PCR, which was conducted in 2 stages. First, 3 cycles (without primers) of denaturation (20 sec at 94°C), annealing (1min at 54°C), and elongation (6min at 68°C). Primers were then added and 25 cycles of denaturation (15 sec at 94°C), annealing (30 at 54°C), and elongation (5min 50sec at 68°C). The resulting SPR-HIS-SUSY-SPR cassette was integrated using the lithium acetate transformation protocol (Gietz *et al.,* 1992) into the *S. cerevisiae* BY4742Δ*suc*2 strain and integration confirmed with colony PCR using primers (Susy_Diag_down and SPR3_Diag_Down_Rv). SUT was amplified from pHVXII-SUT plasmid using primers (SPR-FW-PGKp and SPR-RV-HISp) after which the SUT integration cassette was constructed in a fashion similar to the Susy integration cassette. The integration was confirmed with colony PCR using primers (SPR3_Diag_Up and SUT_Diag_Up_Rv). The respective integration cassettes were transformed into the BY4742Δ*suc*2 strain to yield the strains BY4742Δ*suc*2-SUT and BY4742Δ*suc*2-Susy.

3.2.3 Construction of *S. cerevisiae* expression vectors YCplac33-M1FT and YCplac33-M1FTΔsp

The M1FT and truncated M1FTΔsp genes was amplified by the polymerase chain reaction (PCR) from the pBluescript-M1FT plasmid (Rapp, 2012) using Takara Ex Taq DNA polymerase (Takara BIO INC., Japan) and the primers (M1FTc_EcoR1_F, M1FTt_EcoR1_F, and M1FT_Xho1_R respectively). M1FTΔsp was constructed with a truncation of 50bp to remove the signal peptide predicted by SignalP 3.0 software (Bendtsen *et al.,* 2004). DNA amplification was conducted with 30 cycles of denaturation (30 sec at 94°C), annealing (30 sec at 58°C), and elongation (90 sec at 72°C). PCR products were ligated into pGEM-T Easy vector (Promega)

and sequenced at CAF, Stellenbosch University®. Positive clones were digested with *Eco*R1 and *Xho*1 and ligated into $PGK1_{PT}$ promoter/terminator cassette in the YCplac33-PGK1pt plasmid with resulting in the generation of pYCplac33-PGK1pt-M1FT and pYCplac33-PGK1pt-M1FTΔsp yeast expression vectors. Expression cassettes were transformed into the BY4742Δ*suc*2, BY4742Δ*suc*2-Susy and BY4742Δ*suc*2-SUT strains using the lithium acetate yeast transformation protocol (Gietz *et al*., 1992). Positive clones were confirmed by growth on selective YNB media plates supplemented with the required amino acids.

3.2.4 Fructan extraction from strains and analysis by thin-layer chromatography

In order to confirm fructan production in strains, sugars contained in whole cell extracts were separated using thin layer chromatography (TLC). Overnight cultures of strains were inoculated into 100mL of appropriate culture media and grown for 2 days at 30°C to saturation. Cells were harvested by centrifugation at 5000rpm for 2 min. The cells were then resuspended in 1mL distilled H_2O and transferred to 2mL centrifugation tubes. Cells were washed with 1mL mQ water (Millipore) and resuspended in 500µL mQ water. Addition of ~300µL glass beads to suspension was followed by vigorous vortexing for 10min. Cell suspensions were centrifuged at 12000rpm for 30 seconds. 400µL of cell extract was transferred to 1.5mL centrifugation tubes and dried at 55°C. Extracts where then resuspended in 20µL mQ H_2O (20x concentration) and stored at 4°C.

Samples (2µL) were spotted on thin layer chromatography (TLC) silicagel foil (Merck), and separated in butanol:acetic acid:water (50:30:15). The standards used were a mixture of fructose, glucose and levan (*Zymomonas mobilis,* Fluka Biochemika) at 10g/L concentration. Fructose containing sugars were specifically stained with a urea spray (Wise *et al.,* 1955), and developed at 110°C until the stained bands could be clearly visualized.

Acid hydrolysis was used to analyse the polymers being produced. Samples (2µL) were hydrolysed at 100°C for 1 h using 0.1M HCl. Standards were similarly treated. Samples (3µL) was then spotted onto TLC foils and run in butanol:acetic acid:water (50:30:15). Foils were stained using urea spray and developed at 110°C for several minutes.

3.2.5 Quantification of accumulated intracellular sugars

Cell extracts were obtained as previously described. Samples (200µL) were then hydrolysed at 120°C for 1 h using 2M trifloroacetic (TFA) acid. Since TFA evaporates, no neutralization is required after hydrolysis. Sample volumes were adjusted to 500µL by the addition of mQ water after hydrolysis and sent for sugar analysis on Arena 20XT enzyme robot at CAF, Stellenbosch

University®. Fructose, glucose and sucrose within samples were quantified using the respective kits (Thermo Fisher Scientific).

Table 3.2: Description of all the primers used in this study. Underlined sequences indicate the restriction sites introduced.

Primer	Sequence
M1FTc_EcoR1_F	GATCGAATTCATGAAAAGCACCCCTGAGAA
M1FTt_EcoR1_F	GATCGAATTCATGTGGACCCGCGCCGATG
M1FT_Xho1_R	CCTGCTCGAGTTACTTGAGCGTGACGTCG
SPR-FW-PGKp	AAAAGGGAGTCGGTTGTCAACAGACTGTCCTGTCGAATTTCCCAAGGA TCCGTGGCCTCTTATCGAG
SPR-RV-HISp	AGCACTATCTGTGGAATGGCTGTTGGAACTTTTTCCGATTACTGAGAGT GCACCATAAATTCCCG
SPR_HIS_F	AAAAGGGAGTCGGTTGTCAACAGACTGTCCTGTCGAATTTCCCAA`CTG AGAGTGCACCATAAATTCCCGT
SPR_F1	AAAAGGGAGTCGGTTGTCAACAG
HIS3_PGKP_R	CTGAACGAGGCGCGCTTTCCTTTTTTCTTTTTGCTTTTTCTTTTTTTTAGC TTTCTAACTGATCTATCCAAAAC
PGKt_SPR_R	AAAATTCGCTCCTCTTTTAATGCCTAATCGGAAAAAGTTCCAACAGCCAT TCCACAGATAGTGCT
PGK_LacZ_F	GATCCTCGAGAGCTTTCTAACTGATCTATCCAAAACT
SPR_R1	CAGCCATTCCACAGATAGTGCT
SPR3_Diag_Up	GAAGAGGTAAACCAATCAATGGCC
SUT_ Diag_Up_Rv	TTGGGCCGCACAACCAGATGTA
SUT_ Diag_Down	CTACCACCGCTCTGGAAAGTGC
SPR3-Diag-Down-Rv	CGCAGGGTTCTTTGCATTGCCT
Susy_ Diag_down	CACTGTGGGACAATATGAGAGC

High performance liquid chromatography (HPLC) was also used for sugar quantification. Cell extracts were performed as previously described. 200µL of extract was used for HPLC analysis. Proteinase K (20mg/L) was added to extracts (200µL) and incubated at 37°C for 1 h. Samples were hydrolyzed using TFA (2M, 1 h at 120°C), and dried overnight at 55°C. Samples were resuspended in 200µL mQ water and dilutions (5x and 10x) prepared for each with final volumes of 0.5mL and 1 mL respectively. All HPLC standards were prepared as described by Eyéghé-Bickong *et al.* (2012). The internal standard (IS) used for organic acids were adipic acid

(2g/L) and ribitol (2g/L) for the sugars. The quality control consisted of glucose and fructose (2.5 g/L) and organic acids (0.625g/L) (malic acid, succinic acid, acetic acid and tartaric acid). 5X dilutions were made using 100μL sample and adding 150μL mQ water with 250 μL of IS added to make total volume of 500μL. 10X dilutions were made using 100μL of sampled added to 400μL of mQ water and 500μL of IS. Sample dilutions were filtered using 0.22 μm nylon fibre filters into HPLC vials and crimp-sealed for HPLC analysis. An Argilent 1100 series HPLC system using a Aminex HPX-87H column (300mm x 7.8 mm) was used to analyse the sugars. ChemStation Rev. A.10.02 software (Agilent Technologies©) was used to control the system, acquire data and integrate peaks.

Table 3.3: Description of all the plasmids used in this study.

