Kinetic Modelling of Wine Fermentations: Why Does Yeast Prefer Glucose to Fructose?

by

Leanie Mocke

Thesis presented in partial fulfilment of the requirements for the degree of Master of Science in Biochemistry in the Faculty of Science at Stellenbosch University

Department of Biochemistry,
University of Stellenbosch,
Private Bag X1, Matieland 7602, South Africa.

Supervisor: Prof. J.L. Snoep

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Abstract

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L. Mocke

Department of Biochemistry,
University of Stellenbosch,
Private Bag X1, Matieland 7602, South Africa.

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In the present-day competitive global market, wine industries are constantly aiming to improve the wine-making process,including the role of yeast. The most commonly used wine yeast is *Saccharomyces cerevisiae*, which is able to produce high quality wines, but problem fermentations do sometimes arise. The occurrence of stuck and sluggish fermentations pose a serious problem leading to loss of productivity and quality. Although the precise mechanism leading to stuck fermentations is unknown, they are often correlated with high fructose to glucose ratios in the wine-must. *S. cerevisiae* is a glucophylic yeast, indicating its preference for consuming glucose over fructose. Both these hexose sugars are present in unfermented wine must, mostly in equal concentrations. As fermentation progresses, glucose is consumed at a faster rate than fructose, leading to an increase in the fructose to glucose ratio. Yeast are left with the undesirable fructose at the later stages of fermentation, when the environmental stresses on the yeast can lead to stuck or sluggish fermentation. This residual fructose can lead to undesirable sweetness, as fructose is about twice as sweet as glucose. Even with the extensive research into yeast metabolism, there is as yet no definitive explanation as to why yeasts ferment glucose faster than fructose.

This study aimed to investigate the mechanism responsible for the faster consumption of glucose over fructose of a commercially used wine yeast strain *S. cerevisiae VIN 13*. The first two steps of sugar metabolism, uptake and phosphorylation, were investigated as the possible sites of discrepancy in fermentation rates. Enzyme rates and affinities for both glucose and fructose as

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substrates for the relevant enzymes were experimentally determined. These kinetic parameter values were used to improve an existing model of yeast glycolytic pathway to model wine fermentations. The feasibility of constructing and validating a kinetic model of wine fermentations were investigated, by comparing model predicted fluxes with experimentally determined fluxes.

Another aspect of this study was an investigation into the effect of hexose sugar type on fermentation profiles. Wine fermentations were done with only one hexose sugar as carbon source to determine if it has an effect on the flux through metabolism.
This work succeeded in the construction of a kinetic model that distinguished between glucose and fructose as carbon source. The glucose was consumed faster than fructose, with control lying in the hexose transport step. It was also established that fermentation profiles of fermentations with only one sugar was the same for both one sugar type fermentations. Fermentation with either glucose or fructose as the sole carbohydrate source had the same specific production and consumption rates as normal fermentations with both sugars. Construction of detailed kinetic models can aid in the metabolic and cellular engineering of novel yeast strains. By identifying the importance of hexose transport, and thus the glucophilic character of the yeast, in flux control, yeast transporters can be targeted for strain improvement. This may in turn lead to more effective fermentation practices for controlling problem fermentations, or to the development of novel strains that utilizes fructose in the same manner as glucose, and in so doing lower the risk of stuck or sluggish wine fermentation.

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Uittreksel

Kinetiese Modellering van Wyn Fermentasies: Hoekom Sal Wyngis Glukose Bo Fruktose Verkies?

("Kinetic Wine Modelling: Why Yeasts Prefer Glucose to Fructose")

L. Mocke

Departement Biochemie,
Universiteit van Stellenbosch,
Privaatsak X1, Matieland 7602, Suid Afrika.

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In die hedendaagse kompeterende wynmark is wynmakers aanhoudend besig om die wynmaak proses te verbeter en dit sluit die verbetering van wyngis in. Die mees algemeenste gebruikte wyngis is *Saccharomyces cerevisiae*, omdat dit wyn van gehalte produseer, maar probleem fermentasies kom wel voor. Die verskynsel van vasval of stadige fermentasies kan lei tot die verlies van produksie en kwaliteit. Die oorsaak van probleem fermentasies is gewoontlik veelvoudig, maar die verhouding van glukose tot fruktose in die wyn-mos kan ongunstig raak om fermentasies te onderhou. *S. cerevisiae* is 'n glukofiliese gis, wat sy voorkeur om glukose bo fruktose te gebruik beskryf. Albei hierdie heksose suikers is teenwoordig in ongefermenteerde wyn-mos, meestal in gelyke hoeveelhede. Soos fermentasies vorder word glukose vinniger verbruik as fruktose wat lei tot 'n toename in die fruktose tot glukose verhouding. Die gis moet dus die fruktose in die later stadium van fermentasie gebruik wanneer die omgewings druk op die gis kan lei tot probleem fermentasies. Die oorblywende fruktose kan lei tot ongewenste soetheid aangesien fruktose twee keer soeter is as glukose. Selfs met die ekstensiewe navorsing met betrekking tot gis metabolisme is daar nog nie 'n verduideliking hoekom gis glukose vinniger as fruktose gebruik nie.

Hierdie studie het beoog om die meganisme wat lei tot die vinniger verbruik van glukose oor fruktose te ondersoek vir 'n kommersieël gebruikte gis *S. cerevisiae* VIN 13. Die eerste twee stappe van suiker metabolisme, suiker opname en fosforilasie, was ondersoek as die moontlike punt van die verskil in fermentasie tempo. Ensiem snelhede en affiniteite vir beide glukose en fruktose

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as substrate vir die ensieme van belang was eksperimenteel bepaal. Hierdie waardes is gebruik om 'n bestaande model van gis glikolise aan te pas vir wyn fermentasies. Die uitvoerbaarheid van saamstel en valideer van 'n kinetiese model van wyn fermentasies was ondersoek, deur model voorspelde fluksie waardes met eksperimentele fluksie waardes te vergelyk.

'n Ander aspek van die studie was die ondersoek van die effek van heksose suiker tipe op fermentasie profiel. Wyn fermentasies is gedoen met slegs een heksose suiker as koolstof bron om te bepaal of dit 'n invloed het op die fluksie deur metabolisme.

Hierdie werk het daarin geslaag om 'n kinetiese model saamtestel wat onderskei tussen glukose en fruktose as koolstof bron. Die glukose is vinniger verbruik as fruktose, met beheer gesetel in die heksose opname stap. Dit was ook vasgestel dat fermentasie profiele van fermentasies met slegs een suiker nie verskil het vir fermentasies met slegs fruktose of glukose. Fermentasies met slegs een suiker het dieselfde spesifieke produksie en konsumpsie tempo gehad as die normale fermentasie met albei suikers. Die konstruksie van 'n gedetailleerde kinetiese model kan gebruik word in die metaboliese en sellulêre ontwikkeling van nuwe gisstamme. Deur die ontdekking van die belangrikheid van heksose opname in fluksie beheer, wat lei tot die glukofiliese karakter van gis, kan gis opname geteiken word vir gis ontwikkeling. Dit mag om die beurt lei tot meer effektiewe fermentasie praktyk in die beheer van probleem fermentasies, of die ontwikkeling van nuwe stamme wat fruktose in dieselfde manier as glukose benut, en sodoende die risiko van vasval of stadige wyn fermentasies verlaag.

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Dedications

This thesis is dedicated to my wonderful family for their continuous support, love and encouragement. Parents Hendrik and Joleen, brother Nicolaas, and my husband, Pieter.

Hierdie tesis is opgedra aan my wonderlike familie vir hul volgehoue ondersteuning, liefde en aanmoediging. Ouers Hendrik en Joleen,broer Nicolaas, en my man, Pieter.

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Abbreviations

2PGA	2-phosphoglycerate
3PGA	3-phosphoglycerate
ACALD	Acetaldehyde
ADH	Alcohol dehydrogenase (E.C. 1.1.1.1)
ADP	adenosine diphosphate
AK	Adenosine kinase (E.C. 2.7.1.20)
ALD	Fructose bisphosphate aldolase (E.C. 4.1.2.13)
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
DHAP	Dihydroxyacetone phosphate
ENO	Enolase (E.C. 4.2.1.11)
F16BP	Fructose 1,6-bisphosphate
F6P	Fructose 6-phosphate
FRU	Fructose
G3PDH	Glyceraldehyde-3-phosphate dehydrogenase (E.C. 1.2.1.12)
G6P	Glucose 6-phosphate
G6PDH	Glucose-6-phosphate dehydrogenase (E.C. 5.3.1.9)
GAP	Glyceraldehyde phosphate
GFR	Glucose-fructose ratio
GLC	Glucose
GLK	Glucokinase (E.C. 2.7.1.2)
HK	Hexokinase (E.C. 2.7.1.1)
HPLC	High performance liquid chromatography
HXK	Hexokinase (E.C. 2.7.1.1)
HXT	Hexose transporter
NAD+	Oxidised nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
OD	Optical density
PDC	Pyruvate dehydrogenase complex (E.C. 1.2.4.1)
PEP	Phosphoenolpyruvate
PFK	Phosphofructokinase (E.C. 2.7.1.11)
PMSF	Phenylmethulsulfonyl fluoride
PGI	Phosphoglucose isomerase (E.C. 5.3.1.9)

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PGK	Phosphoglycerate kinase (E.C. 2.7.2.3)
PGM	Phosphoglycerate mutase (E.C. 5.4.2.1)
PYK	Pyruvate kinase (E.C. 2.7.1.40)
PYR	Pyruvate
RPM	Revolutions per minute
TDH	Glyceraldehyde-3-phosphate dehydrogenase (E.C. 1.2.1.9)
TPI	Triosphosphate isomerase (E.C. 5.3.1.1)

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Chapter 1

INTRODUCTION

1.1 General Introduction

At the start of fermentation, unfermented grape must contains approximately equal amounts of the two hexose sugars, glucose and fructose [36]. While both are fermented by wine yeasts to ethanol and carbon dioxide and other metabolites, *Saccharomyces cerevisiae* consumes glucose faster than fructose, being a glucophilic yeast [35]. Although fructose is used along with glucose, the latter is consumed faster, giving rise to the discrepancy observed between the amount of glucose and fructose consumed (G/F discrepancy). Therefore, fermented must usually contains more fructose than glucose as residual sugar. Fructose is the sweetest hexose sugar, approximately twice as sweet as glucose, and therefore its effect on the final sweetness of wine is much more pronounced [61, 27]. Residual fructose is the main cause of undesirable sweetness in dry wines, with high residual fructose also yielding lower ethanol concentrations and increasing the risks of microbial spoilage of the wine. Therefore, wine yeast with a higher capability to ferment fructose are of interest to the wine industry.

During the first phase of fermentation, yeast cells are actively dividing, and the G/F discrepancy gives rise to an increasing difference in residual glucose and fructose [15]. As a consequence, in the final stages of fermentation, when nutrients are depleted and ethanol levels are high, the yeast must ferment the non-preferred fructose [82, 10]. Stuck or sluggish fermentation occurring under these conditions are often associated with a high fructose to glucose ratio [17, 41]. It is thought that the low fructose utilization capacity of *S. cerevisiae* contributes to the low fermentation rate in these situations [41, 83, 86]. Problem fermentations signify a significant economic loss to the global wine industry through prolonged duration of fermentations and lower quality wines [17].

Despite the importance of fructose fermentation to the wine industry, few

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studies have been focused on this subject [15]. Glycolysis is the biochemical pathway by which glucose and fructose are intracellularly transformed into pyruvate, and is the main pathway yeasts utilize for sugar catabolism. [42]. Differences in glucose and fructose fermentation rates may be situated either in the differential transport of these sugars across the plasma membrane or the differences in the hexose phosphorylation occuring inside the cell [43, 16]. After the transport and phosphorylation steps, both glucose (as glucose-6-phosphate) and fructose (as fructose-6-phosphate) are metabolised via the same pathway. Both the hexose transporters and kinases have different glucose/fructose affinities and preferences. To the best of our knowledge, the molecular basis for the difference in sugar utilization by *S. cerevisiae* in general is however not known [16].

In this study, an attempt was made to explain the G/F discrepancy with a mathematical model incorporating simple enzyme kinetics. The strategy was based on an existing model of yeast glycolysis by Teusink *et al.* [90]. The model had been adapted for batch fermentations, and kinetic parameters were determined experimentally. Fructose transport and phosphorylation needed to the added to the model. The metabolic pathway of fructose differs only slightly from that of glucose. Both use the hexose transporter family to transport sugars into the cell. After transport, glucose is phosphorylated to glucose-6-phosphate and then converted to fructose-6-phospate by phosphogluco-isomerase, whereas fructose is directly phosphorylated to fructose-6-phosphate. Both are phosphorylated by hexokinase 1 and 2, and glucose additionally by glucokinase [6]. To validate the model, model predicted fluxes need to be compared to real batch fermentation fluxes to assess the effectiveness of modelling with measured enzyme kinetics.

This work also investigate the effect of sugar type on fermentation profiles. Does the sugar, glucose or fructose, influence metabolic flux or growth if the wine-must contains only one of the sugar hexoses?

A better understanding of the mechanism of glucose and fructose discrepancy might help solve the problems associated with high residual fructose levels in finished wines. Selecting for yeast with high fructose consumption capability is very important for the wine industry to solve problems associated with stuck or sluggish fermentations.

1.2 Project Outline

The first and principle aim of this work was to build a kinetic model of wine fermentations of commercially used wine yeast, *Saccharomyces cerevisiae* VIN13. The approach would be very specific, directed on the first two steps of glycol-

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ysis, hexose transport and phosphorylation. To investigate the difference in consumption profiles of glucose and fructose, analytical techniques were combined with computer assisted kinetic modelling. The power of this approach is in its ability to determine the enzymatic steps within glycolysis responsible for the faster consumption of one substrate over the other. The model could potentially explain the difference in consumption profiles on the basis of simple kinetic constants. The model could in turn be used to aid in the construction of models used for the screening of yeasts with desired characteristics to better fructose consumption.

The second aim was to investigate the fermentation profiles of batch fermentations with only one sugar type as carbon source. The profiles of fermentation with 50% glucose and 50% fructose would be compared to fermentations with either 100% glucose or 100% fructose as sole carbon source.

Briefly, the study was comprised of the following tasks:

- Emulate wine fermentations with synthetic wine-must and a commercially used wine yeast in a bioreactor;
- Run batch wine fermentations with either glucose or fructose as sole carbon source;
- Monitor substrate and product formation during fermentations;
- Kinetically characterize the hexose transport and phosphorylation steps of glycolysis with different substrates in enzyme assays;
- Construct a mathematical model to model wine fermentation, distinguishing between glucose and fructose as substrates;
- Validate the model in its capability to predict glucose and fructose consumption during wine fermentations.

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Chapter 2

LITERATURE REVIEW

2.1 Introduction

Winemaking has come a long way since its humble beginnings more than 7 000 years ago. Today the global wine industry is a highly competitive market, representing a billion dollar industry. Technological innovation has insured the rapid advancement on many of the winemaking fronts the past few decades, but winemaking is not without problems. This review will give a brief overview of the change in the focus area of wine research, the problem of stuck and sluggish fermentation faced by the wine industry, and the wine yeast *Saccharomyces cerevisiae*. The use of a good wine yeast strain is of cardinal importance to the success of winemaking. With the focus of this thesis on the difference in hexose metabolism of glucose and fructose by wine yeast the transport and phosphorylation step of metabolism are also reviewed. It is the aim of this literature review to give an encompassing overview of the wine yeast's importance during enological fermentations.

2.2 Winemaking: Old Technology, New Science

Yeast is invariably connected to the ancient art of winemaking. The history of winemaking dates back seven millennia, with alcoholic fermentation possibly the oldest form of a biotechnological application of microorganisms by humans, albeit unwittingly [84, 78]. It was only in 1863 that Louis Pasteur revealed the role of yeast during wine fermentation, proving that it was the primary catalyst [6]. He based his work on Antonie van Leeuwenhoek's first description of individual yeast cells published in 1680 [6]. Today wine is enjoyed all over the world, playing a major role in the economies of many countries [72]. Competition within the global market has had the effect of increasing diversity and innovation within the wine industry, with the most successful wines those meeting the prevailing definition of quality [72, 23].

