USING FT-NIR SPECTROSCOPY TO AUTOMATE CONSTANT SUGAR CONCENTRATION FED-BATCH FERMENTATIONS WITH SACCHAROMYCES CEREVISIAE: EFFECTS ON CELLULAR VIABILITY AND KINETICS OF OSMOTIC STRESS ASSOCIATED METABOLITES

A Dissertation
Presented to the Faculty of the Graduate School
of Cornell University
In Partial Fulfillment of the Requirements for the Degree of
Doctor of Philosophy

by
Charles A Frohman
August 2013
During alcoholic fermentations with *Saccharomyces cerevisiae*, high sugar concentrations lead to growth inhibition or yeast lysis and cause stuck or sluggish fermentations. Even sublethal sugar concentrations stimulate a yeast hyperosmotic stress response and cause increased formation of various byproducts, including glycerol and acetic acid, and decreased product yields.

In order to avoid the problems associated with high substrate concentrations, fed-batch approaches are utilized in some industrial fermentation. However, this technique has not been applied to winemaking, even though the effects of ongoing climate change are causing grapes to be harvested with increasingly high sugar concentrations. Hence, this work focused on the engineering of an automated system for conducting constant, low sugar concentration fed-batch vinifications, and the effects of these fermentations on yeast metabolism and viability.

An initial manually maintained fed-batch fermentation revealed significant reductions (45, 81, and 52 %, respectively) in the final concentrations of glycerol, acetic acid, and
acetaldehyde and improved ethanol production kinetics and yeast viability, relative to a high gravity batch fermentation of the same juice.

A fully automated fed-batch system that uses FT-NIR spectroscopy-based control of fermentation broth sugar levels was then engineered. Calibrations for glucose, fructose, total sugar, and ethanol were created using over 200 natural and semisynthetic fermentation samples. When used to maintain a test fermentation at a sugar concentration of 45 g l\(^{-1}\), the system performed very well, keeping the concentration within 5 g l\(^{-1}\) of this value.

Automated fed-batch-produced wines confirmed earlier findings and demonstrated large reductions in glycerol and acetic acid concentrations relative to a high gravity batch fermentation, the latter of which was below the limit of detection (0.05 g l\(^{-1}\)). Simultaneously, fed-batch fermentations exhibited a 3.4-fold increase in final \(\alpha\)-ketoglutarate levels and modified concentrations of several aroma-relevant compounds.

Automated fed-batch fermentations also rapidly achieve and maintain high ethanol concentrations with minimal volume delivery, and thus may reduce susceptibility to contamination by spoilage bacteria.

An affordable and offline discontinuous fed-batch approach, where discrete additions of must are made to an active fermentation, may also allow for a lessening of the yeast osmotic stress response and decreased osmolyte formation.
BIOGRAPHICAL SKETCH

Charles Alan Frohman was born in Newark, New Jersey, USA on October 11, 1986. He was raised in Livingston, New Jersey, where he graduated from Livingston High School as salutatorian in 2005. Following his high school graduation, Charles enrolled at Brown University in Providence, Rhode Island, to pursue a Bachelor of Science in chemistry. While always interested in food and science, it was not until the summer between his sophomore and junior years of undergraduate study, when he participated in a summer internship in the Department of Food Science at Cornell University, that his fascination with this multidisciplinary field developed. Charles’ determination to pursue an advanced degree in food science was solidified during the fall semester of his junior year, when he studied abroad in Barcelona and personally experienced the intersection of gastronomy and chemistry at some of the world’s best restaurants. Following his undergraduate graduation in May 2009, Charles began his doctoral studies in the laboratory of Dr. Ramón Mira de Orduña, a professor of enology in Cornell University’s Department of Food Science in Geneva, NY. His research focused on the engineering of a FT-NIR spectroscopy-controlled system for conducting automated fed-batch vinifications with *Saccharomyces cerevisiae*, where very high gravity must is fed continuously to a low gravity grape must fermentation, and the effects of this platform on fermentation kinetics, ethanol yields, yeast viability, and by-product formation. Upon completion of his dissertation, Charles plans to pursue a career within the food science industry.
This work is dedicated to my parents, Judy and Larry Frohman, and my brother Daniel, for their unfaltering love and support, and for nurturing my interest in science from an early age. It is also dedicated in loving memory of my childhood dog, Scrappy-Doo, for all the happy moments I shared with her during her 17 amazing years of life.
ACKNOWLEDGMENTS

I would like to acknowledge my former adviser, Dr. Ramón Mira de Orduña, for his guidance and teachings during my first three years of study. I would also like to thank my current adviser, Dr. Gavin Sacks, for his remarkable scientific insight, impressive teaching abilities, and constant academic support. I am unspeakably grateful for all you have taught me about science and research, and for the many opportunities your advisership has afforded me. I also wish to extend my thanks to my biological and environmental engineering adviser, Dr. Larry Walker, for his excellent teachings on fermentation kinetics and yield coefficients, and my food science adviser, Dr. Randy Worobo, for his academic and emotional support. I would also like to acknowledge Dr. Olga Padilla-Zakour for her incredibly caring demonstration of support and guidance throughout my studies, and especially during my transition between advisers. I also wish to thank Michael Surgeary and Dr. Stephen Medlin of Bruker Optics, for helping me with many of the technical aspects associated with the FT-NIR spectrometer and the fermentation control scheme.

I am also extremely grateful of my past laboratory members, for their help in conducting and maintaining countless experiments, and for their humor and support, which allowed me to maintain my sanity during challenging times. Thank you to Nick Jackowetz, Alison Sudano, Michele Humiston, Erhu Li, Tatsuya Miyawaki, and Meng Ding. Thank you, too, to my closest friends, Calvin Tse, Anthony Wong, Jordan Winderman, Madeline Weiss, Carra Glatt, Austin Blonde, Chris Tyler, Kevin Neal, and Julia Beamesderfer, for all the loving and hysterical memories we have shared throughout the years, and for encouraging me to always pursue my dreams.

Very importantly, I would like to extend my most heartfelt thanks to my parents, Judy and Larry, and to my brother Daniel, for always supporting and believing in me. I love you all very much, and feel extremely fortunate to call you my family. I would also
like to thank the rest of my family, including Grandma Arlene and all of my other aunts, uncles, and cousins, as well as Auntie Joy, my feline and canine family members Phyllo and Lucy, and those members of my family who are no longer alive, for their constant love and encouragement. Finally, I wish to thank the love of my life, Joshua Hatcher, for teaching me that you are never too old to feel and act like a kid, and for always making me feel truly special.
# TABLE OF CONTENTS

## CHAPTER 1 - LITERATURE REVIEW

1.1. Introduction ........................................................................................................... 1

1.2. Glossary of Relevant Terms .................................................................................... 2

1.3. Fermentation Process Control ................................................................................ 5

  1.3.1. Winemaking and Process Control ...................................................................... 6

1.4. High gravity fermentations ...................................................................................... 7

1.5. The Hyperosmotic stress response of *S. cerevisiae* ............................................. 8

  1.5.1. Impact of Elevated Glycerol, Acetic Acid, and Acetaldehyde Levels in Wine .......................................................................................................................... 11

1.6. Winemaking and Climate Change .......................................................................... 12

1.7. Fed-Batch Fermentations ......................................................................................... 16

1.8. Sugar Monitoring Technologies .............................................................................. 17

  1.8.1. Physical Properties of Multicomponent Systems .................................................. 17

  1.8.2. Stoichiometric and Mathematical Approaches ....................................................... 19

  1.8.3. Chemical Properties and Reactivities of Target Molecules ................................. 20

1.9. Dissertation Objectives .......................................................................................... 30

1.10. Potential Impact of Research ................................................................................ 32

## CHAPTER 2 - CELLULAR VIABILITY AND KINETICS OF OSMOTIC STRESS ASSOCIATED METABOLITES OF *SACCHAROMYCES CEREVISIAE* DURING TRADITIONAL BATCH AND FED-BATCH FERMENTATIONS AT CONSTANT SUGAR CONCENTRATIONS ........................................................................................................ 47

2.1. Abstract .................................................................................................................... 47

2.2. Introduction ............................................................................................................. 48
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.3. Materials and Methods</td>
<td>50</td>
</tr>
<tr>
<td>2.3.1. Juice Composition</td>
<td>50</td>
</tr>
<tr>
<td>2.3.2. Fermentations</td>
<td>50</td>
</tr>
<tr>
<td>2.3.3. Sample Taking and Analyses</td>
<td>51</td>
</tr>
<tr>
<td>2.3.4. Replications and Statistical Analysis</td>
<td>53</td>
</tr>
<tr>
<td>2.4. Results</td>
<td>53</td>
</tr>
<tr>
<td>2.4.1. Sugars and Ethanol</td>
<td>53</td>
</tr>
<tr>
<td>2.4.2. Osmotic Stress Response-Related Metabolites</td>
<td>56</td>
</tr>
<tr>
<td>2.4.3. Yeast Viability</td>
<td>59</td>
</tr>
<tr>
<td>2.5. Discussion</td>
<td>61</td>
</tr>
<tr>
<td>2.6. Conclusions</td>
<td>63</td>
</tr>
<tr>
<td>2.7. Acknowledgements</td>
<td>63</td>
</tr>
</tbody>
</table>

CHAPTER 3 - A NIRS-BASED AUTOMATED SYSTEM FOR FED-BATCH FERMENTATIONS AT CONSTANT SUBSTRATE CONCENTRATIONS... 68

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1. Abstract</td>
<td>68</td>
</tr>
<tr>
<td>3.2. Introduction</td>
<td>69</td>
</tr>
<tr>
<td>3.3. Materials and Methods</td>
<td>74</td>
</tr>
<tr>
<td>3.3.1. Juice Composition</td>
<td>74</td>
</tr>
<tr>
<td>3.3.2. Training Fermentations</td>
<td>76</td>
</tr>
<tr>
<td>3.3.3. Semisynthetic Standards Preparation</td>
<td>77</td>
</tr>
<tr>
<td>3.3.4. FT-NIR Spectra Acquisition</td>
<td>78</td>
</tr>
<tr>
<td>3.3.5. Sampling and HPLC Analysis of Fed-batch Fermentations</td>
<td>79</td>
</tr>
<tr>
<td>3.3.6. Calibration</td>
<td>80</td>
</tr>
</tbody>
</table>
3.3.7. Fed-Batch Fermentation Automation Scheme ........................................ 82
3.3.8. Automated Fed-Batch Validation Fermentation .................................. 85
3.3.9. Statistical Analysis ............................................................................. 85
3.4. Results ..................................................................................................... 85
3.4.1. Spectra ................................................................................................. 85
3.4.2. Calibrations .......................................................................................... 92
3.4.3. Automated Fed-Batch Training Fermentation #1 .............................. 102
3.4.4. Automated Fed-Batch Validation Fermentation ............................... 104
3.5. Discussion ................................................................................................ 106
3.6. Conclusions .............................................................................................. 111
3.7. Acknowledgements .................................................................................. 111

CHAPTER 4 - METABOLISM OF SACCHAROMYCES CEREVISIAE DURING FED-BATCH ALCOHOLIC FERMENTATION WITH CONSTANT SUGAR CONCENTRATIONS .................................................................................................................. 118

4.1. Abstract .................................................................................................... 118
4.2. Introduction ............................................................................................... 119
4.3. Materials and Methods ........................................................................... 121
4.3.1. Juice Composition ................................................................................ 121
4.3.2. Fermentations ....................................................................................... 122
4.3.3. Sampling During Fermentations .......................................................... 123
4.3.4. Replications and Statistical Analysis .................................................. 126
4.4. Results ....................................................................................................... 126
4.4.1. Sugars and Ethanol................................................................................ 126
4.4.2. Osmotic Stress Response-Related Metabolites .................................. 129
4.4.3. Organic Acids

4.4.4. SO₂-Binding Compounds

4.4.5. Volatile Compounds

4.4.6. Yeast Viability

4.5. Discussion

4.5.1. Ethanol and Yeast Viability

4.5.2. Osmotic Stress Response-Related Metabolites

4.5.3. Organic Acids

4.5.4. SO₂-Binding Compounds

4.5.5. Volatile Compounds

4.6. Conclusions

4.7. Acknowledgements

CHAPTER 5 - METABOLISM OF SACCHAROMYCES CEREVISIAE DURING DISCONTINUOUS AND CONTINUOUS, AUTOMATED FED-BATCH ALCOHOLIC FERMENTATIONS

5.1. Abstract

5.2. Introduction

5.3. Materials and Methods

5.3.1. Juice Composition

5.3.2. Fermentations

5.3.3. Sampling and Analyses

5.3.4. Replications and Statistical Analysis

5.4. Results

5.4.1. Preliminary Fermentation of Cabernet Franc Juice
8.1.3. Offline Sugar Control Techniques ................................................................. 232

8.1.4. Time Maintained Under and Magnitude of High Gravity Conditions During Fed-Batch Fermentations ................................................................. 233

8.1.5. Separate Glucose and Fructose Feeds During Fed-Batch Fermentations 234


8.1.7. Sensory Testing of Batch and Fed-Batch Wines .................................. 236

8.1.8. Automation of Fed-Batch Malolactic Fermentation and Other Industrial Fermentations .................................................................................................. 237

8.2. General Conclusions ....................................................................................... 238
CHAPTER 1 - LITERATURE REVIEW

1.1. Introduction

Due to the combined effects of ongoing climate change and modified viticultural practices, grapes are being harvested with increasingly high sugar concentrations (Keller, 2009; Keller, 2010; Webb et al., 2007a; Webb et al., 2008). However, the fermentation of high gravity juices can present significant challenges. During batch vinifications, elevated sugar concentrations may result in growth inhibition and a significant decrease in yeast viability (Bai et al., 2004). Simultaneously, the alcoholic fermentation of high sugar containing media induces a hyperosmotic stress response in \textit{S. cerevisiae} (Erasmus et al., 2004; Ferreira et al., 2006) which results in increased synthesis of fermentation byproducts, including glycerol, acetic acid, and acetaldehyde (Erasmus et al., 2004; Kontkanen et al., 2004; Li & Mira de Orduña, 2011; Nurgel et al., 2004; Pigeau & Inglis, 2005a). While such a fermentation platform leads to decreased ethanol production yields (Bai et al., 2004; Cheng et al., 2009; Laopaiboon et al., 2007) and may contribute negative sensory attributes to a finished wine, acetic acid, itself, further decreases fermentation efficiency and yeast viability (Edwards et al., 1998; Edwards et al., 1999; Hunag et al., 2012; Rasmussen et al., 1995).

As such, the development of a fed-batch fermentation system, where media is continuously fed in at calculated rates so as to maintain low sugar concentrations throughout fermentation and thereby avoid incidence of an osmotic stress response, would be highly valuable. Such an approach is widely used in various bioindustrial fermentations (Bae & Shoda, 2004; Berraud, 2000; Kim et al., 1993; Kim et al., 1994;
Moeller et al., 2011; Nomura et al., 1989; Selvarasu et al., 2009; Wang & Shyu, 1997; Watteeuw et al., 1979), but has not seen previous application in the wine industry.

This dissertation focuses on the engineering of an automated FT-NIR spectroscopy-controlled fed-batch fermentation system, and its utilization to ferment high gravity juices at constant low sugar concentrations. The effects of this platform on fermentation kinetics, ethanol yields, yeast viability, and byproduct formation are discussed. The objectives of this project are to improve yeast viability and decrease the incidence of stuck or sluggish fermentations during the vinification of high gravity musts, and to modify yeast metabolic transformations in such a way as to obtain wine containing lower concentrations of compounds with negative organoleptic attributes, such as acetic acid.

1.2. Glossary of Relevant Terms

Due to their usage throughout this work, the definitions of several relevant terms are provided below:

- **Bioprocess**
  - The use of microbial, animal, and plant cells, and components of cells such as enzymes, to obtain desired products or destroy wastes (Doran, 2013)

- **Fermentation**
  - A type of bioprocess; may refer to:
    - 1) A process in which chemical changes are brought about in an organic substrate through the action of enzymes elaborated by microorganisms (Jay, 2000).
2) A mode of energy-yielding metabolism that involves a sequence of oxidation-reduction reactions in which an organic substrate and the organic compounds derived from that serve as the primary electron donor and terminal electron acceptor (Atlas, 1984).

- Alcoholic Fermentation
  - The anaerobic transformation of sugars, mainly glucose and fructose, into ethanol and carbon dioxide, with concurrent production of ATP via substrate level phosphorylation. The process is carried out by yeast and some bacteria such as Zymomonas mobilis, can be summarized by the overall reaction (Moreno-Arribas and Carmen Polo, 2009):

\[
C_6H_{12}O_6 \rightarrow 2 CH_3CH_2OH + 2 CO_2
\]

- Saccharomyces Cerevisiae
  - A species of yeast (eukaryotic microorganisms classified in the kingdom Fungi), abbreviated S. cerevisiae, commonly used in the winemaking, baking, and brewing industries. Along with a large variety of indigenous yeast species, commercial S. cerevisiae strains can grow and perform alcoholic fermentation in wine (Moreno-Arribas and Carmen Polo, 2009).

- Very High Gravity
  - A designation commonly used in the winemaking, brewing, and biofuel industries to describe fermentable medias that exhibit high specific
gravities due to high concentrations of suspended solids. In the winemaking industry, high gravity juices (musts) result from elevated sugar concentrations. Very high gravity technology is defined as “the preparation and fermentation to completion of mashes containing 27 g or more dissolved solids per 100 g mash (Thomas et al., 1993).”

- In the current work, the term “very high gravity” is used to refer to juices containing more than 300 g l\(^{-1}\) of sugars. The terms “high gravity,” “moderately high gravity,” and “normal gravity” are also used throughout as relative designations, and associated with stated sugar concentrations.

- Fed-Batch Fermentation
  - A fermentation strategy commonly used in bioindustrial processes in which a highly concentrated solution of growth-limiting substrate or nutrient is fed continuously or intermittently to an active culture without removal of the product. This technique allows for better control over substrate concentration variations and differentiation of growth, leading to improved overall process productivity (Shetty et al., 2006; Doran, 2013).
  - In the current work, fed-batch vinifications were conducted, whereby the rate at which high gravity juice was fed in to an active fermentation was controlled. This was achieved in two different ways:
    - Manual Fed-Batch
Manual fed-batch fermentations were conducted by regularly measuring the sugar concentration of an active fermentation, determining the instantaneous rate of sugar consumption based on adjacent measurements, and manually adjusting the output speed of a peristaltic pump delivering additional high gravity juice such that the sugar delivery rate matched the sugar consumption rate (Chapter 2).

- Automated Fed-Batch

  Automated fed-batch fermentations were achieved by combining in-line FT-NIR spectroscopy-based sugar measurements with a programmable peristaltic pump and process controllers (Chapter 3).

1.3. Fermentation Process Control

Monitoring and controlling fermentative bioprocesses greatly increases reproducibility and the early detection of problems, and is therefore essential to the success of most microbial cultivations. Different types of fermentations possess distinct priorities in terms of the process parameters that are to be controlled. Often, the consumption of the main substrate is monitored (e.g. sugars in brewing and winemaking or ethanol in acetifications), as well as the formation of the main metabolic product (e.g. ethanol in brewing and winemaking or citric acid/glutamic acid). In the majority of industrial fermentations, however, several of the most important production conditions are ideally measured and regulated. For example:
• Maintenance of an appropriate medium pH is integral to all fermentations (Arroyo-Lopez et al., 2009; Fang & Liu, 2002; Pena et al., 1972), and this parameter may be modified or controlled through the addition of acids and bases throughout the fermentation process.

• Proper culture temperature is essential for the growth behavior of organisms (Barata et al., 2008; Charoenchai et al., 1998; McVeigh & Morton, 1964) and thus has to be carefully maintained, with deviations from the target temperature being corrected through heating or cooling.

• Biomass should be monitored, either because it is the product itself, as in the production of baker’s yeast, or the catalyst of the product formation.

• The dissolved oxygen concentration is crucial to all aerobic fermentations, and very low levels may also be important in some otherwise anaerobic processes (Durner & Fischer, 2009; Gonzalez-del Pozo et al., 2010; Hansen et al., 2001), with deviations from the desired value being fed directly back into an aeration control scheme.

1.3.1. Winemaking and Process Control

Though the range of industrial fermentations encompasses many different sectors, those with relevance to the food area, including the vinification of grape juice to yield wine, as traditionally enacted by *S. cerevisiae*, are some of the most common, and yet, until recently, least well controlled fermentations. The production of wines is among the traditional food fermentations that have accompanied humanity since ancient times (Phillips, 2000). In fact, recent archeological excavations in Georgia have uncovered 8000-year-old ceramic jars that tested positive for wine residues (Keys, 2003).
Winemaking comprises several distinct steps and phases, including grape and must processing, the vinification processes *per se*, and post-fermentation operations, such as fining, stabilization, filtration and bottling. Interestingly, even though the history of winemaking extends back through 8 millennia, automated control features were implemented in the wine industry only in the second half of the 20th century and went along with the mechanization of grape and wine processing equipment, e.g. to control grape presses, separators and filters. More recently, temperature control has been identified as a crucial component of managing pre-fermentation grape treatments, fermentations and wine storage (Bader, 1997; Gerbaux et al., 2002; Ibeas et al., 1997; Marais et al., 1992; Ough, 1985; Tegmo-Larsson & Spittler, 1990). In the last few years, a growing number of systems have been offered that allow one to monitor the actual fermentation progress and direct vinification and wine quality. In spite of these advances, many wineries, especially in traditional vitivinicultural areas, continue to produce wines without any type of automated process control, for cultural or commercial reasons. Indeed, parameters commonly measured during most vinifications are usually limited to density (as an indirect measurement of sugar concentration), temperature, and in the case of malolactic fermentations, malic acid.

1.4. High gravity fermentations

For certain applications in the bioethanol and winemaking industries, it can be useful or necessary to ferment media that contain very high sugar concentrations. For example, during bioethanol production, the fermentation of high gravity media is needed to increase downstream efficiency, since distillation and waste costs per unit amount of ethanol produced are higher at low ethanol concentrations (Bai et al., 2008;
Thomas et al., 1996; Wang et al., 1999; Zacchi & Axelsson, 1989). In the wine industry, juices containing very high sugar concentrations may be intentionally used for the elaboration of certain specialty products, such as icewines, late harvest wines, or those made from botrytized grapes. In the production of dry wines, too, winemakers may choose to ferment high gravity musts for stylistic reasons or out of necessity as a result of climate change-mediated increase in grape sugar content.

1.5. The Hyperosmotic stress response of *S. cerevisiae*

However, the presence of high sugar concentrations during alcoholic fermentations can cause growth inhibition, loss of yeast viability, or yeast lysis (Bai et al., 2004) and be responsible for sluggish or stuck fermentations. Furthermore, even less severe conditions stimulate a strain dependent hyperosmotic stress response in *S. cerevisiae* (Erasmus et al., 2004; Ferreira et al., 2006). The metabolic implications of this phenomenon have been extensively studied during the vinification of high gravity juices used in the production of wines from *Botrytis cinerea* infected grapes (Bely et al., 2005) and the elaboration of ice wines from musts containing up to 350 g l$^{-1}$ of sugars. High sugar-induced osmotic stress was found to up-regulate glycolytic and pentose phosphate pathway genes (Erasmus et al., 2003), thereby leading to increased formation of fermentation by-products, including glycerol and acetic acid (Erasmus et al., 2004; Kontkanen et al., 2004; Nurgel et al., 2004; Pigeau & Inglis, 2005a), the latter of which exceeded 1.5 g l$^{-1}$ in some wines (Kontkanen et al., 2004; Nurgel et al., 2004; Pigeau & Inglis, 2005a).

Specifically, during high gravity fermentations, increased formation of glycerol results from heightened expression of the glycerol-3-phosphate dehydrogenase enzyme
encoded by GPD1. This enzyme catalyzes the key reaction in the glycerol synthesis pathway, the reduction of dihydroxyacetone phosphate (DHAP) to glycerol-3-phosphate, with concurrent oxidation of NADH to NAD$^+$. Synthesized glycerol serves as an osmoprotectant that helps maintain cell volume and fluid balance during conditions of high external osmotic pressure (by preventing the efflux of water from the cell into the environment) (Blomberg, 2000; Blomberg & Adler, 1989; Erasmus et al., 2003; Nevoigt & Stahl, 1997; Pigeau & Inglis, 2005a). In line with the above discussion, previous analysis of icewines, which are traditionally produced from very high gravity must, has identified elevated glycerol concentrations (Erasmus et al., 2004; Pigeau et al., 2002; Pigeau et al., 2007; Pitkin et al., 2002). Simultaneously, during conditions of osmotic stress, the increased formation of glycerol shifts the intracellular redox balance by creating an excess of NAD$^+$. To maintain NADH/NAD$^+$ redox balance, the NAD$^+$-dependent aldehyde dehydrogenase encoded by ALD3 is upregulated, resulting in increased formation of acetic acid via oxidation of acetaldehyde (Pigeau & Inglis, 2005a; Pigeau & Inglis, 2007; Pigeau & Inglis, 2005b). Indeed, monoseptic fermentations of previously sterile filtered musts (Ferreira et al., 2006; Kontkanen et al., 2004) have revealed that substantial amounts of acetic acid may be exclusively derived from $S.~cerevisiae$, and not contaminating lactic acid bacteria. A greatly simplified alcoholic fermentation diagram, which highlights the pathways responsible for glycerol and acetic acid production, is shown in Figure 1.1.
Figure 1.1 Simplified pathway highlighting the transformations responsible for the production of glycerol and acetic acid during the alcoholic fermentation of very high sugar-containing broths. DHAP, dihydroxyacetone phosphate; G3P, glyceraldehydes-3-phosphate; GPDH, glycerol phosphate dehydrogenase, encoded by GPD1; ACDH, acetaldehyde dehydrogenase, encoded by ALD3.
High sugar concentrations have also been shown to increase residual concentrations of acetaldehyde, the major carbonyl formed during alcoholic fermentation (Li & Mira de Orduña, 2011).

The metabolic shunting associated with such physiological yeast stresses causes a decrease in overall ethanol yields (Bai et al., 2004; Bai et al., 2008), and elevated levels of acetic acid and acetaldehyde may have a negative organoleptic impact on the finished wine. In addition, acetic acid, itself, has been shown to cause inhibition of yeast viability and decreased fermentation efficiency (Edwards et al., 1998; Edwards et al., 1999; Huang et al., 1996; Rasmussen et al., 1995). Furthermore, fermentation of high-sugar juice results in high ethanol concentrations, which induce different cellular behaviors and processes related to cell death, including the stress responses, changes in membrane fluidity and protein structure, and mRNA export from the nucleus (Sanchez et al., 1992; Takemura et al., 2004; van Voorst et al., 2006; You et al., 2003).

1.5.1. Impact of Elevated Glycerol, Acetic Acid, and Acetaldehyde Levels in Wine

Though glycerol is widely thought to have a positive impact on a wine’s mouthfeel, recent evidence suggests that the magnitude of this effect is strongly overestimated. Indeed, work by Yanniotis et al. (2007) and Noble and Bursick (1984) showed that in order to produce a detectable increase in perceived wine viscosity, a minimum addition of 25.8 g l⁻¹ glycerol is required. Therefore, at the concentrations regularly found in wines, which are usually below this value, glycerol has a negligible impact on both the viscosity and the perceived viscosity of wine. This finding has been confirmed by additional studies (Runnebaum et al., 2011). The primary effect of
glycerol on a wine’s organoleptic properties, then, is a slight increase in perceived sweetness (Noble & Bursick, 1984; Nurgel & Pickering, 2005; Yanniotis et al., 2007). Conversely, final acetic acid concentrations reached during very high gravity fermentations may be in the range of applicable legal limits for table wines in various markets (Anonymous, 2012). Acetic acid and ethyl acetate, which is formed via the esterification of the former with ethanol, can also contribute undesirable sensory characteristics to wines. The thresholds for acetic acid and ethyl acetate in white table wine were previously reported as 1.1 g l\(^{-1}\) and 0.17 g l\(^{-1}\), respectively (Corison et al., 1979). In ice wines, however, the detection threshold for acetic acid is approximately three times higher, at 3.185 g l\(^{-1}\), while that of ethyl acetate remains largely unchanged (Cliff & Pickering, 2006). In wines produced from very high gravity musts, then, ethyl acetate is more likely to provide off-aromas, and these may be solvent-like and reminiscent of the characteristic smell of nail polish remover.

Similarly, high concentrations of acetaldehyde, the final electron acceptor during alcoholic fermentation, may contribute unpleasantly strong grassy or oxidized aromas to wines. In addition, due to its ability to strongly bind sulfur dioxide (SO\(_2\)) and thereby reduce the antimicrobial and antioxidant properties of this compound, elevated acetaldehyde levels may necessitate increased additions of this wine preservative (Clarke and Bakker, 2004; Miyake & Shibamoto, 1993; Somers & Wescombe, 1982).

1.6. Winemaking and Climate Change

The effects of ongoing climate change have exacerbated winemaking problems and challenges caused by high sugar concentrations. Figure 1.2 shows the magnitude of
global temperature anomalies over the last 130 years and demonstrates a trend towards warmer temperatures (NOAA - National Climatic Data Center, 2013).
Figure 1.2 Magnitude of global temperature anomalies relative to a 100-year (1901-2000) historical period.
Studies have shown that such modifications to the world climate will affect and challenge wine production in all regions of the world in both a viticultural and oenological sense (Webb et al., 2008). Specifically, the formation of warmer viticultural regions will speed up the entire annual grape growth and development cycle, giving rise to earlier harvests. In Alsace, for example, mean annual temperatures increased 1.8 °C from 1972 to 2002 such that in 2002, there were 33 more days with a mean daily temperature above 10 °C compared with 1972, and harvest was 2 weeks earlier (Duchêne & Schneider, 2005; Mira de Orduña, 2010). Similar effects have been observed in viticultural regions throughout the world (Ganichot, 2002; Nemani et al., 2001; Sigler, 2008; Stock et al., 2005). Furthermore, various regional climate models have suggested that the magnitude of this climate change-driven phenomenon will intensify throughout the course of the 21st century (Lebon, 2002; Webb et al., 2007b).

An important implication of this observation is the dual impact of climate change on grape ripening temperature. In addition to higher temperatures caused by global warming, earlier maturation means that temperatures during ripening will increase even further, because grapes will ripen during an earlier, naturally warmer month (Webb et al., 2007a). In addition to accelerating grape maturation, higher growing temperatures do stimulate grape berry sugar accumulation, via increased photosynthesis and sugar transport, though this effect halts at suspended solids concentrations of 24-25 Brix.

Higher sugar concentrations are predominantly due to concentration by evaporative loss (Coombe, 1987; Keller, 2009; Keller, 2010). Consequently, the harvest of grapes
with very high sugar concentrations often results from the winemaker’s decision to delay harvest to optimize technical or polyphenolic and aromatic maturity (Mira de Orduña, 2010).