Plasmid	Genotype	Reference
YCplac33-PGK1$_{PT}$	CEN4 *URA3* PGK1$_P$ PGK1$_T$	This laboratory
YCplac33-pGK1-M1FTΔsp	CEN4 *URA3* PGK1$_P$ -M1FTΔsp –PGK1$_T$	This study
YCplac33-pGK1-M1FT	CEN4 *URA3* PGK1$_P$ -M1FT–PGK1$_T$	This study
pHVXII-SUSY	CEN4 *URA3* PGK1$_P$ -SuSy –PGK1$_T$	This study
pHVXII-SUT	CEN4 *URA3* PGK1$_P$ -SUT–PGK1$_T$	This study
pPVΔI	*HIS3*	This laboratory
pBluescript –M1FT	M1FT	Rapp, 2012

3.2.6 Quantification of total protein and levan produced

Total protein quantification was done at Stellenbosch University's Central Analytical Facility (CAF) using 2-D Quant Kit (GE Healthcare). Cells were grown and harvested as previously described. Cells were washed with 1mL mQ water and resuspended in 500μL mQ. Addition of ~300μL glass beads to suspension was followed by vigorous vortexing for 10min. 100μL of cell extract was aspirated into new 1.5 mL centrifugation tubes for each sample and sent for analysis using 2-D Quant Kit (GE Healthcare). Assays were performed along the following experimental outline: Proteins were pelleted by centrifugation and resuspended in a saturated alkaline solution of cupric ions. The cupric ions bind to the polypeptide backbones of any protein present. A colorimetric agent which reacts with unbound cupric ions is then added. The colour density is inversely related to the concentration of protein in the sample. Protein concentration can be accurately estimated by comparison to a standard curve 2 mg/mL Bovine serum albumin (BSA). Quantification was done using kit specifications. Samples were prepared in duplicate and averages reported.

Levan quantification was done as described previously (Banguela *et al.,* 2011). Cell extract for TLC analysis was done as reported before. Samples (1µL) were developed on a TLC, together with 2.5, 5, 10, 20, 30 µg of levan from *Zymomonas mobilis* (Sigma) as well as fructose and sucrose used as standard curve. The stained fructans in the foils were visualized and photographed using gel camera (G-Box, SynGene) with GeneSnap software v. 7.09.11 (SynGene). A densitometric analysis was done on the spots on the TLC plates to plot a standard curve using the software Genetools v.4.01.04 (Synoptics Ltd). The concentration of the spotted levan samples was then calculated using the generated standard curve.

3.2.7 Production of levan during alcoholic fermentation

BY4742Δ*suc2*-Susy-M1FTΔsp was used as levan producing strain. The control strains were BY4742Δ*suc2*-Susy, BY4742Δ*suc2*-M1FTΔsp and BY4742Δ*suc2*-YCplac33-PGK1pt. Fermentations were done in triplicate in MS300 synthetic wine media (Bely *et al.,* 1990) with 100g/L sugar at 30°C with 100mL media in 250mL Erlenmeyer flasks. Strains were inoculated into media to OD_{600} of 0.1 from respective overnight cultures. Fermentation progress was monitored as weight loss (CO_2 loss) and was measured daily for duration of fermentation (26 days). After completion of fermentation, cells were harvested by centrifugation at 5000rpm for 2 min and stored at 4°C for further analysis. The supernatant was aliquoted and stored at -20°C (Fermentation product analysis) and 4°C (GCFID analysis), respectively, for further analysis.

Cells were resuspended in 1mL distilled H_2O and transferred to 2mL centrifugation tubes. Cells were washed with 1mL mQ water and resuspended in 500µL mQ. Addition of ~300µL glass beads to suspension was followed by vigorous vortex for 10min. Cell suspension was centrifuged at 12000rpm for 30 seconds. 400µL of cell extract was transferred to 1.5mL centrifugation tubes. 100µL of extract was transferred into new 1.5 mL centrifugation tubes for TLC analysis. 100 µL samples were dried overnight at 55°C, and resuspended in 5µL mQ water (20x concentrations). Samples (1µL) were spotted on TLC silica gel foil (Merck, Germany) and separated in butanol:acetic acid:water (50:30:15). Fructose containing sugars were specifically stained with a urea spray (Wise *et al.,* 1955), and developed at 110°C for until the sugars could be clearly visualized.

3.2.8 Analysis of residual and intracellular sugar produced during fermentation

The cells from fermentations were harvested and processed and 400µL of extract was recovered as mentioned previously. 100µL of extract was used for sugar analysis and the remaining sample was stored at 4°C for further experimental analysis. 100µL of sample was aspirated into 1.5 mL centrifugation tubes, and hydrolysed at 120°C for 1 h using 2M TFA. Samples were dried overnight at 55°C. Dried samples were resuspended in 500µL distilled

water and sent for analysis. Glucose, sucrose and fructose in the hydrolyzed cell extract was analysed using the Arena 20XT enzyme robot and the respective kits (Thermo Fisher Scientific).

The completeness of the fermentation was analysed by determining the residual sugar in the fermented MS300 must. 500µL of fermented MS300 was pipetted into 1.5 mL centrifugation tubes and sent for analysis. Sugar (glucose, fructose and sucrose) quantification was done using the Arena 20XT enzyme robot and respective kits (Thermo Fisher Scientific).

3.2.9 High performance liquid chromatography analysis of ethanol and glycerol

Fermented MS300 was analysed using HPLC to determine the ethanol and glycerol produced by fermentations. At completion of fermentation, cells were harvested and the supernatant was stored at -20°C for HPLC analysis as mentioned previously. The supernatant was defrosted and vortexed briefly to homogenise solution. 200 µL was then aspirated into 2 mL centrifugation tube and 800µL mQ water was added to final volume of 1 mL. All HPLC standards were prepared as stated in described previously (Eyéghé-Bickong *et al.,* 2012). The internal standard (IS) used for organic acids was adipic acid (2g/L) and ribitol (2g/L). The quality controls consisted of glucose and fructose (2.5 g/L) and organic acids (0.625g/L) (malic acid, succinic acid, acetic acid and tartaric acid). 1 mL of IS was added to samples (1mL) to a final 10x dilution of samples. Samples were mixed by brief vortexing. Resulting dilutions were filtered using 0. 22 µm nylon fibre filters into HPLC vials and crimp-sealed for HPLC analysis. An Argilent 1100 series HPLC system using an Aminex HPX-87H column (300mm x 7.8 mm) was used for glycerol and ethanol analysis. ChemStation Rev. A.10.02 software (Agilent Technologies©) was used to control the system, acquire data and integrate peaks.

3.2.10 GC-FID analysis of aroma compounds

The volatile compounds in the fermented MS300 were analysed using GC-FID method described by Malherbe *et al*., 2012. 5 mL of fermented MS300 with internal standard, 4-Methyl-2-Pentanol, (100µL of 0.5mg/L solution in soaking solution) was extracted with 1 mL of diethyl ether by placing the ether/wine mixture in an ultrasonic bath for 5 minutes. The quality control synthetic must (4mL) was at pH 3.5, with 12% EtOH and 2.5g/L tartaric acid, and contained 1 mL aroma compound mixture. The synthetic wine/ether mixture was centrifuged at 4000 rpm for 3 minutes. The ether layer was removed and dried on $NaSO_4$. Samples were then placed into GCFID vials and crimp-sealed for GCFID analysis. The samples were injected into the GC-FID with DB-FFAP capillary GC column (20 m length x 0.1 mm id x 0.2 um film thickness) for analysis. Each synthetic wine was extracted once, but injected into the instrument three times from the same extract. The average of the three amounts (three injections) for each compound present in the synthetic wine must was then calculated and reported.

3.2.11 Nuclear magnetic resonance analysis of levan

Levan produced by BY4742Δ*suc2*-Susy-M1FTΔsp and BY4742Δ*suc2*-SUT-M1FTΔsp strains were analysed using nuclear magnetic resonance (NMR). Cells were harvested after they grew to saturation (400mL and 300mL respectively) by centrifugation at 5000 rpm for 2 min. Levan was then extracted as mentioned previously. The cell extract was not dried but treated with DNAse and RNAse and incubated at 37°C for 1 h. Samples were then treated with Proteinase K (20mg/L) and again incubated at 37°C for 1 h. Samples were then centrifuged at 10 000rpm at 4°C for 10 min and the supernatant carefully aspirated into new 1.5 mL eppi. Ice cold 100% EtOH was then added to samples to make up 1.5 mL and polymer was the precipitated overnight at -20°C. Levan was precipitated by centrifugation at 12 000rpm for 10 min at 4°C. Supernatant was carefully aliquoted and the pellet was dried in a 55°C oven for 40 min followed by 5 min drying in a vacuum centrifuge (SpeedyVac). The pellets were resuspended in 10 mL mQ water. Samples underwent dialysis using Snakeskin dialysis tubing (Spectrum Laboratories, USA) with 3.5 kDa molecular cut-off. Dialysis was performed in 2 L dH_2O containing 0.02% sodium azide (NaN_3) for 24 h at 8°C. Dialysed samples were removed from the dialysis tubing in 50mL Falcon tubes. Ice cold 100% EtOH was added to samples to make up to 50mL and levan was precipitated at -20°C for 1 h. Biopolymer was then pelleted by centrifugation at 5000rpm for 10 min and allowed to dry at 55°C overnight. Samples were then resuspended in 2mL mQ water and transferred to 2 mL centrifugation tubes. Samples were centrifuged at 15000 rpm for 4 min and supernatant aspirated into new 2 mL centrifugation tubes. Ice cold 100% EtOH was added to make up 2mL and samples precipitated overnight at -20°C. Precipitation step was repeated 3 times and dried at 55°C overnight.