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A simple definition of fermentation is the chemical transformation of food-products by microorganisms [10]. In turn, alcoholic fermentation is the conversion of sugar into alcohol and CO_2.

$$C_6H_{12}O_6 \rightarrow 2\,CH_3CH_2OH + 2\,CO_2$$

This process relies almost exclusively on yeast, with the most commonly encountered species *Saccharomyces cerevisiae*, known as the baker's, brewer's or wine yeast. With the knowledge that yeast was responsible for the fermentation process, winemakers could now control the process of winemaking. Yeasts with improved characteristics could be selected for alcoholic fermentation. By 1890 the concept of inoculating wine fermentations with pure yeast cultures, displaying desired characteristics, was introduced by Müller-Thurgau, and the quality of winemaking vastly improved [72]. The use of pure yeast inocula insured a more rapid and reliable fermentation with more consistent flavour and better predictability of quality [72]. Fermentations are largely inoculated with single-strain pure cultures added to the grape must soon after crushing [30].

During the past 25 years major advances have been made in the understanding of the biochemistry, physiology, ecology and molecular biology of the yeasts involved in wine making and how they impact on wine chemistry and sensory properties adding to the appeal of the final product [37]. The process of developing new strains has the main goal of achieving a better than 98% conversion of sugar into alcohol and carbon dioxide at a controlled rate with no development of off-flavours [45]. *S. cerevisiae* has been at the forefront of scientific research for decades for being a model organism for studies in genetics, biochemistry and cell biology [26, 30]. Not only is it of scientific value, but it has tremendous economic importance in the food and beverage industries.

Up to know yeast research has mostly been following a reductionist approach, deconstructing complex systems into smaller pathways pliable to study [26]. However, technological advances have given way to a "whole-genome" era as opposed to a single-gene, reductionist study (Figure 2.1) [26]. Out of the combination of whole-genome sources and computational modelling, a new discipline of systems biology is emerging, characterized by modelling cellular functions in such a way that realistic predictions of how the the cell will function can be made under specific conditions or perturbations [26]. Being able to have a systems-level understanding of yeast growth and metabolism has great potential in an industrial context [26]. Computational models of genomic and metabolic systems are already available for *S. cerevisiae*, with the regulation of glycolysis having been modelled by Teusink *et al.* [90] [34].

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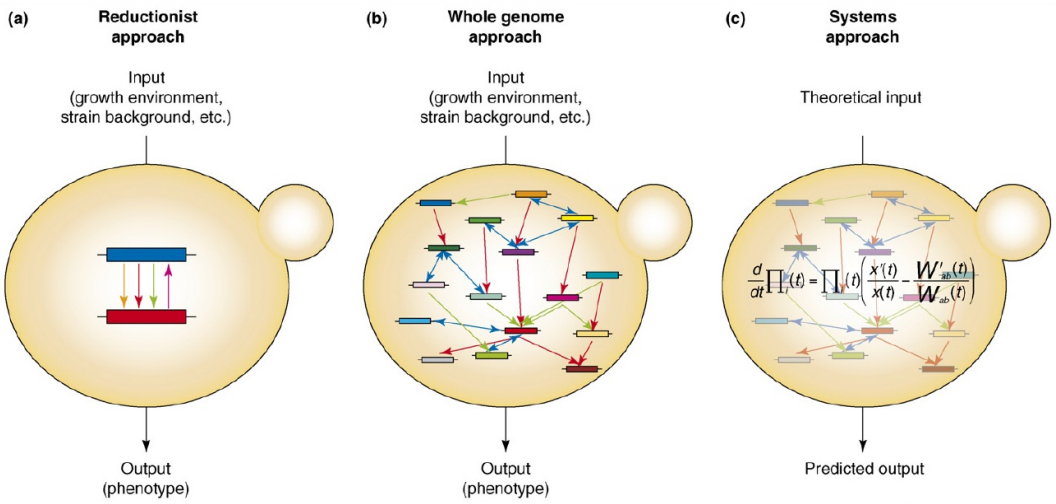

Figure 2.1: The transition from classical genetics to systems biology. (a) A reductionist approach, relying on thorough investigation of the interactions between small numbers of components (genes, proteins etc.;presented by boxes). (b) Whole genome (-omic) approaches identifies interactions (coloured arrows) for all components (boxes) simultaneously. (c) Systems biology, combining reductionist and whole genome studies, and using this with mathematical modelling such that the theoretical behavior of the system can be investigated computationally [26].

Winemaking in particular could benefit tremendously from the applications that systems biology research offer, due to the impact of yeast on wine quality and production [26]. Being able to predict what effect specific mutations will have through the use of computational models of metabolic pathways on wine production can give rise to an array of different wines from the same grapes with different strains of yeast [26].

Wine yeasts genetic make-up is far better understood than those of the grapevine [73]. Wine yeasts are predominantly diploid or aneuploid, occasionally polyploid, with a relatively small genome and a large number or chromosomes. They also have little repetitive DNA and few introns. The haploid strains containt 12-13 megabases (mb) of nuclear DNA on 16 linear chromosomes, with each chromosome 200-2200 kilobases (kb) long [72, 73]. Work for this thesis was done on a commercially used wine yeast, *S. cerevisiae* VIN13. The *S. cerevisiae* VIN 13 strain was engineered by Swiegers [89] to constitutively express a carbon-sulphur lyase gene, tnaA, from *Escherichia coli*, exhibiting the release of volatile thiols from Sauvignon Blanc grape juice. *S. cerevisiae* VIN 13 has also had its genome sequenced by The Australian Wine Research Institute [30].

Goals of wine scientists are to better understand the complex inner workings of wine yeast to be able to develop more informed and inovative ways of developing improved strains. With robust mathematical models describing cellular functions it will be possible to design and trial the performance of the new yeast

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strain *in silico*, eliminating the need of costly and time-consuming fermentations [29, 30]. The complexity of biological systems can make the development of novel strains a very challenging endeavour. With the use of systems biology to understand yeast metabolism, there is the possibility of more accurately modelling metabolic processes for better-informed manipulations to ultimately achieve targeted and predictable outcomes.

2.3 Stuck and Sluggish Fermentations

A central goal during winemaking is the achievement of complete alcoholic fermentation. This is however not always the outcome and the occurrence of premature arrest of alcoholic fermentation is one of the most challenging problems faced by the wine industry. Problem fermentations cause economic losses through loss of fermentation space, increased duration of fermentations and spoilage of wines. Causes of stuck and sluggish fermentations are numerous and sometimes difficult to pinpoint and rectify. Numerous factors can cause problem fermentations, such as high initial sugar content, vitamin or nitrogen deficiencies (nutrient limitations), excessive temperatures (high or low), enological practices, anaerobic conditions, high ethanol content, occurrence of spoilage micro-organisms or toxic compunds (fungicides or ethanol), excessive clarification of the must, presence or toxic fatty acids and high concentrations of volatile acidity have all been linked to stuck and sluggish fermentations [2, 17, 18]. It is therefore very difficult to pinpoint a problem, due to the multiple factors and the possibility of interactions between these factors [2].

Wines with high residual sugar content are susceptible to microbial spoilage and are unacceptable for the market due to the sweetness of the wine. [17] Excessive residual fructose in particular can compromise the quality of the wine, as fructose is about twice as sweet as glucose and adds to undesired sweetness [15].

Stuck, or incomplete, enological fermentations are those that, at the end of alcoholic fermentation, leave a higher than desired residual sugar content. A complete or "dry" fermentation is only reached when sugar levels are lower than 0.4% (4 g/L), with typical sugar concentrations below 0.2%. Slow and sluggish fermentations need a longer fermentation time to reach dryness, with normal fermentation reaching dryness within 7 to 10 days, while sluggish fermentations take considerably longer, even months to complete [17]. Slow or sluggish fermentation is thus characterized by a low fermentation rate throughout fermentation and stuck fermentation in turn is the premature completion of fermentation, with higher than desired residual sugar left in the wine must [24].

Often accompanied by a high fructose to glucose ratio, it is not clear whether

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the yeasts glucophilic character can lead to stuck fermentation or if it simply accompanies it. It has been recorded that very low glucose-fructose ratio (GFR) can lead to sluggish- or stuck fermentations [41]. When the ratio falls below 0.1, with fructose at least ten times higher than glucose, stuck or sluggish fermentation can occur [41]. Problem fermentations can be prohibited with the addition of glucose to better the GFR, but the addition of glucose is under strict legal limitations [41].

The rate of fermentations is a function of the total viable biomass as well as the rate of sugar utilization by the individual cell [66]. When growth is limited by factors in the grape juice and cell death occurs, sugar utilization decreases along with a decrease of viable biomass, which can result in stuck fermentation [17]. Sluggish fermentation can also arise when the rate of fermentation per cell decreases with viable biomass still high [17]. It has been established that a decrease in sugar consumption is correlated with a decrease in sugar uptake capacity [27, 59, 64, 81, 82], while the glycolytic pathway remains functional and intact [17].

Free intracellular glucose is toxic to the yeast cell and so the rate of sugar uptake must be carefully coordinated with the rates of sugar utilization and other metabolic activities, to prevent a build-up occurring if flux through glycolysis downstream were reduced[17, 19, 27, 91]. Loss of transport activity in response to environmental and physiological stress is a vital survival mechanisms [17, 57, 64, 81, 82]. The reversal of this loss of transport is however difficult for the cell, which is why stuck and sluggish fermentations are so difficult to rectify[18].

As mentioned, glucose and fructose consumption are reduced in response to various stress conditions, impacting transport expression and activity, with the rate of sugar entry into the yeast cell is balanced with the rate of catabolism [19, 11]. Examples of such stress conditions are: low pH, lack of oxygen, lack of adequate agitation, temperature extremes, presence of toxic substances, presence of other microorganisms and imbalance of cations.

Fermentation difficulties remain a major problem, adding to production costs. Alcoholic fermentations that cease prematurely or proceed too slowly lead to financial losses due to the inefficient utilisation of fermentor space and wine spoilage due to the low rate or protective carbon dioxide evolution as well as high residual sugar content [72]. General targets to improve fermentation performance include increased resilience and stress resistance, improved nutrient uptake and assimilation, enhanced resistance to ethanol and inhibitory metabolites, resistance to sulfite and antimicrobial compounds and tolerance to environmental stress factors [72, 73].

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2.4 Hexose Metabolism during Alcoholic Fermentation

S. cerevisiae is an industrially important yeast, as it is inclined to perform alcoholic fermentation even under aerobic conditions, known as the Crabtree effect. Although alcoholic fermentation yields less energy than respiration it proceeds at higher rates rapidly producing ethanol giving the ethanol tolerant wine yeast a competitive advantage over ethanol-sensitive organisms. During alcoholic fermentation, hexose sugars in grape must is metabolized to pyruvate via the glycolytic pathway, which is then decarboxylated to acetaldehyde and finally reduced to ethanol. The theoretical conversion during glycolysis would yield two molecules of ethanol and carbon dioxide for one molecule of glucose or fructose. However, that would only be in the absence of any growth and production of other metabolites, with only about 95% sugar converted into ethanol and carbon dioxide in real fermentation. 1% is converted into cellular material and 4% into other secondary products[72, 27].

The most simplistic view of alcoholic fermentation is the anaerobic transformation of hexose sugars in grape must to ethanol and carbon dioxide by yeast and some bacteria (Figure 2.2) [97]. To begin this process, the first essential step of sugar breakdown is the uptake of the sugars into the yeast cell. *S. cerevisiae* uses several hexose transporters, which transport glucose and fructose amongst other sugars, by facilitated diffusion. The two main sugars in grape juice, or grape must, are glucose and fructose. Sucrose is hydrolyzed by invertase in the grape berries, synthesized during photosynthesis in the vine leaves, and yield one glucose and one fructose molecule [44]. They are therefore present in about equimolar concentrations. Of the total carbohydrates in the *Vitis vinifera* berry, 99% is comprised of glucose and fructose [3]. The ratio of glucose to fructose is however not always 1:1, changing during fruit maturity [87]. In overripe grapes, fructose constitutes the major sugar. In unripe berries glucose predominates, while when berries reach maturity (ripe stage) the glucose/fructose ratio is about 1[47, 48, 49, 51, 50].

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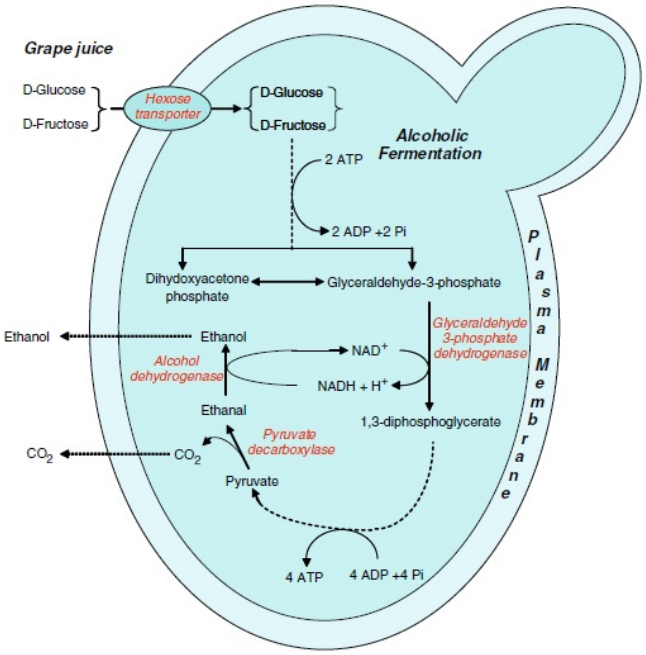

Figure 2.2: Alcoholic fermentation [97].

Glycolysis is the main pathway used for sugar catabolism by yeasts [42]. With sugar concentrations higher than 1%, catabolism is solely facilitated by glycolysis, not entering the tricarboxylic acid cycle [5]. Glycolysis consists of 11 chemical reactions in sequence for the breaking down of hexoses to pyruvate to release energy in the form of ATP [8] (see Figure 2.3).

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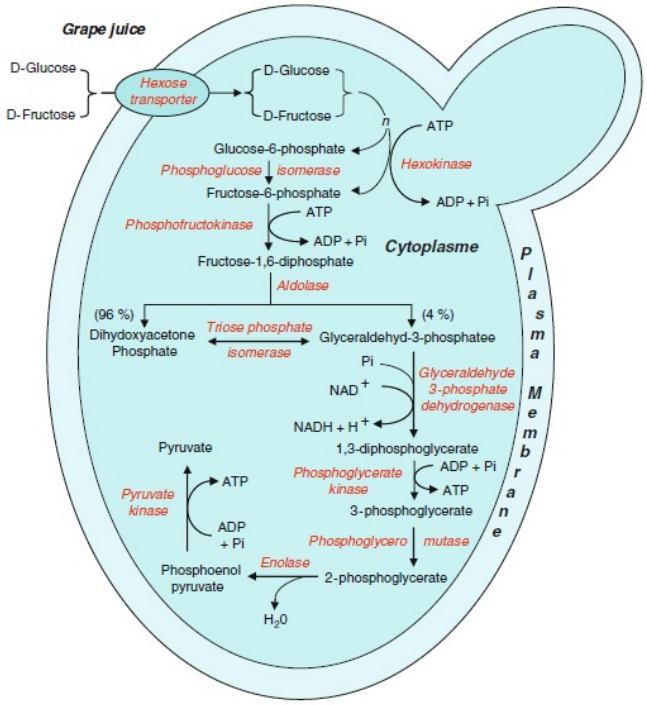

Figure 2.3: Biochemical mechanism of glycolysis [97].