In addition to enhancing the osmotic stress response of *S. cerevisiae*, elevated must sugar concentrations lead to the production of high alcohol content wines. Between 1971 and 2001, for example, average alcohol levels in Napa Valley wines rose from 12.5 % to 14.8 % (v/v) (Vierra, 2004), with over 50 % of this trend being caused by climate variability and change (Jones & Goodrich, 2008).

1.7. Fed-Batch Fermentations

Considering the many problems caused by the fermentation of high sugar-containing media, the utilization of control strategies to continuously measure fermentation broth sugar concentrations and maintain low gravity conditions throughout alcoholic fermentations would be extremely useful and allow the aforementioned high sugar challenges to be avoided. This, in turn, would necessitate control of substrate feed rates, which has long been applied to the successful implementation of fed-batch fermentations, as used in the production of acetic acid (Berraud, 2000; Nomura et al., 1989), citric acid (Moeller et al., 2011), bacterial cellulose (Bae & Shoda, 2004), biodegradable thermoplastics (Kim et al., 1993; Kim et al., 1994), monoclonal antibodies (Selvarasu et al., 2009), ethanol (Wang & Shyu, 1997), and single cell protein (SCP) (Watteeuw et al., 1979). In a fed-batch fermentation, one or more nutrients or substrates is supplied continuously or intermittently to the fermentor (Park, 2004). Such an approach may improve cell densities and fermentation productivity (Berraud, 2000; Kim et al., 1994; Wang & Shyu, 1997).
Recently, the fed-batch approach has been applied to alcoholic fermentations by *S. cerevisiae* (Arndt & Hitzmann, 2004; Bideaux et al., 2006; Laopaiboon et al., 2007; Nilsson et al., 2002; Pham et al., 2010; Seo et al., 2009; van Kleeff et al., 1998), but the success of this strategy has been limited by the utilization of under-performing sugar measurement techniques. Therefore, applying the fed-batch platform to the vinification of very high gravity musts first requires that an appropriate method be identified for rapid, in-line measurement of fermentation broth sugar levels.

### 1.8. Sugar Monitoring Technologies

Over the last three decades, there has been considerable innovation in the field of fermentation analysis, with the development of new technologies and instrumentations, as well as advancements in the fields of spectroscopy, acoustics, mathematical modeling, and enzymatic analysis allowing for enhanced monitoring and control of fermentation parameters (Becker et al., 2007). An overview of these new methodologies, with a consideration of their unique advantages and disadvantages as a tool for rapid, in-line measurement of fermentation broth sugar levels, is presented in the following paragraphs.

#### 1.8.1. Physical Properties of Multicomponent Systems

1.8.1.1. Ultrasound

The application of ultrasound, in which a signal is emitted by a transducer towards a fluid of interest and then either captured by a second transducer or reflected back to the original device, to the real-time determination of fermentation broth sugar concentration has been suggested (Hoche et al., 2011; Krause et al., 2011a; Krause et al., 2011b; Schoeck & Becker, 2010; Sint Jan et al., 2008). While several different
strategies for the employment of this technique have been evaluated, they are all based on the general principle that dissolved sugar increases the density and decreases the compressibility of the fluid. These changes have opposing effects on the speed of sound waves. However, the latter effect is dominant, causing a significant rise in the speed of ultrasound. In some of these systems, it should be possible to distinguish between glucose and fructose concentrations using frequency dispersion measurements. In addition, ultrasound-based determination of fermentation sugars has the advantages of being very fast and inexpensive, requiring minimal sample preparation, and having no effect on the fermentation itself. Unfortunately, several important disadvantages significantly detract from the current usefulness of this technology. Measurements are temperature sensitive, thereby requiring the liquid being measured to be maintained at a precise temperature. Previous research on the ultrasonic determination of the sugar content in juices and drinks demonstrated that temperature must be controlled to +/- 0.1 °C to achieve a predictive accuracy of 0.5 % w/v (Contreras et al., 1992). In addition, to prevent acoustic coupling (e.g. impedance due to a barrier), the walls of the fermentation tank need to be acoustically isolated from other surfaces. Perhaps most importantly, however, the deposition of thin material layers under industrial conditions may cause changes in the signal amplitude and phase. Consequently, this technique would only work if tank surfaces were always perfectly clean and identical from run to run, and if buildup (i.e. due to yeast sediment or proteinaceous foam) did not occur. Furthermore, transfer of calibrations between fermentation vessels would not be possible due to differences in tank material densities, curvature, shape, and size.
1.8.1.2. Must Density and Osmotic Pressure

Other currently available systems for fermentation sugar analysis are based on must density or osmotic pressure measurements (El Haloui et al., 1988; Sablayrolles, 2009). Theoretically, these systems would allow the determination of sugar concentrations based on initial Brix values and fermentation progress. However, differences in yeast growth, transformation efficiency, and by-product formation render this impracticable.

1.8.2. Stoichiometric and Mathematical Approaches

1.8.2.1. CO₂ Mass Flow

Sugar concentrations can also be estimated by measuring CO₂ gas released during fermentation and applying stoichiometric conversion ratios (Kim et al., 1993; Sablayrolles, 2009; Suzuki et al., 1988). While this technique allows for on-line estimations of density, sugar, and ethanol concentrations and can be used to calculate instantaneous fermentation rates, it also fails to account for differences in yeast metabolism.

1.8.2.2. Kinetic and Growth Models

Several authors have attempted to track sugar concentration during active fermentations by devising complex kinetic and growth models (Hong, 1986; Hunag et al., 2012; Wang & Shyu, 1997). This information was then used to determine optimal feeding rates during ethanolic fermentations. While this approach “eliminates” the need for expensive technologies that may require frequent calibration or adjustment, many of these models assume that yeast growth is only limited by glucose...
concentration while disregarding the importance of nutrient availability and other factors. Mathematical models also assume a constant environment, whereas in reality fermentations are dynamic processes. Consequently, any unpredicted factor or change in the fermentation broth may result in an increased prediction error.

1.8.3. Chemical Properties and Reactivities of Target Molecules

1.8.3.1. Biosensors

Several biosensors for glucose detection have been created (Lidgren et al., 2006; Moeller et al., 2011; Phelps et al., 1995), most of which rely on an antibody or enzyme as the biological detection component and an amperometric or optical signal transducer. With regards to glucose measurements, the majority of biosensors work by using glucose oxidase (GOx) to oxidize glucose to hydrogen peroxide and D-glucono-δ-lactone, which then hydrolyzes to gluconic acid. In this reaction, the hydrogen peroxide formed is measured by the biosensor and correlated to a glucose concentration. The applicability of these sensors, however, especially to automated systems, is significantly limited by their short lifetime. In addition, a high percentage of biosensors utilize selective membranes which may clog, particularly when installed in turbid liquids.

1.8.3.2. Chemosensors

A variety of chemical sensors (termed “chemosensors”) selective for glucose detection have also been created. The majority of these glucose chemosensors are based on smart materials that have one or more easily measurable physical properties (i.e. volume, viscosity, conductivity, permittivity) that change in response to a chemical stimulus (Cai et al., 2004; Guan et al., 2005; Lee et al., 2004). Compared to
biosensors, chemosensors have the advantage of increased robustness (including sterilizability) and active lifetime. Contrarily, however, they demonstrate lower sensitivity and selectivity, which is required for accurate measurement and control of substrate levels. In addition, most existing glucose chemosensors can only reliably detect and quantify very low concentrations of sugar (< 25 g l\textsuperscript{-1}), well below those concentrations expected in certain high gravity fermentations.

1.8.3.3. Flow Injection Analysis

For bioprocess monitoring, flow injection analysis (FIA) remains one of the most important analytical techniques, because it can be coupled to multiple sensor types and therefore can be used to measure many different substrates, metabolites, and products (Becker et al., 2007). In terms of functionality, these units are similar to more traditional segmented flow analysis systems but do not inject air into the sample or reagent streams. Several FIA systems have been developed for on-line monitoring of glucose and other sugars during fermentations or in finished wines (Arndt & Hitzmann, 2004; Canizares-Macias et al., 2001; Karayanni-Tzouwara & Crouch, 1990; Rocha & Ferreira, 2002; Ulasova et al., 2003).

For sugars measurement, the automated FIA system mixes a precise volume of sample with known quantities of hexokinase, glucose-6-phosphate isomerase, glucose-6-phosphate dehydrogenase, ATP, and NADP\textsuperscript{+}. Hexokinase catalyzes the phosphorylation of glucose and fructose to form glucose-6-phosphate and fructose-6-phosphate, respectively. Glucose-6-phosphate isomerase then catalyzes the conversion of fructose-6-phosphate to glucose-6-phosphate. Finally, NADP\textsuperscript{+} oxidizes the total pool of glucose-6-phosphate to gluconate-6-phosphate, with concurrent
formation of the colored NADPH. Sugar concentration is then calculated via a measured increase in absorbance at 340 nm.

A common disadvantage of all FIA systems, however, is that they require frequent maintenance and adjustment, making automation difficult. Perhaps more importantly, however, complex sample solutions (e.g. fermentation broth) can significantly influence the detection reaction in the FIA system. For example, proteases excreted by certain cells can inactivate the biosensor component of some FIA systems. Furthermore, suspended solids present in the culture broth can sediment on the sensor, thereby changing its characteristics or blocking system flow. Additional pitfalls and limitations of the FIA method have been discussed in detail (Schugerl, 1993).

1.8.3.4. Spectroscopic Techniques

Perhaps the greatest advancements in fermentation monitoring technology have been in the field of optics, and specifically, in the realms of mid infrared and near infrared spectroscopy (MIR and NIR spectroscopy, respectively). Compared to other analytical techniques, spectroscopy offers the advantage of permitting rapid, non-invasive, non-destructive, continuous, and simultaneous multianalyte monitoring. In very general terms, IR spectroscopy characterizes molecules based on the way their functional groups absorb and respond to infrared radiation, by engaging in different vibrational modes. In terms of wavenumbers, the NIR portion of the electromagnetic spectrum covers the 12,500 – 4,000 cm\(^{-1}\) range, and the MIR region includes the 4,000 – 400 cm\(^{-1}\) range. When matter is exposed to IR radiation, the radiation can be absorbed, reflected, transmitted, scattered, or undergo photoluminescence. Analysis of the absorption spectrum of a given material or mixture can provide both qualitative and
quantitative information about its composition, depending on the particular technique employed (Burns and Ciurczak, 2013).

1.8.3.4.1. Mid Infrared Spectroscopy

The use of MIR spectroscopy for the analysis of wine parameters, including reducing sugars, has been demonstrated (Patz et al., 2004; Shen et al., 2011; Sivakesava et al., 2001). Importantly, absorption of electromagnetic radiation in the mid-infrared range causes organic molecule bonds to experience fundamental vibrational modes, which, in a MIR spectrum, is represented by the existence of sharp, distinct, characteristic absorption bands in the so-called “fingerprint” region. This facilitates the identification of molecules and simplifies matters of calibration. However, mid infrared radiation is a weaker source than NIR light, and therefore may be a suboptimal technique (i.e. yield a lower signal-to-noise (S:N) ratio) for the measurement of turbid or highly absorbing fermentation or wine samples (Urbano et al., 2005). In addition, water is strongly absorbing in the MIR region, so important spectral information may be overshadowed by high intensity –OH peaks in aqueous samples.

1.8.3.4.2. Near Infrared Spectroscopy

In contrast, if higher energy NIR radiation is absorbed by a molecule, then it may jump from the ground state to the 2nd or 3rd or 4th energy levels, which are termed the 1st, 2nd, and 3rd overtones, respectively (Figure 1.3). Combination bands, caused by the combination of different fundamental vibrations in the MIR region, are also possible. However, in order for a molecule to absorb in the NIR, it must also absorb in the MIR
(Ozaki et al., 2007; Osborne and Fearn, 1986). An overview of common NIR band absorbances is provided in Figure 1.4.
Figure 1.3 Energy diagram representation of absorbances in the MIR and NIR regions of the electromagnetic spectrum.
Figure 1.4 Table of common near infrared band absorbances (Siesler et al., 2002; Medlin et al., 2012a)
While NIR spectra contain a great deal of information, absorption bands are severely overlapped and nonselective (e.g. they do not correspond to the presence of specific functional groups). In addition, due to their higher energy requirements, NIR absorbances are much less intense than their corresponding MIR band absorbances. In general, the 1<sup>st</sup> overtone exhibits an absorbance intensity that is 10% of the primary absorption, and the 2<sup>nd</sup> overtone has an absorbance intensity that is 10% of that, or 1% of the original absorption (Burns and Ciurczak, 2013). Due to these challenges, a large pool of training samples and advanced chemometric modeling is required to develop robust and accurate calibrations. However, as a higher energy and therefore more penetrating light source, NIR spectroscopy more readily lends itself to the analysis of non-filtered wine and must samples. In addition, spectra acquisition via NIR spectroscopy can be achieved using long fiber optic cables, which may be directly inserted into a fermentation tank. Indeed, NIR sources are so strong that it can be used for the characterization of solids and powders (Siesler et al., 2002; Aldridge et al., 1994; Nicolai et al., 2007; Pogue et al., 2001).

1.8.3.4.2.1. Fourier Transform Spectrometers

Though dispersive NIR spectrometers are still produced, most modern units utilize Fourier transformation (FT) to devolve a sample interferogram into its associated absorbance spectrum. Traditional FT instruments depend on the presence of an interferometer, which allows for simultaneous measurement using the entire NIR spectrum. The classic Michelson interferometer consists of a beam splitter, a stationary mirror, and a moving mirror. When light from the NIR source hits the beam splitter, it splits 50:50, with half of the light hitting the fixed mirror and the other half
of the light hitting the moving mirror. The light then reflects off the mirrors, returns to the beam splitter, and recombines before passing through the sample and on to the detector. For every single wavelength of light that passes through the interferometer, depending on the instantaneous positioning of the moving mirror and the resulting path difference of the light, the recombined light may be perfectly in phase, perfectly out of phases, or somewhere in between. Thus, with an FT instrument, for each wavelength of light, a plot of signal amplitude vs. mirror displacement can be plotted. This is done very rapidly for the entire wavelength range, but if every one of these theoretical individual plots were added up, it would generate the sample interferogram, which is then mathematically transformed into the entire absorbance spectrum (Smith, 2011).

Compared to other infrared spectroscopy instruments, including those that utilize dispersive (scanning), filter, or diode array-based techniques, FT-NIR spectroscopy presents three primary advantages. Referred to as the Felgett Advantage, FT spectroscopy simultaneously collects light over the entire NIR region, whereas dispersive and filter spectroscopy focus on single wavelengths at a given time point and therefore mask most of the light. Consequently, FT spectroscopy results in an increase in S:N ratio and a decrease in scan acquisition time. The Jacquinot Advantage is used to explain the further increase in S:N ratio of FT spectroscopy as compared to dispersive spectroscopy, which uses slits to select for specific wavelengths of light, and therefore once again masks the majority of incoming light. Finally, FT spectroscopy uses the interferogram produced by a monochromatic HeNe laser to continuously measure the position of the moving mirror, thereby resulting in high x-
axis position certainty. This is referred to as the Connes Advantage (Medlin et al., 2012b; Saptari, 2004). The aforementioned advantages of spectroscopy-based measurement techniques, and in particular FT-NIR spectroscopy, identify this as an ideal method for rapid, in-line measurement of fermentation analyte concentrations.

1.8.3.4.2.2. FT-NIRS Calibration Development

Though a more robust and versatile analytical tool than most univariate analysis-based methods such as HPLC or enzymatic assays, FT-NIR spectroscopy and the associated development of strong multivariate calibrations require the use of a much larger pool of representative training standards. This is because, whereas univariate methods describe a single variable based on one measurable parameter (e.g. analyte concentration via peak height or area, as in HPLC), multivariate analysis involves the simultaneous consideration of more than one variable. In the case of FT-NIR spectroscopy, chemometric software is used to find a statistical correlation between a large amount of spectral information and the corresponding reference values of the training standards. While it would be considerably easier to build such calibrations using hydroalcoholic or sugar water solutions, successful development of FT-NIR spectroscopy calibrations depends on the use of highly representative standards that accurately reflect sample conditions (Conzen, 2006). Therefore, to develop a working method for fermentation analysis using FT-NIR spectroscopy, it is essential that the training samples be generated from similarly conducted fermentations. In addition, to prevent any collinearity effects caused by compounds whose concentrations are related, which would in turn result in highly erroneous predictions whenever process yields vary, it is highly beneficial to include semisynthetic samples, samples that have been
altered by the addition of varying quantities of the compounds of interest, in FT-NIR calibration models (Finn et al., 2006; Petersen et al., 2010; Riley et al., 1998).

1.9. Dissertation Objectives

While multiple studies have applied NIR and FT-NIR spectroscopy to fermentation analysis and monitoring (Cozzolino et al., 2006; Di Egidio et al., 2010; Manley et al., 2001; Niu et al., 2008; Scarff et al., 2006; Tosi et al., 2003; Yu et al., 2008), to the knowledge of the author, this powerful technique has not yet been used to control and direct alcoholic fermentation processes. This project proposes to study the effects of fed-batch vinifications with *S. cerevisiae* - where very high gravity must is fed to a continuously low gravity fermentation - on fermentation kinetics, ethanol yields, by-product formation, and yeast viability.

Initially, fed-batch fermentations were manually achieved by adjusting the output speed of a peristaltic pump delivering additional high gravity juice to an active fermentation, so that process sugar concentrations were maintained at a constant, low target value through vinification (Chapter 2). Samples were removed throughout the course of the fermentation and analyzed via HPLC for glucose, fructose, ethanol, glycerol, and acetic acid content. Yeast viability measurements were also obtained using flow cytometry. The results were compared to those obtained for normal and high gravity batch fermentations to determine the effects of the fed-batch platform on metabolite concentrations, formation kinetics, and yeast viability.

Based on its identification as an ideal technique for rapid fermentation metabolite analysis, FT-NIR spectroscopy was then used in combination with developed chemometric models and process controllers to establish in-line control loops
measuring both substrate (e.g. sugars) and product (e.g. ethanol) concentrations, and to maintain constant low gravity conditions with total sugar concentrations of 3 - 7 % (w/v). To develop calibration models, over 200 unmodified fermentation samples, obtained from batch and manual fed-batch vinifications, and semisynthetic standards, were utilized. Chapter 3 details the creation of these models, the optimization of the spectrometer’s configuration and acquisition settings for the obtainment of high quality spectra, and the utilization of the FT-NIRS with process controllers and appropriate equipment to create a fully automated fed-batch fermentation system. Chapter 3 also demonstrates the ability of this system to successfully maintain sugar levels at a target value during vinifications.

Chapter 4 used the NIRS controlled fermentation system to evaluate the effects of fed-vs. non-fed batch fermentations on yeast viability, fermentation kinetics, and byproduct formation patterns. The fermentation was carried out on Cabernet franc. In addition to the analytes and parameters measured in Chapter 2, this experiment also measured concentrations of volatile and non-acetaldehyde SO₂-binding compounds during fermentation, as well as the final concentrations of several organic acids.

The effectiveness of an offline and affordable discontinuous fed-batch fermentation strategy for decreasing osmotic stress response-related metabolism and improving yeast viability is investigated in Chapter 5. In this experiment, discrete additions of juice were made to an active fermentation at specific density readings. This strategy was compared to two separate automated FT-NIRS-directed fed-batch strategies. The addition of a volume-measuring ultrasound sensor to the must storage tank allowed for the determination of more precise process yield information.
Finally, Chapter 6 details additional experiments and analyses, including:

- Another discontinuous fed-batch fermentation, where sugar concentrations were rapidly spiked between 50 and 150 g L\(^{-1}\) multiple times, and the effects on yeast metabolism and viability were measured.
- An examination of the effects of various GO-FERM\textsuperscript{®} and yeast concentrations on yeast viability and osmolyte production during alcoholic fermentation with *S. cerevisiae*
- Thiol and sensory analysis of the Cabernet Franc wines discussed in Chapter 4.

This final chapter also describes the evolution of the automated fed-batch fermentation system, provides recommendations for its further improvement, and outlines recommendations for the continuation of this research.

1.10. Potential Impact of Research

The new fermentation strategies and control technologies are expected to have a positive impact in the fermentation of high gravity musts (hot climates, late harvest, and Icewines), by reducing the incidence of stuck fermentations and the formation of undesirable fermentation products. This technology should also result in greater process flexibility, since enhanced control of fermentation conditions through substrate feeding could equally be applied to a wider range of feedstock compositions without causing major system disturbances.
Reference List


75. Medlin, S., Surgeary, M., & DeVerse, B. (2012b). NIR training course day 1: it has begun. 1-156.


icewine fermentation and its relation to gene expression and metabolites specific to this wine. In Bachus To The Future - The Inaugural Brock University Wine Conference (pp. 177-178).


CHAPTER 2 - CELLULAR VIABILITY AND KINETICS OF OSMOTIC STRESS ASSOCIATED METABOLITES OF SACCHAROMYCES CEREVISIAE DURING TRADITIONAL BATCH AND FED-BATCH FERMENTATIONS AT CONSTANT SUGAR CONCENTRATIONS

2.1. Abstract

In alcoholic fermentations with Saccharomyces cerevisiae, high sugar concentrations lead to an osmotic stress response that increases the formation of by-products such as acetic acid, glycerol and acetaldehyde, as well as the risk of fermentation failures. This work compared the traditional batch fermentation of a high sugar containing grape juice with a fed-batch fermentation where the same juice was added at such rates as to keep sugar concentrations constant at 50 g/l during the fermentation.

The final ethanol concentrations reached were similar in both treatments, but higher ethanol formation rates were observed in the fed-batch fermentation. The kinetics of metabolite formation differed markedly, and significantly lower acetic acid, glycerol and acetaldehyde concentrations were observed after fed-batch fermentations. In addition, yeast viability levels as assessed by flow cytometry were higher during fed-batch fermentations.

The results demonstrate fundamental differences in the metabolite formation and reutilization patterns of osmotic stress response related key metabolites of alcoholic fermentation in batch and fed-batch fermentations. The implementation of fed-batch fermentations at constant substrate concentrations may be a suitable technique for increasing fermentation success and efficiency and decreasing byproduct formation in alcoholic fermentations by S. cerevisiae.
2.2. Introduction

During alcoholic fermentation (AF) by *Saccharomyces cerevisiae*, the central metabolic transformation is the conversion of sugars to ethanol and CO$_2$. Fermentation by-products such as glycerol, organic acids and carbonyls are formed in varying concentrations depending on the yeast strain and medium and fermentation conditions (Boulton et al., 1996). For some applications in the bio-ethanol or winemaking industries, it can be useful or necessary to apply fermentation media (mashes or juices) that have very high sugar concentrations. Such fermentations of high gravity media can increase downstream efficiency (Thomas et al., 1996; Wang et al., 1999) or be obligatory, e.g. in the production of specialty wines such as Icewine, and late harvest wines. In the wine industry, there has been a general trend towards higher gravity juices because of the combined effects of climate change and vineyard management and harvest decisions (Mira de Orduña, 2010; Webb et al., 2012).

However, high sugar concentrations in production media may lead to growth inhibition or yeast lysis (Bai et al., 2004) and be responsible for sluggish or stuck fermentations where yeast activity slows or completely stalls. Even sublethal sugar concentrations cause a hyperosmotic stress response in *Saccharomyces cerevisiae* (Ferreira et al., 2006). This has been well studied in the context of very high sugar containing juices used in the production of wines from *Botrytis cinerea* infected grapes (Bely et al., 2005) or the production of Icewines from grape juices presenting up to 350 g l$^{-1}$ of sugars. In such fermentations, high sugar stress was found to up-regulate glycolytic and pentose phosphate pathway genes (Erasmus et al., 2003) and lead to increased formation of fermentation by-products, including glycerol and acetic
acid, which exceeded 1.5 g l\(^{-1}\) in some wines (Kontkanen et al., 2004; Nurgel et al., 2004; Pigeau & Inglis, 2005a). High sugar concentrations have also been shown to increase production of acetaldehyde, the major carbonyl formed during AF (Li & Mira de Orduña, 2011). The formation of glycerol and acetic acid, but also carbonyl compounds leads to reduced ethanol production yields. In addition, acetic acid, itself, has been shown to cause inhibition of yeast viability and fermentation efficiency (Edwards et al., 1999; Ludovico et al., 2001; Rasmussen et al., 1995) and may lead to aromatic quality degradation.

In order to avoid inhibitory effects caused by high substrate concentrations, fed-batch approaches are utilized in some industrial fermentations. Their application can improve cell densities and fermentation productivity (Berraud, 2000; Kim et al., 1994; Wang & Shyu, 1997). The purpose of this work was to study the effect of low constant substrate levels during fed-batch alcoholic fermentations (AF) by \textit{Saccharomyces cerevisiae} on the kinetics of several osmotic stress response related metabolites. A Chardonnay must was used either without modification (224 g l\(^{-1}\) of sugar) as a control treatment, or with sugar added to high gravity conditions (343 g l\(^{-1}\) of sugar) to elicit a strong osmotic stress response. The high gravity musts were fermented as a traditional batch, or as a fed-batch by keeping sugar concentrations constant at 50 g l\(^{-1}\) for reduced osmotic stress. In addition to sugars and ethanol, the kinetics of glycerol, acetic acid and acetaldehyde were considered as representatives of the most important sugar alcohol, organic acid and carbonyl by-products of alcoholic fermentation.
2.3. Materials and Methods

2.3.1. Juice Composition

Flash-pasteurized Chardonnay juice from the Languedoc region of France (Kamil Juices, Canada) was used for all fermentations. Its sugar concentration was 223.8 g l\(^{-1}\) combined glucose and fructose, the pH was 3.25 and the titratable acidity was 9.94 g l\(^{-1}\) as tartaric acid. High gravity juice was prepared by adding equal quantities of ACS grade anhydrous D-glucose and D-fructose (Fisher Scientific International Inc, NH) to reach 343.3 g l\(^{-1}\) of combined sugars. For yeast nutrition, a complex supplement (Fermaid K, Lallemand, Canada) and ACS grade (NH\(_4\))\(_2\)HPO\(_3\) (Fisher Scientific International Inc, NH) were added at 0.25 g l\(^{-1}\). Following preparation, all juices were sterile filtered (0.20 μm nylon filter, Millipore, Ireland).

2.3.2. Fermentations

All fermentations were conducted in 5 L glass bottles (Kimble Chase, NJ) at 20°C. Containers were sealed with air locks (Buon Vino Manufacturing, Canada) to allow for fermentation gas release and to prevent air ingress. Both batch fermentations had a volume of 4 L. The fed batch treatment had a starting volume of 80 mL and was fed with high gravity must afterwards. Yeast were inoculated at 40 g hl\(^{-1}\) with respect to the starting volume using the enological *Saccharomyces cerevisiae* strain EC1118 (Lallemand, Canada). The active dry yeast were prepared according to manufacturer’s recommendations by rehydrating yeast and a complex yeast nutrient (GO-FERM®, Lallemand, Canada) for 15 minutes at 40°C in ASTM Class I water (Arium 611UV, Sartorius, Germany).
Traditional batch fermentations were conducted by adding the rehydrated yeast starter to the entire amount of juice. For the fed-batch fermentation, a small volume of high gravity juice was mixed with the starter to reduce the sugar concentration to 160 g l\(^{-1}\). Following inoculation, sugars were allowed to be consumed to 50 g l\(^{-1}\) (approximately for 100 h), after which point they were maintained at this concentration. For this purpose, regular sugar measurements were obtained by HPLC, and the data thus acquired was used to manually adjust the delivery rate of a peristaltic pump (Masterflex L/S, Cole-Parmer, IL) supplying additional high gravity juice. All fermentations were carried out at 20°C and regarded as finished when the sugar consumption was less than 0.5 g l\(^{-1}\) in 24 hours. In fed-batch fermentations, the sugar consumption rate was calculated from the feeding pump speed required to maintain the sugar concentration constant.

2.3.3. Sample Taking and Analyses

Samples were taken regularly under a constant stream of nitrogen to prevent air ingress and sample oxidation. Flow cytometric and HPLC analyses were carried out immediately. The remainder was immediately frozen at –18°C after separation of the biomass by centrifugation (5 min, 15,000 g) for subsequent analysis of acetaldehyde. Sugars, ethanol, glycerol, and acetic acid were analyzed with a high pressure liquid chromatography system (Shimadzu, Japan) consisting of a binary LC-20AB pumping unit, a DGU-20A3 degasser, a SIL-10AD VP autosampler, a CTO-20AC column oven, a SPD-M20A diode array detector, and a RID-10A refractive index detector after isocratic separation. Data acquisition and analysis was performed with the instrument software provided (LCSolution v.1.23). The mobile phase consisted of
ASTM Class I water with 1% (w/v) HPLC grade phosphoric acid and 5% (v/v) HPLC grade acetonitrile and was filtered prior to utilization (0.22 µm, nylon, Millipore, Ireland). After sample injection (5.0 µl), separation occurred at a flow rate of 0.35 ml min⁻¹ on a sulfonated polystyrene/divinyl benzene stationary phase with 9.0 µm particle size (250 x 4.6 mm, Supelcogel H, Sigma Aldrich, MO) with a corresponding 50 x 4.6 mm guard column (Supelguard C610H, Sigma Aldrich, MO), both of which were held at 60°C. Sugars, ethanol, and glycerol were quantified by refractive index while acetic acid was measured by UV spectroscopy at 210 nm. All analytes were quantified using external calibration standards.

Acetaldehyde was determined enzymatically using a commercial test kit (Megazyme, Ireland).

Total live yeast numbers were determined by flow cytometry (Thornton et al., 2002). For this, 0.5 ml samples were centrifuged for 7 minutes at 7,000 g. The clear supernatant was removed, and cell pellets were resuspended in a volume of sterile phosphate buffered saline equal to the culture supernatant removed. The buffer composition was 0.14 M NaCl, 2.7 mM KCl, 10.2 mM Na₂HPO₄·2H₂O, 1.8 mM KH₂PO₄, 1 mM EDTA and 0.2% w/v Pluronic-F68, pH 7.4. The resuspended cells were mixed, diluted 1:100 with the same buffer, and then stained by addition of 16.5 µL of 1.5 mM propidium iodide (PI) (Sigma Aldrich, MO) and 40 µL of 17 µM thiazole orange (TO) (Santa Cruz Biotechnology, Ca) to final assay concentrations of 41 µM PI and 1.1 µM TO, respectively. 50 µl of a counting bead solution (SPHERO™ AccuCount Blank Particles, 7.3 µm, Spherotech Inc, IL) were also added to each
sample as an internal standard for the quantification of cell numbers with a bench-top flow cytometer (Accuri C6, BD Biosciences, NJ).

2.3.4. Replications and Statistical Analysis

All fermentations were conducted in duplicate. Student’s t-tests and ANOVA were conducted with JMP 7.0 (SAS, North Carolina) to determine statistical significance of differences observed between sample populations at the 0.05 confidence level.