Dried biopolymer was prepared by dissolving 10mg of purified polymer in 1 mL deuterium monoxide (D_2O) and adding tetramethylsilane (TMS) to a final concentration of 4.5 mM. Samples were placed in NMR tubes and sent for analysis at the Central Analytical facility (CAF) at Stellenbosch University. A 600MHZ Varian INOVA NMWR was used to create the proton (^{1}H) spectra of the samples.

3.3 RESULTS AND DISCUSSION

3.3.1 De novo synthesis and accumulation of sucrose and levan in modified yeast strains

The intracellular accumulation of sucrose requires a yeast strain that has no or significantly reduced invertase (encoded by *SUC2*) activity. For this purpose, the BY4742Δ*suc2* genetic

background was used as the receiver strain. To ensure synthesis of sucrose, the sucrose synthase (Susy) and the sucrose transporter (SUT) genes from potato and spinach, respectively, were separately introduced into this background resulting in two sucrose producing strains used in this study, namely BY4742Δ*suc2*-Susy and BY4742Δ*suc2*-SUT.

The data confirmed that sucrose accumulation occurs in both BY4742Δ*suc2*-Susy and BY4742Δ*suc2*-SUT, while no sucrose was detected in BY4742Δ*suc2* control strain with empty YCplac33 plasmid (Figure 3.3.1). The control strain showed only some intracellular fructose accumulation. The Susy strain is of particular interest in that it produces intracellular sucrose, thereby negating the need for sucrose uptake from the medium, as is the case for the BY4742Δ*suc2*-SUT strain. This would allow for levan production in a grape must environment, where glucose and fructose are the only carbon sources.

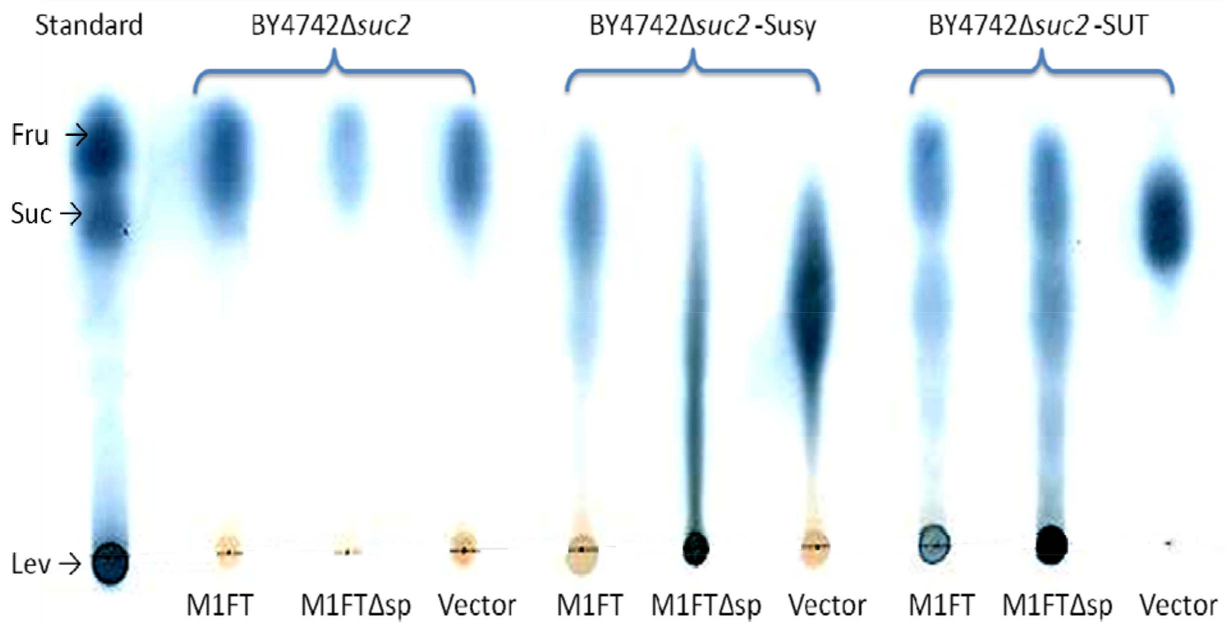

Figure 3.3.1: TLC analysis of levan, fructose and sucrose accumulation in BY4742Δ*suc2*/Susy/SUT strains containing either YCplac33 (vector), M1FT or M1FTΔsp expression cassettes. Fructose and sucrose given smaller molecular structure migrates easily with mobile phase whereas larger levan remains were spotted. Levan from *Zymomonas mobilis*, sucrose and fructose at 10g/L was used as control. Levan production can be seen in strains BY4742Δ*suc2*-Susy-M1FTΔsp, BY4742Δ*suc2*-SUT-M1FTΔsp and BY4742Δ*suc2*-SUT-M1FT. BY4742Δ*suc2*-Susy-M1FTstrains showed minute barely detectable levan production on TLC. Fru-Fructose; Suc- Sucrose; Lev-Levan; Vector- YCplac33

The sucrose-accumulating strains and the control strains were transformed with the two forms, with and without the native secretion signal, of the *L. mesenteroides* levansucrase, M1FT. This bacterial FTFs, is an extracellular fructosyl-transferase (FTF), and an M1FT clone (M1FTΔsp) without the predicted signal peptide was therefore included in the analysis. Strains were inoculated into selective (SCD) media and grown to saturation. Cells were harvested, processed

and the resulting cell extracts were analysed using thin layer chromatography (TLC). The strains were also analysed to determine intracellular fructose and sucrose accumulation.

The BY4742Δ*suc2*-Susy and BY4742Δ*suc2*-SUT strains expressing M1FT and M1FTΔsp exhibited varying degrees of the intracellular levan accumulation. When comparing the strains expressing the complete M1FT gene and strains expressing M1FTΔsp (Figure 3.3.1), it was apparent that M1FTΔsp expressing strains generally appeared to be stronger levan producers. The absence of the signal peptide is likely to explain this increase in levan production. In a study by Scotti and co-workers, the hydrophobicity of SacB levansucrase was correlated to its efficiency in entering the secretion pathway of *S. cerevisiae* (Scotti *et al.,* 1994). Intracellular SacB accumulated in an unprocessed form which remained weakly associated with the cytoplasmic membrane. Therefore, it is likely that the M1FT signal peptide may, in a similar fashion, be interfering with efficient M1FT enzyme folding. This can be expected to result in suboptimal enzymatic activity. Further studies by Scotti and co-workers, however, reported the ability of signal sequences of various *Bacillus* or yeast secreted proteins to direct *B. subtilis* SacB levansucrase into the secretion pathway of *S. cerevisiae* (Scotti *et al.,* 1996). The possibility thus exists that the M1FT signal peptide may be active and allowing for extracellular levan production. It has been shown that glycosylation does not modify the trans-fructosylation polymerase activity of these enzymes (Scotti *et al.,* 1996; Trujillo *et al.,* 2004; Kang *et al.,* 2011). Thus, a significant fraction of the enzymes may be extracellularly active resulting in lower production of intracellular levan as compared to truncated M1FT. Extracellular levan production may impact wine sensory properties thus for the scope of this study, it was not measured. However, it will be of importance in future studies to investigate the precise localization of enzymes and activities in the levan producing yeast strains. The M1FTΔsp expressing strains were selected for further analysis based on increased levan production phenotypes.

To confirm the identity of the produced polymer, acid hydrolysis was employed. Indeed, levan polymers are notably more vulnerable to acid hydrolysis than inulin type fructans. The combination of a fructan specific stain (Wise *et al.,* 1955) and sensitivity to acid hydrolysis was used to identify the fructan polymer produced as a putative levan. However inulin hydrolysed samples could be used in future studies as a possible control. Cell extracts of the all nine strains were prepared and hydrolysed to analyse the produced fructan polymer. The samples were then run on a TLC to ascertain the completeness of the hydrolysis method. The TLC analysis showed only free fructose (Figure 3.3.2). No levan or sucrose was detected for all strains, except for the BY4742Δ*suc2*-Susy strain. The detected sucrose resulted from an incomplete hydrolysis reaction as subsequent TLCs showed complete hydrolysis in all strains. By using a fructose specific stain in combination with the characteristic, complete hydrolysis of levan by HCl to fructose, allows for the putative identification as a levan and not inulin type fructan polymer. This is to be expected, since M1FT was previously characterized as a levansucrase,

producing polymers that are specifically detected by anti-levan-antibody, which is specific for β-2,6 linkages (Kang *et al*., 2005).