Firstly, sugars are transported inside the cell by facilitated diffusion, from where it enters glycolysis [59]. Efficient utilization of sugars is dependent on functional alleles of the transporters and key glycolytic enzymes, namely hexokinase (HXK) and glucokinase (GLK), phosphoglucose isomerase (PGI), phosphofructokinase (PFK), aldolase (FBA), triosephosphate isomerase (TPI), glyceraldehyde-3-phosphate dehydrogenase (TDH), phosphoglycerate kinase (PGK), phosphoglycerate mutase (PGM), enolase (ENO) and pyruvate kinase (PYK) [72].

Wine yeast is capable of fermenting various sugars to ethanol and carbon dioxide under anaerobic or aerobic conditions [5]. They are facultative anaerobic microorganisms as they possess the genetic equipment to metabolize sugars aerobically or anaerobically [27]. Yeasts can therefore consume sugars through respiration and fermentation, but at sugar concentrations higher than approximately 2 g/l, *S. cerevisiae* channels the sugars into alcoholic fermentation [58] (see Figure 2.2). This effect is known as the Crabtree effect. After glycolysis, pyruvate is converted to ethanol to regenerate the NAD^+ consumed during glycolysis and produces a net gain of two ATP molecules [9]. Enzymes responsible for this conversion include pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH)[72].

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The production of ethanol is not the only pathway to regenerate NAD^+ although it is the most important. The alternative pathway is glyceropyruvic fermentation, generating glycerol as final product [74]. Although used to compensate for the NAD^+ deficit in the cell, it is also produced by yeasts as a protector against high osmotic pressures [74]. After water and ethanol, glycerol is the third major component of dry wines, ranging in concentrations of between 6 and 10 g/l and may improve wine quality as it extends sweet and mouthfeel sensations [97].

Unsuccessful attempts have been made to increase glycolytic flux in yeast by over-expression of individual and combinations of glycolytic genes [85]. Overproduction of the enzymes had no effect on the rate of ethanol formation, indicating that the control site for glycolytic flux under anaerobic conditions is situated in the uptake step, with the remaining steps not appearing to be rate limiting[27]. Therefore, the rate of alcohol production is primarily limited by the rate of hexose sugar uptake, with the loss of transport towards the end of fermentation resulting in reduced ethanol yields [93]. Evidently the glycolytic pathway is tightly controlled, illustrating that sugar utilization is already highly optimized with little room for improvement.

Glucose and fructose are the preferred sugars of *S. cerevisiae*. When glucose is present, a wide range of genes involved in utilizing alternative carbon sources are repressed, but fructose utilization is not repressed [31]. Glucose and fructose can be consumed at the same time by yeast, although glucose utilization is faster than fructose utilization. *S. cerevisiae* is a glucophilic yeast, displaying a preference for utilizing glucose. Even though fructose is used along with glucose, the latter is depleted first, giving rise to the discrepancy between the amounts of sugars consumed during fermentation (Figure 2.4). This preference results in a difference in consumption profiles [35]. Consequently the residual sugar left after the completion of fermentation contains more fructose than glucose. Fructose is approximately twice as sweet as glucose, with residual fructose having a stronger effect on the final sweetness of wine, and wine makers often have to content with high residual fructose levels (>2 g/l), accounting for undesirable sweetness in finished dry wine [15, 61]. Intented dry wines have a residual sugar level below 4 g/l. Glucose and fructose are simple reducing sugars, both mono-saccharides with the empirical formula $C_6H_{12}O_6$, but with different structures. Grape musts total hexose sugar concentration can range between 160 and 300 g/L[36].

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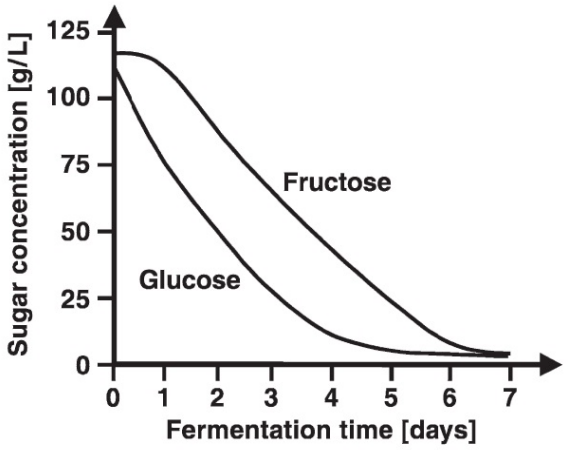

Figure 2.4: Typical kinetics of sugar degradation during fermentations in must [79].

The traditional production of wine is by natural fermentation of grape juice, with yeasts strains originating from the grapes and winery environment (natural flora). These species included the genera *Kloeckera*, *Hansensiaspora*, *Candida*, *Pichia*, and sometimes *Hansenula*, growing during the early stages of fermentation but eventually dying off, leaving *S. cerevisiae* to dominate the rest of fermentation [13, 38, 54]. However, the desired flora may not be established during natural fermentations, so fermentations are inoculated with selected yeast cultures to ensure a more rapid and predictable fermentation with more consistent wine quality. Inoculating with a single strain of *S. cerevisiae* will dominate the fermentation, out-competing unwanted natural yeast species [13].Other members of the *Saccharomyces* group are also used in winemaking, but *S. cerevisiae* is widely preferred for starting wine fermentations, fittingly known as the wine yeast. The fermentation profile of different starter strains has led to significant improvements in the control of fermentation and quality. Nowadays it is common practice to inoculate grape juice with a specific active, dried yeast starter culture, aiding in making a predetermined style of wine [73].

Yeast development during alcoholic fermentation exhibits different phases. Yeasts metabolize sugars and nutrient present in grape must to obtain energy for growth [27]. During the first few hours the cells have to adapt to the new environment and there is no increase in yeasts population, known as the latency or lag phase. In the second phase, the exponential growth phase or log phase, the yeasts have adapted to the environmental conditions and begin to grow. This phase can be influenced by temperature, ammonia, amino acids and other nutrients as well as oxygen [68, 55, 80]. The yeast populationg eventually reach stationary phase. When the decline phase begin the cells have started to die because of a lack of nutrients and the ethanol and other substances produced during alcoholic fermentation are toxic [56]. The

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success of an alcoholic fermentation rests on the viable yeast population being maintained up to the point where all the fermentable sugars are consumed [17].

2.4.1 Hexose Transport

The obligate first step in sugar metabolism is sugar transport across the plasma membrane into the yeast cell. *S. cerevisiae* is capable of accomplishing high rates of hexose transport, with the complexity of the transport regulation reflected in the large number of sugar transport genes in the genome [53]. Transporter genes comprise a family encoding 20 different hexose transporter-related proteins (Hxtp), thought to be involved in transport and regulation [53]. The need for such a vast number of similar hexose transporter proteins can be explained by the broad range of sugar concentrations the yeast is exposed to under natural conditions. To adapt to these varying environments requires the transporters to be differentially regulated, with the proteins having specific individual characteristics and transport kinetics [76]. During fermentation of fruit juices dramatic changes are seen in the physicochemical environment, and to sustain growth yeasts have to adapt to these changes. Sugar concentrations may decline from 1 M to 10^{-5} and the overall composition of the medium changes, and the sugar transport activity of the cell that mediate sugar transport need to be responsive to these changes [53].

The hexose transporters transport glucose, fructose and mannose by passive, facilitated diffusion along the sugar concentration gradient. Two uptake mechanisms have been proposed for yeast: high-affinity and low affinity-uptake, operating under low and high external sugar concentration respectively [20, 21]. These are two kinetically distinct systems, with the high-affinity system having a K_m of about 1 mM for glucose and 6 mM for fructose, and the other constitutive low-affinity system a K_m of about 20 mM and 50 mM for the two sugars respectively [20]. The existence of the low-affinity component has been questioned by some. It has been suggested that the low-affinity transport is nothing more than diffusion of the sugar through the plasma membrane or uptake by a more or less unspecific pore [39, 40]. The affinity of the transport system is seemingly always higher for glucose than for fructose, with the maximum velocity of transport of fructose generally higher than that of glucose [17].

The multigene family of transporters of *S. cerevisiae* are called the hexose transporter (HXT) genes [19, 25, 53, 52]. The HXT family is comprised of 18 members (HXT1 to HXT17 and GAL2) with high identity in coding sequence (65% - 99%) sharing common functional motifs and secondary structure with the same structural features of 12 membrane spanning domains [19, 25, 53, 59]. There are also two glucose sensors Snf3p and Rgt2p that are closely related to the transporters. It has been shown that Hxt1-Hxt4, Hxt6 and Hxt7 are

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the major hexose transporters for transporting glucose, fructose and mannose [77, 76]. Hxt6 and Hxt7 are high affinity carriers (K_m 1-2 mM for glucose), Hxt2 and Hxt4 display intermediate affinity (K_m for glucose 10 mM) and Hxt1 and Hxt3 are low-affinity carriers (K_m values for glucose 100 mM and 30-60 mM, respectively) [76, 63]. The key regulator of HXT gene expression is glucose itself [19, 98, 95, 96]. Genes are regulated by both glucose induction and glucose repression. Transport genes regulated by glucose induction are not expressed in the absence of glucose whereas repressed genes are not expressed at high glucose levels, and becoming derepressed upon glucose depletion.

Expression of high-affinity hexose transporter proteins is dependent on the presence of hexokinases and an active SNF3 gene and is repressible by glucose. The low-affinity uptake is a constitutive, kinase-independent facilitated diffusion process[21, 22, 60, 75]. In media with high concentrations of sugar, cells only exhibit low-affinity uptake[65].

During alcoholic fermentation the most physiologically relevant hexose transporters (Hxt1-Hxt4, and Hxt6/7), accepting both glucose and fructose as substrates, have distinct expression patterns [76, 71]. During alcoholic fermentation yeast faces an ever changing environment, with sugar concentrations dropping and ethanol content increasing. Throughout the fermentation yeast adapts its hexose carrier expression to ensure optimal hexose transport with respect to the environmental and physiological conditions [62]. It is the low-affinity carriers Hxt1 and Hxt3 that play a predominant role during fermentation. Hxt1 is expressed at the beginning of fermentation to ensure initial sugar uptake during the growth phase, whereas Hxt3 is expressed throughout fermentation, with maximal expression at the point of growth arrest, decreasing during stationary phase. The high affinity carriers Hxt6 and Hxt7 are expressed at the end of alcoholic fermentation with Hxt2 involved in growth initiation.

Sugar uptake and assimilation affects fermentation performance of starter cultures. Sugar uptake appears to limit complete sugar utilisation during vinification and is influenced by factors such as ethanol concentration and nitrogen availability [73]. It is of vital importance that the grape sugars are efficiently utilised by *S.cerevisiae* with a rapid rate of glycolytic flux relying on functional alleles of the glycolytic enzymes [72]. Since wine yeasts are glucophilic it may be possible that overexpressing transporters together with fructose-specific transporters (from fructophilic yeasts such as *S. pasteurianus* and *Zygosaccharomyces bailii*) will help alleviate the occurrence of stuck fermentation [73].

In a study done by Guilaume *et al.* [43] it was found that a mutated HXT3 allele enhanced fructose utilization in *S. cerevisiae*. Expression of the allele

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alone increased fructose utilization during fermentation, and the glycolytic flux increased with the overexpression of the mutant gene. This work demonstrated that it is possible to alter the pattern of fructose utilization during fermentation and the importance of the hexose transporter in determining the glucose/fructose utilization ratio.

2.4.2 Hexose Phosphorylation

After transport of glucose and fructose into the cell they are rapidly phosphorylated by the hexose kinase family of enzymes into glucose-6-phosphate and fructose-6-phosphate respectively[42]. This is the first irreversible step of glycolysis [32]. Glycolysis is a sequence of 11 chemical reactions breaking down high energy hexoses for the release of Gibbs free energy in the form of ATP [7]. This first reaction uses ATP and is important in keeping the intracellular free sugar concentrations low (<2.5mM), favouring continuous transport of sugars into the cell [79]. The family of hexokinases in *S. cerevisiae* are glucokinase (Glk1), hexokinase 1 (Hxk1) and hexokinase 2 (Hxk2) [79]. Glk1 can phosphorylate glucose, wherease the two isoenzymes Hxk1 and Hxk2 are able to phosphorylate glucose as well as fructose [32]. Hxk1 and Hxk2 share a high degree of homology (77% identical amino acids) with glucokinase being less than 40% identical to either. The two hexokinases differ in their glucose/fructose preference despite their high degree of sequence similarity. Hxk1 has a higher V_{max} with fructose over glucose (threefold), while Hxt2 has a slightly higher V_{max} for glucose than fructose [14, 33]. The affinity of Hxk1 for glucose (K_m = 0.12 mM) is higher than for fructose (K_m = 1.5 mM), with Hxk2 also having a higher affinity for glucose (K_m = 0.25 mM) than fructose (K_m = 1.5 mM) [14, 33].

During the first phase of fermentation, when cells are growing, HXK2 expression is the highest. In the second phase, where cell growth is much lower, HXK2 expression drops and HXK1 and GLK1 expression increases [92].

The conversion to glucose-6-phosphate is followed by the conversion to fructose-6-phosphate by phosphoglycoisomerase (PGI). All subsequent reaction steps are identical for glucose or fructose metabolism. Therefore there are only two steps in the fermentation pathway, namely transport and phosphorylation, in which differences could explain the glucose/fructose consumption discrepancy.

2.5 Modelling Yeast Metabolism

The glycolytic pathway is one of the best understood metabolic pathways in biochemistry. It has been extensively studied, and its individual steps well described and characterized. However, when viewed as an integrated pathway

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of multiple steps, our understanding leaves much to be desired [46]. In order to gain a better understanding of the glycolytic biochemical pathway in general, several models of glycolysis in *S. cerevisiae* have been constructed [46]. Most of these models use fitting of experimental data to model glycolysis, thereby describing the metabolic system in relation to the conditions under which the data was collected [90]. This puts a severe limitation on these models as they are only able to describe the system under the measured conditions.

Insight into glycolysis as a whole can be achieved through modelling by describing a complete pathway quantitatively. Such a model was constructed by Teusink *et al.* [90]. It is significantly different to other models as it uses *in vitro* measured kinetic data to describe glycolysis and was not fitted to the observed behaviour of the pathway. The aim of the Teusink model was to test if an *in vivo* system could be described in terms of the *in vitro* determined kinetics of its individual components. Most modelling papers aim to describe metabolic behaviour without reference to the molecular mechanisms. Simplified kinetic equations are used and rate constants fitted until the model reproduces the observed behaviour of the pathway. For the Teusink model, enzyme kinetics were experimentally determined from the same yeast source under the same conditions while refraining from adjusting parameters to obtain best fit.

However, this approach has its own set of disputes regarding the use of kinetic properties determined *in vitro* to model the behaviour of the living cell. The conditions in the living cell may be very different to conditions in a test tube [69]. As for regulation, the activity of enzymes controlled by metabolites produced elsewhere in the cell can be overlooked, and enzymes usually found in defined compartments may be subcompartmented due to binding to other structures such as membranes, cytoskeleton or other enzymes [69]. The concentration of enzymes is also much higher in a living cell than in the test tube experiment. Furthermore, all kinetic data to be used must be obtained under the same conditions.

Mathematical modelling of glycolytic pathways can be an important tool in metabolic engineering. Metabolic engineering is the targeted improvement of the cellular properties achieved from the interplay of theoretical analysis, relying on biochemical information, and the application of optimizing genetic and regulatory processes through genetic engineering [4]. It makes use of a directed, rational approach which can only be done with an in-depth understanding of the cellular system in question. The ultimate goal of metabolic engineering is to increase the production of valuable or targeted substances on an industrial scale in a cost effective manner.

Kinetic models are built on the description of individual reaction steps within a pathway. Enzyme characteristics are used to describe kinetic behaviour.

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Kinetic equations with kinetic parameters are used to construct ordinary differential equations (ODE's). ODE's can then be integrated over time to model changes in metabolite concentrations. The output of these mathematical models give changes of metabolite concentrations over time in relation to biochemical characteristics.

2.6 Summary

This literature review has given a general overview of winemaking, problem fermentations, yeast hexose metabolism and mathematical modelling.