2.4. Results

2.4.1. Sugars and Ethanol

The traditional batch fermentation of non-modified Chardonnay juice (217 g l⁻¹ sugars) reached dryness after 332 hours (Figure 2.1). In contrast, both treatments with high gravity juice resulted in stuck fermentations, as expected from the juice sugar concentration (343 g l⁻¹), which corresponds to a theoretical ethanol concentration of 22.2% (v/v) (Boulton et al., 1996). Fermentation parameters for the three treatments are summarized in Table 2.1. While the high gravity batch and fed-batch fermentations reached similar final ethanol concentrations, the fed-batch treatment showed faster production kinetics, as evidenced by the maximum ethanol formation rate (Table 2.1).
Figure 2.1 Time course of sugar and ethanol concentrations in a traditional batch fermentation of Chardonnay juice (●, ▲) and high gravity batch (○, ▼) and fed-batch fermentations (●, ▲) of the same but high gravity juice. Average data of duplicate fermentations shown.
Table 2.1 Final sugar and ethanol concentrations in wines and maximum ethanol formation rates during fermentations. Average of duplicate measurements shown. The presence of different superscript letters indicates that the difference between averages is statistically significant at p=0.05.

<table>
<thead>
<tr>
<th></th>
<th>Fermentation duration (h)</th>
<th>Final Sugar (g L⁻¹)</th>
<th>Final Ethanol (% v/v)</th>
<th>Max. Ethanol Formation Rate (% v/(v·h))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Batch</td>
<td>308±12³⁺</td>
<td>3±0.01ᵃ⁺</td>
<td>12.2±0.01ᵃ⁺</td>
<td>0.091±0.01ᵃ⁺</td>
</tr>
<tr>
<td>High-Gravity Batch</td>
<td>666±24.5ᵇ⁺</td>
<td>70±0.98ᵇ⁺</td>
<td>14.8±0.29ᵇ⁺</td>
<td>0.045±0.002ᵇ⁺</td>
</tr>
<tr>
<td>High-Gravity Fed-Batch</td>
<td>543±10ᶜ⁺</td>
<td>50±0.67ᶜ⁺</td>
<td>14.9±0.10ᵇ⁺</td>
<td>0.070±0.007ᶜ⁺</td>
</tr>
</tbody>
</table>
2.4.2. Osmotic Stress Response-Related Metabolites

Figure 2.2 shows the kinetics of several fermentation by-products in batch and fed-batch fermentations of non-modified and high gravity Chardonnay juice. Overall, the formation patterns and final concentrations of glycerol, acetic acid and acetaldehyde were similar in the batch fermentation of non-modified Chardonnay and the fed-batch fermentation of high gravity juice. In contrast, final concentrations were considerably higher in the high-gravity batch fermentation (Table 2.2). The fed-batch fermentation of high gravity juice led to a 45% reduction of final glycerol levels relative to the high-gravity batch fermentation, and a 14% reduction relative to the batch fermentation of non-modified Chardonnay juice. In fed-batch fermentations, the amount of acetic acid initially produced was similar to the control fermentation with low gravity juice. In addition, acetic acid was reutilized once the feeding phase began. Accordingly, the final acetic acid levels were 50% lower than in wines produced by batch fermentation of non-modified Chardonnay juice, and 80% lower than in wines produced by batch fermentation of high gravity juice. Across all three treatments, acetaldehyde levels rapidly increased and reached a peak concentration within the first 70 hours of fermentations. Subsequently, partial re-uptake occurred, and concentrations decreased before reaching stable values between 200 and 400 hours. Peak and final acetaldehyde concentrations were similar in fermentations of non-modified Chardonnay juice and the fed-batch treatment of high gravity juice, but were twice as high in the batch fermentation of high gravity juice.
Figure 2.2 Time course of glycerol, acetic acid, and acetaldehyde concentrations in a traditional batch fermentation of Chardonnay juice (◊) and high gravity batch (◆) and fed-batch fermentations (×) of the same but high gravity juice. Average data of duplicate fermentations shown.
Table 2.2 Final glycerol, acetic acid, and acetaldehyde concentrations in wines and peak acetaldehyde levels during fermentations. Average of duplicate measurements shown. The presence of different superscript letters indicates that the difference between averages is statistically significant at p=0.05.

<table>
<thead>
<tr>
<th></th>
<th>Final Glycerol Concentration (g l⁻¹)</th>
<th>Final Acetic Acid Concentration (mg l⁻¹)</th>
<th>Peak Acetaldehyde Concentration (mg l⁻¹)</th>
<th>Final Acetaldehyde Concentration (mg l⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Batch</td>
<td>7.4±0.06ᵃ</td>
<td>390±15ᵃ</td>
<td>89±3ᵃ</td>
<td>23±1ᵃ</td>
</tr>
<tr>
<td>High-Gravity Batch</td>
<td>11.7±0.24ᵇ</td>
<td>1000±25ᵇ</td>
<td>173±8ᵇ</td>
<td>49±2ᵇ</td>
</tr>
<tr>
<td>High-Gravity Fed-Batch</td>
<td>6.4±0.08ᵃ</td>
<td>190±8ᶜ</td>
<td>83±4ᵃ</td>
<td>22±2ᵃ</td>
</tr>
</tbody>
</table>
2.4.3. Yeast Viability

Figure 2.3 shows the time course of viable yeast numbers in batch and fed-batch fermentations of non-modified and high gravity Chardonnay juice. Again, the batch fermentation of non-modified Chardonnay and the fed-batch fermentation of high gravity juice were similar. The viable yeast titers peaked at $4.7 \times 10^7$ (170 h) and $6.3 \times 10^7$ yeast/ml (160 h), respectively. In contrast, the maximum viable yeast titer reached during the batch fermentation of high gravity juice was $1.6 \times 10^7$ yeast/ml (408 h).
Figure 2.3 Live yeast titers during traditional batch fermentation of Chardonnay juice (○) and high gravity batch (□) and fed-batch fermentations (△) of the same but high gravity juice. Average data of duplicate fermentations shown.
2.5. Discussion

The current work compares the traditional batch fermentation of a high gravity medium with a fed-batch approach, where the same medium was added at such rates as to keep sugar concentrations constant during fermentations. Both fermentations were contrasted with a lower gravity control. Chardonnay grape juice and a commercial standard yeast for AF were used. Fundamental differences in the kinetics of metabolites and yeast viability between batch and fed-batch fermentation of high gravity Chardonnay were revealed.

Final ethanol concentrations were similar in batch and fed-batch fermentations of high gravity juice. However, the fed-batch approach resulted in a significantly higher ethanol formation rate. The elevated rate was associated with a higher live yeast concentration in the fed-batch treatment, as assessed by flow cytometric analysis. Nagodawithana et al. (1974) had previously shown that reduced sugar concentrations led to enhanced viability in AF by *S. cerevisiae*.

High salt or sugar concentration induced osmotic stress results in increased formation of glycerol by *S. cerevisiae* (Erasmus et al., 2003; Pigeau & Inglis, 2005a). Glycerol serves as an osmoprotectant that helps maintain cell volume and fluid balance during conditions of high external osmotic pressure (Blomberg & Adler, 1989; Nevoigt & Stahl, 1997). In Icewines, which are traditionally produced from very high gravity juice, glycerol concentrations may exceed 15 g l\(^{-1}\) (Erasmus et al., 2004; Pigeau et al., 2002; Pigeau et al., 2007; Pitkin et al., 2002). In the present study, the batch fermentation of high gravity juice led to similarly high concentrations exceeding 10 g l\(^{-1}\). However, the fed-batch fermentation of the same high gravity juice resulted in a
large decrease in glycerol production. This is in line with results obtained by Bideaux et al. (2006), who demonstrated that limited glucose concentrations led to greatly reduced final glycerol levels in alcoholic fermentations with *S. cerevisiae*.

Increased glycerol formation is associated with a concurrent increase in acetic acid production in *S. cerevisiae* (Michnick et al., 1997; Pigeau & Inglis, 2005a; Pigeau & Inglis, 2007; Pigeau & Inglis, 2005b). In this work, too, a high final acetic acid concentration (~1 g l⁻¹) was reached in the high gravity batch fermentation which is in the range of applicable legal limits for table wines in various markets (Anonymous, 2012). In contrast, fed-batch fermentations of high gravity juice led to significantly lower acetic acid production followed by partial reuptake. Metabolism of acetic acid by *S. cerevisiae* has been previously shown at low glucose concentrations (1-50 g/L) (Moreira dos Santos et al., 2003; Vilela-Moura et al., 2008). Ultimately, the fed-batch fermentation led to a five-fold reduction in final acetic acid levels relative to the high gravity batch fermentation.

Acetaldehyde, the final electron acceptor in the alcoholic fermentation pathway, is important to wine aroma and stability. High acetaldehyde concentrations necessitate increased additions of sulfur dioxide (SO₂) to ensure sensory acceptability and microbial and chemical stability (Boulton et al., 1996; Jackowetz et al., 2012). Previous research with resting cells of *S. cerevisiae* showed that high medium sugar concentrations result in elevated acetaldehyde excretion by yeasts (Li & Mira de Orduña, 2011). The results of the current study confirm these findings, and show that fermenting a high gravity juice in a fed-batch mode led to final acetaldehyde levels which were similar to those obtained with a lower gravity control.
This work demonstrates that implementing a feeding strategy which maintains constant sugar concentrations during the fermentation of high gravity juice led to increased live yeast numbers and reduced excretion of osmotic stress response related metabolites as compared with a traditional batch fermentation. The changes in the final metabolite concentrations observed are likely to have implications for wine chemical and microbiological stability and organoleptic quality. Achieving constant sugar concentrations during fermentations required frequent sample taking, measurement of sugar concentrations by HPLC and manual readjustment of the juice delivery pump. This method successfully maintained sugar concentrations within ±4 g l⁻¹ of the target value of 50 g l⁻¹, but such an approach may be impractical for industry. Accordingly, further developments will require a system capable of continuously measuring sugar and automatically adjusting the feeding rate. Such process automation would allow further evaluation of the technique and its suitability in vinifications and other industrial fermentations.

2.6. Conclusions

The application of a fed-batch technique that maintains constant sugar concentrations to the fermentation of a high gravity grape juice by *S. cerevisiae* revealed fundamental changes with regards to yeast viability and the production of osmotic stress response related metabolites. The application of this technology will benefit from process automation.

2.7. Acknowledgements

The authors wish to acknowledge financial support from the Nolan, Dyson and Canandaigua Wine Co. graduate support funds.
Reference List


CHAPTER 3 - A NIRS-BASED AUTOMATED SYSTEM FOR FED-BATCH FERMENTATIONS AT CONSTANT SUBSTRATE CONCENTRATIONS

3.1. Abstract

In the production of grape wines, high sugar concentrations present in musts from late harvest or ice wine grapes or those from hot climates lead to a hyperosmotic stress response in yeast, which increases the risk of sluggish and stuck fermentations and increases byproduct formation. The utilization of control strategies to continuously measure fermentation broth sugar concentrations and maintain low gravity conditions during alcoholic fermentations would allow the aforementioned high sugar challenges to be avoided. This work details the creation and external validation of FT-NIR spectroscopy calibration models for in-line quantification of glucose, fructose, total sugars, and ethanol in actively fermenting wine, and the utilization of the total sugars model with process controllers to engineer a fully automated system capable of conducting constant substrate-level fed-batch vinifications at a low target sugar concentration. Calibrations were created using a set of 240 natural and semisynthetic standards from fermentations conducted using varying concentrations of yeast and yeast nutrient. Calibration models for glucose, fructose, total sugars, and ethanol demonstrated $R^2$ values $> 0.93$ and RMSEP values of 12.3 g l$^{-1}$, 10.2 g l$^{-1}$, 11.6 g l$^{-1}$, and 0.328 % v/v, respectively. When used on a test fed-batch fermentation the automated system maintained sugar concentrations within 5 g l$^{-1}$ of the 45 g l$^{-1}$ setpoint. Calibrated for other substrates, the system can be used in other food and non-food fermentations, too.
3.2. Introduction

Typical batch alcoholic fermentations are performed at <27 % soluble solids, or roughly 16 % potential alcohol. In certain bio-industries, however, it is oftentimes useful or necessary to ferment very high gravity media that contain more than 27 % dissolved solids (Thomas et al., 1993). For example, in the production of bioethanol, the fermentation of broths containing highly elevated sugar concentrations may increase the efficiency of downstream processing, since distillation and waste costs per unit amount of ethanol produced are lower at high ethanol concentrations (Bai et al., 2008; Zacchi & Axelsson, 1989). Conversely, in the wine industry, high gravity vinifications may be obligatory, as in the elaboration of ice wines and other dessert wines produced from highly concentrated grapes, or they may result from stylistic decisions. As well, they may be necessitated by the climate change-driven increase in grape sugar content (Keller, 2009; Mira de Orduña, 2010; Webb et al., 2008).

While allowing for increased ethanol concentrations and more efficient distillation, very high gravity conditions can cause a number of physiological yeast stresses that decrease overall ethanol yields (Bai et al., 2004; Bai et al., 2008). Specifically, high sugar concentrations stimulate a strain dependent hyperosmotic stress response in *Saccharomyces cerevisiae* (Erasmus et al., 2004; Ferreira et al., 2006) which causes upregulation of the glycolytic and pentose phosphate pathway genes (Erasmus et al., 2003). The main result of this metabolic process is an increase in the formation of various fermentation byproducts, including glycerol and acetic acid (Frohman & Mira de Orduña, 2013; Pigeau & Inglis, 2005). For example, in the production of certain dessert wines, the yeast hyperosmotic stress response has been shown to lead to final
acetic acid concentrations above 1.5 g l\(^{-1}\) (Pigeau et al., 2002; Pigeau & Inglis, 2005). High sugar concentrations have also been shown to increase residual concentrations of acetaldehyde, the major carbonyl formed during alcoholic fermentation (Frohman & Mira de Orduña, 2013; Li & Mira de Orduña, 2011). Furthermore, even under less severe osmotic conditions, high gravity fermentations may lead to growth inhibition or yeast autolysis (Bai et al., 2004) and be responsible for sluggish or stuck fermentations.

To avoid challenges associated with high initial substrate concentrations, many industrial fermentations are conducted using a fed-batch approach, whereby a highly concentrated feed solution is intermittently or continuously fed to an active ferment (Park, 2004). Such a technique has previously been applied to the production of various fermentation-derived substances including acetic acid (Berraud, 2000; Nomura et al., 1989), citric acid (Moeller et al., 2011), and ethanol (Wang & Shyu, 1997). In general, utilization of a fed-batch technique allows for better control over substrate concentration variations and differentiation of growth, leading to improved overall process productivity and cell densities (Shetty et al., 2006; Doran, 2013; Wang & Shyu, 1997).

The benefits associated with conducting fed-batch vinifications have been previously demonstrated (Frohman & Mira de Orduña, 2013). By maintaining low sugar concentrations throughout fermentation, fed-batch fermentations achieved 81, 52, and 55 % reductions in the final concentrations of acetic acid, glycerol, and acetaldehyde relative to a high gravity batch fermentation of the same juice. However,
implementation of the fed-batch platform required continuous manual adjustments of
the juice feed rate, and as such, was too tedious for practical implementation.
Automation of substrate feed rates during wine fermentations could be achieved
through rapid, inline measurements of the two major fermentable sugars: fructose and
glucose. Previously described approaches for inline measurement of sugars suffer
from poor robustness, selectivity, or sensitivity. For example, biosensors (Lidgren et
al., 2006; Phelps et al., 1995) and chemosensors (Cai et al., 2004; Guan et al., 2005)
designed for in situ glucose measurements suffer from membrane fouling and poor
sensitivity and selectivity, respectively. Specifically, many chemosensors will exhibit
identical or similar responses to structurally related but chemically distinct species.
Other methods based on changes in fermentation broth density or osmotic pressure, or
on CO₂ gas formation (Kim et al., 1993; Sablayrolles, 2009; Suzuki et al., 1988), are
inexpensive and fairly simple to implement, but may be inaccurate due to differences
in production organism growth, transformation efficiency, and byproduct formation.
Recent work has demonstrated the potential application of ultrasound for rapid sugar
measurements (Krause et al., 2011; Schoeck & Becker, 2010; Sint Jan et al., 2008),
but this technique is temperature sensitive and requires complete acoustic isolation of
the system being measured, which would be challenging for an industrial setting.
Other proposed systems, based on flow injection analysis systems (Becker et al., 2007;
Schugerl, 1993; Ulasova et al., 2003) and complex mathematical models (Hunag et al.,
2012; Wang & Shyu, 1997) exhibit similar challenges. An overview of currently
available sugar monitoring technologies, as well as a brief summary of their specific
advantages and disadvantages, is provided in Table 3.1.
<table>
<thead>
<tr>
<th>Method</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Biosensors</strong></td>
<td>High selectivity</td>
<td>Short lifetime</td>
</tr>
<tr>
<td>(Lidgren et al., 2006; Phelps et al., 1995)</td>
<td></td>
<td>Possible membrane fouling</td>
</tr>
<tr>
<td><strong>Chemosensors</strong></td>
<td>High robustness</td>
<td>Lower sensitivity &amp; selectivity</td>
</tr>
<tr>
<td>(Cai et al., 2004; Guan et al., 2005)</td>
<td>Long lifetime</td>
<td>Small detectable concentration range</td>
</tr>
<tr>
<td><strong>CO2 mass flow</strong></td>
<td>Inexpensive</td>
<td>Determine sugar concentrations based on initial substrate levels and fermentation progress; lower accuracy</td>
</tr>
<tr>
<td><strong>Must density</strong></td>
<td>Simple</td>
<td>Differences in production organism growth, transformation efficiency, and byproduct formation increase estimation error</td>
</tr>
<tr>
<td><strong>Osmotic pressure</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Kim et al., 1993; Sablayrolles, 2009; Suzuki et al., 1988)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Ultrasound</strong></td>
<td>Inexpensive</td>
<td>High temperature sensitivity</td>
</tr>
<tr>
<td>(Krause et al., 2011; Schoeck &amp; Becker, 2010; Sint Jan et al., 2008)</td>
<td>Minimal sample preparation</td>
<td>Tank needs to be acoustically isolated and perfectly clean</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Calibration transfer between tanks impossible</td>
</tr>
<tr>
<td><strong>Flow injection analysis</strong></td>
<td>High versatility</td>
<td>Require frequent maintenance and calibration</td>
</tr>
<tr>
<td>(Becker et al., 2007; Schugerl, 1993; Ulasova et al., 2003)</td>
<td></td>
<td>Complex sample solutions can influence detection reactions</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Potential flow blockages</td>
</tr>
<tr>
<td><strong>Mathematical models</strong></td>
<td>No equipment needed</td>
<td>Assume production organism growth is limited only by substrate availability</td>
</tr>
<tr>
<td>(Hunag et al., 2012; Wang &amp; Shyu, 1997)</td>
<td>No calibration required</td>
<td>Assume constant environment</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Assume fermentation repeatability</td>
</tr>
</tbody>
</table>
Conversely, infrared spectroscopy, which characterizes molecules based on the way their functional groups (primarily O-H, N-H, C-H, and C=O bonds) absorb and respond to infrared radiation, by engaging in different vibrational modes, allows for rapid, non-invasive, non-destructive, continuous, and simultaneous multianalyte monitoring. In particular, near infrared spectroscopy (NIRS) uses penetrating high energy light and is thus better suited for analysis of highly turbid, unfiltered fermentation samples (Burns and Ciurczak, 2013). By combining NIR spectral information with chemometric techniques (e.g. partial least squares regression), NIRS allows for the measurement of compositional parameters of food systems that are not easily determined using conventional analytical techniques and chemical analysis (Ozaki et al., 2007; Osborne and Fearn, 1986).

While previous work has demonstrated the application of NIRS to the quantitative and qualitative analysis of finished or filtered wines (Cozzolino et al., 2006; Cozzolino et al., 2007; Manley et al., 2001), it has not been previously used for inline monitoring of active vinifications, or to control and direct fed-batch alcoholic fermentations.

The goal of the current work was to engineer a fully automated system for conducting fed-batch vinifications at constant, low, target sugar levels, using Fourier transform near infrared spectroscopy (FT-NIRS) in combination with developed chemometric models, process controllers, and automation software.

Robust calibrations for glucose, fructose, total sugars, and ethanol in turbid, fermenting wine were created by collecting representative spectra from multiple batch, non-automated and discontinuous fed-batch, and automated fed-batch fermentations. A relationship between spectral features and target analyte concentrations was
established through the application of chemometric methods, and the final total sugars model was combined with automation software and equipment to create a system for conducting automated fed-batch vinifications. Finally, the ability of the engineered system to maintain sugar concentrations at a low target value throughout fermentation was evaluated.

3.3. Materials and Methods

3.3.1. Juice Composition

Flash-pasteurized Chardonnay juice from the Languedoc region of France (Kamil Juices, Canada) was used for all training fermentations. For the validation fed-batch fermentation, unpasteurized Cabernet Franc juice obtained from grapes grown at the Cornell University Vineyards (Ithaca, NY) was utilized. In all cases, high and low gravity juice was prepared by adding equal quantities of ACS grade anhydrous D-glucose and D-fructose (Fisher Scientific International Inc, NH) or by diluting with ASTM Class I water (Arium 611UV, Sartorius, Germany), respectively. For yeast nutrition, a complex supplement (Fermaid K, Lallemand, Canada) and ACS grade (NH₄)₂HPO₃ (Fisher Scientific International Inc, NH) were added to all juices at 0.25 g l⁻¹. Following preparation, only the Chardonnay juice was sterile filtered (0.22 μm nylon filter, Millipore, Ireland). The final sugar concentrations of the juices used for the various training fermentations as well as the automated fed-batch fermentation used for validation are reported in Table 3.2.
Table 3.2 Chardonnay juice composition and fermentation parameters

<table>
<thead>
<tr>
<th>Fermentation</th>
<th>Sugars (g l⁻¹)</th>
<th>Chaptalized?</th>
<th>Water Added?</th>
<th>Yeast Inoculation (g hl⁻¹)</th>
<th>GO-FERM® (X Yeast Inoc.)</th>
<th>Rehydration H₂O (X Yeast Inoc.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Training Batch #1</td>
<td>310.2</td>
<td>Yes</td>
<td>No</td>
<td>40</td>
<td>1.25</td>
<td>25</td>
</tr>
<tr>
<td>Training Batch #2</td>
<td>121.2</td>
<td>No</td>
<td>Yes</td>
<td>40</td>
<td>1.25</td>
<td>25</td>
</tr>
<tr>
<td>Training Batch #3</td>
<td>223.8</td>
<td>No</td>
<td>No</td>
<td>40</td>
<td>1.25</td>
<td>25</td>
</tr>
<tr>
<td>Training Batch #4</td>
<td>217.3</td>
<td>No</td>
<td>No</td>
<td>40</td>
<td>1.25</td>
<td>25</td>
</tr>
<tr>
<td>Training Batch #5</td>
<td>334.4</td>
<td>Yes</td>
<td>No</td>
<td>40</td>
<td>1.25</td>
<td>25</td>
</tr>
<tr>
<td>Non-automated Fed-Batch Training</td>
<td>334.4</td>
<td>Yes</td>
<td>Yes</td>
<td>2200</td>
<td>1.25</td>
<td>25</td>
</tr>
<tr>
<td>Automated Fed-Batch Training #1</td>
<td>334.4</td>
<td>Yes</td>
<td>No</td>
<td>40</td>
<td>1.25</td>
<td>25</td>
</tr>
<tr>
<td>Automated Fed-Batch Training #2</td>
<td>368.4</td>
<td>Yes</td>
<td>No</td>
<td>400</td>
<td>0.125</td>
<td>10</td>
</tr>
<tr>
<td>Automated Fed-Batch Training #3</td>
<td>303.8</td>
<td>Yes</td>
<td>Yes</td>
<td>1200</td>
<td>0.042</td>
<td>10</td>
</tr>
<tr>
<td>Automated Fed-Batch Validation</td>
<td>261.1</td>
<td>Yes</td>
<td>No</td>
<td>1200</td>
<td>0.042</td>
<td>10</td>
</tr>
</tbody>
</table>
3.3.2. Training Fermentations

3.3.2.1. Batch Fermentations

All training batch fermentations were conducted in 2 L glass bottles (Kimble Chase, NJ) at 20°C. Containers were sealed with air locks (Buon Vino Manufacturing, Canada) to allow for fermentation gas release and to prevent air ingress. These fermentations were inoculated with yeast at 40 g hl\(^{-1}\) with respect to the starting volume using the enological *Saccharomyces cerevisiae* strain EC1118 (Lallemand, Canada). The active dry yeast were prepared according to manufacturer’s recommendations by rehydrating yeast and a complex yeast nutrient (GO-FERM®, Lallemand, Canada) for 15 minutes at 40°C in ASTM Class I water (Arium 611UV, Sartorius, Germany). The batch training fermentations were conducted by adding the rehydrated yeast starter to the entire amount of juice, and were allowed to go to completion.

3.3.2.2. Non-Automated Fed-Batch Fermentations

To generate additional training standards, a non-automated fed-batch training fermentation was conducted in a 2 L glass bottle at 20°C, and was initiated by diluting high gravity must with enough ASTM Class I water to lower the starting sugar concentration to 150 g l\(^{-1}\). The diluted juice was inoculated using EC1118 yeast at 2200 g hl\(^{-1}\), calculated with respect to the starting volume, and rehydrated as described above. Each time the sugar level dropped via yeast metabolism to a target lower threshold of 50 g l\(^{-1}\), additional high gravity must was poured into the fermentation to raise the sugar concentration back to 150 g l\(^{-1}\). This process was then repeated several more times, until metabolic activity halted.
3.3.2.3. Automated Fed-Batch Fermentations

Automated training fed-bath fermentations were conducted in an anaerobic 28 L cylindroconical stainless steel fermentation tank (Glacier Tanks, Oregon) at 20°C, utilizing EC1118 yeast concentrations of 40, 400, and 1200 g hl⁻¹, calculated according to the starting volume, and rehydrated as described above. For these fermentations, progressively updated calibrations for total sugar (calculated as the sum of glucose and fructose) were created using the FT-NIR spectroscopic data acquired from already completed training fermentations. The automated system was initiated, and must was pumped into the fermentation tank, where it combined with a rehydrated yeast slurry, until the sugar level reached 45 g l⁻¹, which was chosen as a decidedly low gravity (Brix) concentration. Fermentations were then maintained at this concentration via an engineered automation scheme for a determined period of time, and then allowed to go to dryness.

The wide range of yeast and nutrient concentrations utilized across all fermentations was chosen to simulate the large differences in matrix turbidity that could be encountered during fed-batch fermentations, and corresponded to a fermentation NTU range of 173 – 24,900. All fermentations were regarded as finished when the sugar consumption was less than 0.5 g l⁻¹ in 24 hours. A summary of the utilized fermentation parameters is found in Table 3.2.

3.3.3. Semisynthetic Standards Preparation

For the preparation of the semisynthetic training standards, previously isolated samples from the training batch fermentations were sterile-filtered (0.22 µm, nylon, Millipore, Ireland) and then spiked with varying quantities of ASTM Class I water,
ACS grade anhydrous D-glucose and D-fructose (Fisher Scientific International Inc, NH) powders, and 200 proof HPLC/Spectrophotometric grade ethanol (Sigma-Aldrich, MO). Additions were made in such a way as to cover a wide range of sugar and ethanol concentrations, and combinations thereof. Standards were then carbonated by purging them with carbon dioxide and/or combined with different quantities of rehydrated GO-FERM® and deactivated yeast to simulate real fermentation process conditions (e.g. carbonation and varying turbidity due to biomass formation, respectively).

Additional standards were generated by placing a 0.5 L of the filtered, dry (< 1 g l⁻¹ residual sugar), batch fermentation wine on a rotary evaporator (Rotavapor R-200, BUCHI, Switzerland) at 91.42 in KPa of pressure and 38°C for 5 hours. A volume of ASTM Class I water (Arium 611UV, Sartorius, Germany) equivalent to that of the isolated condensate was added back to the wine retentate, and dealcoholization was verified via HPLC. The dealcoholized wine was then combined with varying quantities of glucose, fructose, ethanol, GO-FERM® and deactivated yeast in order to create high sugar/high alcohol standards as well as low sugar/low alcohol standards, which would not normally occur in a traditional batch fermentation. A total of 56 semisynthetic samples was created.

### 3.3.4. FT-NIR Spectra Acquisition

All spectra were acquired using a Multi Purpose FT-NIR Analyzer spectrometer (Bruker Optics, MA) equipped with a 5m fiber optic cable and transflectance probe with a 2 mm fixed optical path length (1 mm slit) and a high-sensitivity InGaAs detector with a 12,500 – 4,000 cm⁻¹ detection range. The fermenting wine was directly
analyzed in an in-line mode, without any pretreatment. For the batch and non-automated fed-batch fermentations, the transflectance probe was manually submerged into the fermenting wine at regular intervals to acquire absorbance spectra. Spectra were also manually acquired for all semisynthetic samples. For the fed-batch training fermentations, the probe was installed directly into the fermentation system, and spectra were continuously obtained as part of the automated process, as described below. All scans were done over the instrument’s entire spectral range at a resolution of 16 cm$^{-1}$, and consisted of 1 minute of consecutive single scan spectra (~700), which were subsequently averaged. Sample and preamp signal gain settings were set to X1 and X30, respectively, and zero-filling factor was set to 8. The sample spectra were referenced against an air background spectrum that was collected immediately prior to the start of the fermentation.

3.3.5. Sampling and HPLC Analysis of Fed-batch Fermentations

For all batch and non-automated fed-batch training fermentations, whenever a spectrum was acquired, a corresponding sample was immediately isolated for later analysis. Samples were taken under a constant stream of nitrogen to prevent air ingress and sample oxidation. Samples from the fed-batch training fermentations were acquired at regular intervals from a sampling valve positioned below the liquid level of the tank. In both cases, after separation of the biomass by centrifugation (5 min, 15,000 g), the supernatant was immediately frozen at –18°C for subsequent HPLC analysis.

A high pressure liquid chromatography system (Shimadzu, Japan) consisting of a binary LC-20AB pumping unit, a DGU-20A3 degasser, a SIL-10AD VP autosampler,
a CTO-20AC column oven, a SPD-M20A diode array detector, and a RID-10A refractive index detector was used for isocratic separation and analysis of sugars and ethanol. Data acquisition and analysis was performed with the instrument software provided (LCSolution v.1.23). The mobile phase consisted of ASTM Class I water with 1% (w/v) HPLC grade phosphoric acid and 5% (v/v) HPLC grade acetonitrile and was filtered prior to utilization (0.22 µm, nylon, Millipore, Ireland). After sample injection (5.0 µl), separation occurred at a flow rate of 0.35 ml min⁻¹ on a sulfonated polystyrene/divinyl benzene stationary phase with 9.0 µm particle size (250 x 4.6 mm, Supelcogel H, Sigma Aldrich, MO) with a corresponding 50 x 4.6 mm guard column (Supelguard C610H, Sigma Aldrich, MO), both of which were held at 60°C (Frohman & Mira de Orduña, 2013). Sugars and ethanol were quantified by refractive index. Both analytes were quantified using external calibration standards. Eight standards were utilized for each calibration curve. Glucose and fructose standard concentrations ranged from 1 to 200 g l⁻¹, and ethanol standard concentrations ranged from 0.5 to 20 % (v/v).