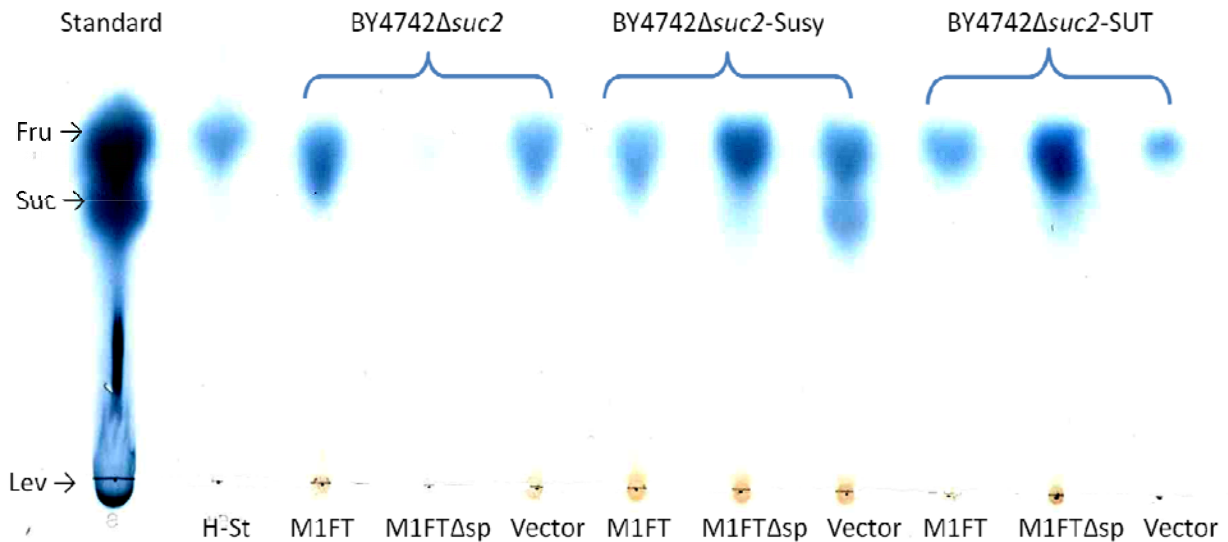

Figure 3.3.2: TLC analysis of acid hydrolyzed cell extracts of BY4742Δ*suc2*/Susy/SUT strains containing either YCplac33 (vector), M1FT or M1FTΔsp expression cassettes. Levan and sucrose was completely hydrolyzed with only free fructose stained. There is no levan as compared to standard. BY4742Δ*suc2*-Susy however still shows sucrose. The hydrolysis was thus incomplete for this strain. Levan from *Zymomonas mobilis*, sucrose and fructose at 10g/L was used as control. Fru-Fructose; Suc- Sucrose; Lev-Levan, H-St-Hydrolyzed Levan standard; Vector- YCplac33

3.3.2 Extracellular fructose, and not fructose-6-phosphate, is preferentially utilized as substrate for the heterologously expressed sucrose synthase (Susy)

The data generated by the TLC analysis clearly illustrates that the heterologous system introduced into yeast effectively functions to firstly accumulate intracellular sucrose, which can subsequently be used as substrate for levan synthesis by M1FT. This is achieved by either importing sucrose, through the action of SUT, or by sucrose synthesis, by the activity of the cloned sucrose synthase (Susy). Endogenous sucrose synthesis is of specific interest to the overall goal of the project, which is to reroute carbon flux away from glycolysis in a grape must based growth medium. In grape must, the major sugars are glucose and fructose, with sucrose generally only present in trace amounts (Liu *et al*., 2006). It would therefore be critical to generate a strain that can produce its own sucrose, by taking from the existing extracellular pool of sugars. Furthermore, it is also of interest whether this strain, and the expressed Susy, would prefer fructose that originates from the medium or fructose-6-phosphate, derived from both glucose and fructose at entry into glycolysis. In fermentation conditions, utilization of fructose would be desirable since this could result in a more specific utilisation of fructose and positively impact on the glucose:fructose ratio.

To determine the source of fructose utilized by Susy for sucrose synthesis and subsequently the utilization of sucrose by M1FT for the production of levan, the BY4742Δ*suc2*-Susy and BY4742Δ*suc2*-Susy-M1FTΔsp strains were cultivated in YNB medium with 8% (w/v) glucose as only carbon source. BY4742Δ*suc2*-SUT and BY4742Δ*suc2*-SUT-M1FTΔsp were grown in SCD sucrose glucose medium and BY4742Δ*suc2*-Susy, BY4742Δ*suc2*-Susy-M1FTΔsp, BY4742Δ*suc2* and BY4742Δ*suc2*-M1FTΔsp were grown in glucose and fructose containing SCD medium for use as control strains. Strains were grown to saturation at 30°C, harvested and processed as mentioned previously. Cell extracts were concentrated and separated on TLC.

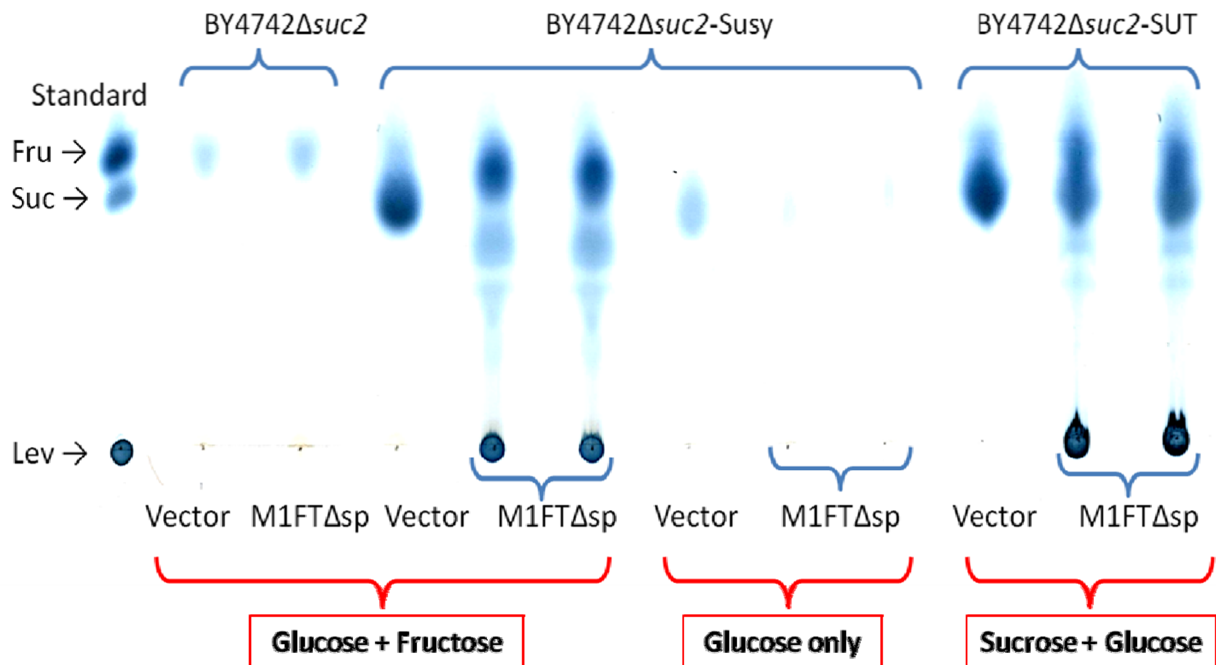

Figure 3.3.3: TLC of BY4742Δ*suc2*-Susy strains grown in media with glucose as sole carbon source with BY4742Δ*suc2*-Susy, BY4742Δ*suc2* and BY4742Δ*suc2*-SUT strains as controls grown in glucose+ fructose and sucrose+fructose SCD respectively. No detectable levan production or fructose accumulation could be seen in strains grown in glucose only media, Furthermore, decreases in intracellular sucrose accumulation was seen. BY4742Δ*suc2* control strains exhibited fructose accumulation phenotype. BY4742Δ*suc2*-Susy-M1FTΔsp control strains showed levan sucrose and fructose accumulation. Levan from *Zymomonas mobilis*, sucrose and fructose at 10g/L was used as standard. Fru-Fructose; Suc- Sucrose; Lev-Levan, Vector- YCplac33

Levan production could be seen in the expected control strains but not in strains grown in glucose only media (Figure 3.3.3). The BY4742Δ*suc2*-Susy strain with empty vector however showed slight sucrose accumulation. This would indicate that Susy has a limited ability to utilize fructose produced during glycolysis. There was a decrease in detectable sucrose accumulation in BY4742Δ*suc2*-Susy-M1FTΔsp strains in comparison to the BY4742Δ*suc2*-Susy strain. This could likely be attributed to the sucrose hydrolysis activity of M1FTΔsp. It has been illustrated that, at lower sucrose concentrations, levansucrase activity shifts toward hydrolysis activity (Olivera *et al.,* 2012). Therefore, lack of detectable levan production by strains in glucose only medium indicates that Susy preferentially utilizes fructose from the extracellular media as

substrate, with only a small amount detectable when cultures are grown only on glucose, and that the decreased intracellular sucrose concentrations is likely to have shifted M1FTΔsp specificity from trans-fructosylation towards hydrolysis activity.