The yeast *S. cerevisiae* is an industrially important organisms. It is the driving force behind alcoholic fermentation and has an enormous impact on wine quality and production.

S. cerevisiae is a glucophilic yeast, consuming glucose at a faster rate than fructose. Although a link exists between stuck fermentation and sugar ratio, with glucose/fructose ratio becoming unfavourable to sustain fermentation, other factors can also lead to problem fermentations. Irrespective of the cause of problem fermentation, it is the high concentration of residual sugar that can lead to loss of productivity and quality. Fructose is the main sugar left during the final stages of fermentation, and the yeast has to use this undesirable sugar in an increasing stressful environment. Residual fructose can lead to undesirable sweetness in dry wines as it is about twice as sweet as glucose.

Glucose and fructose are both hexose sugars. Difference in consumption must be situated in the uptake or phosphorylation, or both, steps. This is the only steps where glucose and fructose metabolism is different.

With the occurrence of stuck and sluggish fermentations posing economic losses to the industry the development of wine fermentations with more predictable outcomes is needed. The development of more robust wine yeasts can aid in preventing problem fermentations.

The ability of the yeast to co-ferment fructose at the same rate as glucose is of particular interest. Lowering residual fructose concentrations would ad to the desired quality of wines. The power of systems biology as an engineering tool can be applied to yeast strain development.

The first step is understanding the mechanism responsible for the difference in consumption rates. This knowledge could in turn be used for the selection or engineering of novel wine yeast with a higher ability of fructose utilization.

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One such tool to determine the mechanism responsible for higher glucose utilization is systems biology. The availability of a kinetic model, describing molecular interaction can be used to an enhanced fundamental understanding and be used as an analytical tool for yeast strain development.

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Chapter 3

METHODS

3.1 General Overview

The aim of this project was to construct a kinetic model of glycolysis for a batch fermentation of *S. cerevisiae* under enological conditions, with both glucose and fructose explicitly modelled as substrates.The kinetic model would be based on the glycolytic model by Teusink *et al.* [90] adapted by van Nuland to simulate batch fermentations. As such, *S. cerevisiae* had to be cultured, batch fermentations completed and the transport and hexokinase steps kinetically characterised from live cells and cell extracts. Additionally, validation data in the form of glycolytic fluxes from different batch fermentations had to be determined. The effect of single sugar on fermentations were also investigated by monitoring fermentations with either only glucose or fructose in the media. Growth media components were either obtained from Sigma, Merck or Saarchem (South Africa). All enzymes were obtained from Sigma (South Africa). Radiolabelled substrates were obtained from AEC-Amersham.

3.2 Growth Conditions

3.2.1 Culturing of Wine Yeast

S. cerevisiae was grown from glycerol stocks kept at -80°C by streaking out on YPD agar plates (2% glucose, 2% agar, 2% peptone powder, 1% yeast extract). YPD plates were incubated at 30°C for $\geq$ 48 hr before single colonies were picked for growth in liquid media. Pre-cultures were grown in YPD liquid media (2% glucose, 2% peptone powder, 1% yeast extract) in erlenmeyer flasks on a shaking incubator (30°C, 125 rpm). The densities of the cells in culture were determined spectrophotometrically by measuring optical density (OD) at 600nm.

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3.2.2 Alcoholic Batch Fermentations

To characterise an alcoholic wine fermentation, small scale batch fermentations were completed with wine yeast strain *S. cerevisiae* VIN 13 on artificial wine must MS300. Growth of the yeast as well as the consumption and production profiles under batch fermentation conditions were monitored. This was done with OD600 readings for growth and High Performance Liquid Chromatography (HPLC) samples for metabolic fluxes. Biomass readings were also included to get an relationship between OD and dry weight. The consumption of glucose and fructose as well as the production of ethanol and glycerol were determined with HPLC. To simulate oenological fermentations, the sugar composition of the synthetic wine must consisted of 50% glucose and 50% fructose (50/50 fermentation). Two 50/50 fermentations were completed and profiled.

Batch fermentations with 100% glucose (100% glucose fermentation) and 100% fructose (100% fructose fermentation) were performed in duplicate and profiled in the same way as the normal fermentations.

3.2.3 Synthetic Wine Media (Culture Media)

Synthetic wine must MS300 (20% wt/vol hexose sugar) was used as medium to simulate a standard grape juice for batch fermentations [12]. The medium composition was obtained from the Institute of Wine Biotechnology, Stellenbosch University, South Africa. It contained the following components (expressed per liter): glucose 100g, fructose 100g, citric acid 6g, D-L malic acid 6g, mineral salts (mg): KH_2PO_4 750, KH_2SO_4 500, $MgSO_4 \cdot 7\,H_2O$ 250, $CaCl_2 \cdot 2\,H_2O$ 155, NaCl 200, $MnSO_4 \cdot H_2O$ 4, $ZnSO_4$ 4, $CuSO_4 \cdot 5\,H_2O$ 1, vitamins (mg): Myo-inositol 20, nicotinic acid 2, calcium panthothenate 1.5, thiamine hydrochloride 0.25, pyridoxine hydrochloride 0.25, biotin 0.003, anaerobic growth factors: ergosterol 15 mg, sodium oleate 5 mg, Tween 80 0.5 ml, nitrogen source: 120mg/L N ammoniacal nitrogen (NH4Cl 0.46 g) and amino acids (mg): L-proline 612.61, L-alanine 145.30, L-glutamic acid 120.43, L-serine 78.54, L-threonine 75.92, L-leucine 48.43, L-aspartic acid 44.51, L-valine 44.51, L-phenylalanine 37.96, L-isoleucine 32.73, L-histidine 32.73, L-methionine 31.42, L-tyrosine 18.33, L-glycine 18.33, L-lysine 17.02, L-cysteine 13.09. For fermentations with only one hexose sugar as carbon source, total sugar concentrations were either 200 g/l glucose (100% glucose fermentation) or 200 g/l fructose (100% fructose fermentation). For a normal 50/50 fermentation, concentrations were 100g/l glucose and 100g/l fructose.

Batch fermentations with 100% glucose and 100% fructose had either 200g/l glucose or 200g/l fructose as total sugar.

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3.2.4 Batch Fermentations in Bioreactor

Batch fermentations were performed in 1 L BioFlo 110 reactors (New Brunswick) at 30°C, 100 rpm, anaerobic, until all fermentable sugars were depleted, ranging between 50 and 100 hours. Cell growth was monitored with OD600 readings throughout fermentations.

3.2.4.1 Pre-culture for Batch Fermentations

YPD pre-culture were used to inoculate diluted synthetic media MS300 (50% water, 50% media). Cells were grown to mid-exponential phase (OD600 between 4 and 6) in YPD before inoculating diluted synthetic starter cultures with an OD600=0.1 (0.83- 2.5ml) and grown in erlenmeyer flasks (volume 50-100ml). Cells were again grown to mid-exponential phase (OD600 between 4 and 6) and used to inoculate the bioreactor to a starting OD of 0.1 (13.3-20ml). Synthetic media volumes were 800 mL in bioreactors.

3.2.5 Metabolite Fluxes

In order to follow sugar consumption and ethanol and glycerol production rates, external metabolite concentrations had to be determined for the duration of fermentation. HPLC was used to determine the concentrations. For HPLC, 2 ml samples were taken from bioreactor throughout the course of fermentation. The sample was centrifuged (14000 rpm, 5 min, 4C) whereafter 1.8 ml supernatant was transferred to a fresh tube. Perchloric acid (35%) was added (108.9 μl) and stored at -20°C for later use. When ready, samples were thawed and potassium hydroxide (7 M) added (99 μl) and kept on ice for 10 minutes. After centrifugation (14000 rpm, 5 min, 4°C) the supernatant was filtered (Hydrophilic PVDF 0.45 μm; Millipore millex-HV filters) and used for HPLC (Aminex HPX-87H column from Biorad, 65°C, mobile phase 0.005 M H_2SO_4 at 0.6ml/min).

3.3 Kinetic Parameter Determination

Literature (See Literature Review section 2.4) yielded kinetic parameters for transport and phosphorylation steps for *S. cerevisiae* in various conditions. For this study kinetic parameters for the uptake of sugars across the plasma membrane was determined using living cells. Phosphorylation kinetic parameters were determined *in vitro* using cell extracts.

3.3.1 Hexose Transport Assay

Glucose and fructose uptake assays were performed as described by Walsh *et al.* [94] from the original method of Bisson Fraenkel [20]. Cells were grown in

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synthetic wine media MS300 (50% glucose, 50% fructose) to mid-exponential growth phase (OD600 between 5 and 6) in erlenmeyer flasks in a rotary shaker (30°C, 125 rpm). Cultured cells, typically 200 mL of culture, were centrifuged (5000 rpm, 5min, 4°C) in 50ml tubes, supernatant discarded and resuspended in 100 mM potassium phosphate buffer (pH 6.5). This wash step was repeated twice. Pellet was then resuspended in buffer to a final volume of 1 mL.

Biomass readings were taken for the cells grown in synthetic media. Volumes of 20 mL were filtered on a Millipore filter (dried and weighed), rinsed with water, and dried in a dessicator for two days before weighing.

Uptake was measured at glucose and fructose concentrations ranging from 1.25 to 120 mM in final assay volume (specific radioactivity, 111 GBq.μmol^{-1} to 1,156 GBq.μmol^{-1}). Radiolabelled mixture (10 μL) and yeast cells (30 μL) were preincubated at assay temperature (30°C) and then mixed and incubated for 5 s (measured with stop-watch). Uptake of sugars by cells was terminated by quenching with 15 ml 100 mM potassium phosphate buffer (pH 6.5) containing 500 mM unlabelled sugar (either glucose or fructose) kept at a temperature below -5°C on salt-ice mixture. Cells were collected on filters with an additional 15 ml quenching solution. Filters were transferred to scintillation vials containig 5 ml scintillation fluid and radioactivity was measured with a liquid scintillation counter. The control consisted of labelled sugar added to quenching solution at the same time as the yeast cells.

Each sugar concentration experiment was done in triplicate. Two of the experiments were done with samples taken from cells cultures from one batch fermentation, and a final one with cells cultured from a different fermentation.

3.3.2 Hexokinase Enzyme Assay

The hexokinases (hexokinase 1, hexokinase 2 and glucokinase) were kinetically characterised in terms of their affinity and maximal rate for both glucose and fructose as substrate. The three iso-enzymes were analysed together and the determined parameters are thus weighed averages of the individual kinases. Cells were cultured in YPDF media (1% glucose, 1% fructose, 2% peptone powder, 1% yeast extract), typically 100 mL of culture volume, to mid-exponential phase and spinned down (5 min, 5000 rpm, 4°C) on a centrifuge. Cell pellets were resuspended in 2 ml extraction buffer, containing 20 mM KH_2PO_4 (pH 7) and 1 mM freshly prepared PMSF (protease inhibitor, stock: 0.1 M PMSF in DMSO). Glass beads (0.25-0.55 mm) were prepared by cleaning overnight in 5.8 M HCl and washing 5 times in H_2O and dried overnight at 30°C. One gram of the clean glass beads was added to 1 ml of cell suspension. Samples were vortexed for 30 seconds and kept on ice for 30 seconds alternately for 8 cycles. Samples were centrifuged afterwards (10 min, 14000 rpm, 4 °C) and super-

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natant kept on ice for enzyme assays. Assays were performed in assay buffer containing PIPES (50 mM), KCl (0.1 M), $MgSO_4$ (5 mM) and KH_2PO_4 (50 mM). The pH was set to 7. NADP/NADPH linked enzyme assays were performed to determine the Vmax and Km values for the hexokinase step for either glucose or fructose as substrate. The assays were performed at OD340 in 96 well plates (Greiner bio-one flat bottom microplates) on a spectrophotometer (VarioSkan microplate reader, Thermo Electron Corporation). Hexokinase was measured with 2 mM NADP, 1.5 mM ATP, 2.8 U/ml glucose-6-phosphate dehydrogenase (G6PDH) and glucose substrate concentrations ranging between 0-10 mM. For fructose as substrate, with concentrations ranging between 0-10 mM, 2 U/ml PGI was added. All reagents and enzyme dilutions were made up in assay buffer.

3.3.2.1 Protein Determination

Protein concentrations of cell lysate were determined with the use of the Bradford method [28]. The protocol was adapted for use in 96 well plates, where 190 μL of Bradford reagent was added to 5 μL of sample or standard and incubated for 15 minutes before reading the absorbance at 595 nm. The standard was a BSA calibration curve in the range of 0-1 mg/mL.

3.3.2.2 Binding Constant Determination

For each substrate concentration, initial maximum reaction rates were determined over a minimum period of 1 minute by using the slope of maximum rate ($R^2 > 0.90$) and the extinction coefficient for NADPH (6.22 $L^{-1}.mol^{-1}.cm^{-1}$) with the Beer-Lambert Law. The pathlength of the 100 μl assay working volume was taken to be 3.0419 mm [70]. By plotting substrate concentration versus corresponding maximal rates and normalised to protein concentration, a curve was obtained. The curve was analysed with nonlinear regression, Michaelis-Menten, to obtain binding and Vmax values. The program GraphPad Prism 5 was used for all calculations.

3.4 Mathematical Modelling

3.4.1 Model Construction

Kinetic models aim to be virtual representations of enzyme-catalyzed reactions of living cells, reproducing metabolism *in silico*. This is accomplished by constructing a system of interdependent differential equations according to the properties of the pathway and its enzymes.

Wine fermentation was described through the construction of a kinetic model. For this project a previous model was refined to separately model the uptake

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of fructose and glucose rather than as a single entity. The model aimed to be capable of accounting for the discrepancy in the consumption of the sugars.

For the hexose uptake and hexokinase phosphorylation steps values were experimentally determined with glucose or fructose as substrates. Other kinetic parameters were taken from previous work by Teusink, Van Nuland and Abrie [90, 67, 1]. The kinetic model was constructed in Wolfram Mathematica 8.0. using NDSolve function.

3.4.2 Model Validation

Model validation is an important part of kinetic modelling. The constructed model uses parameters of enzymes that have been characterised in isolation to predict the consumption and production of certain metabolites over the time span of a batch fermentation. Through comparison of the predicted values with experimentally determined batch fermentation consumption and production fluxes, one can critically test whether a proposed mechanism can explain observed behaviour.

This model is however not completely generic, needing specific inputs of growth rates, cell volume and metabolite concentrations at a certain time point of fermentation. These variable values are experimental determined during batch fermentations.

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Chapter 4

RESULTS

In this chapter the results of the experimental and modelling investigation into wine fermentation are presented. The results are presented in three parts; wine fermentations, kinetic parameter estimation and mathematical modelling.

4.1 Wine Fermentation

In total six batch fermentation with synthetic wine must, inoculated with *S. cerevisiae* VIN 13, were completed. During the wine fermentations, biomass and external metabolite concentrations were measured.

4.1.1 50/50 Fermentation

In grape juice, glucose and fructose are present at equal concentrations. The 50/50 fermentation with 100 g/L glucose and 100 g/L fructose serves as our reference condition. Two 50/50 batch fermentations were completed, distinguished as Fermentation 1.1 and Fermentation 1.2.

4.1.1.1 Cell growth

Growth of yeast cells were monitored during fermentation with optical density measurements. An exponential curve was fitted to the experimental data points describing exponential growth in log scale. Specific growth rate (μ) of Fermentation 1.1 was $\mu = 0.131\ h^{-1}$ and for Fermentation 1.2 $\mu = 0.125\ h^{-1}$. Exponential growth phase was approximately between 10 and 15 hours, with growth ceasing after about 40 hours.