3.3.6. Calibration

Separate PLS models were generated for the prediction of glucose, fructose, total sugars, and ethanol based on the collected FT-NIR spectra. Models were created using the chemometric model development platform in the OPUS software package (Bruker Optics, Germany). In developing the models, the wavenumber regions below 5000 cm⁻¹ and above 11100 cm⁻¹ were excluded due to the high contribution of spectral noise in these regions. In addition, the two predominant water absorption bands in the NIR spectral region, which are located in the 6600 - 7100 cm⁻¹ and
4800 – 5300 cm\(^{-1}\) ranges, were excluded to avoid negative affects caused by the gradual diffusion of ambient water vapor into the instrument. Spectral preprocessing was utilized to enhance spectral features, with options being limited to vector normalization (SNV), first derivative (Savitzky Golay, 17 smoothing points), and a combination of these two methods. Once these limitations were made, the software was allowed to automatically identify and exclude redundant spectra using a PCA factorization algorithm, and then to choose the wavenumber intervals, number of factors (rank), and pretreatment method so as to minimize the Root Mean Square Error of Cross Validation (RMSECV). Generated models were ranked by RMSECV, and for each analyte, several of the lowest RMSECV models were saved. Potential model outliers (automatically identified by the OPUS software) were viewed on a graph of Mahalanobis distance vs spectral residue. The spectra of samples presenting high values for both of these parameters were carefully analyzed, and removed from the model if they appeared to result from an erroneous measurement. In addition, spectral loadings were viewed and the model rank was decreased if a factor appeared to only contribute random noise to the calibration. The adjusted models were then tested against an external validation set consisting of 28 randomly selected samples which were excluded from the calibrations, and the best performing model (lowest Root Mean Square Error of Prediction; RMSEP) for each analyte was saved. As training fermentations were completed, all calibration models were progressively expanded and revalidated. The initial total sugars model was termed ‘A’ and the final and externally validated model, created using the obtained spectra and corresponding
HPLC data from all completed batch and fed-batch training fermentations, was named ‘B’.

3.3.7. Fed-Batch Fermentation Automation Scheme

Automated fed-batch fermentations are initiated by adding a rehydrated yeast slurry to the fermentation tank, where the fermentation liquid is continuously recirculated via a large external peristaltic pump (704 U/R, Watson-Marlow, England). A process file within the spectroscopic software package (OPUS 7.2, Bruker Optics, Germany) signals the MPA FT-NIR spectrometer to acquire and average approximately 700 single sample scans (sample scan time = 1 minute), and glucose, fructose, ethanol, and total sugar (glucose + fructose) are calculated using the aforementioned chemometric models. The predicted total sugar value is written to an OPC server (OPC Server, Advantech, CA) and sent to a programmable logic controller (PLC) (ADAM-5000/485, Advantech, CA) via an RS232 cable, which in turn sends a scaled 4-20 mA signal through a four-channel analog output module (ADAM-5024, Advantech, CA) to a PID controller (2216e, Invensys Eurotherm USA, VA). The PID controller compares the measured total sugar to the setpoint value and sends a scaled 4-20 mA output signal to a digitally programmable peristaltic pump (Masterflex L/S, Cole-Parmer, IL) to adjust the juice delivery speed and maintain constant sugar concentrations. This overall process continuously repeats until the control system is turned off and the wine is allowed to go to dryness, or until all of the must to be fermented has been delivered.

Fermentation temperature is maintained at the target value (20°C) using a similar control scheme. A 3-wire PT100 RTD probe (ProSense, Automation Direct, GA)
installed in the fermentation tank recirculation loop measures the fermentation temperature and transmits this to a second PID unit, which controls the positioning of a modulating electrically actuated ball valve (BI-TORQ, IL). When opened to varying degrees, the ball valve allows 15°C water to flow through a stainless steel cylindroconical coil installed inside the fermentation tank. To inhibit microbial growth, the juice tank is maintained at 2°C by circulating water of the same temperature through a similarly designed coil, without PID control.

The process and setpoint values for both PID units are continuously recorded by a data logging software (iTools OPC Scope, Invensys Eurotherm USA, VA) during all automated fermentations. This overall automation scheme is detailed in Figure 3.1.
Figure 3.1 Fed-batch fermentation automation scheme. Thick solid lines indicate liquid flow; thin solid lines indicate data flow; dotted lines indicate electrical (mA) signals.
3.3.8. Automated Fed-Batch Validation Fermentation

Following external validation of the final total sugars model, B, its performance was tested by conducting an automated fed-batch validation fermentation. The same general procedure used for conducting the automated fed-batch training fermentations was also applied to this fermentation. The system was allowed to raise the sugar content of the fermentation to the target setpoint of 45 g l\(^{-1}\). It was then maintained at this concentration for approximately 250 hours, at which point the pump was turned off and the fermentation was allowed to go to dryness.

3.3.9. Statistical Analysis

All fermentations were conducted in duplicate. Average values are displayed and reported. All duplicate measurements were within 5% of one another.

3.4. Results

3.4.1. Spectra

Sample spectra demonstrated a wide range of baseline absorbances, ranging from approximately \(-1\) to 1.8 (Figure 3.2). The higher baseline absorbance values were associated with the most visually turbid samples, which contained elevated concentrations of yeast and GO-FERM®. In contrast, the lower absorbance values are associated with fairly clear samples that contained low concentrations of yeast and nutrient, with the negative values being the mathematical result of an electronic amplification of the signal. A high degree of spectral noise can be seen at both extremes of the acquisition range, and the two main water absorption bands are centered around 5200 cm\(^{-1}\) and 6900 cm\(^{-1}\).
Figure 3.2 Sample spectra before preprocessing
Figure 3.3 shows the sample spectra following vector normalization. Vector normalization divides spectra by the square root of the sum of all squares of all Y-values, thereby “eliminating” height information that results from differences in sample turbidity or optical path length. As a result, the primary differences observed in the resulting processed spectra are structural in nature. Calculating the first derivative, which was used in combination with SNV for the fructose model, enhances pronounced but small spectral features by emphasizing signals with steep edges (Conzen, 2006).
Figure 3.3 Spectra following vector normalization
Figure 3.4 shows the sample spectra following vector normalization, and the utilized spectral regions for the total sugars calibration model. A summary of the different calibration models, including preprocessing options and calibration spectral regions, is detailed in Table 3.3.
Figure 3.4 Sample spectra following vector normalization with spectral regions for total sugars model shown. Empty regions were excluded from the model.
Table 3.3 FT-NIRS calibration model preprocessing technique and calibration regions

<table>
<thead>
<tr>
<th>Model</th>
<th>Preprocessing</th>
<th>Calibration Regions (cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>SNV</td>
<td>10229.4 – 9388.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8709.7 – 8115.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7683.6 – 7120.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6557.3 – 5323.0</td>
</tr>
<tr>
<td>Fructose</td>
<td>SNV</td>
<td>11031.7 – 7120.5</td>
</tr>
<tr>
<td></td>
<td>1ˢᵗ Derivative</td>
<td>6557.3 – 5647.0</td>
</tr>
<tr>
<td>Total Sugars</td>
<td>SNV</td>
<td>11031.7 – 10136.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9442.5 – 7120.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6557.3 – 5323.0</td>
</tr>
<tr>
<td>Ethanol</td>
<td>SNV</td>
<td>9442.5 – 7621.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6557.3 – 5647.0</td>
</tr>
</tbody>
</table>
3.4.2. Calibrations

Figure 3.5 shows the effect of calibration size (number of standards) on total sugar RMSEP, as tested via the external validation set. The initial model (A), which consisted of only 33 standards, performed poorly, demonstrating an RMSEP of > 25 g l\(^{-1}\). However, approximately doubling the number of standards drastically improved the RMSEP, decreasing it to 18 g l\(^{-1}\). The addition of all remaining standards generated from subsequent training fermentations resulted in a final RMSEP of 11.6 g l\(^{-1}\). With 242 training standards utilized in the final model (B), RMSEP has not asymptotically approached a lower limit, thereby suggesting the potential for further improvement via the inclusion of additional standards.
Figure 3.5 The effect of the number of training standards, N, on RMSEP for the total sugars model
The results of final PLS model calibration and external validation for the prediction of glucose, fructose, total sugars, and ethanol are shown in Figure 3.6. Cross- and external validation statistics for the created models are detailed in Table 3.4.
Fructose

Predicted Fructose (g/L) vs. True Fructose (g/L)

Predicted Fructose (g/L) vs. True Fructose (g/L)
Total Sugar
Figure 3.6 Results of final PLS model calibration and external validation for the prediction of glucose, fructose, total sugars, and ethanol
Table 3.4 Final FT-NIRS calibration model statistical parameters

<table>
<thead>
<tr>
<th>Model</th>
<th>N</th>
<th>Rank</th>
<th>$R^2$</th>
<th>RPD</th>
<th>RMSECV</th>
<th>RMSEP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (g L$^{-1}$)</td>
<td>235</td>
<td>8</td>
<td>93.17</td>
<td>3.83</td>
<td>10.1</td>
<td>12.3</td>
</tr>
<tr>
<td>Fructose (g L$^{-1}$)</td>
<td>236</td>
<td>7</td>
<td>93.25</td>
<td>3.86</td>
<td>10.7</td>
<td>10.2</td>
</tr>
<tr>
<td>Total Sugars (g L$^{-1}$)</td>
<td>242</td>
<td>8</td>
<td>97.23</td>
<td>6.01</td>
<td>13</td>
<td>11.6</td>
</tr>
<tr>
<td>Ethanol (% v/v)</td>
<td>236</td>
<td>4</td>
<td>98.80</td>
<td>9.14</td>
<td>0.534</td>
<td>0.328</td>
</tr>
</tbody>
</table>
In creating the calibration models, the OPUS software resulted in the election of a fairly high rank for all three sugar models. However, upon inspection of the spectral loadings, it became evident that the number of factors should be reduced for both the total sugars and fructose models (from 9 and 8, respectively) so as to prevent these calibration models from being over-fitted and including a high degree of spectral noise. Reducing the rank of both models was achieved without a significant increase in RMSECV (+0.5 and +0.0, respectively). A rank of 7 or 8 is still high but not unusual given the complexity of the sample matrix.

All four models demonstrated very high coefficients of determination (>0.93) and residual prediction deviation (RPD) values >3, the latter of which serves as a statistical indication of an acceptable model (Conzen, 2006). The higher $R^2$ and RPD values of the total sugars and ethanol models suggest a greater level of robustness and accuracy in these calibrations.

All three sugar models exhibited RMSECV and RMSEP values of approximately 10 g l$^{-1}$, which was considered satisfactory given the large range of concentrations being measured (0-360 g l$^{-1}$ for total sugars), and high variation in sample turbidity. Specifically, the average absolute prediction error of the final total sugars model (B) was greatest towards the lower (0-30 g l$^{-1}$) and higher (181-230 g l$^{-1}$) ends of the measurement range and smallest in the middle (121-150 g l$^{-1}$) (Figure 3.7).
Figure 3.7 Average absolute total sugar prediction errors at different concentration range for model B.
In addition, the external validation indicated a bias towards overprediction for glucose over the entire range of concentrations (average error = 7.2 g l$^{-1}$). However, a significant trend was not exhibited in the fructose, ethanol, or total sugar models (average errors = 1.9 g l$^{-1}$, -0.13 % v/v, and 1.1 g l$^{-1}$, respectively). The model for the prediction of ethanol utilized a much lower rank (4), and yet exhibited the highest $R^2$ and RPD values. This model predicted alcohol concentrations with a high degree of accuracy over the entire measurement range (approximately 0-20% v/v), indicating that the varying levels of turbidity in the calibration and validation samples did not negatively impact prediction ability.

3.4.3. Automated Fed-Batch Training Fermentation #1

To improve early calibration models, and to test the ability of the developed models to control sugar concentrations, automated fed-batch fermentations were conducted. Figure 3.8 shows total sugar and ethanol concentrations as a function of time for the first automated fed-batch training fermentation, conducted using model A. Due to the low number of utilized training standards and the high model RMSEP, the system performed very poorly, with some sugar levels being approximately 25 g l$^{-1}$ from the target setpoint of 45 g l$^{-1}$. The model also demonstrated a negative bias (actual = 65 g l$^{-1}$, model = 45 g l$^{-1}$).
Figure 3.8 Total sugar and ethanol concentrations, measured via HPLC, as a function of time for the first automated fed-batch fermentation, conducted using total sugars model A. Target sugar setpoint = 45 g l\(^{-1}\).
3.4.4. Automated Fed-Batch Validation Fermentation

The final calibration model (B) was tested by the performance of an automated fed-batch validation fermentation, the results of which are displayed in Figure 3.9. In this case, total sugar levels were maintained within 5 g l\(^{-1}\) of the target value of 45 g l\(^{-1}\). During the feeding phase of the fermentation, the average error of prediction was -0.45 g l\(^{-1}\).
Figure 3.9 Total sugar and ethanol concentrations, measured via HPLC, as a function of time for the validation automated fed-batch fermentation, conducted using total sugars model B. Target sugar setpoint = 45 g l\(^{-1}\).
3.5. Discussion

The current work details the creation and validation of FT-NIR spectroscopy-based calibration models for the quantification of glucose, fructose, total sugars, and ethanol in actively fermenting and turbid wine. It also demonstrates the application of the total sugars calibration model for automated control of constant substrate-level fed-batch wine fermentations.

A series of 5 batch and 4 fed-batch fermentations were conducted with sterile-filtered Chardonnay in order to generate a total of approximately 240 natural and semisynthetic calibration standards. Using chemometric model development software, the metabolite concentration data for these standards was combined with the obtained spectral data to generate and externally validate the calibration models. An automated fed-batch fermentation system was engineered, and the accuracy and robustness of the finalized model for total sugars was demonstrated through its ability to maintain the sugar concentration at a target concentration during a fed-batch fermentation with non-filtered Cabernet Franc juice.

NIR spectroscopy-based calibrations have previously been developed for the quantification of various compounds in wines, including sugars (Di Egidio et al., 2010; Manley et al., 2001; Niu et al., 2008; Urbano-Cuadrado et al., 2004). While many of the developed models demonstrated unsatisfactory statistical parameters, likely due to the exceedingly low concentration of the target analytes in wine, several of them exhibited low average prediction errors and high RPD values. However, all of the previously cited models were developed using filtered, centrifuged, or chemically clarified samples, or finished wines which likely underwent one of these treatments,
and consequently exhibited low turbidity levels. The creation of highly accurate calibration models is fairly straightforward and readily achievable under such conditions. A pre-experiment by the authors found that it was possible to create models for the quantification of glucose, fructose, total sugars, and ethanol in fermenting wines with $R^2$ values >0.999, RMSECV values <1 (<0.15 for ethanol), and RPD values >40 when 50 sterile-filtered fermentation samples were utilized (data not shown). Such models, however, have the limitation of only being useable for offline analysis.

In contrast, active fermentations exhibit a wide range of variability, including turbidity, which makes the acquisition of high quality spectra and the generation of accurate and robust models very challenging. In the current work, 33- and 55-fold differences in GO-FERM® and yeast concentrations, respectively, were used among the 8 training fermentations to build differences in sample turbidity into the developed models. Simultaneously, the FT-NIR spectrometer’s spectral acquisition settings were fine-tuned such that the highest possible quality spectra could be obtained.

Accordingly, sample spectra collection was achieved using a transflectance-type NIR spectroscopy probe, which has the benefit of simultaneously measuring transmitted and back-scattered radiation (Ozaki et al., 2007; Osborne and Fearn, 1986; VonBargen, 1996). Compared to test spectra recorded using a transmission probe, the transflectance probe spectra demonstrated a 1-log decrease in baseline absorbance levels and a significant reduction in background noise.

To further improve the signal to noise (S:N) ratio, sample scan time was set to 1 minute (approximately 700 scans), spectral resolution was adjusted to 16 cm$^{-1}$, and
sample signal and preamp gain were set as high as possible (X1 and X30, respectively) without saturating the detector (Cervera & Petersen, 2009). To ensure no loss of important spectral information, spectral resolution was virtually re-increased by upping the zero-filling factor from 2 to 8.

In addition to optimizing the FT-NIR spectrometer’s settings, the fermentation vessel had to be designed so as to allow for good quality spectra collection. Particulate or CO₂ bubble build-up on the transfectance probe head would result in unrepresentative spectra. To avoid such a problem, a recirculation loop was added to the system, and the transfectance probe was installed directly into this loop, such that fast-moving fermentation liquid continuously flushed the probe head and prevented build-up. The inclusion of a recirculation loop also ensured rapid and thorough mixing and minimized the time delay between feed addition and fermentation homogeneity during fed-batch fermentations.

Following instrument and tank adjustment, 9 separate training fermentations were conducted, and progressively updated total sugars calibration models were generated using the acquired fermentation and semisynthetic standard spectra. Previous work has demonstrated the importance of including semisynthetic samples in FT-NIR spectroscopy calibration models (Finn et al., 2006; Petersen et al., 2010; Riley et al., 1998) to prevent the creation of models based on compounds whose concentrations are correlated, which provide highly erroneous predictions whenever process yields are not constant.

As expected, the initial total sugars model (A) exhibited a very high RMSEP value and performed quite poorly. This may be attributed to the fact that the initial calibration
model contained very few (33) standards, and was therefore not robust enough to describe the matrix variation encountered during fed-batch fermentations.

In contrast, the finalized models consisted of approximately 240 standards and included spectra from several automated and continuous fed-batch fermentations. When the PLS algorithm was applied to the full set of spectra from all 9 training fermentations, acceptable models were created for all four compounds. Calibration model RPD values ranged from 3.83 (glucose) to 9.14 (ethanol), with the total sugars model (B) demonstrating an intermediate value of 6.01. All of these values are above the accepted screening limit (3), with 6.01 (total sugars) being above the limit for quality control (5) and 9.14 (ethanol) being beyond the cut-off for all analytical tasks (8) (Conzen, 2006). The number of factors was 4 for the ethanol model and 7 or 8 for the various sugar models. The lower number of variables for the ethanol model may be due to the more distinct ethanol absorption bands in the NIR region of the electromagnetic spectrum. While the number of variables utilized for the 3 sugar models is comparatively high, it is low in comparison to other developed sugar NIRS models (Arnold et al., 2003; Chung et al., 1996; Petersen et al., 2010; Urbano-Cuadrado et al., 2004) which may exhibit an increased risk of over-fitting due to the inclusion of noise features in the model. When tested via cross and external validation, RMSECV and RMSEP values were approximately 10 g l\(^{-1}\) for the sugar models and 0.5 % v/v for the ethanol model. The larger average absolute error (~ 12 g l\(^{-1}\)) exhibited by the final total sugars model (B) at the low end of the concentration range (0-30 g l\(^{-1}\)) may be explained by the approach of these values to the instrument’s limit.
of detection, which is generally accepted as 1 % for complex matrices (Niemoeller & Conzen, 2013).

The significant reduction in RMSEP value for the final total sugars model (B) as compared to its earlier iterations emphasizes the benefit of using a large number of representative standards to create robust and accurate calibrations. Although the non-asymptotic nature of the curve in Figure 3.5 suggests that it would be possible to further improve the total sugars model through the inclusion of additional training standards, the models and their associated statistical parameters were considered acceptable for the purpose of maintaining total sugar concentrations within a set point during fed batch fermentation. Using this calibration model in combination with our newly developed automated fed-batch fermentation system, we were able to maintain the total sugar concentration within +/- 5 g l\(^{-1}\) of a target value (45 g l\(^{-1}\)) during a fermentation. The robustness of this calibration model was further demonstrated by the fact that the validation fed-batch fermentation utilized red wine, whereas the calibration models were developed using all white wine samples.

By combining integrated process control with in-line sugar analysis, the FT-NIRS-based system presented herein enables the automation of constant substrate-level fed-batch vinifications. While NIRS is widely used as an analytical tool in various bioprocesses, its use in combination with automation modules to control and direct fermentations is still very limited (Berraud, 2000; González-Vara y R.A. et al., 2000; Macaloney et al., 1996; Navrátil et al., 2005; Tosi et al., 2003). To the authors’ knowledge, this is the first time NIRS has been used to control a fed-batch alcoholic fermentation process.
We have demonstrated that this system can be used to ferment an initially high gravity must (>280 g l\(^{-1}\) sugars) under low gravity conditions (<50 g l\(^{-1}\) sugars). This approach should be useful for avoiding the hyperosmotic stress response of *S. cerevisiae* may be lessened or avoided, and result in a significant reduction in the final concentration of various osmolytes and related metabolites, including glycerol, acetic acid, and acetaldehyde, and an increase in live yeast numbers (Frohman & Mira de Orduña, 2013). Adapted for the quantification of other metabolites, the described system would also allow for the automatic control of fed-batch malolactic fermentations or other industrial bioprocesses that depend on or benefit from the maintenance of low substrate levels.

3.6. Conclusions

The development of FT-NIRS calibration models for glucose, fructose, total sugars, and ethanol in turbid and actively fermenting wine was demonstrated. The FT-NIR spectrometer and associated total sugars model was combined with process controllers to create a fully automated system for conducting constant substrate-level fed-batch vinifications. By maintaining low gravity conditions during the fermentation of high gravity musts, the engineered system may prevent induction of the hyperosmotic stress response in *S. cerevisiae*.

3.7. Acknowledgements

The authors acknowledge financial support from the Nolan, Dyson and Canandaigua Wine Co. graduate support funds, as well as Stephen Medlin, PhD and Michael Surgeary, MS of Bruker Optics for their hardware and programming support.
Reference List


resins and cross-flow filtration in a fully automated pilot plant controlled via NIR. *Biotechnology and Bioengineering, 67*, 147-156.


54. VonBargen, K. P. (1996). Transfectance probe having adjustable window gap adapted to measure viscous substances for spectrometric analysis and
MD, USA.

fermentation of ethanol production by Zymomous mobilis. Bioprocess
and Biosystems Engineering, 17, 63-68.

between climate, winegrape price and winegrape quality in Australia.
Climate Research, 36, 89-98.

production of ethanol from dilute sugar solutions. Biotechnology and
Bioengineering, 34, 223-233.
CHAPTER 4 - METABOLISM OF SACCHAROMYCES CEREVISIAE DURING FED-BATCH ALCOHOLIC FERMENTATION WITH CONSTANT SUGAR CONCENTRATIONS

4.1. Abstract

During alcoholic fermentations, high sugar concentrations stimulate an osmotic stress response in Saccharomyces cerevisiae (S. cerevisiae) that increases byproduct formation and the risk of sluggish or failed fermentations. This work compared a traditional batch fermentation of a high sugar containing grape juice with a FT-NIRS-controlled automated fed-batch fermentation that used the same juice but was maintained at 45 g l\(^{-1}\) during the fermentation.

While final ethanol concentrations were similar in both treatments, the maximum ethanol formation rate was 13 % higher in the fed-batch fermentation. Metabolite formation patterns differed markedly, and significantly lower final concentrations of acetic acid (<-86 %), glycerol (-20 %), acetaldehyde (-67 %), acetoin (-67 %), 3-methylbutyl acetate (-25 %), 2-phenylethanol (-21 %), and several medium chain saturated fatty acid esters were observed after fed-batch fermentations. The fed-batch wine also exhibited higher final levels of lactic acid (+71 %), 2-methylbutylacetate (+142 %), 2-phenylethyl acetate (+48 %), and ethyl propanoate (+247 %).

In addition, yeast viability levels as assessed by flow cytometry were higher during fed-batch fermentations. The overall results demonstrate significant differences in the metabolite formation patterns of osmotic stress response-related metabolites and aroma compounds formed during batch and fed-batch alcoholic fermentations.
4.2. Introduction

*Saccharomyces cerevisiae* plays a preeminent role as a production organism in many fermentations, including the manufacture of biofuels, foods, and wines. For some of these applications, such as the production of bio-ethanol, it is advantageous to use high sugar-containing feedstocks in order to achieve low distillation and waste costs (Bai et al., 2008; Zacchi & Axelsson, 1989). In other industries, the fermentation of high gravity media may be conducted for stylistic reasons, as in the elaboration of certain specialty wines such as ice wine and late harvest wines. In the wine industry in general, very high gravity fermentations (> 27 % soluble solids) (Thomas et al., 1993) are becoming increasingly common due to the effects of ongoing climate change and viticultural decisions, such that grapes are harvested at higher sugar concentrations (Coombe, 1987; Keller, 2009; Mira de Orduña, 2010; Webb et al., 2008).

However, as the fermentation of high-sugar containing feedstocks becomes increasingly common, so may the occurrence of fermentation challenges or failures, as the traditional batch fermentation of high gravity mashes or juices stimulates a strain dependent hyperosmotic stress response in *S. cerevisiae* (Bai et al., 2004; Erasmus et al., 2004; Ferreira et al., 2006). In addition to causing growth inhibition or yeast autolysis, high sugar stress causes upregulation of glycolytic and pentose phosphate pathway genes (Erasmus et al., 2003) and increases formation of undesirable fermentation by-products including glycerol, acetic acid, and carbonyls (Frohman & Mira de Orduña, 2013; Li & Mira de Orduña, 2011; Pigeau & Inglis, 2005). While the enhanced synthesis of secondary metabolites decreases ethanol yields (Bai et al., 2004;
Cheng et al., 2009), elevated acetic acid concentrations may further reduce yeast viability and fermentation efficiency (Edwards et al., 1999; Rasmussen et al., 1995). To avoid the challenges associated with an osmotic stress response, juice or broth sugar concentrations may be maintained at low levels throughout fermentation by a fed-batch approach. Such an approach is commonly utilized in industrial fermentations, as in the production of acetic acid (Berraud, 2000; Nomura et al., 1989), citric acid (Moeller et al., 2011), and ethanol (Wang & Shyu, 1997). The fed-batch fermentation platform has also been applied to alcoholic fermentations by \textit{S. cerevisiae} (Arndt & Hitzmann, 2004; Bideaux et al., 2006; Laopaiboon et al., 2007; Seo et al., 2009; van Kleeff et al., 1998), but non-robust or poorly performing sugar control strategies have limited the success of this strategy (Chapter 3).

Frohman et al. (2013) showed that the utilization of a manually achieved fed-batch approach during the vinification of high gravity Chardonnay juice leads to 55 and 81% reductions in the final concentrations of glycerol and acetic acid, respectively, compared to a batch fermentation of the same juice. A 55% reduction in residual acetaldehyde levels was also observed. While this experiment demonstrated the advantages of fermenting under low gravity conditions, the approach presented therein is too tedious for application in a real winery setting due to the lack of automation.

Recently, an in-line FT-NIR spectroscopy-based system capable of maintaining constant low sugar concentrations during totally automated fed-batch fermentations was described and tested (Chapter 3). The engineered system continuously scans the fermenting wine to determine sugar concentrations in real time, which are then used to adjust the juice delivery speed so that total sugar levels remain constant at a low target
value. In contrast with other analytical techniques that can be used for control schemes, FT-NIRS does not require frequent recalibration, is a rapid and readily automatable technique capable of in-line analysis, has minimal consumables, and can be used for the simultaneous quantification of multiple parameters. Furthermore, the strong NIRS light source allows for the analysis of turbid samples, so that sample preparation is unnecessary (Conzen, 2006; Osborne and Fearn, 1986).

The purpose of the current work was to use the FT-NIRS-based system to conduct automated fed-batch alcoholic fermentations of a high gravity wine must (Cabernet franc, 286 g l\(^{-1}\)) maintained at constant low sugar concentrations and compare them to a traditional batch fermentation. Yeast viability, fermentation kinetics, and the kinetics and production of osmotic stress response related metabolites, organic acids, SO\(_2\)-binding compounds, and several volatile compounds were monitored.

### 4.3. Materials and Methods

#### 4.3.1. Juice Composition

Cabernet Franc juice from the Cornell University Vineyards (Ithaca, NY) was used for all fermentations. Whole clusters of previously frozen grapes from the 2007 harvest were partially thawed and pressed on their stems using an 80 L inflatable bladder press to yield a juice with a sugar content of 19.9 °Brix, measured via refractometry (Atago 3415 WM-7, WA). Equal quantities of ACS grade anhydrous D-glucose and D-fructose (Fisher Scientific International Inc, NH) were then added to the juice to raise the sugar concentration to 286 g l\(^{-1}\), as measured via HPLC. The pH of the adjusted juice was 3.97 and the titratable acidity was 5.75 g l\(^{-1}\) as tartaric acid. For yeast
nutrition, a complex supplement (Fermaid K, Lallemand, Canada) and ACS grade 
\((\text{NH}_4)_2\text{HPO}_3\) (Fisher Scientific International Inc, NH) were added at 0.25 g l\(^{-1}\).

4.3.2. Fermentations

All fermentations were conducted in duplicate in an anaerobic 28 L cylindroconical 
stainless steel fermentation tank (Glacier Tanks, Oregon) at 20°C, using the enological 
\textit{Saccharomyces cerevisiae} strain EC1118 (Lallemand, Canada) at a concentration of 
1.35 g l\(^{-1}\), calculated according to the final volume (11.5 L). The active dry yeast were 
prepared according to manufacturer’s recommendations by rehydrating the yeast and a 
complex yeast nutrient (GO-FERM®, Lallemand, Canada) for 15 minutes at 40°C in 
ASTM Class I water (Arium 611UV, Sartorius, Germany).

Traditional batch fermentations were conducted by adding the rehydrated yeast starter 
to the entire volume of juice. For the fed-batch fermentations, the yeast starter was 
added directly to the fermentation tank, and the automated system was initiated so that 
high gravity must was added to the tank until the setpoint of 45 g l\(^{-1}\) total sugars was 
achieved.

Automation of must delivery during fed-batch fermentations was achieved using a 
previously described FT-NIR spectroscopy-based system (Chapter 3). Briefly, a 
transflectance probe with a 2 mm fixed optical path length (Bruker Optics, MA) 
installed in the fermentation tank continuously scans the fermenting wine and 
quantifies total sugar and ethanol values by processing the acquired spectra against 
previously generated calibration models. The predicted total sugar value is compared 
to the target concentration, and the output speed of a peristaltic pump (Masterflex L/S, 
Cole-Parmer, IL) delivering additional fresh, high gravity must is adjusted accordingly
by a programmable logic controller (2216e, Invensys Eurotherm USA, Virginia). Must
delivery ends when the total volume of juice to be fermented has been delivered to the
tank. Both the fed-batch and the traditional batch fermentations achieved temperature
control using a second programmable logic controller which controls the flow of cool
(15°C) water through a stainless steel immersion coil submerged in the fermentation
volume. To ensure an oxygen-free environment, fermentations were maintained under
3 PSI of nitrogen gas at all times. All fermentations were regarded as finished when
the sugar consumption was less than 0.5 g l⁻¹ in 24 hours.