An interesting aspect of the TLC's is the streaks that can be seen in levan accumulation strains grown in selective YNB (Figure 3.3.1 and Figure 3.3.3.). The streaks occur slightly below sucrose. This would indicate that levan type fructans of various degrees of polymerisation (DP) are being formed, more notably the smaller 6-Kestose and Nystose types. In a study by Ozimek and colleagues, a similar phenotype was observed (Ozimek *et al.,* 2006). 6-Kestose, nystose and large levan polymers were detected but no significant accumulation of larger intermediate fructo-oligosaccharides was observed. This study proposed a model that levan production occurs as a processive reaction as levansucrases exhibited an increased affinity for fructan acceptors with a higher degree of polymerization (DP).

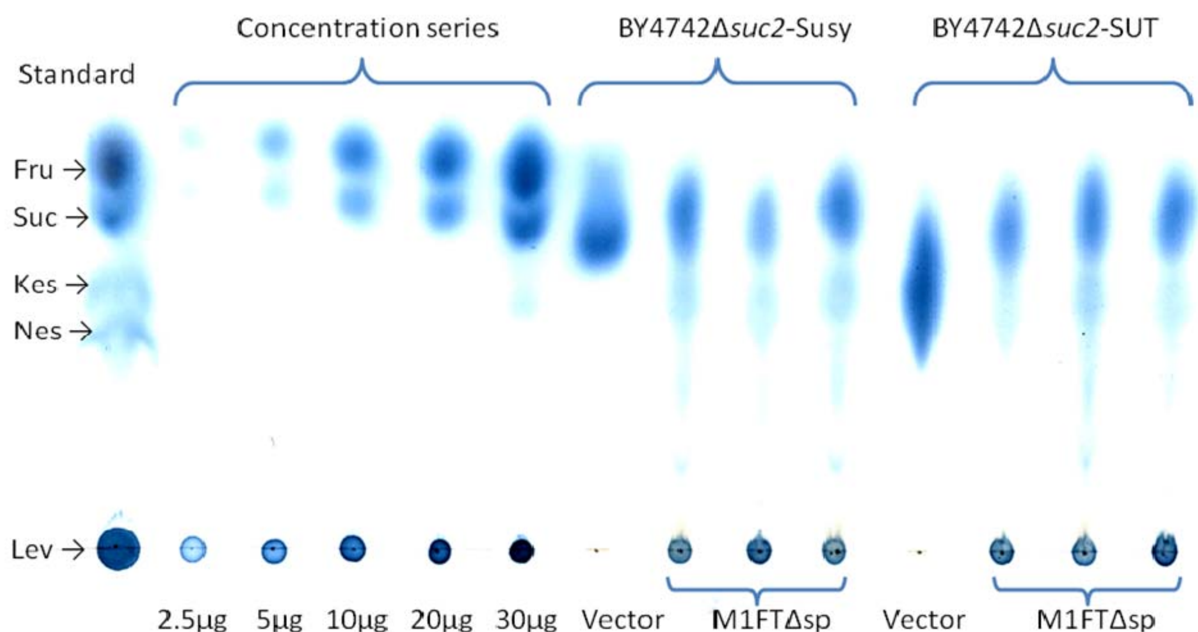

Figure 3.3.4: TLC to quantify levan production via concentration series and densitometry. BY4742Δ*suc2*-Susy-M1FTΔsp and BY4742Δ*suc2*-SUT-M1FTΔsp strains were analysed in triplicate, and their respective control strains were also analysed. Levan from *Zymomonas mobilis*, sucrose, fructose, kestose and nystose at 10g/L was used as standards. Fru-Fructose; Suc- Sucrose; Lev-Levan, Vector- YCplac33; Kes-1-Kestose; Nes-Nystose

3.3.3 Quantification of produced Levan using densitometric analysis of TLC plates

The production of levan by the BY4742Δ*suc2*-Susy-M1FTΔsp and BY4742Δ*suc2*-SUT-M1FTΔsp strains was quantified by correlating the densities of spotted samples to the standard curve of known levan concentrations that where spotted on TLC plates. The concentrated extract samples (1µL) were run on a TLC together with 2.5, 5, 10, 20 and 30µg of levan from *Zymomonas mobilis*, sucrose and fructose (Figure 3.3.4). The stained levan was then analysed using image densitometry and a standard curve plotted. The concentration of levan in samples

was then determined using the standard curve. The total protein in samples was analysed using the 2-D Quant Kit (GE Healthcare). The levan concentration was calculated by correlating total levan to the total protein concentrations in the samples. Correlation to total protein can give a good indication of cell growth and metabolism.

The BY4742Δ*suc2*-Susy-M1FTΔsp strains exhibited an average productivity of 0.136mg levan/mg protein (SD 0.078). BY4742Δ*suc2*-SUT-M1FTΔsp strains exhibited an average productivity of 0.178mg/mg protein (SD 0.061). The difference between strains was not statistically significant. In future, optimisation of the media conditions could be crucial to optimized levan productivity. Various authors have critically emphasised the importance of biochemical and medium optimisation strategies for levan production (Anathalakshmi and Gunasekaran., 1999; Jang *et al.,* 2001; Senthilkumar and Gunasekaran., 2004; Song and Rhee., 1994). This novel approach of combining intracellular sucrose production with levan production in *S. cerevisiae* has further emphasised this point.

3.3.4 Chemical analysis of the produced levan

The chemical analysis of the sugar and polymer produced was performed using HPLC and NMR based techniques. The samples were analysed as 10x and 5x dilutions of the hydrolysed levan cell extracts. The data however, suggested that the amount of levan was below the limit of detection. In order to extract a sufficient amount of levan for analysis, substantial up scaling of the yeast production cultures was required.

NMR was done to determine the magnetic resonance of the polymer and compare it to the reported resonance of levan in an effort to positively identify the fructan polymer. Polymer produced by BY4742Δ*suc2*-Susy-M1FTΔsp and BY4742Δ*suc2*-SUT-M1FTΔsp strains during aerobic growth in glucose and fructose containing media were analysed. Cells were grown to saturation at 30°C, harvested crude extracts prepared. Levan was purified from cell extract and analysed by NMR. The ^{1}H proton NMR analysis of the two samples showed a significant amount of contamination within the samples (Figure 3.3.5). This made identification on proton level difficult. The control levan from *Zymomonas mobilis* was identical to reported ^{1}H NMR spectra for levan (Figure 3.3.5), thus ^{1}H NMR is a reliable method for levan identification.

Key peaks in the BY4742Δ*suc2*-Susy-M1FTΔsp and BY4742Δ*suc2*-SUT-M1FTΔsp samples correspond to the identifiable/unique peaks in the control sample. The presence of additional peaks however does not allow for substantiated positive identification of the polymer being produced. However, closer analysis of the BY4742Δ*suc2*-Susy-M1FTΔsp and BY4742Δ*suc2*-SUT-M1FTΔsp polymer spectra's showed that they overlap to a large extent. Furthermore, the concentration of levan in test samples as compared to the internal standard

was significantly lower. This further substantiates the need to optimize levan production and extraction methods to obtain sufficient concentrations for chemical analysis. The purification methods also need to be optimized as there is a high degree of background signal present which likely originates from the cell extract, containing residual cell debris, proteins and DNA which makes positive identification difficult. The use of ultra filtration or column chromatography would likely be required to optimize purification methods. Ultra filtration approaches has been reported to be reliable methods of levan purification (Banguela *et al.,* 2012).