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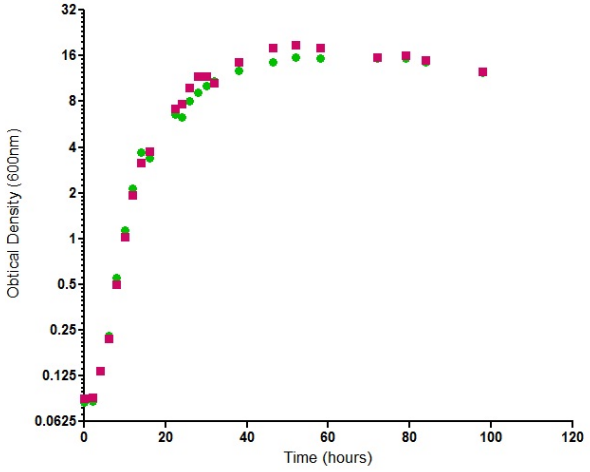

Figure 4.1: Optical density (600nm) measurement of 50/50 fermentations to monitor cell growth. An exponential fit between time points 3 and 14 hours was used to determine specific growth ($\mu = 0.131\ h^{-1}$ (Fermentation 1.1, green dots); $0.125\ h^{-1}$ (Fermentation 1.2, pink dots)).($R^2 > 0.96$)

4.1.1.2 Fermentation Fluxes

The rate of consumption of the two hexose sugars and production of both ethanol and glycerol was measured for the two batch fermentations (Figure 4.2 and 4.3). Both fermentations reached dryness (consumed all the sugars) between 50 and 70 hours, taking a little bit longer to consume all the available fructose. Both fermentations had a faster consumption of glucose over fructose, confirming the glycophilic character of the wine yeast *S.cerevsiae* VIN 13. Starting total sugar concentrations were 1043 and 1130 mM, and final ethanol concentrations 1906 and 1932 mM for Fermentation 1.1 and 1.2 respectively.

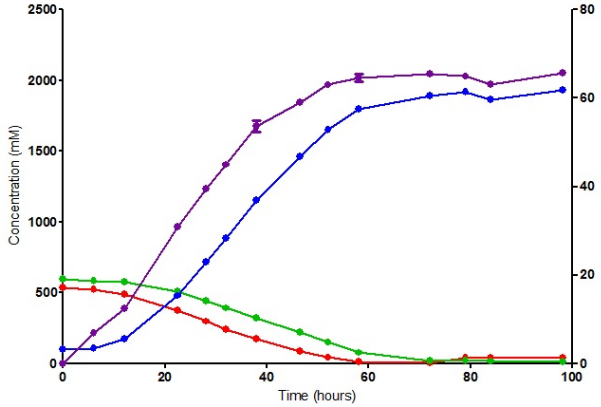

Figure 4.2: Substrate and product fluxes for fermentation with 50% glucose and 50% fructose (Fermentation 1.1). On left Y-axis is glucose (red), fructose (green), and ethanol (blue) and on the right Y-axis is glycerol (purple).

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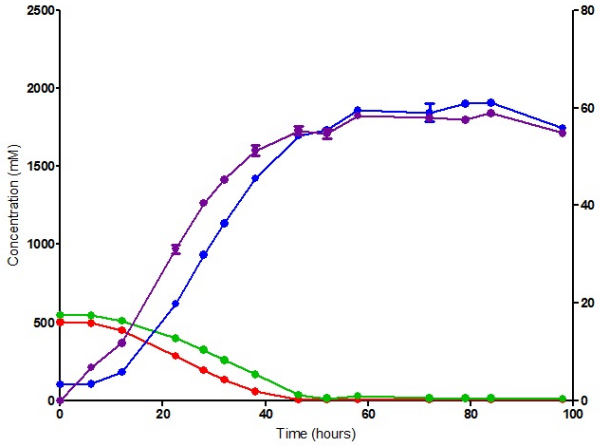

Figure 4.3: Substrate and product fluxes for fermentation with 50% glucose and 50% fructose (Fermentation 1.2). On left Y-axis is glucose (red), fructose (green), and ethanol (blue) and on the right Y-axis is glycerol (purple).

Specific substrate consumption and production formation rates of the two fermentations were very similar (Figure 4.4 and 4.5). During the exponential growth phase (10 to 15 hours) sugars were rapidly consumed and ethanol rapidly formed. As fermentation progressed specific consumption and production rates declined.

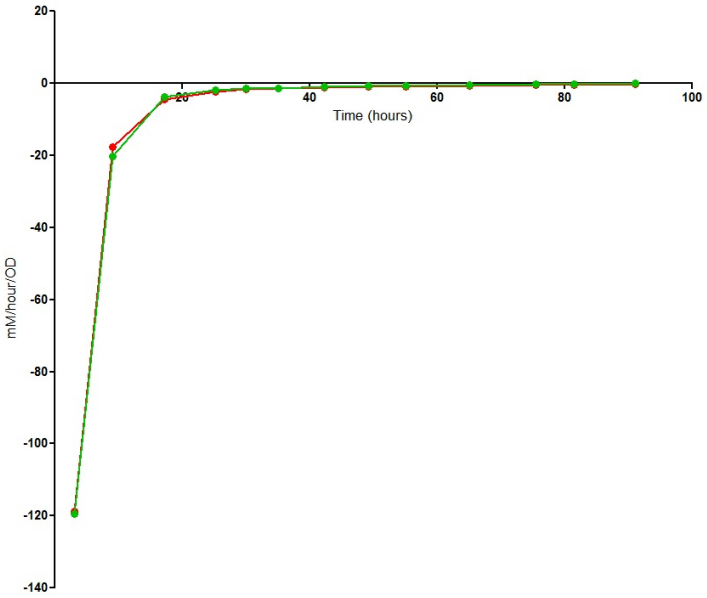

Figure 4.4: Specific hexose sugars consumption rates of 50/50 fermentations.

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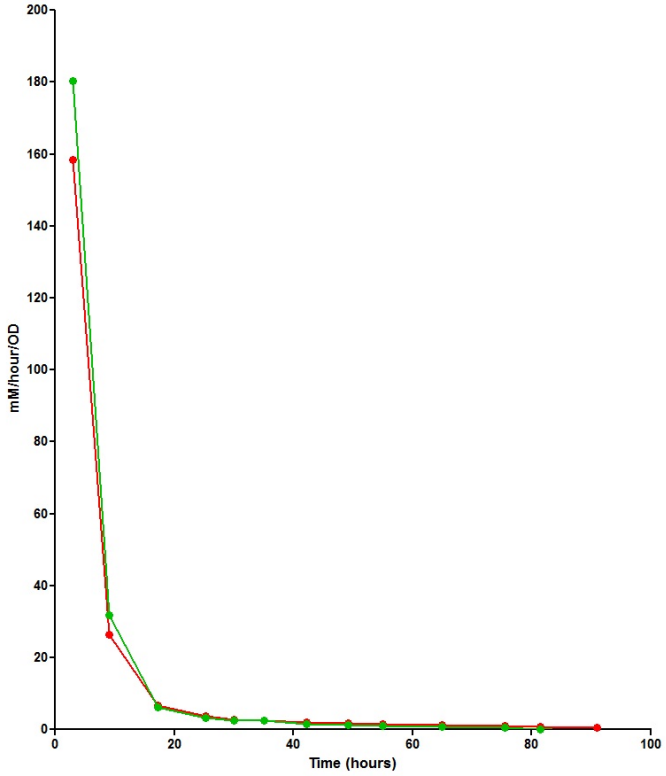

Figure 4.5: Specific ethanol formation rates of 50/50 fermentations.

4.1.2 Effect of Carbon Source on Wine Fermentation

In addition to the 50/50 fermentations, we also investigated the yeast's ability to consume the individual sugars in isolation. Alcoholic fermentations were completed for two batch fermentations with 200 g/L glucose (Fermentation 2.1 and Fermentation 2.2) and two batch fermentations with 200 g/L fructose (Fermentation 3.1 and Fermentation 3.2).

4.1.2.1 Cell growth

Optical density measurements were fitted with an exponential equation to determine the specific growth rate for the different fermentations (Figure 4.6). Fermentation 2.1 and 2.2 had specific growths of 0.136 h^{-1} and 0.122h^{-1} and Fermentation 3.1 and 3.2 specific growths of 0.133 h^{-1} and 0.124 h^{-1} (Figure 4.7) respectively. These specific growth rates were comparable to the 50/50 fermentations.

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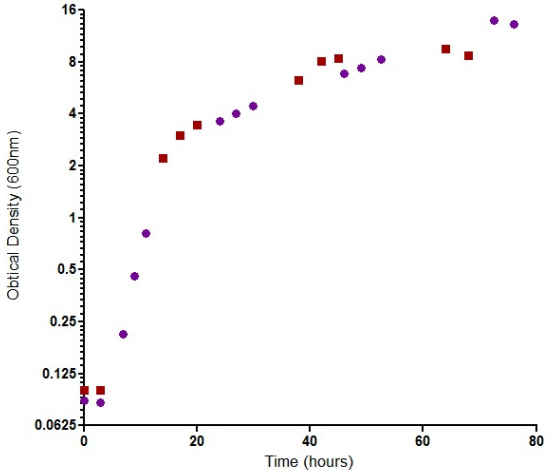

Figure 4.6: Optical density (600nm) measurement of 100% glucose fermentations to monitor cell growth. Exponential fit between time points 3 and 14 hours was used to determine specific growth (mu = 0.136 h^{-1} (Fermentation 2.1, purple dots); 0.122 h^{-1} (Fermentation 2.2, red dots)). ($R^2 >$ 0.98)

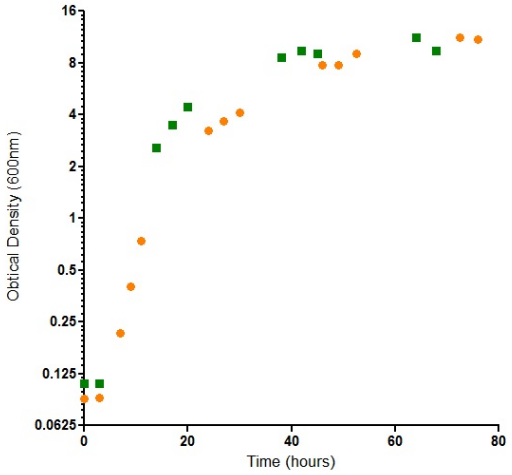

Figure 4.7: Optical density (600nm) measurement of 100% fructose fermentations to monitor cell growth. Exponential fit between time points 3 and 14 hours was used to determine specific growth (mu = 0.133 h^{-1} (Fermentation 3.1, yellow dots);0.124 h^{-1} (Fermentation 3.2, green dots)). (R^2 $>$ 0.98)

4.1.2.2 Fermentation Fluxes

The consumption rate of glucose and/or fructose and production of ethanol was monitored for the different fermentations. In these experiments we investigated the ability of yeast to consume glucose or fructose if it is the only consumable sugar present. Metabolite concentration changes during fermentations were monitored (Figure 4.8 and 4.9). Both the fermentation with 100% glucose and

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100% fructose consumed all the sugars in approximately 120 hours. There is no observable difference in either growth rates or fermentation profiles between single sugar fermentations.

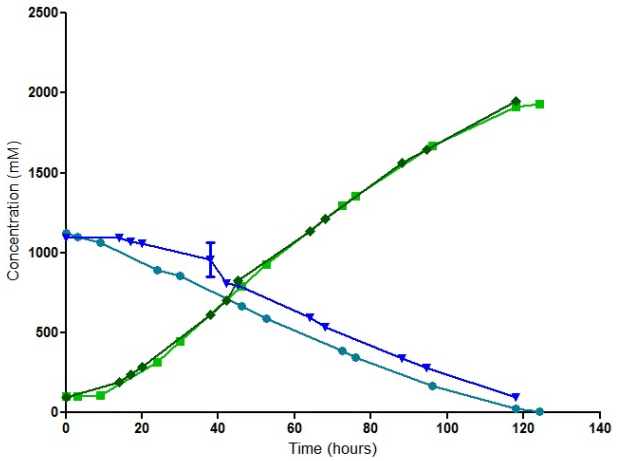

Figure 4.8: Substrate and product changes over time for fermentation with 100% glucose (Fermentation 2.1 and 2.2). Glucose (blue) and ethanol (green).

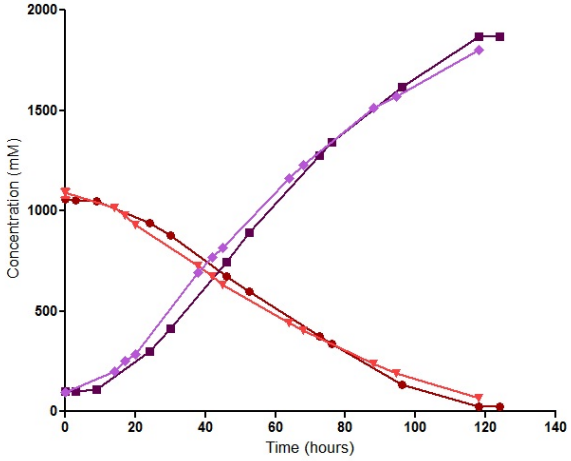

Figure 4.9: Substrate and product changes over time for fermentation with 100% fructose (Fermentation 3.1 and 3.2). Fructose (red) and ethanol (purple).

4.1.3 Comparing Fermentations

The single sugar fermentations (glucose or fructose) showed very similar growth curves and fermentation profiles. However, when the single sugar fermentations were compared to a 50/50 fermentation, differences in both growth and flux patterns were observed. Fermentations with only one sugar showed much

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slower fermentation rates, taking more than twice the time from start to dryness compared to the 50/50 fermentations. Another difference between fermentation with 50/50 sugars and single sugar fermentations is the final cell densities that are reached during alcoholic fermentation (Figure 4.10). The maximal optical density measured for 50/50 fermentations is between 16 and 19 and for the fermentations with only glucose or fructose maximal OD readings are between 9 and 11.

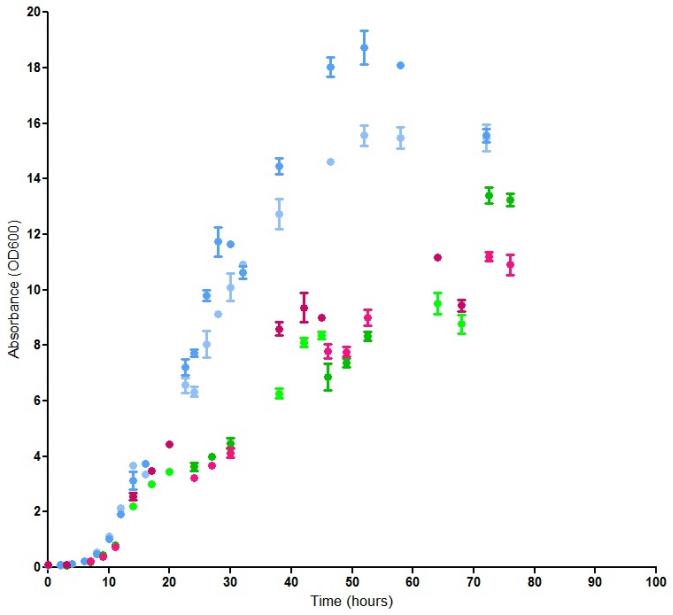

Figure 4.10: Growth curves of all wine fermentations. 50/50 fermentation (blue dots), 100% glucose (green dots) and 100% fructose fermentation (pink dots).

It should be noted that in the early phases of the fermentations, up to 20 hours, the specific growth rate for single sugar and 50/50 fermentations are very similar. However, after 20 hours a marked difference in biomass variance is observed between the two types of fermentations. The lower biomass concentration and longer fermentation times raise the question whether the specific substrate consumption rates and product formation rates are different between the two fermentation types.

Specific production of ethanol and the specific consumption of sugar was calculated by taking the time derivative of the substrate/product concentrations divided by the optical density at different time points (mM/hour/OD). Specific production rates were calculated from change in total sugar concentration divided by optical density. Similarly production rates were calculated from the change in ethanol concentrations divided by optical density. These data revealed very similar trends for all the fermentations (Figure 4.11 and 4.12).

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Thus, normalising for cell density shows that, although the final biomass concentration is different for the three fermentation types, the glycolytic flux through each cell is comparable.