4.3.3. Sampling During Fermentations

Samples were taken at regular time intervals from a sampling valve at the bottom of
the tank. Flow cytometric analysis was carried out immediately. The remainder was
immediately frozen at −18°C after separation of the biomass by centrifugation (5 min,
15,000 g) for subsequent HPLC and GC-MS analysis.

4.3.3.1. HPLC Analysis of Fermentations

A high pressure liquid chromatography system (Shimadzu, Japan) consisting of a
binary LC-20AB pumping unit, a DGU-20A3 degasser, a SIL-10AD VP autosampler,
a CTO-20AC column oven, a SPD-M20A diode array detector, and a RID-10A
refractive index detector was used for isocratic separation and analysis of sugars,
ethanol, glycerol, and acetic acid. Data acquisition and analysis was performed with
the accompanying instrument software (LCSolution v.1.23). The mobile phase
consisted of ASTM Class I water with 1% (w/v) HPLC grade phosphoric acid and
5% (v/v) HPLC grade acetonitrile and was filtered prior to utilization (0.22 µm, nylon,
Millipore, Ireland). After sample injection (5.0 µl), separation occurred at a flow rate
of 0.35 ml min\(^{-1}\) on a sulfonated polystyrene/divinyl benzene stationary phase with 9.0 µm particle size (250 x 4.6 mm, Supelcogel H, Sigma Aldrich, MO) with a corresponding 50 x 4.6 mm guard column (Supelguard C610H, Sigma Aldrich, MO), both of which were held at 60 °C (Frohman & Mira de Orduña, 2013). Sugars, ethanol, and glycerol were quantified by refractive index using external standards while acetic acid was measured by UV spectroscopy at 210 nm using propionic acid, which was added to all samples at 1.41 g l\(^{-1}\), as an internal standard. Eight standards were utilized for each calibration curve. Glucose and fructose standard concentrations ranged from 1 to 200 g l\(^{-1}\), and ethanol standard concentrations ranged from 0.5 to 20 % (v/v).

For the isocratic separation and analysis of citric, malic, succinic, lactic, and tartaric acids, the same HPLC system was used, but the mobile phase contained sterile filtered 6% (v/v) HPLC grade acetonitrile and ASTM Class I water adjusted to pH 1.7 with HPLC grade sulfuric acid. Injection volume was 20 µl, and separation occurred at a flow rate of 0.5 ml min\(^{-1}\) at 45 °C on a sulfonated polystyrene/divinyl benzene stationary phase with 9.0 µm particle size (300 x 7.8 mm, Aminex HPX-87H, BIO-RAD, CA) with a corresponding 30 x 4.6 mm guard column (Micro-Guard Cation H\(^{+}\), Bio-Rad, CA) (Frayne, 1986). All organic acids were measured via UV spectroscopy at 210 nm and quantified using external calibration standards.

To quantify the SO\(_2\)-binding compounds α-ketoglutarate, pyruvate, acetoin, and acetaldehyde, samples were first derivatized with 2,4-dinitrophenylhydrazine (Jackowetz & Mira de Orduña, 2013a). Separation of DNPH-derivatised wine carbonyls then occurred using an ultra high pressure liquid chromatography system (UHPLC) (Shimadzu, Japan) consisting of a binary LC-20AD XR pumping unit, a
DGU-20A₃ degasser, a SIL-20AC XR autosampler, a CTO-20AC column oven, and a SPD-20A UV/VIS detector. Analysis was achieved via a gradient elution of solvent A, consisting of water acidified to pH 2.50 using perchloric acid, and solvent B, HPLC grade acetonitrile. Both solvents were sterile filtered prior to utilization. Following 5 μL sample injection, separation occurred on a C18 stationary phase with 2.6 μm particle size (100 x 3.0 mm, Kinetex, Phenomenex, CA) held at 37 °C with a flow rate of 0.75 ml min⁻¹. The analytes were quantified at 365 nm using external calibration standards.

4.3.3.2. Flow Cytometric Analysis

Live yeast percentages were determined by flow cytometry (Accuri C6, BD Biosciences, NJ). For this, 0.5 ml samples were centrifuged for 7 minutes at 7,000 g. The clear supernatant was removed, and cell pellets were resuspended in a volume of sterile phosphate buffered saline equal to the culture supernatant removed. The buffer composition was 0.14 M NaCl, 2.7 mM KCl, 10.2 mM Na₂HPO₄·2H₂O, 1.8 mM KH₂PO₄, 1 mM EDTA and 0.2% w/v Pluronic-F68, pH 7.4. The resuspended cells were mixed, diluted 1:100 with the same buffer, and then stained by addition of 16.5 μL of 1.5 mM propidium iodide (PI) (Sigma Aldrich, MO) and 40 μL of 17 μM thiazole orange (TO) (Santa Cruz Biotechnology, Ca) to final assay concentrations of 41 μM PI and 1.1 μM TO, respectively. 50 μl of a counting bead solution (SPHERO™ AccuCount Blank Particles, 7.3 μm, Spherotech Inc, IL) were also added to each sample as an internal standard for the quantification of cell numbers (Thornton et al., 2002).
4.3.3.3. Volatile Compound Analysis

Prior to volatile compound analysis, wine samples were spiked with 2,6-dimethyl-5-hepten-2-ol (DMH, 1151 µg l⁻¹) and isopropylbenzene (259 µg l⁻¹) as internal standards, and then subjected to liquid-liquid discontinuous extraction with 1,1,2-Trichloro-1,2,2-trifluoroethane (Kaltron) (Sigma Aldrich, MO). Following extraction and drying with Na₂SO₄, 2 µL samples were injected in splitless mode at 30°C into a 5890 Series II Gas Chromatograph (Hewlett Packard, CA) with a Varian VF-5MS column (Agilent, CA) coupled to a 5972 Mass Selective Detector (Hewlett Packard, CA) operating in scan mode. A modified procedure based on the chromatography and mass spectral conditions described by Rapp et al. was used for GC-MS analysis (Rapp et al., 1994; Rauhut & Fischer, 2005). Data processing was carried out by MSD ChemStation software (Agilent, CA).

4.3.4. Replications and Statistical Analysis

All fermentations were conducted in duplicate. Student’s t-tests and ANOVA were conducted with JMP 7.0 (SAS, North Carolina) to determine statistical significance of differences observed between sample populations at the 0.05 confidence level. All duplicate measurements were within 5 % of one another.

4.4. Results

4.4.1. Sugars and Ethanol

The traditional batch fermentation of Cabernet Franc juice (286 g l⁻¹ sugars) reached dryness (<3 g l⁻¹ total sugars) after approximately 240 hours, while the fed-batch fermentation lasted 325 hours (Figure 4.1). Fermentation parameters for the three treatments are summarized in Table 4.1.
Figure 4.1 Time course of sugar and ethanol concentrations in fed-batch (ients) and traditional batch (ents) fermentations of Cabernet Franc juice. The initial sugar concentration of the batch fermentation corresponds to the juice following addition of the rehydrated yeast starter.
Table 4.1 Final sugar and ethanol concentrations in wines and maximum ethanol formation rates during fermentations. Average of duplicate measurements shown. Different letters indicate statistical difference of means at p=0.05.

<table>
<thead>
<tr>
<th></th>
<th>Final Sugar (g l⁻¹)</th>
<th>Final Ethanol (% v/v)</th>
<th>Max. Ethanol Formation Rate (% v/(v·h))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Batch</td>
<td>1.2ᵃ</td>
<td>14.5ᵃ</td>
<td>0.086ᵃ</td>
</tr>
<tr>
<td>Fed-Batch</td>
<td>1.3ᵃ</td>
<td>14.4ᵃ</td>
<td>0.097ᵇ</td>
</tr>
</tbody>
</table>
Although the batch and fed-batch fermentations reached similar final ethanol concentrations, the fed-batch fermentation demonstrated a 13 % faster rate of formation during the first 150 hours of fermentation. During the next 100 hours, the fed-batch fermentation demonstrated slower ethanol production as the alcohol level asymptotically approached its limit during the feeding phase. When must delivery ended at 250 hours, an immediate increase in ethanol concentration was seen in the fed-batch fermentation as the residual 45 g l⁻¹ of sugar was consumed.

4.4.2. Osmotic Stress Response-Related Metabolites

Figure 4.2 shows the kinetics of glycerol and acetic acid in batch and fed-batch fermentations of Cabernet Franc juice. Final concentrations of both metabolites were considerably higher in the batch-produced wine (Table 4.2). The fed-batch fermentation led to a 20% reduction of final glycerol levels relative to the batch fermentation, and a reduction in acetic acid levels to below the limit of detection (0.05 g l⁻¹). The small increase in glycerol concentration observed in the fed-batch fermentation starting at 250 hours corresponds with the end of must delivery, when the fermentation was allowed to go to dryness. The molar increase in final glycerol concentration experienced under high gravity conditions (0.0196 M) was approximately 3.8 times greater than that of acetic acid (0.00516 M).
Figure 4.2 Time course of glycerol and acetic acid concentrations in a traditional batch (■) and fed-batch (○) fermentation of Cabernet Franc juice. Concentrations of acetic acid were below the detection threshold (0.05 g l\(^{-1}\)) throughout the fed-batch fermentation.
Table 4.2 Final glycerol and acetic acid concentrations in wines. Average of duplicate measurements shown. Different letters indicate statistical difference of means at p=0.05.

<table>
<thead>
<tr>
<th></th>
<th>Final Glycerol Concentration (g L⁻¹)</th>
<th>Final Acetic Acid Concentration (g L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Batch</td>
<td>9.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;0.05&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Fed-Batch</td>
<td>7.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.36&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
4.4.3. Organic Acids

Final concentrations of several organic acids in both the batch and fed-batch wines are displayed in Table 4.3. Malic, citric, and succinic acid levels were statistically equivalent in both finished wines, with succinic acid levels being very high compared to average reported values (Moreno-Arribas and Carmen Polo, 2009). In contrast, the fed-batch wine contained 70% more lactic acid than the batch wine, although both values were low (<0.3 g/L) because no malolactic fermentation was performed.
Table 4.3 Final organic acid concentrations in wines. Average of duplicate measurements shown. Different letters indicate statistical difference of means at p=0.05.

<table>
<thead>
<tr>
<th></th>
<th>Final Malic Acid Concentration (g l⁻¹)</th>
<th>Final Lactic Acid Concentration (g l⁻¹)</th>
<th>Final Citric Acid Concentration (g l⁻¹)</th>
<th>Final Succinic Acid Concentration (g l⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Batch</td>
<td>1.8ᵃ</td>
<td>0.14ᵃ</td>
<td>0.15ᵃ</td>
<td>7.7ᵃ</td>
</tr>
<tr>
<td>Fed-Batch</td>
<td>1.7ᵃ</td>
<td>0.24ᵇ</td>
<td>0.16ᵃ</td>
<td>7.6ᵃ</td>
</tr>
</tbody>
</table>
4.4.4. **SO$_2$-Binding Compounds**

During the batch fermentation, acetaldehyde levels rapidly increased and reached a peak concentration within the first 40 hours of fermentation (Figure 4.3). Subsequently, partial re-uptake occurred, resulting in a dryness concentration of 17.2 mg l$^{-1}$. In contrast, the fed-batch fermentation of the same juice did not produce a detectable peak in acetaldehyde, and resulted in an acetaldehyde concentration at dryness that was 45% lower as compared to the batch fermentation. Final acetaldehyde concentrations followed a similar pattern, and were 67% lower in the fed-batch fermentation. Overall, both fermentation treatments resulted in very low final acetaldehyde levels, as compared to reported averages in New York State table wines (Jackowetz & Mira de Orduña, 2013b).

As with acetaldehyde, a very high peak acetoin concentration was observed in the batch fermentation at 40 hours, while a corresponding peak was not detected in the fed-batch fermentation. Similarly, the batch fermentation exhibited reutilization of acetoin, resulting in dryness and final concentrations of 6.3 mg l$^{-1}$ and 4.6 mg l$^{-1}$, respectively, which were 1.4 and 3 times greater than corresponding concentrations in the fed-batch wine.

A peak in pyruvate concentration was observed in both fermentations at approximately 50 hours, shortly after maximum acetaldehyde and acetoin levels were achieved in the batch fermentation. While the peak pyruvate concentration was 23% higher in the batch fermentation, dryness and final concentrations were similar between the two treatments.
The most dramatic difference in SO₂-binder concentration between the batch and fed-batch fermentations was that of α-ketoglutarate. While levels of this metabolite gradually increased throughout the course of the batch fermentation, a very rapid increase was observed during the first 55 hours of the fed-batch fermentation. Its concentration then leveled off for the next approximately 200 hours of fermentation, before further increasing following the completion of the feeding phase, when the residual 45 g l⁻¹ sugar was consumed. Dryness and final α-ketoglutarate levels were approximately 3.4 times higher in the fed-batch wine as compared to the batch wine. Peak, dryness, and final concentrations of measured SO₂-binders are displayed in Table 4.4.
Figure 4.3 Time course of SO$_2$-binder concentrations in a traditional batch (■) and fed-batch (△) fermentation of moderately high gravity Cabernet Franc juice.
Table 4.4 Peak, dryness (<3 g l\(^{-1}\) total sugars), and final SO\(_2\)-binder concentrations in wines. Average of duplicate measurements shown. Different letters indicate statistical difference of means at p=0.05.

<table>
<thead>
<tr>
<th></th>
<th>Peak Batch</th>
<th>Peak Fed-Batch</th>
<th>Dryness Batch (238 h)</th>
<th>Dryness Fed-Batch (325 h)</th>
<th>Final Batch</th>
<th>Final Fed-Batch</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetaldehyde (mg l(^{-1}))</td>
<td>45.4(^a)</td>
<td>N.D.(^b)</td>
<td>17.2(^a)</td>
<td>9.5(^b)</td>
<td>14.1(^a)</td>
<td>4.7(^b)</td>
</tr>
<tr>
<td>Acetoin (mg l(^{-1}))</td>
<td>64.6(^a)</td>
<td>N.D.(^b)</td>
<td>6.3(^a)</td>
<td>4.4(^b)</td>
<td>4.6(^a)</td>
<td>1.5(^b)</td>
</tr>
<tr>
<td>Pyruvate (mg l(^{-1}))</td>
<td>45.8(^a)</td>
<td>37.3(^b)</td>
<td>36.5(^a)</td>
<td>35.1(^a)</td>
<td>35.8(^a)</td>
<td>34.3(^a)</td>
</tr>
<tr>
<td>α-ketoglutarate (mg l(^{-1}))</td>
<td>N.D.(^a)</td>
<td>N.D.(^a)</td>
<td>40.1(^a)</td>
<td>135.9(^b)</td>
<td>43.3(^a)</td>
<td>146.5(^b)</td>
</tr>
</tbody>
</table>
4.4.5. Volatile Compounds

A summary of peak and final concentrations of several important volatile organic compounds potentially relevant to wine aroma is provided in Table 4.5. Overall, the batch fermentation exhibited highly varied formation patterns, with the measured analytes displaying maximum concentrations at different timepoints throughout the fermentation. In contrast, with the exception of a few compounds whose concentrations remained unchanged throughout fermentation, maximum analyte concentrations during the fed-batch fermentation occurred either immediately after the end of the feeding phase (250 hours) or upon completion of fermentation (354 hours). Significant concentration differences were observed for several of the medium chain saturated fatty acid ethyl esters (e.g. ethyl hexanoate, ethyl octanoate, and ethyl decanoate), which demonstrated considerably higher maximum and final concentrations in the batch-produced wines. Levels of the parent saturated fatty acid compounds, however, were only slightly higher in the batch fermentation wines. The batch fermentation also exhibited significantly higher final concentrations of 3-methylbutyl acetate (+33 %) and 2-phenylethanol (+26 %). In contrast, the fed-batch fermentation resulted in higher final concentrations of 2-methylbutyl acetate (+142 %), 2-phenylethyl acetate (+48 %), and ethyl propanoate (+247 %). While hexanol concentrations were very high at the start of the batch fermentation, final levels were nonetheless 96 % higher in the fed-batch wine. Maximum and final concentration differences between the two fermentations were not significant for all other measured compounds.
Table 4.5 Peak and final aroma compound concentrations in wines. N.Q. = not quantifiable (traces), N.D. = not detected, Static = Constant (+/- 10%) throughout fermentation.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Batch Max Concentration (hours)</th>
<th>Fed-Batch Max Concentration (hours)</th>
<th>Batch Final Concentration</th>
<th>Fed-Batch Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid ethylester (mg L⁻¹)</td>
<td>47 (261)</td>
<td>52 (354)</td>
<td>45</td>
<td>52</td>
</tr>
<tr>
<td>i-Butanol (mg L⁻¹)</td>
<td>37 (261)</td>
<td>33 (354)</td>
<td>34</td>
<td>33</td>
</tr>
<tr>
<td>Ethyl propanoate (µg L⁻¹)</td>
<td>186 (261)</td>
<td>630 (260)</td>
<td>175</td>
<td>607</td>
</tr>
<tr>
<td>3-Methyl-butanol (mg L⁻¹)</td>
<td>192 (261)</td>
<td>174 (354)</td>
<td>178</td>
<td>174</td>
</tr>
<tr>
<td>2-Methyl-butanol (mg L⁻¹)</td>
<td>66 (261)</td>
<td>87 (354)</td>
<td>62</td>
<td>87</td>
</tr>
<tr>
<td>i-Butyric acid ethylester (µg L⁻¹)</td>
<td>8 (261)</td>
<td>16 (354)</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td>Butyric acid ethylester (µg L⁻¹)</td>
<td>129 (261)</td>
<td>120 (354)</td>
<td>122</td>
<td>120</td>
</tr>
<tr>
<td>Lactic acid ethylester (mg L⁻¹)</td>
<td>9 (287)</td>
<td>9 (354)</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>Hexanol (µg L⁻¹)</td>
<td>730 (2.68)</td>
<td>452 (249)</td>
<td>196</td>
<td>385</td>
</tr>
<tr>
<td>3-Methylbutyl acetate (µg L⁻¹)</td>
<td>533 (164)</td>
<td>325 (354)</td>
<td>433</td>
<td>325</td>
</tr>
<tr>
<td>2-Methylbutylacetate (µg L⁻¹)</td>
<td>64 (164)</td>
<td>128 (354)</td>
<td>53</td>
<td>128</td>
</tr>
<tr>
<td>Hexanoic acid (mg L⁻¹)</td>
<td>5 (187)</td>
<td>3 (static)</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Ethyl Hexanoate (µg L⁻¹)</td>
<td>183 (200)</td>
<td>50 (260)</td>
<td>130</td>
<td>35</td>
</tr>
<tr>
<td>Acetic acid hexylester (µg L⁻¹)</td>
<td>9 (48)</td>
<td>N.Q.</td>
<td>N.Q.</td>
<td>N.Q.</td>
</tr>
<tr>
<td>trans-Linalool oxide (µg L⁻¹)</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>cis-Linalool oxide (µg L⁻¹)</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Linalool (µg L⁻¹)</td>
<td>N.D.</td>
<td>2 (static)</td>
<td>N.D.</td>
<td>2</td>
</tr>
<tr>
<td>2-Phenylethanol (mg L⁻¹)</td>
<td>48 (200)</td>
<td>34 (354)</td>
<td>43</td>
<td>34</td>
</tr>
<tr>
<td>Octanoic acid (mg L⁻¹)</td>
<td>3 (52)</td>
<td>1 (static)</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Succinic acid diethylester (µg L⁻¹)</td>
<td>N.Q.</td>
<td>N.Q.</td>
<td>N.Q.</td>
<td>N.Q.</td>
</tr>
<tr>
<td>Ethyl Octanoate (µg L⁻¹)</td>
<td>362 (44)</td>
<td>N.Q.</td>
<td>38</td>
<td>N.Q.</td>
</tr>
<tr>
<td>α-Terpineol (µg L⁻¹)</td>
<td>N.D.</td>
<td>5 (static)</td>
<td>N.D.</td>
<td>5</td>
</tr>
<tr>
<td>Benzenacetic acid ethylester (µg L⁻¹)</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>2-Phenylethyl Acetate (µg L⁻¹)</td>
<td>88 (164)</td>
<td>102 (354)</td>
<td>69</td>
<td>102</td>
</tr>
<tr>
<td>Decanoic acid (mg L⁻¹)</td>
<td>0.5 (164)</td>
<td>0.2 (260)</td>
<td>0.2</td>
<td>0.1</td>
</tr>
<tr>
<td>Ethyl Decanoate (µg L⁻¹)</td>
<td>138 (140)</td>
<td>39 (260)</td>
<td>24</td>
<td>18</td>
</tr>
</tbody>
</table>
4.4.6. *Yeast Viability*

Figure 4.4 shows the time course of viable yeast percentage in batch and fed-batch fermentations of Cabernet Franc juice. In both treatments, the live yeast percentage peaked around 170 h. However, while both fermentations were inoculated at the same concentration, the fed-batch fermentation exhibited a higher viability throughout the entire fermentation, reaching a maximum of >90 % live yeast, while the batch fermentation started with a significantly lower live yeast titer, and achieved a lower maximum. In addition, yeast viability in the batch fermentation began to decline immediately after a maximum was achieved, whereas the percentage of live yeast in the fed-batch fermentation only began to decline after 250 hours, when the feeding phase ended.
Figure 4.4 Percentage of live yeast during a traditional batch (△) and fed-batch (□) fermentation of Cabernet Franc juice.
4.5. Discussion

The current work compares the traditional batch fermentation of a moderately high gravity medium with a fed-batch approach, where the same medium was added at such rates as to keep sugar concentrations constant during fermentations. Control of fermentation broth sugar levels and automation of must delivery was achieved using a previously described FT-NIRS-based system (Chapter 3). Cabernet Franc grape juice and a commercial standard yeast for alcoholic fermentation were used. Fundamental differences in metabolite formation patterns and yeast viability between batch and fed-batch fermentation produced wines were revealed.

4.5.1. Ethanol and Yeast Viability

Final ethanol concentrations were similar in batch and fed-batch fermentations of Cabernet Franc juice. However, the fed-batch approach resulted in a slightly (13%) higher maximum ethanol formation rate. This effect has been previously reported by the authors (Frohman & Mira de Orduña, 2013), and is associated with a higher live yeast concentration in the fed-batch treatment, as assessed by flow cytometric analysis. Nagodawithana et al. (1974) and Erasmus et al. (2003) had previously shown that reduced sugar concentrations led to enhanced viability in AF by *S. cerevisiae*.

Another interesting result of the fed-batch platform is that the system achieves a moderately high ethanol concentration when only a small percentage of the juice to be fermented has been delivered. In the current study, the ethanol concentration of the fed-batch fermentation was 80 % of the final ethanol concentration after approximately 200 hours and <15 % juice delivery (data not shown). Once the feeding phase’s maximum ethanol level is reached, which depends on the sugar
content of the must as well as the concentration at which the fermentation is maintained, it remains fairly constant as additional juice is delivered, due to the balancing rates of ethanol formation and dilution (hours 150-250 in (Figure 4.1). A final increase in alcohol level is then observed when the juice delivery stops and the fermentation goes to dryness. While microbial challenge studies were not an objective of the current work, the fed-batch system may reduce the risk of microbial contamination during fermentation since the majority of the must is added to a high alcohol wine.

4.5.2. Osmotic Stress Response-Related Metabolites

During alcoholic fermentation, high salt or sugar concentration-induced osmotic stress causes up-regulation of the genes responsible for glycerol production in \textit{S. cerevisiae}, thereby resulting in elevated levels of this metabolite (Pigeau & Inglis, 2005). The increased glycerol concentration helps protect against the external osmotic pressure by preventing cell water loss and thereby maintaining cell volume and fluid balance (Blomberg, 2000; Blomberg & Adler, 1989; Nevoigt & Stahl, 1997). In ice wines, which are traditionally produced from very high gravity juice, glycerol concentrations may exceed 15 g l\(^{-1}\) (Erasmus et al., 2004; Pigeau et al., 2007). In contrast, limited glucose concentrations led to >50 % reductions in final glycerol levels in alcoholic fermentations with \textit{S. cerevisiae} (Bideaux et al., 2006; Frohman & Mira de Orduña, 2013). The current work is in agreement with this finding, and demonstrated a 20 % reduction in final glycerol levels in the fed-batch fermentation as compared to the batch fermentation of Cabernet Franc juice.
The osmotic stress response of *S. cerevisiae* also causes increased formation of acetic acid (Frohman & Mira de Orduña, 2013; Pigeau et al., 2002; Pigeau & Inglis, 2005). Previous work has demonstrated the ability to reduce final acetic acid levels in wines produced from high gravity musts through application of a manually maintained fed-batch fermentation system (Frohman & Mira de Orduña, 2013). In the present study, also, the batch fermentation of Cabernet Franc juice led to final acetic acid levels of 0.36 g l\(^{-1}\), while the automated fed-batch fermentation of the same juice produced only trace quantities of this metabolite, which were below the limit of detection (0.05 g l\(^{-1}\)). Relative to the fed-batch fermentation, the high gravity batch fermentation also demonstrated a molar increase in final glycerol concentration that was 3.8 times greater than that of acetic acid. As the formation of these two compounds produces and consumes NAD\(^+\), respectively, unequal rates of synthesis would result in a redox imbalance. In order to maintain redox balance then, it is possible that substantial amounts of pyruvate, whose formation consumes NAD\(^+\) and regenerates NADH, is being diverted for growth related processes.

4.5.3. Organic Acids

With the exception of lactic acid, whose final concentration was twice as high in the fed-batch wine as compared to the batch wine, levels of the other measured organic acids were very similar. The highly elevated final succinic acid levels observed in both the batch and fed-batch wines prompted a measurement of its concentration in the unfermented Cabernet Franc juice. The initial succinic acid concentration (8.4 g l\(^{-1}\)) was similarly high, suggesting the possibility that the grapes, which had been frozen for several years, underwent some degree of carbonic maceration (Margalit, 2013).
A previous study (Yoshimi & Masazumi, 1981) found that must sugar concentration affects the formation of succinic acid, but not lactic and citric acids. In support of the current results, additional studies by Devantier et al. (Devantier et al., 2005) and Yang (Yang, 2007) also found that low sugar concentrations during alcoholic fermentation lead to elevated lactic acid levels.

4.5.4. SO₂-Binding Compounds

When concentrations of SO₂-binding compounds are high in finished wines, larger additions of SO₂ are required in order to ensure sensory acceptability and microbial and chemical stability (Boulton et al., 1996; Jackowetz et al., 2012). Among the SO₂-binding compounds measured, concentrations of pyruvate at dryness and at the final sampling point were similar in the batch and fed-batch fermentations, while those of acetaldehyde, acetoin, and α-ketoglutarate differed significantly. Specifically, final acetaldehyde and acetoin concentrations were 3 times higher in the finished batch wine as compared to the fed-batch wine, while α-ketoglutarate levels were approximately 3.4 times higher in the fed-batch wine. With the exception of pyruvate, none of the SO₂-binding compounds demonstrated peak concentrations in the fed-batch fermentation, likely due to the effects of dilution.

During alcoholic fermentation, acetaldehyde serves as the final electron acceptor and influences wine aroma and stability. Previous research with resting cells of S. cerevisiae showed that high media sugar concentrations result in elevated acetaldehyde excretion by yeasts (Li & Mira de Orduña, 2011) while low sugar levels lead to decreased residual acetaldehyde levels (Frohman & Mira de Orduña, 2013). The results of the current study agree with these findings, and reveal that fermenting a
juice in a fed-batch mode causes a significant reduction in dryness (-45 %) and final (-67 %) acetaldehyde levels. As acetaldehyde is involved in the formation of acetoin (Moreno-Arribas and Carmen Polo, 2009), the reduction in the final concentration of the latter in the fed-batch wine may be directly related to the observed decrease in the final level of the former. Alternatively, increased glycerol production may decrease the availability of reducing factors to convert acetoin to 2,3-butanediol. Elevated glycerol formation, as seen in the current batch fermentation, has been associated with higher residual acetoin levels in other studies (Michnick et al., 1997; Remize et al., 1999).

The higher α-ketoglutarate concentrations at dryness and at the final sampling point observed in the fed-batch wine is in concordance with earlier research (Devantier et al., 2005; Yang, 2007), which demonstrated that the fermentation of a low sugar medium led to largely elevated levels of this metabolite relative to that of a very high gravity medium.

4.5.5. Volatile Compounds

When Cabernet Franc juice was fermented in a fed-batch mode, the prominent effect on volatile compounds was a large reduction in the final concentrations of 3-methylbutyl acetate and several medium chain saturated fatty acid ethyl esters, as compared to the batch-produced wine. Simultaneously, the fed-batch wine demonstrated higher final levels of 2-methylbutylacetate, 2-phenylethyl acetate, and ethyl propanoate, whose concentrations increased between 48 and 247%. The effect of fermentation sugar concentration on finished wine ester levels has been previously
shown by Houtman et al. (Houtman et al., 1980) and largely agrees with the current finding.

Previous research has also indicated that the addition of nitrogen to a fermentation at stationary phase, as compared to an addition prior to fermentation, results in the production of significantly less saturated fatty acid ethyl esters and more 2-phenylethyl acetate and ethyl propanoate (Barbosa et al., 2009). Since the fed-batch platform described in the current work results in continuous nitrogen supplementation, the observed effects on the measured volatile organic compounds may be due to the dual impact of decreased sugar concentration and the presence of assimable nitrogen at a later stage of fermentation.