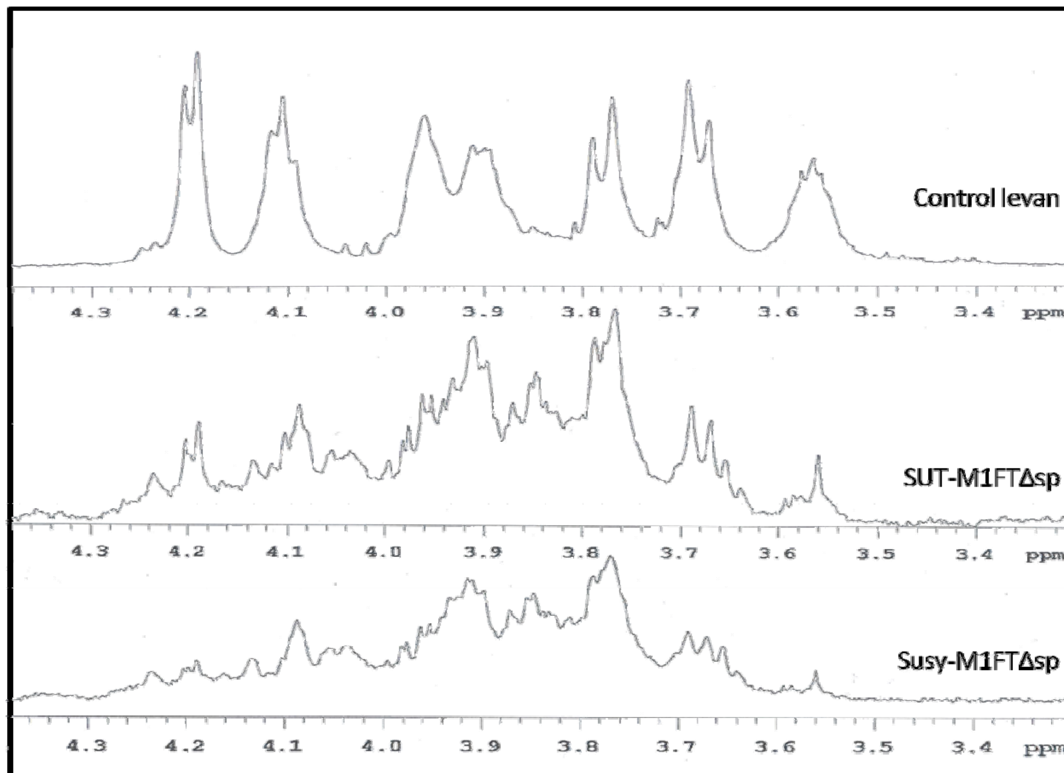

Figure 3.3.5: ^{1}H NMR spectra of polymer produced by BY4742Δ*suc2*-Susy-M1FTΔsp and BY4742Δ*suc2*-SUT-M1FTΔsp strains as compared to control levan polymer from *Zymomonas mobilis*. The control clearly indicates reported spectra of levan in literature. The test samples have corresponding peaks as compared to control; however there are additional peaks in samples that are attributed to background signal.

3.3.5 Fermentation performance and analyses of key metabolites produced by the levan producing yeast strains

The production of levan under fermentative conditions was analysed to ascertain the viability of levan polymer as a carbon sink during ethanol fermentation by the levan producing yeast strains. The fermentation performance and levan productivity of BY4742Δ*suc2*-Susy-M1FTΔsp strain was analysed with BY4742Δ*suc2*-Susy, BY4742Δ*suc2*-M1FTΔsp and BY4742Δ*suc2* as control strains. The strains with Susy gene were used in the fermentations as these strains can produce intracellular sucrose using glucose and fructose, which are the predominate sugars in

grape must. The fermentations were done in the synthetic wine medium MS300 and incubated at 30°C for duration of fermentation. The fermentation progression was monitored as cumulative weight loss over the 26-day duration of fermentation.

The strains exhibited similar fermentation kinetics in general. However the BY4742Δ*suc2*-Susy strain fermentation kinetics was slower than that of the other strains that were tested (Figure 3.3.6). This was also reflected in the residual sugar concentrations for this stain. The average residual fructose concentration in fermentation by this strain was 8.04g/L (Figure 3.3.7). Overall, the BY4742Δ*suc2*-Susy strain also produced less ethanol and glycerol as compared to the other strains. This may be due to decreased/sluggish fermentation performance resulting in higher residual sugar. The fermentation kinetics for the remaining strains were almost identical, thus the heterologous genes did not seem to have a negative metabolic impact on yeast growth. The BY4742Δ*suc2*-Susy-M1FTΔsp strain did not exhibit any significant differences in wet weight as compared to the control strains, further emphasising the lack of distinguishable differences is growth exempting BY4742Δ*suc2*-Susy strain (data not shown).

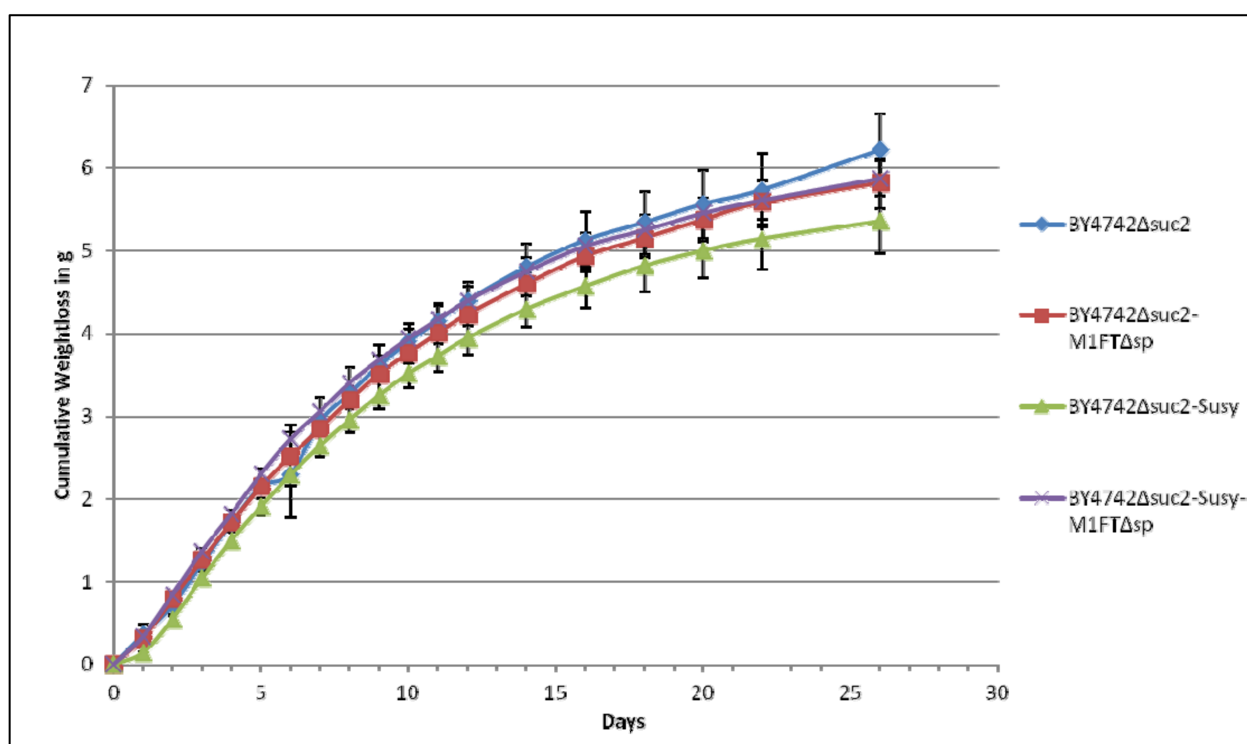

Figure 3.3.6: The cumulative weight-loss reflecting the growth rate of BY4742Δ*suc2*-Susy-M1FTΔsp test strain and controls strains BY4742Δ*suc2*-Susy, BY4742Δ*suc2* and BY4742Δ*suc2*-M1FTΔsp strains during fermentation of MS300. BY4742Δ*suc2*-Susy strains showed more sluggish growth when compared to the almost identical growth curves of remaining strains.

The fermented MS300 was analysed using HPLC and GC-FID to determine the concentrations of residual sugars and fermentation products. The aroma profile was analysed using GC-FID method. No marked differences in the aroma profiles between the strains were observed (Data not shown). This further indicates the minimal impact that expression of the heterologous enzymes appears to have on yeast fermentative metabolism. HPLC was performed to determine the ethanol and glycerol concentrations produced by strains during fermentation. The residual sugars were analyzed on enzyme robot. The ethanol and glycerol concentrations of strains did not show any marked impact on the production of these compounds (Figure 3.3.7.). The BY4742Δ*suc2*-Susy-M1FTΔsp test strain average ethanol was 39.9 g/L whereas the controls strains BY4742Δ*suc2*-Susy BY4742Δ*suc2* and BY4742Δ*suc2*-M1FTΔsp averaged 37.8g/L, 38.8g/L and 42.8g/L respectively. The glycerol concentrations showed similar pattern. BY4742Δ*suc2*-Susy-M1FTΔsp strains average glycerol was 3.38g/L and the controls strains BY4742Δ*suc2*-Susy BY4742Δ*suc2* and BY4742Δ*suc2*-M1FTΔsp averaged 2.55g/L, 3.35g/L and 3.66g/L respectively. Glycerol production was roughly 8% of ethanol concentrations for respective strains. The fermentations were considered to be dry with residual sugar concentration below 4g/L except BY4742Δ*suc2*-Susy fermentations.