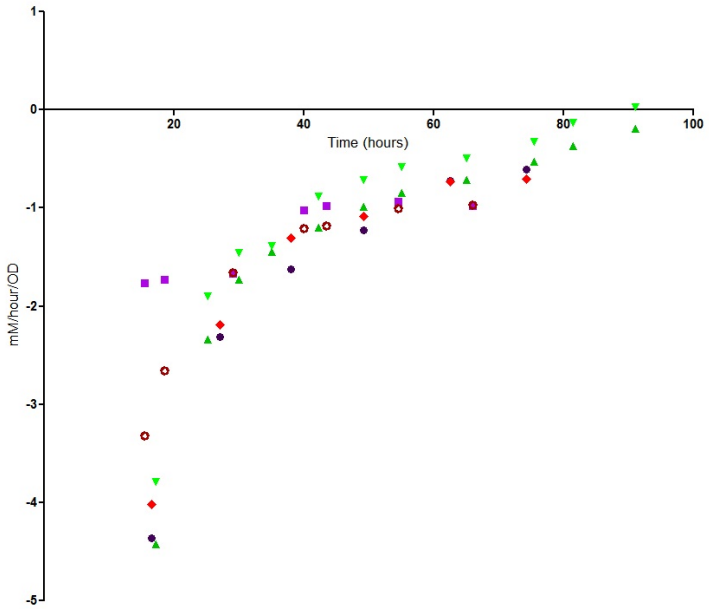

Figure 4.11: Specific sugar consumption rates for all completed fermentations. Two 50/50 fermentations (green), two 100% fructose fermentations (red) and two 100% glucose fermentations (purple) are compared.

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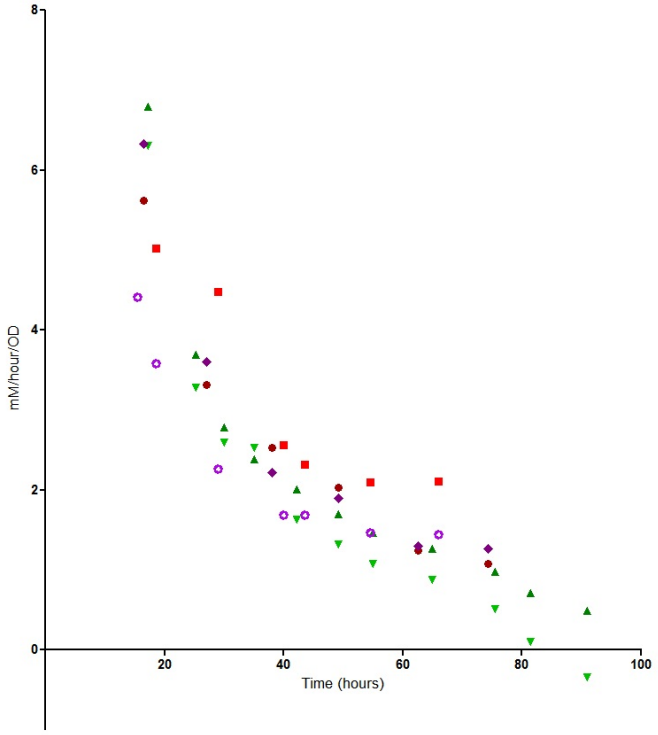

Figure 4.12: Specific ethanol production rates for all completed fermentations.Two 50/50 fermentations (green), two 100% fructose fermentations (purple) and two 100% glucose fermentations (red) are compared.

4.2 Kinetic Parameter Estimation

To estimate enzyme kinetic parameters from the transport and phosphorylation steps for glucose and fructose, enzyme kinetic assays were performed on the isolated enzymes.
The term isolated is used to distinguish the enzyme kinetic experiments from the fermentation studies in these experiments.

4.2.1 Hexose Transport

In rapid, zero trans influx experiments, radio-labelled uptake of glucose and fructose was measured as a function of the carbohydrate concentration.

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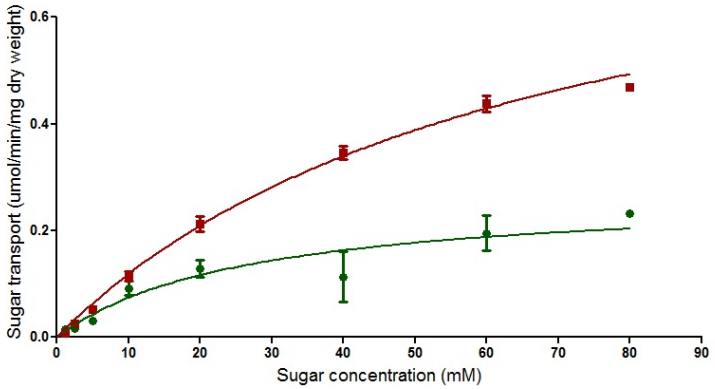

Figure 4.13: Kinetic characterisation of hexose transport in *S. cerevisiae* VIN 13 as a function of the substrate concentrations. Fructose uptake (red) and glucose uptake (green) was evaluated with Michaelis-Menten equation to determine V_{max} and K_m values. Error bars represent standard deviation of the mean. $R^2 > 0.81$ for glucose, $R^2 > 0.99$ for fructose.

The affinity and the maximal rate of transport for either glucose or fructose into the yeast cell were determined by fitting a Michaelis-Menten-equation to the experimental data of the transport rate (umol/min/mg dry weight) against substrate concentrations (mM) (Figure 4.13). For each substrate concentration three data points were collected; two from uptake experiment from yeast cells collected from fermentation number one and another point from uptake by yeast cells from a second batch fermentation, both in mid-exponential growth phase. The data-sets were analysed by fitting the curve with non-linear regression using the irreversible Michaelis-Menten equation to obtain maximal uptake rate and binding constant of the substrate.

It should be noted that quite high errors were obtained from the estimation of the Km values. Specifically for the fructose kinetics we should have included higher substrate concentrations. We now have only one point above the estimated K_m value. This leads to an inaccurate estimation of K_m and V_{max} value for fructose.

The determined V_{max} and K_m values revealed a higher maximum rate for fructose transport compared to glucose, with a higher affinity for glucose as substrate (Table 4.1).

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Table 4.1: Experimentally determined Michaelis-Menten constant and maximal transport rate for *S. cerevisiae* VIN 13. Rates are normalised to dry weight with standard deviation of triplicate values determined from two sample sets.

Substrate	Km *(mM)*	Vmax *(µmol/min/mg dry weight)*
Glucose	26.40 ± 11.66	0.27 ± 0.05
Fructose	66.15 ± 8.937	0.9 ± 0.07

4.2.2 Hexose Phosphorylation

Kinetics of hexose phosphorylation were measured for cells grown on 2% sugar YPD media. For cells grown on synthetic wine-must a saturation curve could not be fitted on a glucose phosphorylation assay. In cells grown on wine-must, without the addition of substrate, the reaction measured occurred at maximum velocity for a prolonged time period (> 10 min). It was thus decided to perform the hexose phosphorylation kinetic experiment with yeast cells grown in YPD as these cells did not give the same problem.

Kinetic parameters for the phosphorylation of glucose and fructose by the hexokinases were obtained from NADP-linked kinetic assays.

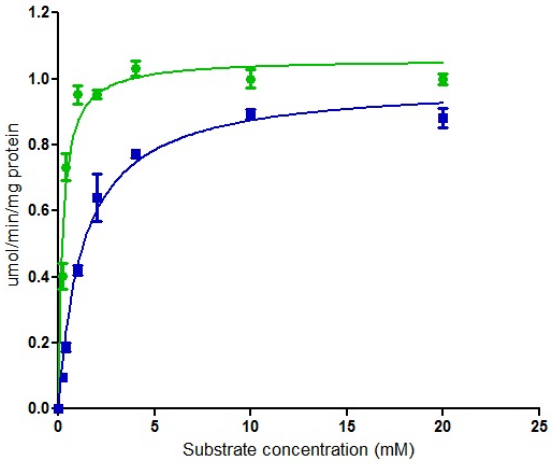

Figure 4.14: Kinetic characterisation of *S. cerevisiae* VIN 13 hexokinase in terms of its sugar substrates, glucose (green) and fructose (blue). Fitting with Michaelis-Menten equation was used to determine V_{max} and K_m values.

To obtain K_m and V_{max} values for the hexose phosphorylation of glucose and fructose, Michaelis-Menten kinetic equations were fitted to the kinetic data of hexokinase rate (umol/min/mg protein) to substrate concentration (mM)

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(Figure 4.14).

Fitting the Michaelis-Menten equation to the experimental data revealed a higher maximal rate and affinity of hexokinase for glucose compared to fructose (Table 4.2).

Table 4.2: Experimentally determined Michaelis-Menten constant and maximal hexokinase enzyme rate for *S. cerevisiae* VIN 13. Rates are normalised to total protein content and standard deviation of triplicate measurements.

Substrate	Km *(mM)*	Vmax *(µmol/min/mg protein)*
Glucose	0.222 ± 0.028	1.059 ± 0.028
Fructose	1.279 ± 0.145	0.985 ± 0.029

4.3 Mathematical Modelling

4.3.1 Model Construction

In the following section an outline is given of the model structure of glycolysis and how it was adapted for batch wine fermentations. We adapted an existing model of Teusink *et al.* [90], to include variable biomass concentrations. The model was constructed from the enzyme kinetic rate equations and the pathway stoichiometry, leading to a set of ordinary differential equations. The original model can be obtained from the JWS Online database (http://jjj.biochem.sun.ac.za).

An existing model of the glycolytic pathway constructed by Teusink *et al.* [90] was specifically adapted for *S. cerevisiae* VIN 13 fermentations on MS300 media. The existing kinetic model was built to describe a non-growing budding yeast (Koningsgist), under anaerobic conditions, to investigate whether the *in vivo* behaviour of yeast glycolysis could be understood in terms of the *in vitro* kinetics of the glycolytic enzymes. Several changes had to be incorporated into the Teusink model to simulate wine fermentations. The extended model had to describe growth, volume change, metabolite transport and included experimentally determined V_{max} and K_m values for the hexose transport and phosphorylation steps with either glucose or fructose.

4.3.1.1 Differential equations

A set of ordinary differential equations was used to describe the time-dependent changes in metabolite concentrations. In this study, the model was extended

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to incorporate glucose and fructose as substrates of the pathway, and the following equations were adapted or added into the original Teusink model;

$$d[Glc_{in}]/dt = \upsilon_{transportglc} - \upsilon_{HKglc} \tag{4.3.1}$$

$$d[Fru_{in}]/dt = \upsilon_{transportfru} - \upsilon_{HKfru} \tag{4.3.2}$$

$$d[G6P]/dt = \upsilon_{HKglc} - \upsilon_{PGI} - 2\upsilon_{trehalose} - \upsilon_{glycogen} \tag{4.3.3}$$

$$d[F6P]/dt = \upsilon_{PGI} + \upsilon_{HKfru} - \upsilon_{PFK} \tag{4.3.4}$$

A significant adaptation of the Teusink model is the modelling of glucose and fructose as two substrates, as opposed to the original single substrate. Parameters for the uptake and phosphorylation steps for the different substrates were experimentally determined. V_{max} and K_m values were included and the initial external metabolite concentrations and biomass were also incorporated.

The rest of this sub-section describes the adaptions made to the Teusink model by Nuland [67]. The adaptations changes the model from describing non-growing cell glycolysis to describing a batch wine fermentation. This incorporated changes in volume and transport in and out of the cell of substrates and products.

External metabolite concentrations of glucose, fructose, ethanol and glycerol, were modelled as variables in our model, this in contrast to the original model where they were parameters. Mass action kinetics for transport of products was adopted from the Hynne *et al.* [46] model. The rate of ethanol and glycerol transport was described by;

$$v(P_{transport}) = ((P_{in}) - (P_{out})) \times K_{PTransport} \tag{4.3.5}$$

P represents the metabolite concentration and $K_{PTransport}$ a rate constant.

During the exponential growth phase, changes in biomass and internal volume were incorporate in the model as follows:

During exponential growth changes in biomass can be described with

$$\frac{d(Biomass[t])}{d[t]} = \mu \times Biomass[t] \tag{4.3.6}$$

Additionally the growth rate (μ) was described as a piecewise function, being equal to zero during lag and stationary phase and equal to experimentally determined specific growth rate during exponential phase.

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Total cytosolic volume was modelled as a compartment of the total reactor volume. Changes in intracellular and extracellular volume during fermentation was modelled with the Teusink assumption that protein constitutes 50% of the dry weight biomass, and 1 mg protein equal to 3.75 ml cytosol, used to determine total cellular volume (in ml).

$$Protein[t] = 0.5 \times Biomass[t] \tag{4.3.7}$$

$$Vol_{internal}[t] = 3.75ml \times Protein[t] \tag{4.3.8}$$

With this information the change in intracellular and extracellular volume was included. The concentration of external glucose, fructose, glycerol and ethanol was modelled as a function of intracellular volume;

$$P_{external[t]} = \frac{A \times v[P_{transport}] \times Volume_{Internal[t]}}{Volume_{Vessel} - Volume_{Internal[t]}} \tag{4.3.9}$$

P represents the metabolite, A is a sign factor, with 1 equal to transport into the cell, and -1 for transport out of the cell. The rate of succinate production was also adjusted by changing the rate constant with the simplified equation;

$$v_{succinate} = k_{succinate} \times [acetaldehyde] \tag{4.3.10}$$

For a schematic representation of the reaction network, refer to Figure 4.15.

4.3.2 Rate equations

4.3.2.1 Transport of glucose and fructose: Hexose Transporter: HXT

The transport of the two hexose sugars, glucose and fructose, across the cell membrane occurs via facilitated diffusion [88, 60]. Reversible Michaelis-Menten equations were used to describe the transport of glucose and fructose into the cell. We assume that both substrates are transported via the same enzyme and that they act as competitive inhibitors for each other.

$$v_{HXTGlc} = \frac{V_{maxGlc}([Glc_{out}] - [Glc_{in}])}{K_{mHXTGlc}\left(1 + \frac{[Glc_{out}]}{K_{mHXTglc}} + \frac{[Glc_{in}]}{K_{mHXTglc}} + \frac{[Fru_{out}]}{K_{mHXTfru}} + \frac{[Fru_{in}]}{K_{mHXTfru}}\right)} \tag{4.3.11}$$

$$v_{HXTFru} = \frac{V_{maxFru}([Fru_{out}] - [Fru_{in}])}{K_{mHXTFru}\left(1 + \frac{[Glc_{out}]}{K_{mHXTglc}} + \frac{[Glc_{in}]}{K_{mHXTglc}} + \frac{[Fru_{out}]}{K_{mHXTfru}} + \frac{[Fru_{in}]}{K_{mHXTfru}}\right)} \tag{4.3.12}$$