4.6. Conclusions

This work demonstrates that the utilization of an automated feeding strategy that maintains constant low sugar concentrations during the fermentation of moderately high gravity juice leads to enhanced yeast viability, decreased production of osmotic stress response related metabolites, and modification of SO$_2$-binder and volatile compound concentrations. The demonstrated changes in compounds are important to wine microbial stability and organoleptic qualities. Future works will provide a sensorial comparison of batch and fed-batch produced wines. Additional research will examine the effects of a more economical non-continuous feeding strategy on yeast metabolic activity and the application of the developed technology to other industrial fermentations.
4.7. Acknowledgements

The authors wish to acknowledge financial support from the Nolan, Dyson and Canandaigua Wine Co. graduate support funds.
Reference List


CHAPTER 5 - METABOLISM OF SACCHAROMYCES CEREVISIAE DURING DISCONTINUOUS AND CONTINUOUS, AUTOMATED FED-BATCH ALCOHOLIC FERMENTATIONS

5.1. Abstract

Saccharomyces cerevisiae experience an osmotic stress response during the fermentation of high sugar-containing juices. This metabolic phenomenon increases formation of byproducts and the chance of sluggish or failed fermentations. The current work compared three approaches to fermentation of the same must: i) a discontinuous fed-batch approach, where discrete additions of juice were made throughout fermentation at specific density measurements, a continuous fed-batch approach, in which fermentation was started with undiluted must and then maintained at 30 g l\(^{-1}\) total sugars by automated additions, and iii) a continuous fed-batch approach, in which the total sugars were initially at 30 g l\(^{-1}\) and were maintained at this concentration during fermentation. Automation was achieved through a recently described FT-NIRS based system. Both continuous fed-batch fermentations reached higher final ethanol concentrations than the discontinuous fed-batch approach, with the fermentation that was started at 30 g l\(^{-1}\) also exhibiting a 36 % higher maximum rate of ethanol formation. The same continuous fed-batch fermentation, which was inoculated with a higher starting yeast concentration than the other two fermentations, also demonstrated approximately constant live yeast numbers throughout the feeding phase of the fermentation, even as volume was exponentially increasing, thereby behaving like a type of continuous culture. While measured osmolyte concentrations
were similar among all 3 fermentations, the continuous fed-batch fermentations demonstrated 5 - 14 % reductions in final glycerol concentrations and a decrease in final acetic acid concentrations to non-detectable levels. Conversely, aside from galacturonic acid, final SO₂-binder concentrations were higher in the continuous fed-batch fermentation wines, with the most significant effects being observed for alpha-ketoglutarate and acetoin, which were up to 5 and 14 times higher in these fermentations, respectively, as compared to the discontinuous fed-batch fermentation. The continuous fed-batch fermentations also demonstrated 59 – 121 % higher SO₂-binding capacities. Differences in organic acid levels were also observed between the different fermentation platforms. The overall results demonstrate significant differences in yeast metabolism during discontinuous and continuous fed-batch alcoholic fermentations, but simultaneously suggest that a more affordable and easily achievable discontinuous approach may also allow for an avoidance or lessening of the osmotic stress response.

5.2. Introduction

In the wine industry and other bioindustries, it is oftentimes necessary or beneficial to ferment media that contain very high sugar concentrations which exceed 27 % soluble solids (Thomas et al., 1993). In the case of winemaking, such an approach may be intentionally used for the elaboration of specialty wines such as ice wine and late harvest wines. However, even when the production of dessert wines is not desired, the vinification of high Brix juices may be unavoidable, as the effects of ongoing climate change and viticultural decisions are resulting in the harvesting of berries with increasingly high sugar concentrations (Mira de Orduña, 2010; Webb et al., 2012). On
the other hand, for the production of bioethanol or other fermentation-derived industrial products, the utilization of a high sugar-containing broths may be required to improve downstream processing efficiency (Thomas et al., 1996; Wang et al., 1999). However, the presence of high sugar concentrations during alcoholic fermentation with *Saccharomyces cerevisiae* can result in growth inhibition or yeast lysis, and in turn cause sluggish or failed fermentations (Bai et al., 2004). In addition, high sugar concentrations stimulate a hyperosmotic stress response in the fermenting yeast that causes increased expression of the glycolytic and pentose phosphate pathway genes and elevated production of glycerol and acetic acid (Erasmus et al., 2003). This phenomenon was previously studied in the context of very high sugar containing juices used in the production of wines from *Botrytis cinerea* infected grapes (Bely et al., 2005) or the production of wines from grape juices containing up to 350 g l⁻¹ sugars, and revealed final acetic acid levels which exceeded 1.5 g l⁻¹ in some wines (Pigeau et al., 2002b; Pigeau & Inglis, 2005a). High sugar concentrations may also increase the production of acetaldehyde (Frohman & Mira de Orduña, 2013; Li & Mira de Orduña, 2011)(Chapter 4), a carbonyl compound important to the aroma and stability of finished wines. While increased concentrations of acetic acid may further inhibit yeast viability and decrease fermentation efficiency (Edwards et al., 1999; Ludovico et al., 2001), in addition to causing degradation of aromatic quality, increased formation of byproducts in general leads to reduced ethanol production yields (Bai et al., 2004; Cheng et al., 2009).

In an effort to circumvent the challenges associated with fermenting high-substrate containing broths, and to improve cell densities and process productivities (Berraud,
2000; Kim et al., 1994; Wang & Shyu, 1997), many industries utilize a fed-batch fermentation approach, whereby substrate is continuously fed to an active fermentation such that concentrations remain low throughout fermentation. Previously, an FT-NIR spectroscopy-based system capable of automatically maintaining sugar concentrations at low target levels during vinifications was engineered (Chapter 3), and the effect of such fermentations on yeast viability and metabolism was tested (Chapter 4). While utilization of this system avoids incidence of an osmotic stress response during the fermentation of high gravity juices, the equipment required to achieve such a set-up is expensive and requires trained operators. Consequently, such technology may not be readily accessible to small winemakers or to companies that do not have the dedicated personnel required to calibrate and operate such equipment. Therefore, it would be advantageous to determine if a discontinuous, non-automated approach to sugar additions during fermentation could also lessen yeast osmotic stress response. Some winemakers already use a discontinuous approach to fermentations, when grapes are harvested over a several week period as they achieve ripeness and their juice is added in aliquots to the same on-going vinification (Heras, 2012). As a result of this approach, every time additional juice is added to the fermentation, concentrations of glycerol and acetic acid decrease due to the effects of dilution. Simultaneously, the sugar concentration of the fermentation will increase, but to a level below that of the pure juice, such that the osmotic stress response experienced by the yeast should be less severe, and osmolyte production should decrease.
Theoretically, the end result would be a decrease in the concentration of these compounds relative to a batch-produced wine.

The purpose of this work was to compare the metabolic implications of such a discontinuous fed-batch fermentation approach, whereby additional must is added to a fermentation in discrete intervals at specific density measurements, to a continuous and FT-NIRS-automated fed-batch approach where sugar levels are maintained at a low target concentration during fermentation. Accordingly, a Chardonnay must was adjusted to a total sugar concentration of 200 g l\(^{-1}\) and subsequently fermented in a discontinuous mode, where additional juice was added at specific density measurements, or using two different fed-batch approaches. In the first continuous fed-batch method, the fermentation was started in a batch mode using the prepared must, and then once the low target sugar concentration of 30 g l\(^{-1}\) was reached via yeast metabolism, additional must was automatically fed in at calculated rates to maintain sugar levels at this value throughout fermentation, until all the must was consumed. Subsequently, the fermentation was allowed to go to dryness. In the second continuous fed-batch approach, the fermentation was immediately started and maintained at the target sugar concentration of 30 g l\(^{-1}\) until all must was consumed. In addition, this fermentation used a lower yeast concentration, calculated based on the final volume, but included a nutrient supplementation partway through the vinification to ensure continued yeast growth. A preliminary fermentation using high gravity (286 g l\(^{-1}\) total sugars) Cabernet Franc was also conducted, where sugar concentrations were rapidly increased from the maintained setpoint of 50 g l\(^{-1}\) to an upper level of 100 g l\(^{-1}\) several times throughout fermentation. In addition to sugars and ethanol,
glycerol and acetic acid formation kinetics were measured as the most important osmotic stress-response related compounds formed during alcoholic fermentation. Yeast viability and the concentrations of several wine-relevant organic acids and SO$_2$-binding carbonyl compounds were also measured to determine the effects of different discontinuous and continuous fed-batch methods on yeast fermentative metabolism.

5.3. Materials and Methods

5.3.1. Juice Composition

For the preliminary experiment, where fermentation sugar concentrations were rapidly raised to 100 g l$^{-1}$ during a fed-batch fermentation that was otherwise maintained at 50 g l$^{-1}$, Cabernet Franc juice from the Cornell University Vineyards (Ithaca, NY) was used. Whole clusters of previously frozen grapes from the 2007 harvest were partially thawed and pressed on their stems using an 80 L inflatable bladder press to yield a juice with a sugar content of 19.9 °Brix, measured via refractometry (Atago 3415 WM-7, WA). Equal quantities of ACS grade anhydrous D-glucose and D-fructose (Fisher Scientific International Inc, NH) were added to the juice to raise the sugar concentration to a 286 g l$^{-1}$, as measured via HPLC. The pH of the adjusted juice was 3.97. For yeast nutrition, a complex supplement (Fermaid K, Lallemand, Canada) and ACS grade (NH$_4$)$_2$HPO$_3$ (Fisher Scientific International Inc, NH) were added at 0.25 g l$^{-1}$.

For the discontinuous and the continuous fed-batch fermentations, flash-pasteurized Chardonnay juice from the Languedoc region of France (Kamil Juices, Canada) was used. The juice was treated with 50 g hl$^{-1}$ bentonite, rehydrated according to the manufacturer’s instructions, to lower the turbidity to 42 Nephelometric Turbidity...
Units (NTU) and combined with enough ASTM Class I water (Arium 611UV, Sartorius, Germany) to lower the density to 1080 g l$^{-1}$ (200 g l$^{-1}$ sugar). The pH of the adjusted juice was 3.02. For the discontinuous fed-batch fermentations, FermaidK was added in two aliquots, at specific density measurements, for a final concentration of 0.106 g l$^{-1}$. For the continuous fed-batch fermentations, FermaidK was added directly to the must at the same concentration prior to fermentation.

5.3.2. Fermentations

Preliminary fermentations were conducted in an anaerobic 28 L cylindroconical stainless steel fermentation tank (Glacier Tanks, Oregon) at 20°C, using the enological Saccharomyces cerevisiae strain EC1118 (Lallemand, Canada) at a concentration of 40 g hl$^{-1}$, calculated according to the final volume. The active dry yeast were prepared according to manufacturer’s recommendations by rehydrating the yeast and a complex yeast nutrient (GO-FERM®, Lallemand, Canada) for 15 minutes at 40°C in ASTM Class I water (Arium 611UV, Sartorius, Germany). To start the fermentation, the yeast slurry was added directly to the fermentation tank, and an automated FT-NIR spectroscopy-based system was initiated so that high gravity must was added to the tank until the setpoint of 50 g l$^{-1}$ total sugars was achieved. Automation of must delivery during fed-batch fermentations via an FT-NIRS-based system has been previously described (Chapter 3). The fermentation sugar concentration was then maintained at this target value throughout fermentation, except when the automated system was temporarily stopped and additional high gravity must was manually added to the fermentation to rapidly raise the sugar content to 100 g l$^{-1}$. The rapid increase in sugar concentration was conducted three times, and each time the sugar level was
allowed to naturally drop back down to the setpoint value and was then maintained there for a specific amount of time before the following addition was implemented. After the third and final addition, and a subsequent period of approximately 250 hours when the fermentation was maintained at 50 g l⁻¹, the automated system was stopped and the fermentation was allowed to go to dryness.

Discontinuous fed-batch fermentations (Fermentation A) were conducted in 2 L glass bottles (Kimble Chase, NJ) at 20°C. Containers were sealed with air locks (Buon Vino Manufacturing, Canada) to allow for fermentation gas release and to prevent air ingress. Yeast were inoculated at 10.6 g hl⁻¹ with respect to the final volume (1.88 L) with EC1118 (Lallemand, Canada). The active dry yeast were prepared according to the manufacturer’s recommendations by rehydrating the yeast for 15 minutes at 40°C in ASTM Class I water (Arium 611UV, Sartorius, Germany) and were then added to a small partial volume (0.2 L) of the total must to be fermented. Following inoculation, discrete additions of nutrients and must were made at specific density measurements throughout fermentation, which were achieved using a handheld densiometer (Density 30PX, Mettler Toledo, OH). An overview of these additions is provided in Table 5.1. Following the final addition, the fermentation was allowed to go to dryness.
Table 5.1 Overview of additions made to discontinuous fed-batch fermentations (Fermentation A); \( \text{Density}_o \) = density before addition, \( \text{Density}_r \) = density following addition. Starting density = 1080 g l\(^{-1}\)

<table>
<thead>
<tr>
<th>Addition</th>
<th>Density(_o) (g l(^{-1}))</th>
<th>Density(_r) (g l(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>80 mg FermaidK</td>
<td>1070</td>
<td>1070</td>
</tr>
<tr>
<td>0.2 L must</td>
<td>1035</td>
<td>1057.5</td>
</tr>
<tr>
<td>0.4 L must</td>
<td>1040</td>
<td>1060</td>
</tr>
<tr>
<td>120 mg FermaidK</td>
<td>1050</td>
<td>1050</td>
</tr>
<tr>
<td>0.4 L must</td>
<td>1040</td>
<td>1053.3</td>
</tr>
<tr>
<td>0.4 L must</td>
<td>1040</td>
<td>1050</td>
</tr>
<tr>
<td>0.28 L must</td>
<td>1035</td>
<td>1042</td>
</tr>
</tbody>
</table>
Both continuous fed-batch fermentations were conducted in the previously described 28 L cylindroconical stainless steel fermentation tank at 20°C. The first (Fermentation B-1) used EC1118 yeast at the same concentration as in Fermentation A (10.6 g hl\(^{-1}\)), calculated according to the final volume. Following rehydration by the same procedure used for Fermentation A, the yeast were added to 2 L of the adjusted Chardonnay must, and fermentation commenced. When the total sugar content decreased to 30 g l\(^{-1}\), it was automatically maintained at this setpoint until the entire volume of juice to be fermented (19.4 L) was delivered, after which the fermentation was allowed to go to dryness.

The second continuous fed-batch fermentation (Fermentation B-2) used a smaller EC1118 yeast concentration of 3 g hl\(^{-1}\), calculated according to the final volume (9.3 L). This fermentation was conducted by automatically adding juice to the rehydrated starter until a sugar concentration of 30 g l\(^{-1}\) was reached, and then maintaining this concentration throughout fermentation, until all must to be fermented was delivered and the fermentation went to dryness. In addition, to stimulate yeast growth and activity, a mix of nutrients (0.25 g l\(^{-1}\) (NH\(_4\))\(_2\)HPO\(_4\), 10 μg l\(^{-1}\) MnSO\(_4\)·H\(_2\)O, 50 μg l\(^{-1}\) MgSO\(_4\), and 50 μg l\(^{-1}\) ZnSO\(_4\)·7H\(_2\)O) (Fisher Scientific International Inc, NH) was added 270 hours into the fermentation.

For continuous Fermentations B-1 and B-2, fermentation volume was constantly measured using an ultrasound distance detector (UNAM 18I6903/S14, Baumer, Switzerland) that was calibrated to the fill volume of the juice storage tank. Specifically, when juice was delivered to the fermentation, an increase in the distance
between the detector and the storage tank fill level was measured and used to calculate the fermentation’s volume increase.

All fermentations were conducted in duplicate and under 3 psi of nitrogen, to ensure a completely anaerobic environment.

5.3.3. Sampling and Analyses

Sampling of the discontinuous fed-batch fermentations occurred at regular time intervals under a stream of nitrogen gas, to prevent oxygen ingress. For fermentations conducted in the 28 L fermenter, samples were regularly drawn from a sampling valve at the bottom of the tank. Flow cytometric analysis was carried out immediately. The remainder was immediately frozen at -18°C after separation of the biomass by centrifugation (5 min, 15,000 g) for subsequent HPLC analysis.

5.3.3.1. HPLC Analysis

A high pressure liquid chromatography system (Shimadzu, Japan) consisting of a binary LC-20AB pumping unit, a DGU-20A3 degasser, a SIL-10AD VP autosampler, a CTO-20AC column oven, a SPD-M20A diode array detector, and a RID-10A refractive index detector was used for isocratic separation and analysis of sugars, ethanol, glycerol, and acetic acid. Data acquisition and analysis was performed with the accompanying instrument software (LCSolution v.1.23). The mobile phase consisted of ASTM Class I water with 1% (w/v) HPLC grade phosphoric acid and 5% (v/v) HPLC grade acetonitrile and was filtered prior to utilization (0.22 µm, nylon, Millipore, Ireland). After sample injection (5.0 µl), separation occurred at a flow rate of 0.35 ml min⁻¹ on a sulfonated polystyrene/divinyl benzene stationary phase with 9.0 µm particle size (250 x 4.6 mm, Supelcogel H, Sigma Aldrich, MO) with a
corresponding 50 x 4.6 mm guard column (Supelguard C610H, Sigma Aldrich, MO), both of which were held at 60 °C (Frohman & Mira de Orduña, 2013). Sugars, ethanol, and glycerol were quantified by refractive index using external standards while acetic acid was measured by UV spectroscopy at 210 nm using propionic acid, which was added to all samples at 1.41 g l⁻¹, as an internal standard.

For the isocratic separation and analysis of citric, malic, succinic, and lactic acids, the same HPLC system was used, but the mobile phase contained sterile filtered 6% (v/v) HPLC grade acetonitrile and ASTM Class I water adjusted to pH 1.7 with HPLC grade sulfuric acid. Injection volume was 20 µl, and separation occurred at a flow rate of 0.5 ml min⁻¹ at 45 °C on a sulfonated polystyrene/divinyl benzene stationary phase with 9.0 µm particle size (300 x 7.8 mm, Aminex HPX-87H, BIO-RAD, CA) with a corresponding 30 x 4.6 mm guard column (Micro-Guard Cation H⁺, Bio-Rad, CA) (Frayne, 1986). All organic acids were measured via UV spectroscopy at 210 nm and quantified using external calibration standards.

To quantify the SO₂-binding compounds α-ketoglutarate, pyruvate, acetoin, and acetaldehyde, samples were first derivatised with 2,4-dinitrophenylhydrazine (Jackowetz & Mira de Orduña, 2013). Separation of DNPH-derivatised wine carbonyls then occurred using an ultra high pressure liquid chromatography system (UHPLC) (Shimadzu, Japan) consisting of a binary LC-20AD XR pumping unit, a DGU-20A₃ degasser, a SIL-20AC XR autosampler, a CTO-20AC column oven, and a SPD-20A UV/VIS detector. Analysis was achieved via a gradient elution of solvent A, consisting of water acidified to pH 2.50 using perchloric acid, and solvent B, HPLC grade acetonitrile. Both solvents were sterile filtered prior to utilization. Following 5
μL sample injection, separation was achieved on a C18 stationary phase with 2.6 μm particle size (100 x 3.0 mm, Kinetex, Phenomenex, CA) held at 37 °C with a flow rate of 0.75 ml min⁻¹. The analytes were quantified at 365 nm using external calibration standards.

5.3.3.2. Flow Cytometric Analysis

Live yeast numbers were determined by flow cytometry (Accuri C6, BD Biosciences, NJ). For this, 0.5 ml samples were centrifuged for 7 minutes at 7,000 g. The clear supernatant was removed, and cell pellets were resuspended in a volume of sterile phosphate buffered saline equal to that of the culture supernatant removed. The buffer composition was 0.14 M NaCl, 2.7 mM KCl, 10.2 mM Na₂HPO₄·2H₂O, 1.8 mM KH₂PO₄, 1 mM EDTA and 0.2% w/v Pluronic-F68, pH 7.4. The resuspended cells were mixed, diluted 1:100 with the same buffer, and then stained by addition of 15 μL of 1.5 mM propidium iodide (PI) (Sigma Aldrich, MO) and 15 μL of 17 μM thiazole orange (TO) (Santa Cruz Biotechnology, Ca) to final assay concentrations of 38.8 μM PI and 0.44 μM TO, respectively. 50 µl of a counting bead solution (SPERO™ AccuCount Blank Particles, 7.3 μm, Spherotech Inc, IL) were also added to each sample as an internal standard for the quantification of cell numbers (Thornton et al., 2002).

5.3.4. Replications and Statistical Analysis

All fermentations were conducted in duplicate. Student’s t-tests and ANOVA were conducted with JMP 7.0 (SAS, North Carolina) to determine statistical significance of differences observed between sample populations at the 0.05 confidence level. All duplicate measurements were within 5 % of one another.

166
5.4. Results

5.4.1. Preliminary Fermentation of Cabernet Franc Juice

The preliminary fermentation of chaptalized Cabernet Franc juice reached dryness after 500 hours, and had a final ethanol concentration of 14.5 % (v/v) (Figure 5.1). Maximum and final concentrations of glycerol, acetic acid, and several SO\textsubscript{2}-binding compounds are displayed in Table 4.1.
Figure 5.1 Time course of sugar (○) and ethanol (□) concentrations in the preliminary fermentation of Cabernet Franc juice. Fermentation sugar concentration spiked between 50 and 100 g l\(^{-1}\).
Table 5.2 Maximum and final concentrations of glycerol, acetic acid, acetaldehyde, acetoin, pyruvate, and alpha ketoglutarate in wine produced from the preliminary fermentation of chaptalized Cabernet Franc juice. Average of duplicate measurements shown.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Max Concentration</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol (g l(^{-1}))</td>
<td>6.1</td>
<td>6.1</td>
</tr>
<tr>
<td>Acetic Acid (g l(^{-1}))</td>
<td>0.07</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Acetaldehyde (mg l(^{-1}))</td>
<td>23.7</td>
<td>8.3</td>
</tr>
<tr>
<td>Acetoin (mg l(^{-1}))</td>
<td>11.8</td>
<td>3.9</td>
</tr>
<tr>
<td>Pyruvate (mg l(^{-1}))</td>
<td>64.6</td>
<td>46.0</td>
</tr>
<tr>
<td>Alpha Ketoglutarate (mg l(^{-1}))</td>
<td>171.1</td>
<td>171.1</td>
</tr>
</tbody>
</table>
With the exception of pyruvate, which had higher maximum and final levels in the current work, concentrations of the measured metabolites were similar to those obtained during a previously performed continuous fed-batch fermentation of the same juice, where the sugar concentration was kept constant at 45 g l\(^{-1}\) throughout the feeding phase (Chapter 4).

5.4.2. Discontinuous and Continuous Fed-Batch Fermentations of Chardonnay Juice

5.4.2.1. Sugars and Ethanol

Final sugar and ethanol concentrations, as well as maximum rates of ethanol formation for the discontinuous Fermentation A and continuous fed-batch Fermentations B-1 and B-2, are displayed in Table 5.3. Fermentation A lasted approximately 500 hours. Each time additional must was added to the fermentation, a corresponding decrease in ethanol concentration was observed due to effects of dilution. While a final ethanol concentration of 11.3% (v/v) was achieved, 89 % of the juice by volume was added when the alcohol content was below 4.5 % (v/v) (Figure 5.2).
Table 5.3 Final sugar and ethanol concentrations and maximum rates of ethanol formation for the discontinuous (A) and continuous (B-1, B-2) fed-batch fermentations of Chardonnay juice. Different letters indicate statistical difference of means at p=0.05.

<table>
<thead>
<tr>
<th></th>
<th>Final Sugar (g L⁻¹)</th>
<th>Final Ethanol (% v/v)</th>
<th>Maximum Rate of Ethanol Formation (% v/(v·h))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fermentation A</td>
<td>0.72ᵃ</td>
<td>11.3ᵃ</td>
<td>0.105ᵃ</td>
</tr>
<tr>
<td>Fermentation B-1</td>
<td>0ᵃ</td>
<td>12.5ᵇ</td>
<td>0.052ᵇ</td>
</tr>
<tr>
<td>Fermentation B-2</td>
<td>0ᵃ</td>
<td>12.2ᶜ</td>
<td>0.143ᶜ</td>
</tr>
</tbody>
</table>
Figure 5.2 Time course of sugar and ethanol concentrations as functions of time and volume in the discontinuous (A) and continuous (B-1, B-2) fed-batch fermentation of Chardonnay juice. ▲, ▼ - Fermentation A; ○, ● - Fermentation B-1; ▲, ▼ - Fermentation B-2
In contrast, both continuous fed-batch fermentations of Chardonnay juice lasted approximately 40 % longer, taking slightly over 700 hours to reach dryness, and reached higher final ethanol concentrations. Fermentation B-1 achieved a final ethanol concentration of 12.5 % (v/v), while Fermentation B-2 ended at 12.2 % (v/v) ethanol. Fermentation B-2 exhibited a significantly higher maximum rate of ethanol formation than either other fermentation, though Fermentation B-1’s seemingly low maximum rate is likely the result of infrequent sampling. Fermentations B-1 and B-2 also achieved a high ethanol content of approximately 10 % (v/v) with less than 10 % juice delivery. Both continuous fed-batch fermentations demonstrated some movement above and below the target sugar concentration, indicating that the FT-NIRS-based system demonstrated a larger than normal error in its predictions. During all discontinuous and continuous fed-batch fermentations, when must delivery ended, an immediate increase in ethanol concentration was seen as the residual sugar was consumed.

5.4.2.2. Osmotic Stress Response Related Metabolites

Figure 5.3 shows the kinetics of glycerol and acetic acid in the three different discontinuous and continuous fed-batch fermentations of Chardonnay juice. Maximum and final concentrations of all measured metabolites are displayed in Table 5.4.
Figure 5.3 Time course of glycerol and acetic acid concentrations and yields in the discontinuous (A) and continuous (B-1, B-2) fed-batch fermentation of Chardonnay juice. ◼- Fermentation A; ◁- Fermentation B-1; ◐- Fermentation B-2
Table 5.4 Maximum and final concentrations of glycerol, organic acids, and SO₂-binders in discontinuous (A) and continuous (B-1, B-2) fed-batch fermentations of Chardonnay juice. Average of duplicate measurements shown. N.D indicates a measurement below the detection limit of the analytical method. N.A. indicates a value was not determined, because relevant measurements were not taken. Different letters indicate statistical difference of means at $p=0.05$.

<table>
<thead>
<tr>
<th></th>
<th>Fermentation A Maximum</th>
<th>Fermentation B-1 Maximum</th>
<th>Fermentation B-2 Maximum</th>
<th>Fermentation A Final</th>
<th>Fermentation B-1 Final</th>
<th>Fermentation B-2 Final</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol (g l⁻¹)</td>
<td>5.8ᵃ</td>
<td>7.4ᵇ</td>
<td>5.8ᵃ</td>
<td>5.8ᵃ</td>
<td>5.5ᵇ</td>
<td>5.0ᵇ</td>
</tr>
<tr>
<td>Acetic Acid (g l⁻¹)</td>
<td>0.24ᵃ</td>
<td>0.06ᵇ</td>
<td>0.24ᵃ</td>
<td>0.09ᵇ</td>
<td>N.D.ᵇ</td>
<td>N.D.ᵇ</td>
</tr>
<tr>
<td>Malic Acid (g l⁻¹)</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
<td>0.95ᵃ</td>
<td>0.89ᵇ</td>
<td>0.80ᵇ</td>
</tr>
<tr>
<td>Lactic Acid (g l⁻¹)</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
<td>0.23ᵃ</td>
<td>0.26ᵃ</td>
<td>0.35ᵇ</td>
</tr>
<tr>
<td>Citric Acid (g l⁻¹)</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
<td>0.17ᵃ</td>
<td>0.22ᵇ</td>
<td>0.20ᵇ</td>
</tr>
<tr>
<td>Succinic Acid (g l⁻¹)</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
<td>1.4ᵃ</td>
<td>1.6ᵇ</td>
<td>1.5ᵇ</td>
</tr>
<tr>
<td>Acetaldehyde (mg l⁻¹)</td>
<td>74.6ᵃ</td>
<td>72.9ᵇ</td>
<td>57.0ᵇ</td>
<td>49.7ᵃ</td>
<td>72.9ᵇ</td>
<td>57.0ᵇ</td>
</tr>
<tr>
<td>Acetoin (mg l⁻¹)</td>
<td>64.8ᵃ</td>
<td>8.9ᵇ</td>
<td>10.3ᶜ</td>
<td>0.27ᵃ</td>
<td>3.8ᵇ</td>
<td>2.2ᶜ</td>
</tr>
<tr>
<td>Pyruvate (mg l⁻¹)</td>
<td>110.7ᵃ</td>
<td>177.0ᵇ</td>
<td>237.2ᶜ</td>
<td>23.2ᵃ</td>
<td>130.6ᵇ</td>
<td>76.2ᵇ</td>
</tr>
<tr>
<td>Alpha Ketoglutarate (mg l⁻¹)</td>
<td>27.3ᵃ</td>
<td>125.7ᵇ</td>
<td>101.0ᶜ</td>
<td>27.3ᵃ</td>
<td>125.7ᵇ</td>
<td>101.0ᶜ</td>
</tr>
<tr>
<td>Galacturonic Acid (mg l⁻¹)</td>
<td>180.0ᵃ</td>
<td>188.7ᵃ</td>
<td>172.3ᵃ</td>
<td>180.0ᵃ</td>
<td>188.7ᵃ</td>
<td>172.3ᵃ</td>
</tr>
</tbody>
</table>
During the discontinuous Fermentation A, the concentrations of both metabolites rapidly increased during the first 50 hours of fermentation, and then fell and slightly oscillated during the discontinuous feeding portion of the fermentation. Following the final addition of must, glycerol and acetic acid levels increased and then leveled off, resulting in final concentrations of 5.8 and 0.09 g l\(^{-1}\), respectively. The quantity of each osmolyte formed per unit of sugar consumed followed a similar pattern, reaching a peak during the early stages of fermentation and then falling and leveling off at relatively low values.

The continuous fermentations B-1 and B-2 also demonstrated a peak and subsequent decline in the concentrations and yields of both osmolytes, but final concentrations of glycerol and acetic acid were lower in these fermentations. While Fermentations B-1 and B-2 exhibited only modest 6 % and 14 % reductions in final glycerol levels, respectively, final acetic acid levels were below the 0.05 g l\(^{-1}\) HPLC detection limit in both of these finished wines. It should be noted that although Fermentation B-1 seems to exhibit lower maximum concentration and yield values than the other two fermentations, this is likely an artifact of the low sampling frequency used during this fermentation. Overall, final concentrations of glycerol and acetic acid were fairly low across all three fermentations (Moreno-Arribas and Carmen Polo, 2009).

5.4.2.3. Organic Acids

Final concentrations of several organic acids in both the discontinuous and continuous fed-batch wines are displayed in Table 5.4. Final succinic and citric acid levels were similar in the continuous fed-batch Fermentations B-1 and B-2, and slightly (7-29 %) higher than final concentrations in the discontinuous Fermentation A. In contrast, final
lactic acid concentrations were comparable in Fermentation A and Fermentation B-1, but significantly (52 and 35 %, respectively) higher in Fermentation B-2. Malic acid levels were the highest in Fermentation A and lowest in Fermentation B-2, which exhibited a 16 % relative reduction. Overall, aside from malic acid, final organic acid levels were higher in the continuous fed-batch fermentations than in the discontinuous fed-batch fermentation.