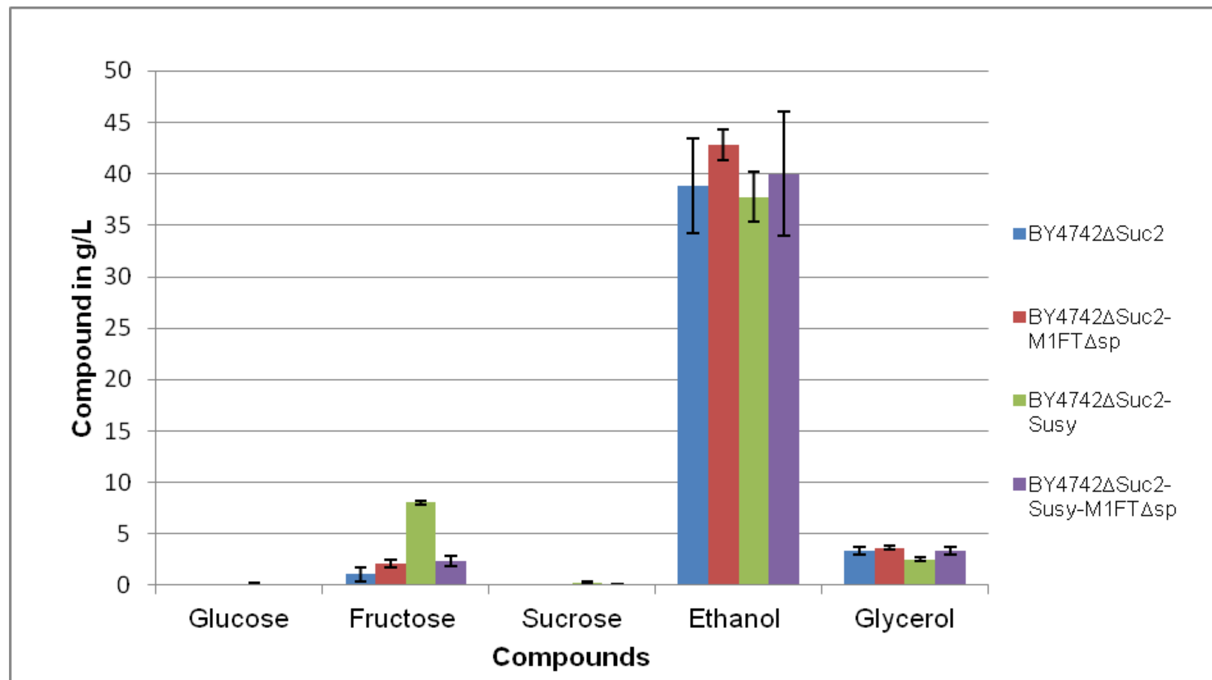

Figure 3.3.7: The concentrations of residual sugar, ethanol and glycerol present at the end of MS300 fermentations by BY4742Δ*suc2*-Susy-M1FTΔsp test strain and controls strains BY4742Δ*suc2*-Susy BY4742Δ*suc2* and BY4742Δ*suc2*-M1FTΔsp. Fructose was only remaining sugar in average concentrations higher then 1g/L, with an average concentration of 8g/L for BY4742Δ*suc2*-Susy strains. This strain produced lowest ethanol and glycerol concentrations. No marked differences were observed in fermentation product for remaining strains.

At the end of fermentation, the cells were harvested and processed to analyse the cell extracts for the presence of fructose based sugars and sugar polymers by using TLC. TLCs showed no discernible levan production or intracellular sucrose accumulation (Figure 3.3.8.). There may be a single or a combination of causes for this result, which will require further investigation. Firstly, the Susy gene and/or M1FT genes may not be functionally expressed during the course of the fermentation. This would, however, be unexpected since the enzymes where already shown to be functionally expressed during aerobic conditions. It would, however, be essential to confirm gene expression using RT-PCR to eliminate this possibility. The lack of observed levan production during fermentation in contrast to aerobic production may indicate that oxygen may play a role in levan production. This however is unlikely as the fermentative levan producer; *Zymomonas mobilis* produces levan under fermentative conditions. Alternatively, the observed differences between aerobic and fermentative growing cultures might be due to metabolic prioritization of activities that are aligned with efficient glycolysis. This would favour the optimized functioning of yeast cells that are geared towards effective conversion of sugars to ethanol and not grant the heterologous enzymes a foothold on their required substrates.

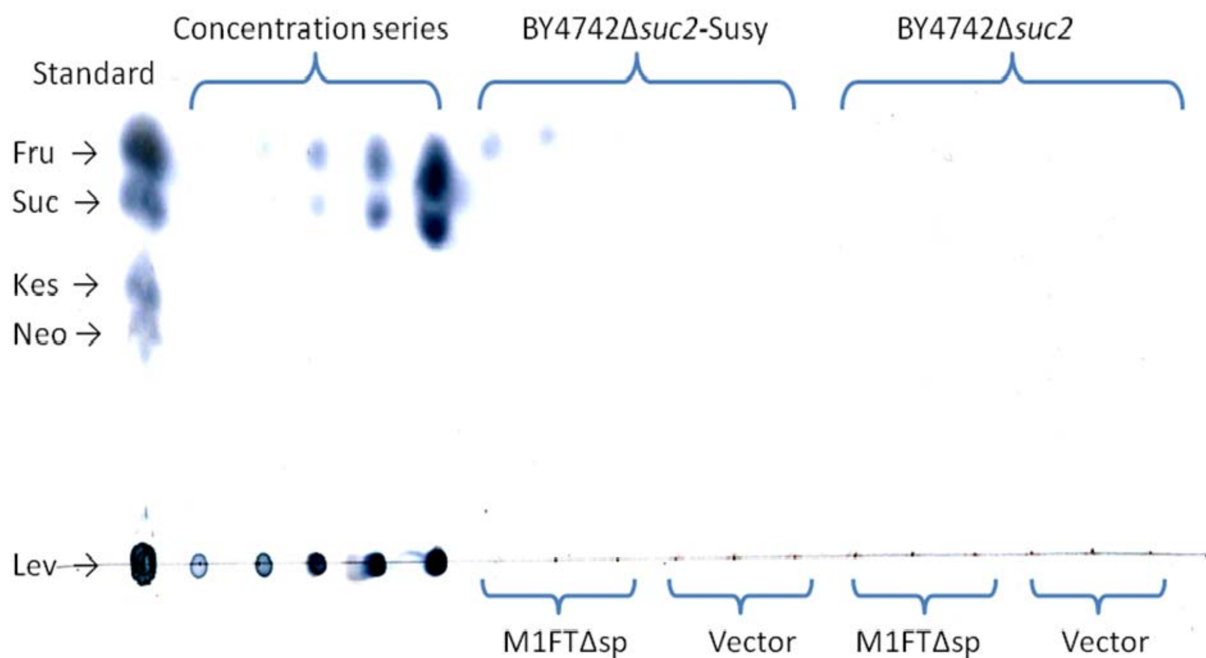

Figure 3.3.8: TLC to determine the levan production by BY4742Δ*suc2*-Susy-M1FTΔsp under fermentative conditions. BY4742Δ*suc2*-Susy, BY4742Δ*suc2*-M1FTΔsp and BY4742Δ*suc2* strains were used as controls. All strains were analysed in triplicate. There is no discernable levan production or intracellular sucrose accumulation seen on TLC. Levan from *Zymomonas mobilis*, sucrose, fructose, kestose and nystose at 10g/L was used as standard. Fru-Fructose; Suc- Sucrose; Lev-Levan, Vector-YCplac33; Kes-1-Kestose; Nes-Nystose

The additional possibility remains that there are enzymatic functions present in *S. cerevisiae* that could degrade the levan polymer. Theoretically, this should have been circumvented as

levan polymer is not native to *S. cerevisiae.* However, there are indeed at least ten glycoside hydrolases in the yeast genome. Some of which remain to be characterized. A recent study demonstrated that one of these enzymes specifically counters the activity of heterologously expressed flavonoid glycosyltransferases (Schmidt *et al*, 2011). It therefore remains a distinct possibility that an enzyme with even low levels of β-2,6-glycosidase activity could, over the course of fermentation, degrade intracellular levan to fructose. It might also be that some of these glycoside hydrolases are expressed in conditions specific to fermentations.

The lack of any stains on the TLC from cell extracts may however indicate that the extraction method used was suboptimal (Figure 3.3.8.). The cells have been grown past saturation thus are recalcitrant. Levan may have been produced but the cell extract method was unable to adequately free intracellular levan, fructose and sucrose. This may explain the absence of stains on TLC. If this is the case, levan as a carbon sink does not adequately redirect carbon from ethanol production as ethanol concentrations were similar for both control and test strains. There may be a combination of decreased gene activity, native degrading agents and insufficient extraction methods responsible for the absence of detectable levan or sucrose production at end of fermentation. However, further optimization of both levan production and extraction methods may lead to levan production under fermentative conditions.