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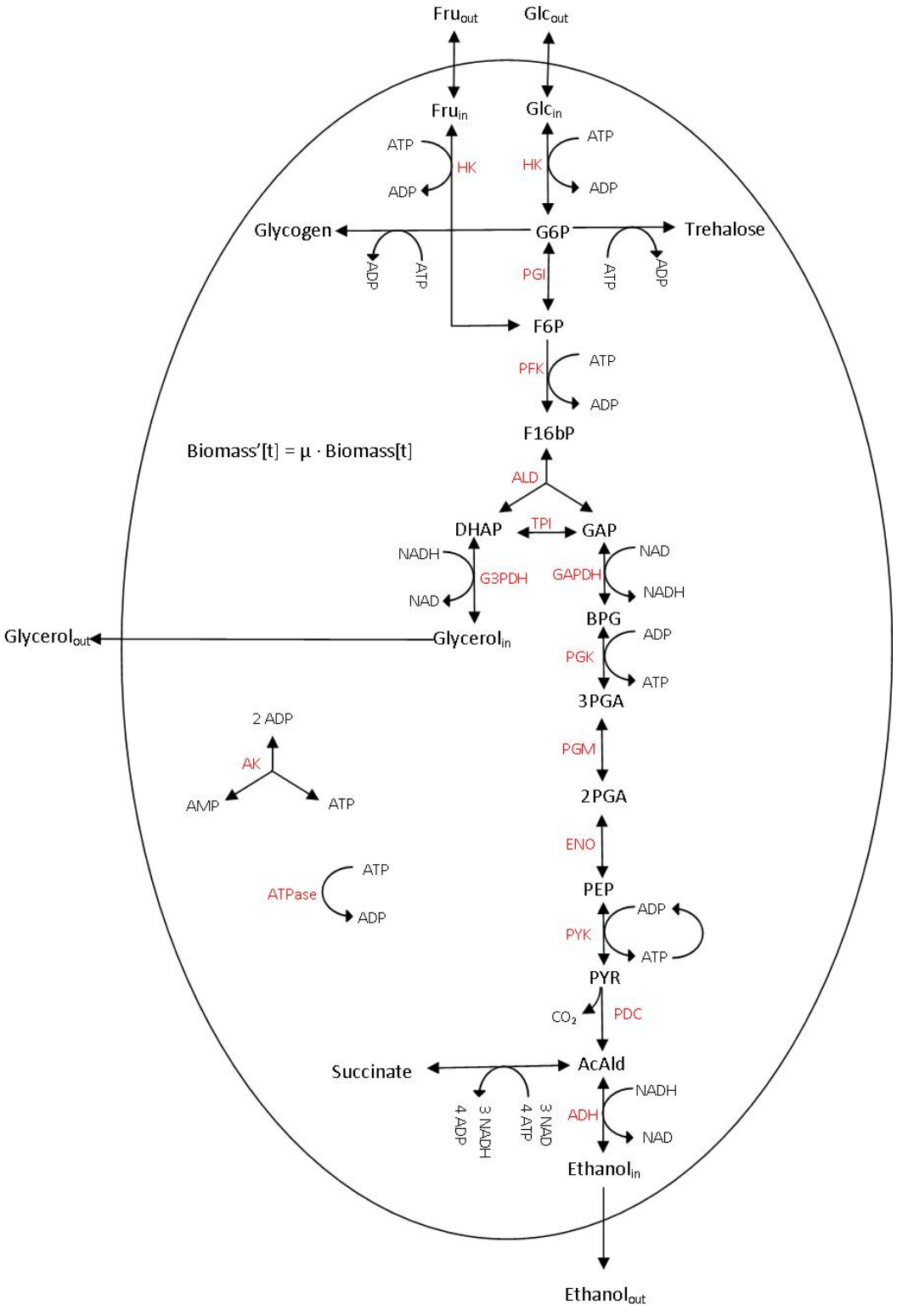

Figure 4.15: Schematic of adapted model.

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4.3.2.2 Phosphorylation of glucose and fructose: Hexokinase: HK

The phosphorylation rate of glucose and fructose by the hexokinases and glucokinase was modelled using reversible Michaelis-Menten kinetics. Also for phosphorylation of glucose and fructose we assume that both substrates act as competitive inhibitors.

$$v_{HKGlc} = \frac{atp \times V_{maxHKGlc}\left(1 - \frac{adp \times [G6P]}{atp \times K_{eqHX} \times Glc_{in}}\right) Glc_{in}}{\left(1 + \frac{adp}{K_{mHKadp}} + \frac{atp}{K_{mHKatp}}\right) K_{mHKatp} \times K_{mHKglc}\left(\frac{[F6P]}{K_{mHKf6p}} + \frac{[Fru_{in}]}{K_{mHKfru}} + \frac{[G6P]}{K_{mHKg6p}} + \frac{[Glc_{in}]}{K_{mHKglc}}\right)} \quad (4.3.13)$$

$$v_{HKFru} = \frac{atp \times V_{maxHKFru}\left(1 - \frac{adp \times [F6P]}{atp \times K_{eqHX} \times Fru_{in}}\right) Fru_{in}}{\left(1 + \frac{adp}{K_{mHKadp}} + \frac{atp}{K_{mHKatp}}\right) K_{mHKatp} \times K_{mHKfru}\left(\frac{[F6P]}{K_{mHKf6p}} + \frac{[Fru_{in}]}{K_{mHKfru}} + \frac{[G6P]}{K_{mHKg6p}} + \frac{[Glc_{in}]}{K_{mHKglc}}\right)} \quad (4.3.14)$$

In our model we used the experimentally determined parameter values of the glucose/fructose transport and phosphorylation steps, determined in this study. Maximal enzyme rates for the rest of glycolysis were experimentally determined for the *S. cerevisiae* VIN 13 yeast by Abrie [1] (See Appendix A.1). These V_{max} values were used for our model as it was determined under wine fermentation conditions. Other parameter values we used were adopted from the Teusink model.

4.3.3 Model Validation

An important part of any kinetic modelling study is model validation. In this study the kinetic model was validated by comparing measured changes in external metabolite concentrations with the fluxes predicted by the model.

4.3.3.1 Mathematical Model Testing

According to literature (See Literature Review, Section 2.4) the control over glycolytic flux is expected to be mostly situated in the hexose transport step. To test the ability of the model to distinguish between glucose and fructose due to different kinetics for the two substrates in the transport step a simple rate equation describing the transport step was constructed (See Appendix A.2). Using experimentally determined kinetic parameters for the transport step the uptake of glucose and fructose over time was modelled. Model simulation predicted a faster consumption of fructose compared to glucose (Figure 4.16).

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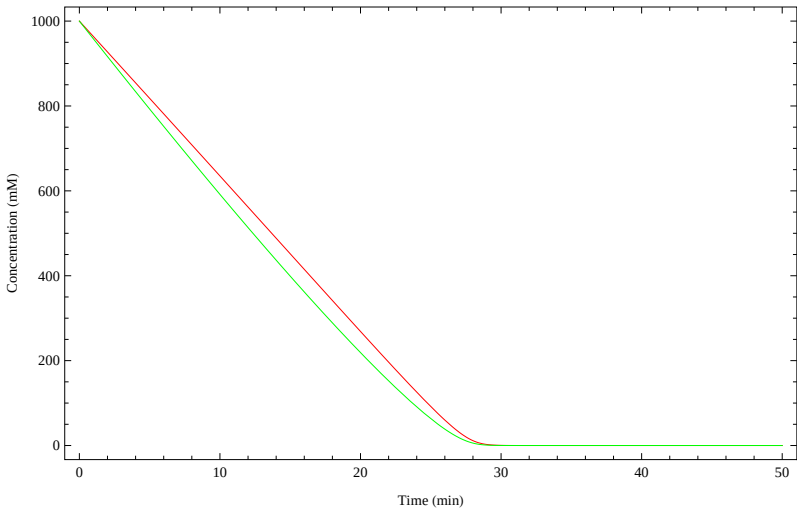

Figure 4.16: Model prediction of sugar transport with glucose (red) and fructose (green) using the experimentally determined kinetic parameter values for the transport step.

As was noted in the discussion of the experimentally measured transport kinetics, there was a large error in the K_m for glucose and fructose and in addition we could not estimate the V_{max} for fructose very well.

To test the sensitivity of the transport step for the experimentally measured kinetic parameters we used model simulations at the measured values $\pm$ the experimental error. When we used the lower K_m for glucose (26.4 - 11.66 = 14.47 mM) and a higher K_m for fructose (66.15 + 8.937 = 75.087) there was a preferred uptake of glucose above fructose (Figure 4.17). Even when a lower K_m for fructose (66.15 - 8.937 = 57.21) was used glucose was preferred (Figure 4.18).

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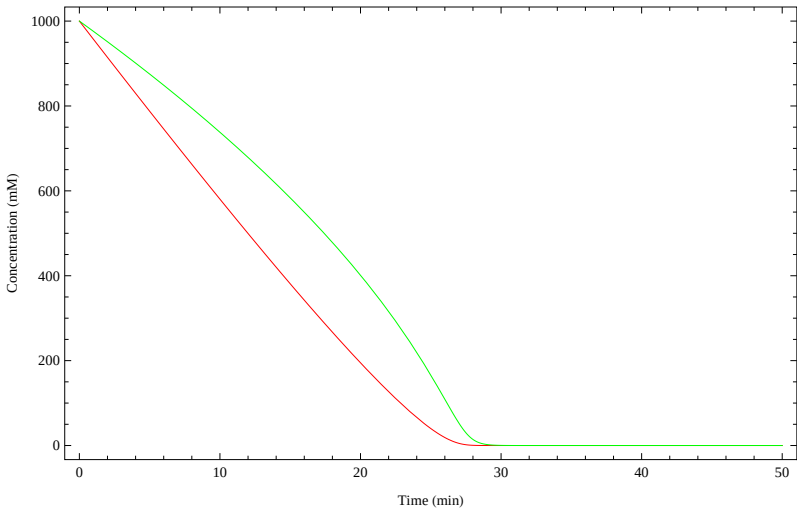

Figure 4.17: Model prediction of sugar transport of glucose (red) and fructose (green) using the adapted kinetic parameter values for the transport step. Maximum and minimum K_m values ($\pm$ experimental error) were used in model.

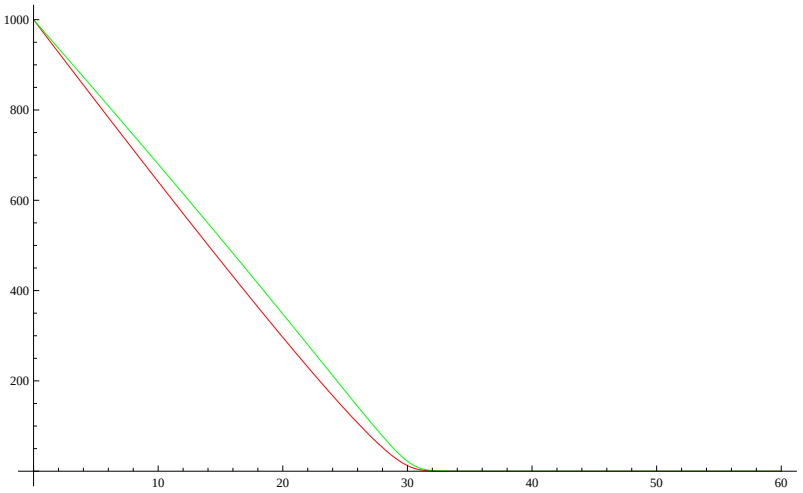

Figure 4.18: Model prediction of sugar transport of glucose (red) and fructose (green) using the adapted kinetic parameter values for the transport step. K_m value for fructose minus experimental error was used in model.

Using lower V_{max} values for fructose transport also had the effect of leading to the preferred uptake of glucose. Lowering the uptake rate by 25% a notable difference in glucose and fructose consumption rates could be observed (Figure 4.19).

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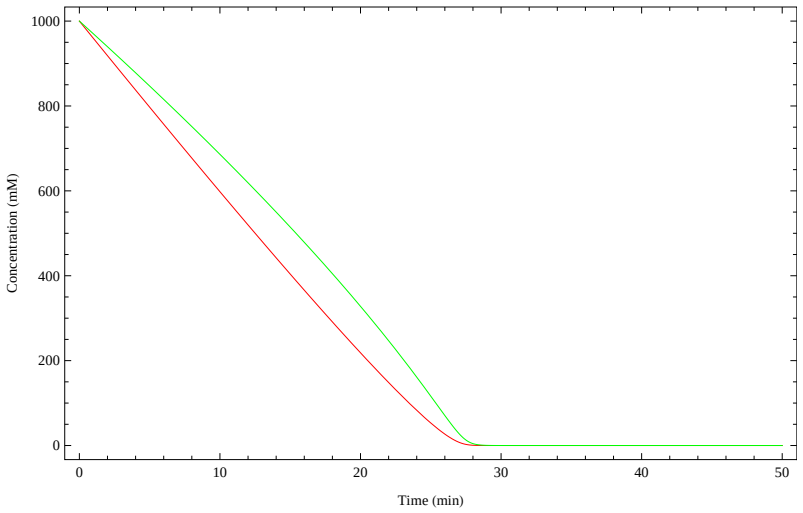

Figure 4.19: Model prediction of sugar transport of glucose (red) and fructose (green) using the adapted kinetic parameter values for the transport step. V_{max} value for fructose transport lowered by 25%.

Experimentally determined affinity values did not succeed in leading to the faster transport of glucose. It would seem that even though the hexose transporters have a higher affinity for glucose the faster maximal uptake rate of fructose has a more pronounced effect.

The ability to model the faster uptake of glucose does however lie within the measured affinity range when standard error are taken into account. For the further construction of the wine fermentation it was decided to use the parameter values that succeeded in predicting the faster consumption of glucose.

From the experimentally measured kinetic parameters for glucose/fructose transport and phosphorylation it would be expected that *S. cerevisiae* has a preference for fructose not glucose. Since a relatively large experimental error was observed for the kinetic parameters of the transport steps it was analyzed whether a set of parameter values exists within the experimental error that would result in carbohydrate transport kinetics similar to those observed in the fermentations. For this the glucose and fructose fluxes during Fermentation 1.1 were used to calculate the ratios of glucose to fructose transport during the fermentation. Subsequently the ratio of the kinetic equations for glucose and fructose was fitted to the observed flux ratios, with the experimental errors as fitting constraints.

The kinetic equations:
Taking Equation 4.3.11 and 4.3.12 result in a ratio of v_{Glc}/v_{Frc} (assuming internal Glc and Fru concentrations that are negligible compared to the external

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concentrations, i.e.

$$Glc_{in}/Glc_{out} = Fru_{in}/Fru_{out} = 0) \tag{4.3.15}$$

then:

$$\frac{v_{Glc}}{v_{Fru}} = \frac{\frac{VmaxGlc \times Glc_{out}}{K_{mGlc}}}{\frac{VmaxFru \times Fru_{out}}{K_{mFru}}} \tag{4.3.16}$$

.

A constrained fit of this equation to the observed flux ratios resulted in the following set of parameter values: V_{maxGlc} = 0.282259 umol/min/mg dry weight, V_{maxFru} = 0.85402 umol/min/mg dry weight, K_{mGlc} = 14.3991 mM, and K_{mFru} = 72.7864 mM.

Note that this is not a unique solution as there are more solutions possible within the experimental error constrains that are equally good. The observed flux ratios can be quite accurately described with the fitted parameter values:

Table 4.3: Flux ratios within parameter constrains from experimental data.

t (h)	Jglc/Jfrc	vGlc/vFrc	[glc] (mM)	[frc] (mM)
6	1.44342	1.5187	494.46	543.94
12	1.39192	1.46793	494.46	511.69
22.5	1.32332	1.19055	449.59	398.7
28	1.03178	1.00865	195.33	323.54
32	0.902144	0.85697	132.4	258.11
38	0.686638	0.5673	56.571	166.6
46.5	0.062324	0.28192	6.0063	35.594

With the measured ratio of fluxes in the second column and the ratio of transport kinetic rates with the fitted parameter values in the third column. In the fourth and fifth column the glucose and fructose concentrations at the respective time points in the fermentation are given.

4.3.3.2 Modelling Wine Fermentation

The constructed model for wine fermentations was simulated with experimentally determined metabolite concentrations and growth rates of two indepen-

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dent fermentations (Fermentation 1.1 and Fermentation 1.2). This was done to test the ability of the model to model wine fermentation.

For use in the model, V_{max} values were converted to units of mmol/min/L-cytosol. Initial metabolite concentrations used in the simulation were taken from experimental data. Specific growth rates used were experimentally determined and initial total biomass in the bioreactor determined to be 0.8 g for the total volume (0.8 g/800 ml).

To access the ability of the model to accurately predict batch fermentations, experimental data and simulations are compared. Model simulation using input values from Fermentation 1.1 was compared to concentration changes over time of the real fermentation. This was also done for the simulation using input from Fermentation 1.2. The model was validated with the data from two fermentations to evaluate the ability of the model to model changes in fluxes due to changes in growth rates and external metabolite concentrations.