5.4.2.4. SO₂-Binding Compounds

With the exception of galacturonic acid, which demonstrated similar maximum and final concentrations across the discontinuous and continuous fed-batch fermentations, SO₂-binder levels varied highly among the three different fermentations (Figure 5.4 and Table 5.4). In general, however, the continuous fed-batch fermentations B-1 and B-2 demonstrated higher final concentrations of all SO₂-binders relative to the discontinuous Fermentation A.
Figure 5.4 Time course of SO₂-binder concentrations in the discontinuous (A) and continuous (B-1, B-2) fed-batch fermentation of Chardonnay juice. - Fermentation A; ♂ - Fermentation B-1; ♀ - Fermentation B-2
In the case of acetaldehyde, levels during the discontinuous fed-batch Fermentation A rapidly increased and reached a peak concentration within the first 50 hours of fermentation. Subsequently, partial re-uptake occurred, resulting in a final concentration of approximately \(50 \text{ mg l}^{-1}\). In contrast, continuous fed-batch fermentations B-1 and B-2 demonstrated a smaller re-uptake phase and a significant increase towards the end of fermentation, when must delivery ended and the remaining residual sugar was consumed, thereby resulting in higher final concentrations. Interestingly, Fermentation B-2 demonstrated a considerably lower starting acetaldehyde concentration (18.8 mg l\(^{-1}\)) than Fermentation B-1 (47.7 mg l\(^{-1}\)) and Fermentation A (47.0 mg l\(^{-1}\)).

In Fermentation A, acetoin levels demonstrated a pattern similar to that of acetaldehyde, first rapidly increasing and then quickly dropping to a low final value. Neither continuous fed-batch fermentation B-1 or B-2 demonstrated a peak in the concentration of this metabolite, likely due to the effects of continuous dilution. While final concentrations were low, they were significantly (8.15 – 14.1x) higher than that of Fermentation A.

Pyruvate concentrations peaked in all three fed-batch fermentations, with each maximum occurring at or immediately following the end of the feeding phase. Maximum and final concentrations, however, were higher in the continuous fed-batch fermentation wines B-1 and B-2. Specifically, maximum levels were 0.6 and 2.14 times greater and final values were 5.6 and 3.3 times greater in Fermentations B-1 and B-2, respectively, as compared to Fermentation A.
The most notable difference between the three fermentation treatments was observed for alpha-ketoglutarate. Although concentrations of this metabolite increased throughout all fermentations, final concentrations were 4.6 and 3.7 times higher in Fermentations B-1 and B-2, respectively, relative to Fermentation A.

5.4.2.5. Yeast Viability

Figure 5.5 shows the time course of live yeast numbers in discontinuous and continuous fed-batch fermentations of Chardonnay juice. In all fermentations, total live yeast numbers increased during the early stages of fermentation and declined towards the end, when must delivery halted and residual sugar was consumed. While the Fermentation A and Fermentation B-1 demonstrated similar starting live yeast numbers, Fermentation B-2 exhibited a much higher initial live yeast concentration. This was expected, however, as the former two fermentations were inoculated with yeast at equivalent concentrations, whereas Fermentation B-2 used a 10 times higher inoculation. Fermentation B-2, which involved nutrient supplementation, also exhibited a fairly constant live yeast concentration during the feeding phase, even as the volume was continuously increasing, with viable yeast numbers tightly oscillating around $1.1 \times 10^7$ CFU ml$^{-1}$. 
Figure 5.5 Time course of viable yeast numbers in the discontinuous (A) and continuous (B-1, B-2) fed-batch fermentation of Chardonnay juice. □ - Fermentation A; ◇ - Fermentation B-1; ◆ - Fermentation B-2
5.5. Discussion

The current works compares the discontinuous fed-batch fermentation of Chardonnay juice with two separate fed-batch approaches. A preliminary fed-batch fermentation of Cabernet Franc juice was also performed, where sugar levels were rapidly spiked several times. Control of fermentation broth sugar levels and automation of must delivery was achieved using a previously described FT-NIRS-based system (Chapter 3). In the first fed-batch approach (Fermentation B-1), 2 L of undiluted must was fermented in a batch mode until it naturally reached a low target setpoint of 30 g l\(^{-1}\) and was then maintained at this value during a feeding phase. When all must to be fermented was delivered, the fed-batch phase ended and the fermentation was allowed to go to dryness. In the second continuous approach (Fermentation B-2), the fermentation was directly started at the low sugar setpoint by adding a small volume of the same Chardonnay juice to the rehydrated yeast starter. Compared to the other two fermentations, Fermentation B-2’s yeast inoculation concentration was 10 times higher calculated according to the starting volume, but 3.5 times lower based on the final volume. While fundamental differences in metabolite formation patterns and yeast viability between the different fermentation platforms were revealed, the results suggest that it may be possible to achieve some of the same benefits of the automated fed-batch approach using a more affordable and widely applicable discontinuous feeding method.

Although the preliminary fermentation of Cabernet Franc juice involved raising the sugar concentration to 100 g l\(^{-1}\) several times, final osmolyte and SO\(_2\)-binder concentrations were still similar to those obtained during a previously conducted fed-
batch fermentation of the same juice (Chapter 4). This initial experiment suggested that fluctuations in sugar concentrations during a fed-batch fermentation may not cause a pronounced osmotic stress response in the fermenting yeast. This hypothesis was then evaluated by performing the subsequent discontinuous and continuous fed-batch fermentations of Chardonnay juice.

The continuous fed-batch fermentation wines (B-1 and B-2) demonstrated significantly higher final ethanol concentrations as compared to the discontinuous fed-batch fermentations (A). In addition, while the infrequent sampling frequency of Fermentation B-1 makes it impossible to draw any conclusions about its formation kinetics, the maximum rate of ethanol production in Fermentation B-2 was 36% higher than that of Fermentation A, indicating an increase in both process yield and efficiency. Higher product yields have previously been associated with a fed-batch fermentation approach (Altýntaþ et al., 2002; Bae & Shoda, 2004; Cheng et al., 2009; Laopaiboon et al., 2007). Similarly, the observed increase in maximum ethanol formation rate during Fermentation B-2 has been previously reported (Frohman & Mira de Orduña, 2013)(Chapter 4), and is related to the higher live yeast concentration in this treatment, as assessed by flow cytometric analysis.

Fermentations B-1 and B-2 also achieved a moderately high ethanol concentration when only a small percentage of the juice to be fermented had been delivered. Due to the balanced rates of formation and dilution, the ethanol levels of these continuous fed-batch fermentations then remained relatively constant around 10 % (v/v) until the feeding phase ended, at which point it rapidly increased as the remaining approximately 30 g l\(^{-1}\) of sugar was consumed. This temporarily sustained ethanol
concentration could be adjusted by altering either the sugar concentration of the must being fermented or the sugar concentration at which the fed-batch fermentation is maintained. This is in contrast with Fermentation A, which only achieved a high ethanol concentration following the addition of the final 11% of its total volume. Implementation of a continuous fed-batch fermentation platform may therefore reduce the risk of microbial contamination, as the majority of the fermentation by volume occurs under high ethanol concentrations. Indeed, previous research has suggested that lactic acid bacterial growth becomes increasingly limited when ethanol concentrations exceed 10% (v/v) (Davis et al., 1988; Wibowo et al., 1985).

Fermentation B-2, which started with a high yeast concentration in the initial volume and involved the addition of a nutrient mixture part way through fermentation to ensure continued yeast growth, also demonstrated fairly constant live yeast numbers throughout the feeding phase of the fermentation. As a result, this fed-batch fermentation behaved like a continuous culture, where substrate, product, and production organism concentrations remained static even as volume exponentially increased. However, even though this fermentation started with a higher yeast concentration, the inoculation concentration based on the final fermentation volume was actually 3.5 times lower than that used in either of the other fermentations of Chardonnay juice. The economic implications of this are significant, as the results suggest the possibility of performing fed-batch fermentations using considerably smaller yeast quantities while simultaneously further reducing the risk of microbial contamination throughout fermentation by maintaining a high yeast titer (Ingram, 1990; Narendranath & Power, 2004; Thomas et al., 2001).
Conversely, live yeast numbers in Fermentation B-1 and Fermentation A gradually increased to a maximum level and then fell at the end of the feeding phase, with Fermentation A exhibiting a somewhat higher maximum viable yeast level. Neither of these fermentations, however, achieved the high maximum live yeast numbers associated with Fermentation B-2. This may be because they were inoculated with a lower yeast concentration, or because nutrient levels weren’t high enough to support yeast growth at an exponential rate equivalent to that of the volume increase.

In addition to affecting product yields and yeast viability, high salt or sugar concentrations present during alcoholic fermentation cause *Saccharomyces cerevisiae* to experience an osmotic stress response that increases expression of the genes responsible for glycerol production (Erasmus et al., 2003; Pigeau & Inglis, 2005a). As a result of this metabolic change, glycerol, which helps maintain redox balance during fermentation and prevents the loss of cell water caused by the higher external osmotic pressure, is synthesized at elevated levels (Blomberg, 2000; Blomberg & Adler, 1989; Nevoigt & Stahl, 1997).

In specialty wines produced from highly concentrated juices, such as those used in the elaboration of ice wines, final glycerol concentrations may exceed 15 g l⁻¹ (Erasmus et al., 2004; Pigeau et al., 2002a; Pigeau et al., 2007; Pitkin et al., 2002). Previous research, however, has indicated that by fermenting with *S. cerevisiae* under maintained low sugar concentration conditions, it is possible to reduce final glycerol levels (Bideaux et al., 2006; Frohman & Mira de Orduña, 2013)(Chapter 4). In the current experiment, all three fermentations demonstrated similar low final glycerol
concentrations below 6 g l$^{-1}$, with Fermentation A having the highest final level and Fermentation B-2 the lowest.

The osmotic stress response of *S. cerevisiae* also causes elevated production of acetic acid, via the oxidation of acetaldehyde (Michnick et al., 1997; Pigeau et al., 2007; Pigeau & Inglis, 2005a; Pigeau & Inglis, 2005b; Remize et al., 1999). As with glycerol, however, implementation of a fed-batch technique that maintains low sugar concentrations throughout fermentation reduces the production of this metabolite (Frohman & Mira de Orduña, 2013)(Chapter 4). In the present study, too, continuous fed-batch Fermentations B-1 and B-2 led to low final concentrations of acetic acid which were below the limit of detection (0.05 g l$^{-1}$) of the HPLC instrumentation used for the analysis. However, Fermentation A also resulted in a low final acetic acid concentration that was below 0.10 g l$^{-1}$.

Examination of the quantity of glycerol and acetic acid formed per unit of sugar of consumed revealed that in all fed-batch fermentations, these metabolites were predominantly synthesized during the early stages of fermentation. After the first approximately 50 hours of fermentation, osmolyte production yields rapidly fell to very low values. Once again, in the case of Fermentation B-1, the absence of a distinct maximum yield for either compound is likely the result of infrequent sampling. Overall, these results suggest that fermenting wine in a discontinuous mode, where additional must is added in discrete steps throughout the process, causes final osmolyte concentrations that are similar to those obtained during continuous fed-batch fermentations.
With regards to final organic acid concentrations, Fermentations B-1 and B-2 exhibited higher final lactic, citric, and succinic acid levels and lower final malic acid levels than Fermentation A. However, some effects were more pronounced than others. In particular, Fermentation B-2, which was started at the setpoint sugar concentration of 30 g l⁻¹, contained a significantly higher final lactic acid concentration than either other fermentation. A correlation between low sugar concentrations during alcoholic fermentation with *S. cerevisiae* and elevated lactic acid levels has been previously demonstrated (Devantier et al., 2005; Yang, 2007) (Chapter 4). The study by Devantier et al. also showed an increase in final succinic acid levels during the fermentation of low-sugar containing media. To the authors’ knowledge, the other effects observed, a decrease in the concentration of malic acid and an increase in the concentration of citric acid in the continuous fed-batch fermentations, have not been previously demonstrated.

Sulfur dioxide is essential for the preservation of wines due to its antimicrobial, antioxidative, and antienzymatic properties. Consequently, if high concentrations of carbonyl compounds that bind SO₂ are present in wine, elevated additions are required to ensure sensory acceptability and microbial and chemical stability (Boulton et al., 1996; Jackowetz et al., 2012). In the current study, significant differences in the final concentrations of all SO₂-binders measured, except for galacturonic acid, were observed between the discontinuous and continuous fed-batch fermentations of Chardonnay juice. Specifically, final concentrations of acetaldehyde, acetoin, pyruvate, and alpha-ketoglutarate were higher in the continuous fed-batch fermentations B-1 and B-2. However, differences in the final levels of acetaldehyde
and acetoin were smaller than those observed for pyruvate and alpha-ketoglutarate. Overall, assuming a target free SO\textsubscript{2} concentration of 30 mg l\textsuperscript{-1}, continuous fed-batch fermentations B-1 and B-2 would require 121 and 59 % higher total SO\textsubscript{2} levels than that of Fermentation A (Jackowetz & Mira de Orduña, 2012).

Acetaldehyde, the final electron acceptor during alcoholic fermentation, influences wine aroma and stability. In most finished wines, its presence is undesirable because of the strong grassy or oxidized aroma it contributes and its ability to strongly bind with SO\textsubscript{2} (Clarke and Bakker, 2004; Miyake & Shibamoto, 1993; Somers & Wescombe, 1982). Previous research has demonstrated that elevated sugar concentrations during vinifications result in increased formation of this metabolite by yeasts (Li & Mira de Orduña, 2011). In contrast, the maintenance of low sugar concentrations using a fed-batch technique leads to decreased acetaldehyde production (Frohman & Mira de Orduña, 2013)(Chapter 4) Surprisingly, in the current study, the discontinuous fed-batch Fermentation A exhibited a lower final acetaldehyde concentration than Fermentations B-1 and B-2. In addition, all of the fermentations started with considerably high concentration of acetaldehyde, whereas levels of this metabolite are usually close to zero prior to fermentation (Li & Mira de Orduña, 2011). As such, it is possible that some of the samples, and particularly those belonging to Fermentations B-1 and B-2, partially oxidized during storage and prior to analysis.

If the continuous fermentation samples did undergo some oxidation, then it would also explain the slightly higher concentrations of acetoin observed in Fermentations B-1
and B-2 relative to Fermentation A, as acetaldehyde is involved in the formation of this compound (Moreno-Arribas and Carmen Polo, 2009).

The most significant differences between the discontinuous and continuous fed-batch fermentations were observed for pyruvate and alpha-ketoglutarate, which were present at significantly higher final concentrations in Fermentations B-1 and B-2. These observations, too, are confirmed by earlier studies that showed that the alcoholic fermentation of a low-sugar containing media results in increased formation of these compounds relative to that of a high gravity fermentation (Devantier et al., 2005; Yang, 2007)(Chapter 4).

The current work demonstrates that the utilization of an automated continuous fed-batch fermentation strategy that maintains low sugar concentrations throughout fermentation leads to low-level production of osmotic stress response related metabolites, but may enhance the synthesis of certain SO₂-binding compounds. A discontinuous fed-batch platform, whereby must is added to an active fermentation in discrete intervals at specific density measurements, also results in low final concentrations of osmolytes, as well as lower final levels of SO₂-binders relative to the continuous fed-batch fermentation. Differences in yeast viability, ethanol formation kinetics and concentrations, and the formation of organic acids were also observed between the discontinuous and continuous fed-batch fermentations. Overall, however, both fermentation strategies allowed for the production of a dry, finished wine, and even the most significant metabolite differences in terms of concentration may not translate into organoleptically detectable differences, thereby suggesting the potential application of the discontinuous fed-batch approach as a feasible and economical
alternative to the FT-NIRS-driven automated and continuous system. Consequently, future works will further investigate the metabolic and sensorial implications of various discontinuous feeding strategies for fed-batch vinifications.

5.6. Conclusions

The discontinuous and FT-NIRS automated and continuous fed-batch fermentations of Chardonnay juice by *S. cerevisiae* demonstrated significant differences with regards to yeast viability and the production of ethanol, certain osmotic stress response related metabolites, organic acids, and SO$_2$-binding compounds. However, implementation of the discontinuous fed-batch approach still prevented the incidence of a pronounced osmotic stress response, as evidenced by the low final concentrations of glycerol and acetic acid. The metabolic and sensorial implications of this offline and affordable approach to sugar concentration management during vinifications require further investigation.

5.7. Acknowledgements

The authors wish to acknowledge financial support from the Nolan, Dyson and Canandaigua Wine Co. graduate support funds.
Reference List


6.1. Discontinuous Fed-Batch Fermentation Experiment: Cycling Sugar Concentrations From 50 – 150 g l⁻¹

Before automatable equipment capable of rapid in-line analysis of fermentation broth sugar levels was available, an initial set of experiments was performed in which the metabolic effects of a discontinuous fed-batch approach to winemaking was tested. For this purpose, a small quantity of the same chaptalized Chardonnay must (343 g l⁻¹ total sugars) described in Chapter 2 was combined with a slurry of rehydrated *S. cerevisiae* EC1118 yeast to a starting concentration of 150 g l⁻¹ total sugars. Fermentations were conducted at 20°C in 2 L glass bottles (Kimble Chase, NJ). Additional high gravity must was manually added to the vinification each time the sugar concentration dropped to 50 g l⁻¹, in order to re-raise it back to 150 g l⁻¹. The quantity of yeast and rehydration nutrient (GO-FERM®) utilized was calculated such that the inoculation concentration based on the target final fermentation volume (10 L) would be 40 g hl⁻¹, the same concentration used in the normal and high gravity batch fermentations detailed in Chapter 2. However, as the starting volume was approximately 180 ml, the initial yeast inoculation and nutrient concentrations (2222 g hl⁻¹ and 2778 g hl⁻¹, respectively) were high. In addition, fermentative activity halted at a final volume of < 2 L, thereby resulting in a final volume yeast inoculation concentration much higher than that of either batch fermentation. The fermentation was conducted in duplicate, and sugar, ethanol, acetic acid, and glycerol concentration measurements were obtained throughout via HPLC, using the method described in
Chapter 2. Sugar and ethanol concentrations for the described discontinuous fed-batch fermentation are displayed as a function of time in Figure 6.1. Data from the normal and high gravity batch fermentations detailed in Chapter 2 are also included for comparison.

While the normal batch fermentation went to dryness, both the high gravity batch and discontinuous fed-batch fermentations were stuck at 70 and 94 g l⁻¹ residual sugar, respectively. Sugar consumption during the initial portion of the feeding phase in the discontinuous fed-batch fermentation was very rapid, likely due to the largely elevated yeast concentration (Frohman & Mira de Orduña, 2013)(Chapter 4,5). This rate continuously slowed, however, as the fermentation volume and ethanol content increased. A similar pattern applies to the ethanol data. Initially, the discontinuous fed-batch fermentation displayed rapid ethanol formation kinetics that were 4.0 and 5.0 times faster than that of the normal and high gravity batch fermentations, respectively. Towards the end of the fermentation, however, the ethanol concentration increased very slowly due to the yeast’s limited fermentative activity. Final ethanol concentrations were higher in the high gravity batch fermentation (14.8 % v/v) than in the discontinuous fed-batch fermentation (13.6 % v/v), but this is likely due to the difference in final sugar concentrations.
Figure 6.1 Time course of sugar and ethanol concentrations for the discontinuous fed-batch fermentation (△) and the normal (○) and high gravity (□) batch fermentations. Average data of duplicate fermentations shown.
High sugar concentrations during alcoholic fermentation with *S. cerevisiae* stimulate an osmotic stress response which increases the production of glycerol and acetic acid (Chapters 4,5)(Pigeau & Inglis, 2005; Nurgel et al., 2004). By maintaining low sugar concentrations throughout fermentation, however, it is possible to limit this metabolic response and the associated osmolyte production (Frohman & Mira de Orduña, 2013)(Chapters 4,5).

In the current study, a discontinuous feeding strategy with high yeast and nutrient concentrations, where sugar concentrations were cycled between 50 and 150 g l$^{-1}$ throughout fermentation was employed, and its effects on osmolyte production was investigated. The results, displayed in Figure 6.2, revealed that such a platform does not significantly lower the production of these compounds relative to a high gravity batch control. Indeed, final glycerol and acetic acid concentrations in the discontinuous fed-batch fermentation were 9.8 and 0.80 g l$^{-1}$, respectively, which represent 32 and 110 % increases relative to final concentrations in the normal batch fermentation and only 16 and 20 % reductions relative to final levels in the high gravity batch fermentation (which ended with a lower sugar concentration, too). In addition, the discontinuous fed-batch fermentation demonstrated fast production of both osmolytes during the first approximately 50 hours of fermentation, which were 2.6 to 3.3 times greater than those of either batch fermentation.
Figure 6.2 Time course of glycerol and acetic acid concentrations for the discontinuous fed-batch fermentation (△) and the normal (○) and high gravity (□) batch fermentations. Average data of duplicate fermentations shown.
This is in contrast to the results of the preliminary and main discontinuous fed-batch fermentation experiments detailed in Chapter 5, which found that implementation of such an approach might allow for a lessening of the osmotic stress response during the fermentation of high gravity musts. In that case, however, sugar concentrations during fermentation were either cycled between a lower range of 50 – 100 g l\(^{-1}\) (as in the preliminary fermentation of Cabernet Franc juice), or the must itself contained a significantly lower sugar concentration (200 g l\(^{-1}\) in the Chardonnay juice). In addition, those fermentations used regular quantities of yeast and the rehydration nutrient GO-FERM®, whereas dosage in the current experiment was highly elevated. As high salt concentrations also induce an osmotic stress response in \textit{S. cerevisiae} (Logothetis et al., 2010; Posas et al., 2000), it is possible that the high concentration of GO-FERM®, which contains mineral nutrients, or the elevated yeast levels partly contributed to the rapid and elevated formation of glycerol and acetic acid. Taken together, these results suggest that yeast and nutrient concentration as well as the upper sugar concentration limit allowed during a discontinuous fed-batch feeding approach and the absolute sugar concentration of the must being fermented may influence glycerol and acetic acid production kinetics during vinifications. A more complete understanding of yeast metabolism during such fermentations will require further investigation.
6.2 Effects of Various GO-FERM® and Yeast Concentrations on Yeast Viability and the Production of Osmotic Stress Response-Related Compounds during Alcoholic Fermentations with *S. cerevisiae*

When conducting fed-batch vinifications, yeast and rehydration nutrient (i.e. GO-FERM®) additions may be determined based on the small starting volume, the final volume, or some in-between volume. If quantities are calculated based on the target final volume, then initial concentrations of yeast and nutrient may be extremely high and result in incredibly turbid and muddy-like fermentations. While such environmental conditions make it quite challenging to obtain high quality NIR spectra, the potential effects of high yeast and nutrient concentrations on fermentation performance and yeast metabolism have not been previously investigated. Consequently, an experiment was designed in which batch fermentations were conducted using varying concentrations of yeast and GO-FERM®, and sugar, ethanol, glycerol, acetic acid, and viability measurements were recorded throughout. For this purpose, flash-pasteurized Chardonnay juice from the Languedoc region of France (Kamil Juices, Canada) with a sugar concentration of 192 g l⁻¹ was sterile filtered (0.22 μm nylon filter, Millipore, Ireland), and a complex supplement (Fermaid K, Lallemand, Canada) and ACS grade (NH₄)₂HPO₃ (Fisher Scientific International Inc, NH) were both added at 0.25 g l⁻¹ for yeast nutrition. Twelve 0.4 L aliquots of the prepared must were then combined with 0.1 L of ASTM Class I water (Arium 611UV, Sartorius, Germany) and varying concentrations of rehydrated *S. cerevisiae* EC1118 yeast and GO-FERM® in 0.5 L glass bottles (Kimble Chase, NJ), as detailed in Table 6.1.
Table 6.1 Overview of fermentation parameters

<table>
<thead>
<tr>
<th>#</th>
<th>Total Volume (L)</th>
<th>Must Volume (L)</th>
<th>[Yeast] (g hl(^{-1}))</th>
<th>Yeast (g)</th>
<th>GO-FERM® (g)</th>
<th>H(_2)O (L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.5</td>
<td>0.4</td>
<td>20</td>
<td>0.1</td>
<td>0.125</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>0.5</td>
<td>0.4</td>
<td>40</td>
<td>0.2</td>
<td>0.25</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>0.5</td>
<td>0.4</td>
<td>40</td>
<td>0.2</td>
<td>0.125</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td>0.5</td>
<td>0.4</td>
<td>100</td>
<td>0.5</td>
<td>0.625</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>0.5</td>
<td>0.4</td>
<td>100</td>
<td>0.5</td>
<td>0.125</td>
<td>100</td>
</tr>
<tr>
<td>6</td>
<td>0.5</td>
<td>0.4</td>
<td>500</td>
<td>2.5</td>
<td>3.125</td>
<td>100</td>
</tr>
<tr>
<td>7</td>
<td>0.5</td>
<td>0.4</td>
<td>500</td>
<td>2.5</td>
<td>0.125</td>
<td>100</td>
</tr>
<tr>
<td>8</td>
<td>0.5</td>
<td>0.4</td>
<td>1000</td>
<td>5.0</td>
<td>6.25</td>
<td>100</td>
</tr>
<tr>
<td>9</td>
<td>0.5</td>
<td>0.4</td>
<td>1000</td>
<td>5.0</td>
<td>0.125</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>0.5</td>
<td>0.4</td>
<td>2500</td>
<td>12.5</td>
<td>15.625</td>
<td>100</td>
</tr>
<tr>
<td>11</td>
<td>0.5</td>
<td>0.4</td>
<td>2500</td>
<td>12.5</td>
<td>0.125</td>
<td>100</td>
</tr>
<tr>
<td>12</td>
<td>0.5</td>
<td>0.4</td>
<td>20</td>
<td>0.1</td>
<td>15.625</td>
<td>100</td>
</tr>
</tbody>
</table>
For each yeast concentration utilized, a pair of fermentations was conducted. In the first, GO-FERM® quantity was calculated based on yeast quantity, using a factor of 1.25. In the second, the amount of GO-FERM® added was always 1.25 g, the dosage that would regularly be used with a 20 g hl⁻¹ yeast inoculation for a 0.5 L fermentation. The exception to this is Fermentation #1, whose pair, Fermentation #12, used a low yeast and high GO-FERM® concentration combination.

All fermentations were conducted at 20°C, and samples were regularly isolated for immediate flow cytometric analysis or subsequent HPLC analysis, using the methods detailed in Chapter 2. Sugar consumption and ethanol formation patterns for the 12 different fermentations are displayed in Figure 6.3.

While all 12 fermentations went to dryness and achieved approximately the same final ethanol concentration (8 % v/v), their relative rates of sugar consumption and ethanol formation widely varied. In general, as yeast concentration increased, so did fermentation rate. Specifically, Fermentations #10 and #11 achieved dryness in < 1 day, whereas Fermentation #1 concluded after > 6 days. Within each pair of fermentations, those that utilized the lower GO-FERM® concentration fermented at a slower rate, but the difference was generally quite small. For example, whereas Fermentation #4 finished after approximately 80 hours, its low GO-FERM® pair, Fermentation #5, took closer to 95 hours. Fermentation #12, however, which utilized a small yeast concentration but an extremely high GO-FERM® concentration, reached dryness in about half the time it took its pair, Fermentation #1.
Figure 6.3 Time course of sugar and ethanol concentrations for 12 different batch fermentations using various concentrations of yeast and GO-FERM®.

- Fermentation 1; □ - Fermentation 2; ⊙ - Fermentation 3; ◊ - Fermentation 4; ○ - Fermentation 5; ✶ - Fermentation 6; ◼ - Fermentation 7; ■ - Fermentation 8; ▲ - Fermentation 9; ◆ - Fermentation 10; ● - Fermentation 11; ◆ - Fermentation 12
Extensive previous research has demonstrated a correlation between high fermentation broth sugar concentrations and increased formation of glycerol and acetic acid via an osmotic stress response in the fermenting yeast (Chapters 4,5)(Pigeau & Inglis, 2005; Nurgel et al., 2004; Frohman & Mira de Orduña, 2013). A few works have also demonstrated that high salt concentrations can cause a yeast osmotic stress response (Logothetis et al., 2010; Posas et al., 2000). Surprisingly, however, no works have examined the effect of high nutrient or yeast concentrations on glycerol and acetic acid production during vinifications. The results of the current work, displayed in Figure 6.4, revealed significant differences in the kinetics and magnitude of osmolyte production among the 12 different fermentation treatments.
Figure 6.4 Time course of glycerol and acetic acid concentrations for 12 different batch fermentations using various concentrations of yeast and GO-FERM®.