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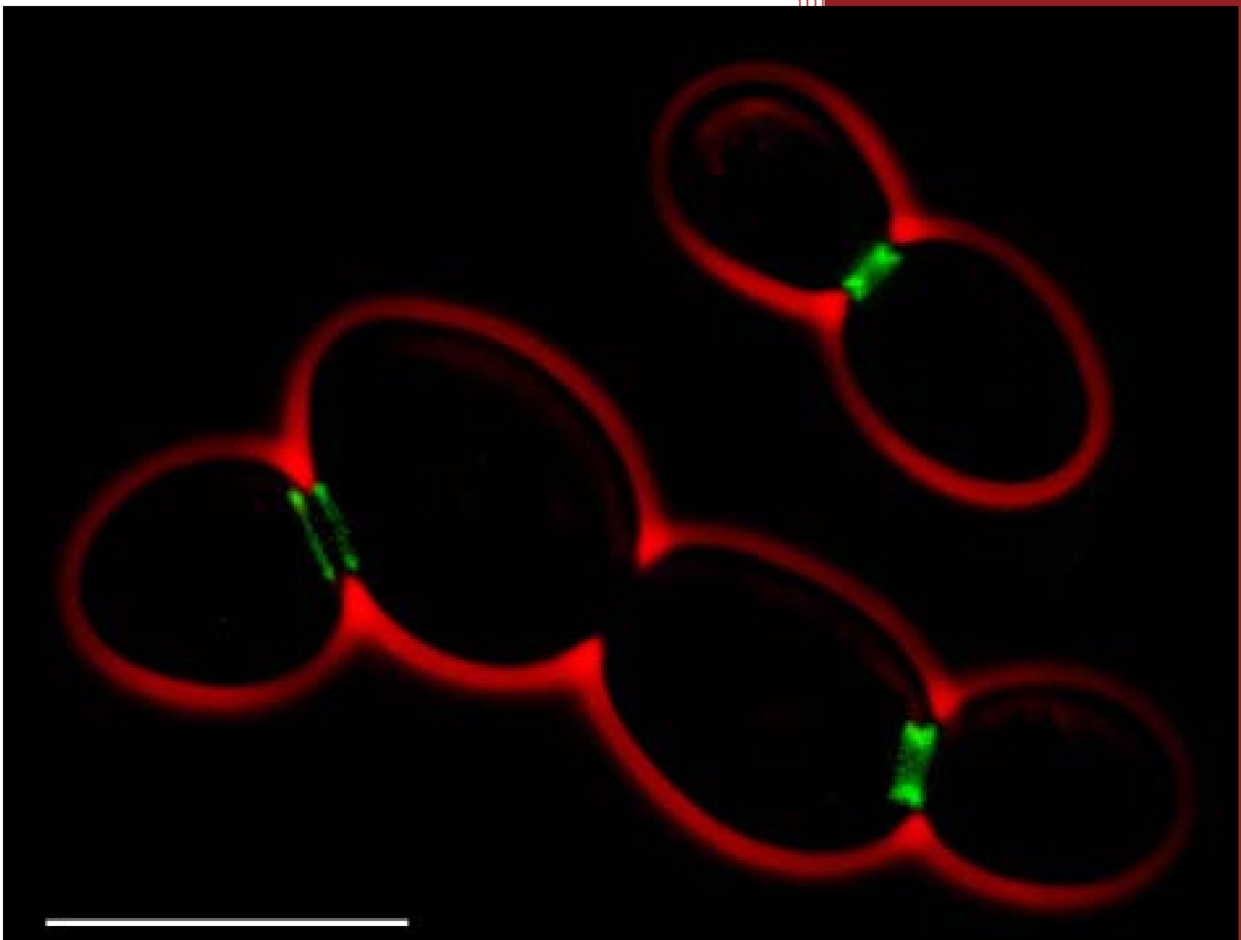
Chapter 4

Conclusions



4. CONCLUSIONS

4.1 CONCLUSIONS

The capacity of *Saccharomyces cerevisiae* to produce and accumulate high concentrations of glycogen polymers without major metabolic burden indicates a potential for intracellular fructan polymer production. Therefore, this study undertook the heterologous expression of a levansucrase M1FT from *Leuconostoc mesenteroides*, an enzyme producing β(2-6) levan-type fructan polymers, in *S. cerevisiae*. M1FT has been reported to be active both in *Escherichia coli* (Kang *et al.,* 2005) and *Pichia pastoris* (Kang *et al.,* 2011), thus similar gene function was expected in *S. cerevisiae*. Using both the complete and truncated gene (M1FTΔsp) allows for novel insight into the expression and activity of M1FT as a levansucrase in *S. cerevisiae.* Furthermore, this study provides insights into the use of storage polymers as carbon sinks to redirect carbon flux. The biological and industrial importance of fructans has been the subject of extensive research, conducted to improve their production or to elucidate their biological role in nature. Thus, insights from this study may be used to further the development of industrial fructan applications.

S. cerevisiae BY4742Δ*suc2* strains with no invertase activity, facilitating higher intracellular sucrose accumulation phenotypes, were constructed as base strains. This was achieved by the heterologous expression of either a sucrose synthase (Susy; cloned from potato) or by growing strains expressing the spinach sucrose transporter (SUT) in sucrose containing media. The extracellular LS M1FT was introduced into Susy and SUT strains as either the complete gene (M1FT) or 50bp truncation (M1FTΔsp) without the predicted signal peptide to facilitate intracellular levan production, as in studies by Scotti and co-workers, it was shown that the SacB (*Bacillis subtilis*) levansucrase when expressed in *S. cerevisiae,* had impaired enzyme folding and function due to the hydrophobicity of its signal peptide (Scotti *et al.,* 1994).

Previous work by this laboratory expressed the levansucrases SacB (*Bacillus subtilis*) along with LsdA (*Glucoacetobacter diazotrophicus*) and FtfA (*Lactobacillus sanfranciscensis*) in *S. cerevisiae* sucrose accumulation strains, without success as levan production was not detected (unpublished data). This is the first time a microbial levansucrase was shown to be expressed and fully functional in *S. cerevisiae,* with intracellular levan production under aerobic conditions. This indicated that intracellular levan accumulation was possible without causing cell toxicity and impaired growth in strains. However, the capacity of levan to act as carbon sink and redirect carbon away from ethanol production during anaerobic fermentations by *S. cerevisiae* BY4742Δ*suc2*-Susy-M1FTΔsp was not confirmed. A possible reason for this result may be the presence of native degradation agents in *S. cerevisiae* cells which could degrade levan

overtime, thereby freeing fructose for ethanol production. There are indeed at least ten glycoside hydrolases, some of which remain to be characterized. It may be that some of these glycoside hydrolases are expressed in conditions specific to fermentations. Furthermore, a recent study has demonstrated that one of these enzymes specifically counters the activity of heterologously expressed flavonoid glycosyltransferases (Schmidt *et al*, 2011). It therefore remains a distinct possibility that an enzyme with even low levels of β-2,6-glycosidase activity could, over the course of fermentation, degrade intracellular levan to fructose. Thus, under current conditions, fructans are not viable metabolic end points for the redirection of carbon from ethanol production during fermentation.

A second possibility is that when sugars become limited, Susy activity may compete with glycolysis. Thus, the resulting sucrose concentrations would be insufficient to allow LS transfructosylation activity. M1FT would thus cleave sucrose to glucose and fructose that can once again be used for glycolysis or sucrose production. Thus, the Susy and M1FT enzymes may effectively be working against each other, with no effective redirection of carbon.

The challenge of levan production in *S. cerevisiae* relates to enzyme compatibility and activity in the heterologous host. Furthermore, the availability of intracellular sucrose as LS substrate is also of import to maintain the transfructosylation activity of LS enzyme (Olivera *et al.,* 2012). The synergistic production of intracellular sucrose in combination with levan production has an advantage; in allows for a broader selection in fructose/carbon sources. Therefore, levan production can occur from intracellular produced sucrose whereas carbon for yeast growth can be varied. As several authors have critically emphasized the importance of medium and biochemical optimization strategies for fructan production (Anathalakshmi and Gunasekaran., 1999; Jang *et al.,* 2001; Senthilkumar and Gunasekaran., 2004; Song and Rhee., 1994), this approach may lower industrial costs for production of levan from sucrose.

There is currently a growing interest in characterization and development of novel fructans for industrial application. However, studies of levansucrases are hampered by the poor availability of relatively stable LSs and limited characterization of the fructan product. Furthermore, the use of native fructan producers for industrial applications is not financially viable, due to low expression levels of LS genes and limited cell density achieved in bioreactors. Therefore, LS expression studies in heterologous hosts and the characterization of the resulting fructan products are ongoing. The successful heterologous expression of a LS showed that fructan production in *S. cerevisiae* is possible with limited genetic manipulation and metabolic burden. This can be used as a stepping stone for future levan production in *S. cerevisiae*. Furthermore, inulin as fructan molecule still remains to be heterologously produced in *S. cerevisiae*. Both inulin and levan fructans are molecules of economic interest. However, there is still no viable industrial fructan production platform. With the biological production of levan type fructans by *S. cerevisiae* shown to be possible under aerobic conditions, the data may be

applied toward generating such platforms. However, further optimization of levan production in *S. cerevisiae* is crucial if it is to be used as viable expression system for levan production. This could result in viable future biotechnological applications.

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