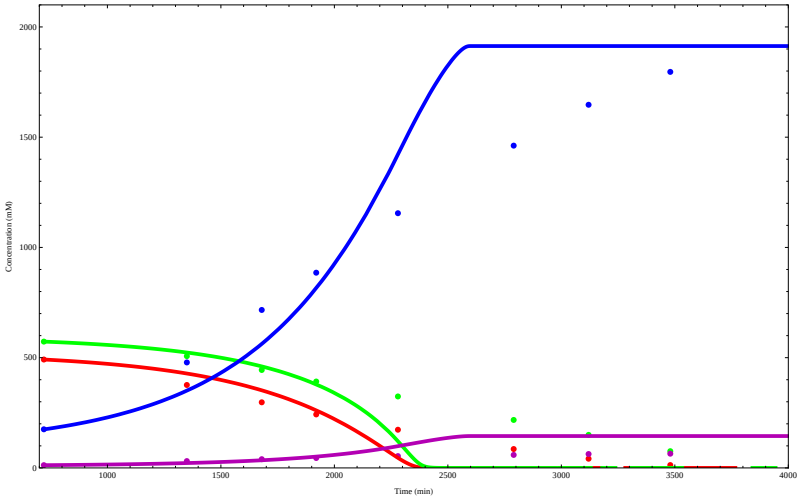

Figure 4.20: Comparing model predicted and experimental fluxes of Fermentation 1.1. Glucose (red), fructose (green), ethanol (blue) and glycerol (purple), with solid lines describing simulated results and dots experimental data.

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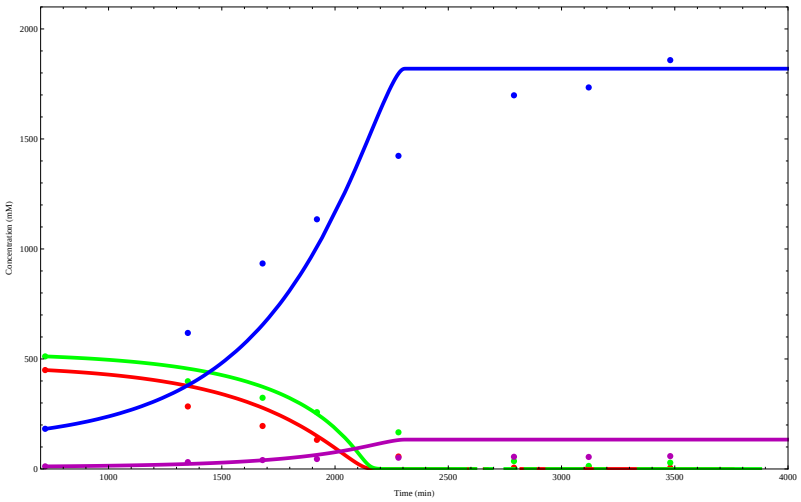

Figure 4.21: Comparing model predicted and experimental fluxes of Fermentation 1.2. Glucose (red), fructose (green), ethanol (blue) and glycerol (purple) with solid lines describing simulated results and dots experimental data.

From Figure 4.20 and 4.21 it can be observed that the changes made to the Teusink model enabled the wine fermentation model to describe batch fermentation dynamics. However, experimentally determined values and modelled determined values of flux did not exactly match-up. Although the overall trend was the same for the different metabolites, there were slight over or under estimations. Model predictions of ethanol concentrations were underestimated at the beginning of fermentation and later on slightly overestimated. Both glucose and fructose was depleted faster in the model simulation. The model also slightly overestimated glycerol production.

4.3.4 Mechanism of Control

We tested the glycolytic model more extensively in terms of the role of hexose transport and hexose phosphorylation in distinguishing between glucose and fructose. This was done by modelling a wine fermentation with equal concentrations of glucose and fructose as starting condition (Figure 4.22).

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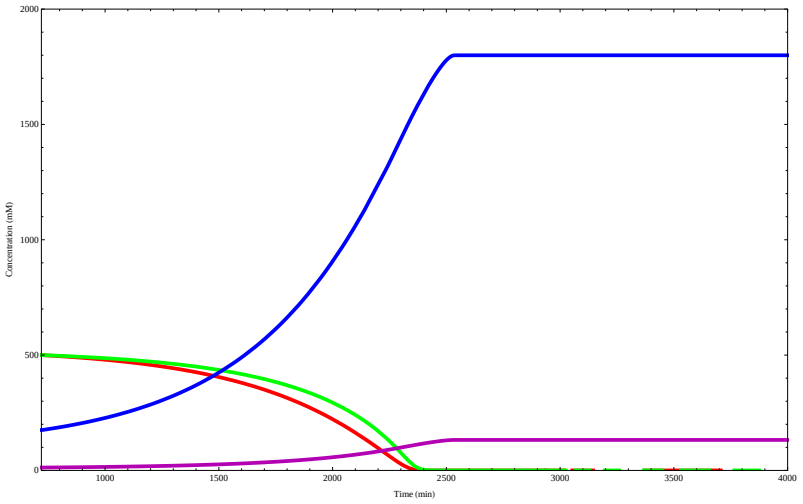

Figure 4.22: Modelling fluxes with equal glucose and fructose concentrations as starting condition. Glucose (red), fructose (green), ethanol (blue) and glycerol (purple).

Changing kinetic parameter values of the phosphorylation step to equal values for both glucose and fructose (K_m and V_{max} values) had no notable effect on the model (Figure 4.23). However, when the transport kinetics are changed to be the same for glucose and fructose transport (Figure 4.24) the effect is evident. After this change it is not possible to distinguished between the rates of glucose and fructose consumption. This points to the control over glycolytic flux residing in the hexose transport step.

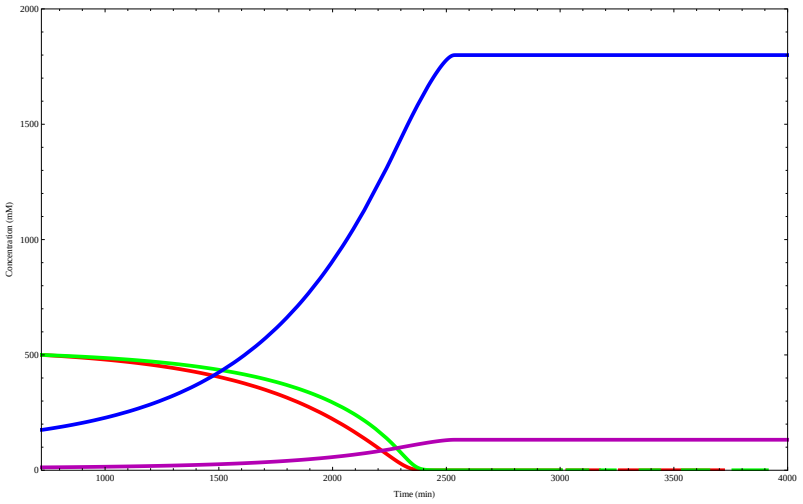

Figure 4.23: Modelling fluxes with equal glucose and fructose concentrations as starting condition. Enzyme parameters changed to same values for hexokinase. Glucose (red), fructose (green), ethanol (blue) and glycerol (purple).

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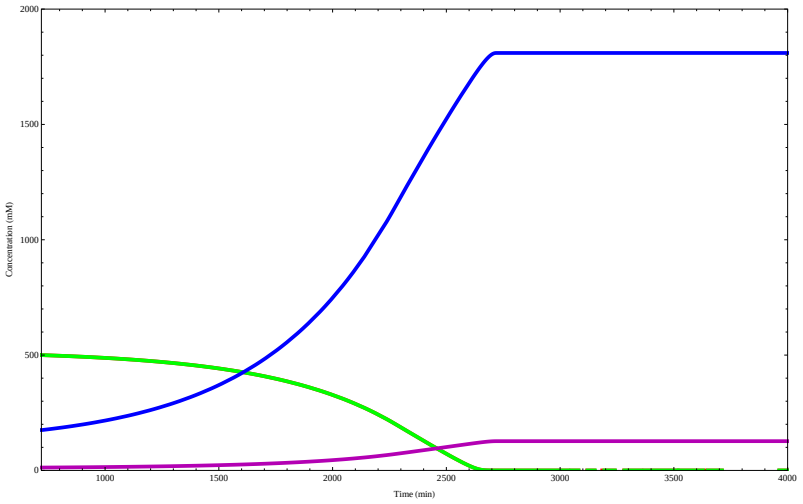

Figure 4.24: Modelling fluxes with equal glucose and fructose concentrations as starting condition. Transport parameters changed to same values for hexose transport. Glucose (red, behind green line), fructose (green), ethanol (blue) and glycerol (purple).

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Chapter 5

DISCUSSION AND CONCLUSIONS

This chapter discusses the three main areas of the Results chapter; wine fermentation; kinetic parameters and mathematical modelling. It gives a brief overview of the findings and a final conclusion on the project.

5.1 Wine Fermentations

To test the glucophilic character of the wine strains S. cerevisiae VIN 13 we performed a number of wine fermentations that function as our reference state. Analysis of the consumption and production rates of external metabolites confirmed the glucophilic character of the yeast; glucose was consumed at a faster rate than fructose.

Batch fermentations with 100% glucose or 100% fructose had more unexpected results. Firstly, both glucose and fructose were consumed at similar rates; i.e. when used as a single substrate there is no difference between consumption of glucose or fructose. The 100% fermentations were significantly slower than the 50/50 fermentations.

There were marked differences in fermentation time (to dryness) between different fermentations (even in duplicate experiments), and to be able to compare them we had to work with specific production and consumption rates. Even though the fermentations had different times to dryness, the specific consumption and production rates of all the different fermentations (50/50 and 100% glucose or fructose) was observed to be comparable. The 100% glucose or fructose fermentation may have taken twice as long to deplete all the consumable sugars, but the total biomass yield was only about half reached by the 50/50 fermentations. Consumption of all the sugars took longer because there are less yeast cells to consume the sugars. Why the yeast would not yield the same

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maximum biomass is unclear.

What is clear is that irrespective of the fermentation type, the specific flux through glycolysis is the same for all the cells. It is only in the case where glucose and fructose are both given to the cells simultaneously that glucose is consumed at a faster rate than fructose. Total sugar consumption for all the fermentations are the same.

5.2 Kinetic Parameters

The hexose transport step and phosphorylation step were kinetically characterized. Maximum velocities and affinities were determined with either glucose or fructose as substrates.

The determined values for the kinetic parameters for affinity determined are comparable to those published in literature (see Literature Review: Section 2.4.1 for comparison). The same trend of higher affinity for glucose is observed. Literature values are slightly less (lower K_m) compared to experimental values. However, these values are the first experimentally determined K_m and V_{max} values of hexose transporters determined for cells grown in synthetic wine must.

Kinetic parameters for the hexose transport showed a higher affinity for glucose, but much higher maximum transport rate for fructose. Analysis of the experimental data did however show high margins of error, especially for the determined values for fructose. The graph obtained for transport rate as a function of substrate concentrations (Figure 4.13) failed to reach a state where the graph plateaued. To be able to more accurately determine the parameter values higher substrate concentrations should have been added. Especially the estimated maximum rate of fructose could be an overestimation. Adding data points could lead to obtaining lower affinity and maximum transport rate values. This was however not done for this study as the cost of repeating the experiment was too high.

The phosphorylation step was also kinetically characterized with regards to either glucose or fructose as substrate. A study done by Berthels *et al* [16] on hexokinase kinetic properties in *S. cerevisaie* VIN 13 had similar findings for the affinity of the hexokinases for the different substrates. In the mentioned study hexokinases had a higher affinity for glucose (0.15 ± 0.01 mM) compared to fructose (1.09 ± 0.002 mM).

In this study the transport and phosphorylation steps were only kinetically characterized during the mid-exponential growth phase in yeast. It could be possible that the change in expression patterns of the different transporters and

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hexokinases during the fermentation stages could lead to different affinities and maximum rates. However, for this study changes in parameter values were not taken into account.

5.3 Mathematical Modelling

The original glycolytic model by Teusink *et al.* [90] was changed to describe batch wine fermentations. The mathematical model was adapted in such a way to distinguish between glucose and fructose as substrates during wine fermentations.

With the use of parameter values fitted to the initial glucose and fructose consumption rates during the fermentation, the model predicted the faster consumption of glucose over fructose. These fitted parameter values fall within the error margins of the experimentally determined values. The sensitivity of the model to the change on these parameters was demonstrated. While the original parameter value estimations did not succeed in predicting the faster consumption of glucose over fructose, it was shown that it is possible to model the faster consumption of glucose over fructose through manipulation of these values. The values that were used in the model still had a higher affinity for glucose and a higher maximum transport rate for fructose. Even though intuitively it would seem that the fructose would be consumed faster due to the higher transport rate, it is the higher affinity of the transporters for glucose that leads to the faster consumption of this sugar into the yeast cell.

The model was also validated by comparing predicted profiles with real batch fermentations. With input from real batch fermentation into the model (initial experimental conditions, biomass, growth rate) the expected consumption and production rates should be the same as the rates seen in real fermentations. Although the same trends were observed as with real batch fermentations, the predicted rates of the model was not exactly the same as the real fluxes. The model exceeded the real fermentation fluxes, completely consuming the sugars in a shorter time period.

The growth function of the model would be better modelled as an equation describing growth with regards to other metabolite concentrations, for example ethanol. This would be better than a piecewise function, as real fermentation growth does not follow a piecewise function. Observing real growth rates (in log scale) shows the steady decrease of the growth rate as fermentation transitions from the log phase to stationary phase.

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5.4 Conclusion

The main aim of this project was to establish the mechanism of control over glucose and fructose flux through glycolysis in wine fermentations. We tested the simplest hypothesis that differences in kinetic parameters for the two substrates were responsible for this difference, by estimating these kinetic parameters and adapting an existing model of yeast glycolysis to model wine fermentation.

The constructed model succeeded in predicting the faster consumption of glucose over fructose. It was established that the hexose transport step has the control over the glycolytic flux in the model.

Another aim of the project to investigate the effect of sugar type on fermentation profile revealed unexpected results. Irrespective of sugar type, specific total sugar consumption rates were the same for all fermentations. It is only when glucose and fructose are fermented together by yeast that glucose is consumed faster.

The model has the ability to be used as an engineering tool to improve the fructophilic character of the yeast by changing the transport parameter values. With such challenges as stuck and sluggish fermentation, finding new and innovative ways to study these problems and rectify them is possible.

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Appendices

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Appendix A

ENZYME KINETICS

A.1 Experimental kinetic data

Apart from the kinetic parameters of the transport and phosphorylation step determined for this study, measured kinetics were used as determined by Abrie *et al.* (Table A.1).

Table A.1: Experimentally determined V_{max} values of glycolytic enzymes by Abrie et al.

Enzyme	Normalized Vmax *mmol/min/L-cytosol*
Alcohol dehydrogenase	578
Aldolase	712
Enolase	196
Glyceraldehyde 3-phosphate dehydrogenase:	
Forward reaction	161
Reverse reaction	469
Glycerol 3-phosphate dehydrogenase	9
Pyruvate decarboxylase	174
Phosphofructokinase	1050
Phosphoglucose isomerase	276
Phosphoglycerate kinase	176
Phosphoglycerate mutase	579
Pyruvate kinase	580

A.2 Hexose Transport

Transport of glucose and fructose was modelled as a single step. Evaluation was done in Wolfram Mathematica 8 using the NDSolve function.

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Ordinary differential equations:

$$d[Glc_{out}]/dt = v_{transportglc} \tag{A.2.1}$$

$$d[Fru_{out}]/dt = v_{transportfru} \tag{A.2.2}$$

Rate equations:

$$v_{transportglc} = \frac{V_{maxGlc}([Glc_{out}])}{K_{mHXTGlc}\left(1 + \frac{[Glc_{out}]}{K_{mHXTglc}} + \frac{[Fru_{out}]}{K_{mHXTfru}}\right)} \tag{A.2.3}$$

$$v_{transportfru} = \frac{V_{maxFru}([Fru_{out}])}{K_{mHXTFru}\left(1 + \frac{[Glc_{out}]}{K_{mHXTglc}} + \frac{[Fru_{out}]}{K_{mHXTfru}}\right)} \tag{A.2.4}$$

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