⩾- Fermentation 1; ⧍- Fermentation 2; ⩾⩾- Fermentation 3; ⩾⩾⩾- Fermentation 4; ⩾⩾⩾⩾- Fermentation 5; ⩾⩾⩾⩾⩾- Fermentation 6; ⩾⩾⩾⩾⩾⩾- Fermentation 7; ⩾⩾⩾⩾⩾⩾⩾- Fermentation 8; ⩾⩾⩾⩾⩾⩾⩾⩾- Fermentation 9; ⩾⩾⩾⩾⩾⩾⩾⩾⩾- Fermentation 10; ⩾⩾⩾⩾⩾⩾⩾⩾⩾⩾- Fermentation 11; ⩾⩾⩾⩾⩾⩾⩾⩾⩾⩾⩾- Fermentation 12
As yeast concentration increased, so did the production of both glycerol and acetic acid. Fermentation #1, which utilized the lowest yeast concentration, exhibited final acetic acid and glycerol concentrations of 0.05 g l\(^{-1}\) and 4.9 g l\(^{-1}\), respectively. In contrast, Fermentation #11, which used a 125 times higher yeast concentration, but the same GO-FERM® concentration, demonstrated final acetic acid and glycerol levels that were 23.8 and 1.8 times greater, respectively. As with fermentation rate, for each pair of vinifications, the one with the elevated GO-FERM® concentration exhibited somewhat higher final osmolyte levels, but the differences were not very large. When a normal (20 g hl\(^{-1}\)) yeast concentration was used in combination with a high GO-FERM® dosage (Fermentation 12), final concentrations of glycerol and acetic acid were still relatively low. For both metabolites, it appears that the utilization of a yeast concentration of 500 g hl\(^{-1}\) or greater results in a significant increase in final osmolyte concentrations. Overall, the results suggest that the utilization of a high yeast concentration during alcoholic fermentation with *S. cerevisiae* causes a pronounced osmotic stress response and increased osmolyte formation. In contrast, application of high GO-FERM® concentrations only causes a slight increase in glycerol and acetic acid production. Understanding of the mechanism involved in this metabolic shift, however, will require further investigation. Regardless, the implications of these findings are significant, and indicate that when conducting fed-batch fermentations, low yeast concentrations (i.e. – calculated based on the small starting volume) should be used in order to prevent high final glycerol and acetic acid concentrations.
In performing this experiment, yeast viability levels were also assessed throughout fermentation using the flow cytometric method detailed in Chapter 2, and the results are displayed in Figure 6.5.
Figure 6.5 Percent live yeast as a function of time for 12 different batch fermentations using various concentrations of yeast and GO-FERM®. ⩾ - Fermentation 1; ⬣ - Fermentation 2; ⋅ - Fermentation 3; ⚫ - Fermentation 4; ⫶ - Fermentation 5; ⋆ - Fermentation 6; ⊥ - Fermentation 7; – - Fermentation 8; ⊽ - Fermentation 9; ⊙ - Fermentation 10; ⚬ - Fermentation 11; ⚫ - Fermentation 12
Aside from Fermentation #1, every fermentation that utilized a low GO-FERM® concentration exhibited fairly similar starting viability levels of 60-75 % live yeast, with those fermentation that involved higher yeast concentrations demonstrating the higher live yeast percentages. In contrast, when GO-FERM® quantities were scaled according to yeast concentrations, percent live yeast measurements were much lower, and centered around 25-35%. Fermentation #12, which used low yeast and high GO-FERM® concentrations, demonstrated an initial viability reading of < 1 %. While these results seem to suggest that high GO-FERM® concentrations reduce yeast viability, this is a misinterpretation of the data. As the sugar and ethanol data revealed, fermentation occurred very rapidly under these conditions. Instead, the low viability measurements recorded by the flow cytometer are caused by the high concentrations of GO-FERM®, which is largely composed of dead yeast. Therefore, any flow cytometry-based viability measurement of a fermentation that contains yeast-derived nutrients will include an error caused by the dead yeast present in that nutrient. The magnitude of that error will depend on the ratio of yeast to nutrient present in the fermentation. To further test the impact of yeast nutrient concentrations on fermentation viability measurements recorded using flow cytometry, a separate experiment was performed in which live yeast percentages were determined in rehydrated yeast solutions with and without GO-FERM®. Specifically, *S. cerevisiae* EC1118 yeast were rehydrated according to the manufacturer’s instructions at a concentration of 40 g hl⁻¹, with (A) and without (B) the addition of 0.5 g GO-FERM®, and added to 1 L of ASTM Class I water (Arium 611UV, Sartorius, Germany) containing 10 g l⁻¹ of ACS grade glucose (Fisher Scientific International Inc, NH) as a
carbon source. A third solution (C) containing rehydrated GO-FERM® but no yeast was also prepared. Flow cytometric measurements were immediately obtained. The results are displayed in Table 6.2.
Table 6.2 Flow cytometry-obtained measurements of percent live and dead yeast in solutions of EC1118 yeast and GO-FERM®

<table>
<thead>
<tr>
<th></th>
<th>% Live Yeast</th>
<th>% Dead Yeast</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>32.9</td>
<td>67.1</td>
</tr>
<tr>
<td>B</td>
<td>82.9</td>
<td>17.1</td>
</tr>
<tr>
<td>C</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>
The results of this experiment confirmed that the presence of yeast-derived nutrients effect yeast viability measurements during vinifications, and cause live yeast determinations to be erroneously low. While percent viability in the solution containing only yeast was measured at 82.9 %, the addition of 0.5 g of GO-FERM® to the 1 L solution reduced this value to 32.9 %. This observed effect is likely much more significant during the early stages of fermentation, when live yeast numbers do not largely outnumber dead yeast supplied by GO-FERM®. In order to obtain accurate measurements, future work could focus on the creation of a calibration that accounts for the contribution GO-FERM® or any other yeast-derived nutrient make to dead yeast measurements at various concentrations.

6.3. Thiol and Sensory Analysis of Batch and Fed-Batch Produced Wines Discussed in Chapter 4

Although not included in Chapter 4, thiol and sensory analysis of the batch and fed-batch Cabernet Franc wines was also performed, and the details of the methods utilized and the results are included below.

6.3.1. Thiol Analysis

When comparing the organoleptic profiles of the batch and fed-batch produced Cabernet Franc wines, experimenters noted that the batch wine smelled very vegetative and herbaceous whereas the fed-batch wine did not present such aromas. However, when a small quantity (<0.5 g) of copper (II) sulfate was added to 100 mL of the batch wine, the intensity of the vegetative aromas was significantly weakened, and the resulting treated wine smelled quite similar to the fed-batch wine. As copper (II) sulfate reacts with thiol functionalities to form non-volatile precipitates,
this preliminary experiment suggested that the predominant aromatic difference between the batch and fed-batch Cabernet Franc wines was caused by thiol concentration differences. To evaluate this hypothesis, the concentration of three representative thiol compounds was measured in each finished wine.

Accordingly, batch and fed-batch wine samples were spiked with deuterated analogs of the compounds of interest, 4-mercapto-4-methyl-2-pentanone (4-MMP), 3-mercaptohexanol (3-MH), and 3-mercaptohexylacetate (3-MHA), and then derivatised under basic conditions (pH 12) with pentafluorobenzyl bromide. Thiol analysis was limited to these three compounds due to the time associated with synthesizing the deuterated analogs. HS-SPME analysis was performed using an automatic CombiPal system (Cohesive technologies, GA) and a 1 cm, 65 µm, SPME fiber (PDMS-DVB) (Supelco, PA). Following extraction for 60 minutes at 70 °C, the compounds were desorbed in the GC injector of an Agilent 6890N (Agilent, CA) which was coupled to an Agilent 5973N mass spectrometer. GC-MS analysis then occurred under the chromatography conditions described by Musumeci et al (Musumeci et al., 2013). Data processing was carried out by MSD ChemStation software (Agilent, CA). Identification of the target volatile thiols was achieved by comparing the peak formed in SIM mode with the ions previously selected using the method described by Musumeci et al.

Neither wine contained detectable quantities of 4-MMP. 3-MHA levels were just around the detection limit of 17 ppt in the batch wine, but not detected in the fed-batch wine. In contrast, both wines displayed large and readily quantifiable 3-MH peaks, with the concentration in the batch wine (1221 ppt) being 22.5% higher than that of
the fed-batch wine (997 ppt). Based on this preliminary finding, it would be interesting to test the concentration of other volatile thiols in both wines, to see if levels are always higher in the batch wine.

Unfortunately, literature relating sugar concentrations or nitrogen feeding strategies during alcoholic fermentation to thiol synthesis and conjugate release is highly limited. However, it is widely known that a shortage of assimilable nitrogen during alcoholic fermentations results in the accumulation of hydrogen sulfide (H$_2$S) (Jiranek et al., 1995; Stratford & Rose, 1985). It is therefore possible that high levels of synthesized H$_2$S in the batch wine resulted in increased nucleophilic attack of aldehyde and ketone species and thus caused elevated final thiol concentrations. In support of this untested hypothesis, very strong rotten egg-type odors, which are generally associated with the presence of H$_2$S, were detected throughout the batch fermentation but not during the fed-batch fermentation of the same Cabernet Franc juice.

6.3.2. Sensory Analysis

Based on these initial sensory observations and chemical findings, the finished batch and fed-batch wines were also evaluated for the intensity of herbaceous aromas by a two-alternative forced choice (2-AFC) test (Bi et al., 1997). A group of 16 panelists with wine evaluation experience, consisting of 10 females and 6 males, ages 23 to 65, analyzed the wines following sterile filtration (0.22 µm, nylon, Millipore, Ireland). Panelists were chosen based on interest and availability, and were familiarized with the evaluation protocol prior to the session. To familiarize panelists with the stemmy, herbaceous aroma of interest, a standard composed of 60 g of yellow bell pepper
blended in 200 ml of deionized water and then passed through a coarse filter (20-25 µm, cellulose, Whatman, UK) to remove solids was provided. For the batch and fed-batch produced wines, one wine sample was randomly selected for sensory evaluation from duplicate wines. To ensure the replicates did not differ, presensory testing was performed. Twenty-four hours prior to testing, wines were moved to the testing area to equilibrate their temperature. Five minutes prior to serving, 30 mL of each wine was poured into clear 50-mL beakers, which were topped with plastic covers to retain aromas. Panelists were presented with a pair of coded samples and asked to select the sample with the stronger herbaceousness. Panelists were not asked to determine the intensity of the herbaceous aroma based on the presented sensory standard, as the panel was not trained for such an evaluation due to time and cost constraints, nor was it a goal of the current study. Sensory evaluation was conducted in the New York State Agricultural Experiment Station Sensory Evaluation Room under red lighting, with order of sample presentation randomized.

Of the 16 panelists, all but one chose the batch wine as the more vegetative and herbaceous sample. Furthermore, the one panelist who elected the fed-batch wine noted that she was suffering from a cold, so the reliability of her evaluation is questionable. Overall, the results suggest a highly significant difference in the aromas of the two finished wines. Additional detail on the organoleptic differences between the batch and fed-batch produced wines will require more sensory testing, including the performance of descriptive analysis and preference testing.
6.4. Calculation of Volumetric Productivity for Batch and Fed-Batch Fermentations Discussed in Chapter 4

Volumetric productivity of a bioreactor is defined as amount of product formed per unit time per liter of reactor volume. A calculation of this parameter for the batch and fed-batch fermentations of Cabernet Franc juice described in Chapter 4 is provided below:

\[
Volumetric \ productivity = P_v = \frac{(g \ of \ product)}{(Time)(Volume)}
\]

\[P_v^{\text{Batch}} = \frac{\left(\frac{145 \ mL \ EtOH}{L \ wine}\right)\left(\frac{0.789 \ g \ EtOH}{1 \ mL \ EtOH}\right)\left(11.5 \ L \ wine\right)}{(238 \ h)(28 \ L)} = \frac{0.197 \ g \ EtOH}{h \cdot L}\]

\[P_v^{\text{Fed-Batch}} = \frac{\left(\frac{144 \ mL \ EtOH}{L \ wine}\right)\left(\frac{0.789 \ g \ EtOH}{1 \ mL \ EtOH}\right)\left(11.5 \ L \ wine\right)}{(325 \ h)(28 \ L)} = \frac{0.144 \ g \ EtOH}{h \cdot L}\]

While the fed-batch fermentation of high gravity (286 g l-1) Cabernet Franc juice led to reduced osmolyte production relative to a batch fermentation, the above calculation demonstrates that the fed-batch fermentation also resulted in a 27% lower volumetric productivity. Traditionally, continuous and fed-batch fermentations demonstrate higher volumetric productivities than batch operations due to the time required for loading, discharging, and cleaning of the bioreactor in the latter. In addition, since volume is continuously drawn off during traditional continuous fermentation, tank size can be greatly reduced, resulting in a further increase in volumetric productivity (Mitchel et al., 2006).

In the current experiment, both the batch and fed-batch fermentations achieved approximately equivalent final ethanol concentrations. Furthermore, since the fed-batch vinification did not involve continuous removal of product, both fermentation
treatments were conducted in the same 28 L tank. Consequently, the observed difference in volumetric productivity was predominantly driven by the difference in fermentation duration, with the fed-batch fermentation lasting 27% longer than the batch fermentation.
Reference List


CHAPTER 7 - SYSTEM DEVELOPMENTS, LIMITATIONS, AND IMPROVEMENTS

7.1. Evolution of the Automated FT-NIRS-Based System for Fed-Batch Fermentation Control

Throughout the course of this research project, the FT-NIRS-driven system for conducting automated fed-batch vinifications has been continuously updated, redesigned and advanced. Initially, a small 5 L Nalgene container was used as a fermentation vessel, and the transflectance probe was installed horizontally through a compression fitting attached to the side of the jar. Must was delivered, via a peristaltic pump (704 U/R, Watson-Marlow, England), through a hole drilled into the cap of the container, such that it dripped down into the active fermentation. Homogenization of the fermentation was achieved by placing the vessel on a magnetic stir plate and suspending a stir bar from the cap into the liquid below. For these small-scale fermentations, the must reservoir was stored in a 5 L glass bottle (Kimble Chase, NJ), which was partly submerged in a water bath (NESLAB RTE-211, Fisher Scientific International Inc, NH) at 2°C for preservation. Temperature control was achieved by combining a thermostat and probe (WIN100, Lux Products, NJ) set to 20°C with a second identical peristaltic pump, with tubing inlet and outlets submerged in the same 2°C water bath. The thermostat turned a the peristaltic pump on and off, as necessary, to control the flow of chilled water through stainless steel coils submerged in the fermentation vessel, so that fermentation temperature was maintained at the target value.
Initially, control of fermentation broth sugar levels was achieved using a VBScript that calculates a 5-point moving block average of sugar concentrations. Specifically, an average of the last 5 sugar measurements obtained by the FT-NIRS is calculated and subtracted from the target value. If the difference is positive, then the pump turns on, and the speed is determined by the magnitude of the difference. If the difference is negative or zero, the pump remains off.

While this control system provided a simple and fast method for maintaining a setpoint, it did not include any predictive capability. As a result, over- and under-shooting the target value were a significant problem. Additional problems associated with this early fed-batch fermentation system design included the partly open lid, which resulted in significant sample oxidation, and the small size of the fermentation vessel itself, which prevented the implementation of larger pilot-plant scale experiments.

Subsequently, the small Nalgene container was replaced with a specially designed 28 L cylindroconical fermenter, as described in Chapter 3. In addition, control of sugar concentrations was achieved via a PID controller (2216e, Invensys Eurotherm USA, VA), which continuously compared the process value to the setpoint value and accordingly adjusted the output speed of a peristaltic pump delivering additional high gravity must to the fermentation. Initially, recirculation of the fermenting media was achieved using a brewing transfer pump (Super Vinpro Transfer Pump, Midwest Supplies, MN). However, these pumps were incapable of running continuously for extended periods of time, and so they were replaced with a large external peristaltic pump (704 U/R, Watson-Marlow, England).
The first fermentations conducted in this larger tank did not implement any type of temperature control. Due to excessive fermentative activity, temperatures during these fermentations often exceeded 26 °C. A second PID controller was then installed and used to maintain fermentation temperatures at the target value by controlling the flow of cool (15 °C) water through a stainless steel immersion coil submerged in the fermentation liquid. The overall details of this control scheme are provided in Chapter 3.

More recently, a third PID controller has been added to maintain fermentations under 5 PSI of nitrogen gas at all times, to prevent oxidation. This PID unit adjusts the positioning of a modulating ball valve (BI-200, Bi-Torq, IL) that in turn controls the flow of gas into the fermentation tank. To further monitor the risk of sample oxidation, a fluorescence lifetime quenching dissolved oxygen meter (Fibox 3 meter with PSt3 sensor spots, Presens, Germany) was implemented to regularly measure oxygen levels within the tank. For this purpose, a PSt3 sensory spot was attached to the inside surface of a watch glass on the tank.

In addition, instead of storing unfermented must in a glass jar in a water bath, an insulated and cooled 20 L Cornelius keg was implemented. To cool the keg and the juice within, a copper coil was tightly wound around the outside of the container, which was then insulated with nitrile butadiene rubber (NBR) foam. Chilled 2 °C water from a water bath flowed through the copper coils to keep the juice cold at all times. An ultrasound distance detector (UNAM 18I6903/S14, Baumer, Switzerland) that was calibrated to the fill volume of the juice storage tank was installed in the lid for continuous volume measurements. The addition of this analytical device, which
was the final modification made to the fermentation system, allows one to track the volume of the fermentation throughout the fed-batch process, and therefore to calculate various yield coefficients (Chapter 5).

A basic animation of the engineered system, which excludes some of the aforementioned details and modifications, can be found at the following address:

http://www.youtube.com/watch?v=kEUvYFA0lj0

7.2. Limitations of the Current System

While the FT-NIRS-directed fed-batch fermentation system has performed quite well, it still presents a few significant limitations. Vinifications can be conducted using different grape varieties, yeast strains, juice compositions, nutrient concentrations, and temperatures. Due to the inherent complexity of fermenting wine, it was quite challenging to generate a single calibration model that accurately describes all theoretically possible sample variation that might be encountered during a given fermentation. Although the calibration models utilized for the described research contained over 200 individual standards, they still failed to provide accurate predictions under all conditions. This is likely because the various models were generated primarily using standards acquired from fermentations of Chardonnay must, instead of a large assortment of red and white wine samples. During the enactment of certain fermentations that utilized conditions different from those included in the calibration models, total sugars predictive accuracy was quite low, resulting in errors of up to +/- 20 g l⁻¹. Fortunately, however, the magnitude of the predictive error was usually consistent throughout a particular fermentation, thereby allowing for relatively
static control of sugar levels, even though the maintained concentration was off from the target setpoint.

Another significant limitation of the current system is that it relies on the acquisition of transflectance spectra, which include measurements not only of back-scattered radiation but also of transmitted light. The utilized probe contains a small 1 mm slit where the liquid being measured flows through. As a result of these considerations, it would not be possible to use such a system for the analysis of red wine fermentations that are conducted on their skins, as not enough light would be able to penetrate through the solid skins and berries. In addition, berry particulate would likely get stuck within the small gap of the fiber optic probe head, preventing the obtainment of representative spectra. It would likely be possible to achieve good measurements in such a fermentation using a reflectance-type probe, but this would require the generation of new calibration models, which was not an objective of the current project.

The engineered system was also not tested on a large-scale fermentation (>1000 L), but instead was primarily used to control sugar concentrations during vinifications in a fairly small 28 L cylindroconical tank. In this relatively small system, it was possible to ensure the homogeneity of the system and to prevent buildup of carbon dioxide bubbles or particulate on the probe head by recirculating the liquid using a single peristaltic pump. In very large industrial style fermentations, however, stratification issues would be more prevalent, making it harder to obtain representative spectra. Very strong and thorough mixing of the fermentation broth would be required, and several probes might need to be installed at different locations within the tank with the
collected spectra then being averaged. However, achieving thorough mixing during large volume fermentations might require a significant redesign of the tanks, which would be quite costly.

Another limitation of the current system is that when starting a fed-batch fermentation, a sufficiently high starting volume is required to ensure that the probe head is always fully submerged. Unless the fermentation tanks are further redesigned to include a very small side loop where the probe could be installed, there are two main ways to achieve this. In the first possibility, a certain volume of the juice to be fermented is inoculated without any prior dilution. Once the fermentation’s sugar concentration naturally drops to the target level, it is maintained here during a fed-batch phase. In the second, a small partial quantity of the juice to be fermented is diluted to the target sugar concentration with the yeast starter and additional water, as needed, so that the probe is submerged. In this second case, the fermentation entirely occurs in a fed-batch mode. Unfortunately, neither of these situations is ideal, as the first involves an initial batch phase, which, if sugar concentrations are significantly high, may initiate an osmotic stress response, and the second requires a substantial addition of water to the juice.

As should be evident from the above discussion, a FT-NIR spectroscopy based system for in-line measurement of target compounds is not a turnkey system that can be purchased from an equipment supplier and immediately used for analysis and control. The calibration development and engineering required necessitates a significant initial time investment. In addition, the base equipment is currently quite expensive, with an estimated cost of $40,000 – 90,000, depending on the particular model and
configuration purchased. As such, until spectroscopic equipment prices fall considerably, application of the described system may be limited to the few largest wine companies that are able to make such an investment.

7.3. Future Improvements of the FT-NIRS System

In order to improve the engineered FT-NIRS-controlled fed-batch fermentation system, there are several steps that can be taken. To improve the predictive accuracy of already developed calibrations, additional standards could be generated and added to the models. Such calibration standards should be obtained from various batch and fed-batch fermentations. Importantly, a variety of different juice types, must and fermentation setpoint sugar concentrations, yeast strains, and nutrient levels should be utilized in order to maximize robustness over a wide range of conditions.

In addition, it may be beneficial to remove the most turbid standards, which are associated with astronomically high yeast and nutrient (i.e. GO-FERM®) concentrations, from the current models. Such standards were included to represent fed-batch fermentations started using a small fraction of the total juice to be fermented, but inoculated with the amount of yeast and nutrient that would be used if the entire volume were fermented in a batch mode. In reality, however, none of the fed-batch fermentations were conducted in this manner. Even when a high yeast concentration (calculated according to the total volume to be fermented) was used, the quantity of yeast nutrient added to the rehydration slurry was not scaled by the same factor. More commonly, yeast and nutrient concentrations were calculated based on the partial starting volume. As a result, while many of the fed-batch fermentations were highly turbid, they were not nearly as muddy or opaque as the most turbid
calibration samples. Since it is unlikely that any fermentation will demonstrate similarly high turbidity levels, such calibration standards may be considered outliers. Removing them from the calibration models may significantly improve predictive accuracy.

To further improve the developed models, it may be useful to remove the small number of calibration standards whose spectra were obtained using the sample compartment. These approximately 30 standards were sent to Bruker Optics during the developmental stages of this research project so that initial calibrations could be developed. Due to a miscommunication, however, the samples were measured in the instrument’s sample compartment, instead of using the fiber optic probe. More importantly, the cuvettes used had a path length of approximately 8 mm, which is much larger than the optimal 1-2 mm range. The large sample gap results in high absorbance levels which may be outside the linear range of the Lambert-Beer law, and would therefore inhibit the creation of accurate calibration models.

In addition to improving the existing calibration models, the developed system could be further enhanced through the creation of additional models for other enological parameters. For example, a calibration for glycerol could be created to help track incidence of an osmotic stress response during high gravity fermentations. Models for malic and lactic acids in wine would allow a similar automated approach to be applied to the enactment of malolactic fermentations. In this case, finished wine would be continuously fed to an active malolactic fermentation such that malic acid concentrations are kept constant and low throughout the process. Furthermore, the creation of calibration models for additional substrates would allow the engineered
system to be applied to the automation and control of other industrial fermentations, such as acetifications, biofuel fermentations, or those used in the production of other products such as antibiotics or polymers.

As a final example, it would be highly advantageous to design a mechanism that allows the fermenting media to be filtered before reaching the FT-NIRS probe head, but simultaneously recaptures and reincorporates any blocked yeast or particulate. Such a system would largely eliminate the challenge of obtaining high quality spectra with low absorbance baselines during active fermentations, as the samples being measured would not be turbid. This, in turn, would likely drastically improve calibration model accuracy and performance. Unfortunately, passive filtration occurs too slowly to be practical, and most active filtration methods involve trapping of particulate. A miniature cross-flow filtration unit might work, as in these systems the flow of the filtrate is tangential to the direction of the incoming liquid. Consequently, with such a system, the filtrate could be directed towards the probe head, while the retentate, which would contain all of the yeast and other particulate (continuously washed free by the liquid flow), could be recycled back into the main volume of the fermentation.
CHAPTER 8 - FUTURE EXPERIMENTS & GENERAL CONCLUSIONS

8.1. Future Experiments

The results obtained throughout the course of this study provide new insight into yeast metabolism during diverse fermentation conditions. Nonetheless, there are still a large number of experiments that could be performed using the engineered system to further expand our knowledge of the effects of various parameters on vinification performance and wine composition and quality.

8.1.1. Fed-Batch Ice Wine Fermentations

While various fed-batch fermentations have been conducted, the system has not yet been utilized for ice wine production. For this purpose, very high gravity ice wine juice could be fermented at a low sugar concentration until a target ethanol concentration is reached. The fermentation would then be immediately halted, via filtration or the addition of sulfur dioxide, and either bottled as is or blended with additional unfermented juice to raise the sugar content of the finished product.

Ice wines usually contain elevated concentrations of acetic acid due to the osmotic stress response of the fermenting yeast, which increases formation of this metabolite during the vinification of high gravity musts (Kontkanen et al., 2004; Pigeau & Inglis, 2005; Nurgel et al., 2004). By maintaining low sugar levels throughout fermentation, however, implementation of a fed-batch approach would theoretically allow for the creation of dessert wines with greatly reduced concentrations of acetic acid and ethyl acetate, the latter of which forms via the esterification of acetic acid and ethanol.
Beyond these expected chemical changes, though, the effect of the fed-batch platform and subsequent blending on levels of other esters and wine aroma-relevant compounds is unknown, and requires further investigation. As such, in addition to studying overall yeast metabolism during fed-batch ice wine fermentations, it would be advantageous to perform thorough sensory and preference testing on the finished products and to compare them to traditional batch-fermented ice wines.

8.1.2. Different Sugar Concentration Setpoints

Most of the previously conducted fed-batch fermentations have maintained sugar levels in the 30-50 g l\(^{-1}\) range. While the metabolic effects of fermentations sustained at these sugar concentrations have been investigated, the implications of performing fed-batch fermentations at other sugar levels has not been examined. Therefore, it would be highly interesting to test the effects of using different sugar concentration setpoints (i.e. 10, 20, 70, 100 g l\(^{-1}\)) on yeast metabolism during vinifications and final wine quality. Such an experiment might identify an osmotic stress response sugar concentration “cut-off,” above which there is a rapid increase in the formation of glycerol and acetic acid and a decrease in yeast viability. It might also reveal the setpoint value that results in the greatest product yields, information that would be valuable to the bio-ethanol industry. Indeed, previous research has indicated that substrate setpoints during fed-batch fermentations have an effect on yield parameters (Nilsson et al., 2002).

8.1.3. Offline Sugar Control Techniques

Since the current cost of process NIR spectrometers is quite high, such instrumentation is not a viable option for fermentation sugar control for most winemakers. As a result,
additional experimentation needs to focus on the implementation of more affordable offline techniques. For example, sugar measurements could be estimated at regular intervals based on density recordings, enzymatic assays, or quick chemical tests such as Clinitest®, and this information could then be used to determine must additions. However, in addition to testing the feasibility of such alternative methods, the effects of less precise sugar control techniques on yeast metabolism need to be investigated. Previous research has not examined the difference (in terms of final wine composition) between using one method that maintains substrate levels very close to the target value throughout fermentation and another method that results in small or large oscillations around the setpoint. Such research might reveal that as long as sugar concentrations are kept low, fluctuations do not have a negative impact on fermentation performance or wine chemistry.

8.1.4. Time Maintained Under and Magnitude of High Gravity Conditions During Fed-Batch Fermentations

It is also currently unknown if the time a fed-batch fermentation is maintained under high gravity conditions or the maximum sugar concentration experienced has a larger influence on yeast metabolism. This information, too, would be useful for the creation of an offline and practicable fed-batch protocol. Accordingly, an experiment could be performed in which a high gravity must is fermented in a standard batch mode and several different fed-batch modes. The first fed-batch approach would maintain sugar levels at a low target concentration throughout fermentation, as has been previously done. A second method would start the fermentation at low gravity conditions, but then rapidly increase the sugar concentration up to some higher limit (i.e. 250 g l⁻¹) via
the addition of more high gravity must. The fermentation’s sugar level would then be immediately allowed to fall back down via yeast metabolism to the lower setpoint, where it would subsequently be maintained. A third fed-batch protocol would also start the fermentation at low gravity conditions, but then rapidly raise the sugar concentration to some intermediate value (i.e. 150 g l$^{-1}$) where it would be maintained for a short period of time (i.e. 3 hours). As in the second approach, the fermentation’s sugar concentration would then be allowed to return to the lower setpoint value. Finally, a fourth fed-batch fermentation would utilize the same procedure as the third, but the upper sugar concentration limit would match that of the second method, so as to serve as a control. Samples would be collected throughout all fermentations, and the formation kinetics and final concentrations of several osmotic stress response-related compounds as well as SO$_2$-binders, esters, and other wine aroma-relevant compounds would be investigated to determine the effects of these different fed-batch platforms on yeast metabolism.

8.1.5. Separate Glucose and Fructose Feeds During Fed-Batch Fermentations

*S. cerevisiae* is a glucophilic yeast, meaning that it preferentially consumes glucose at a higher rate than fructose during alcoholic fermentation. Consequently, the fructose to glucose ratio increases throughout a typical batch fermentation, and fructose becomes the main sugar present during the latter stages of most vinifications (Guillaume et al., 2007; Berthels et al., 2004; Moreno-Arribas and Carmen Polo, 2009). This phenomenon is observed during fed-batch fermentations, too, where the setpoint sugar concentration becomes increasingly dominated by fructose as the fermentation progresses. Using a modification of the engineered fed-batch system, however, it
would be possible to force glucose and fructose concentrations to remain equivalent throughout fed-batch fermentations. In order to perform such an experiment, separate PID-driven peristaltic pumps would be used for glucose and fructose feeds. Each feed would be a model grape juice solution, containing a high concentration of either monosaccharide as well as a mixture of organic acids and nutrients normally found in grape juice. A fed-batch fermentation would be started in a media containing equal concentrations of glucose and fructose, and the rates of both pumps would be automatically adjusted throughout fermentation to maintain a 1:1 concentration ratio. Assuming a faster consumption of glucose throughout fermentation, this would translate into the glucose solution being pumped in faster than the fructose feed. A related experiment could test the metabolic effects of a fed-batch fermentation where the sole carbon source is fructose. Analysis of the resulting model wines would help elucidate the impact of preferential sugar consumption on yeast metabolism.

8.1.6. Semi-Continuous Approach to Fed-Batch Fermentations

In the modern production of acetic acid by the submers process, a semi-continuous approach is utilized whereby upon depletion of the substrate (ethanol), 60-75% of the fermentation broth is removed while the rest remains as a starter for the next batch. In acetifications, there are multiple advantages to this approach. By maintaining the titer of the production organism high, lag-times are significantly reduced. Because the substrate and product concentrations oscillate over a smaller range, more continuous medium conditions are established for the production organism, thereby reducing the incidence of sluggish or stuck fermentations caused by high substrate concentrations.
Finally, by maintaining high product levels (in this case acetic acid) throughout the process, the risk of contaminations is reduced.

The FT-NIRS-driven fed-batch system could also be used to study the effects of such a semi-continuous approach on vinifications or bio-ethanol fermentations. For this, fermentations would be carried out in a semi-continuous modus, where 1/4 - 1/3 of the fermentation broth at the maximum ethanol concentration is retained as starter for the next fed-batch. It is hypothesized that this method will both improve viability and performance of the production yeast by reducing extremes in medium composition. In addition, by keeping yeast populations above $3 \times 10^7$ cfu ml$^{-1}$ and ethanol yields above 5% (v/v) at all times, this approach may reduce the susceptibility to spoilage by lactic acid bacteria (Narendranath & Power, 2004; Thomas et al., 2001; Ingram, 1990), which is difficult to control in continuous multi-stage fuel ethanol production systems (Bayrock & Ingledew, 2001). This hypothesis could be tested by challenging active vinifications and bio-ethanol fermentations with appropriate spoilage lactic acid bacteria.

8.1.7. Sensory Testing of Batch and Fed-Batch Wines

As a further example, more thorough sensory testing of all produced wines could be performed. While significant compositional differences between the batch and fed-batch wines have been identified, minimal effort has been placed on evaluating their organoleptic impact. This is of great significance, as most people purchase wine to consume and enjoy it. A lower final concentration of acetic acid in a fed-batch wine is therefore irrelevant if the wine’s overall aroma and taste is unpleasant, or at least less desirable than that of the batch produced wine. Consequently, for all experiments
performed, it would be advantageous to conduct a control batch fermentation, and to compare the finished wines using a combination of different sensory test protocols such as discrimination testing, descriptive analysis, preference testing, and 2-AFC.

8.1.8. Automation of Fed-Batch Malolactic Fermentation and Other Industrial Fermentations

Another interesting application of the engineered FT-NIRS-driven fed-batch system would be the automation of malolactic fermentations, a secondary fermentation process used for some wine styles and enacted by lactic acid bacteria whose main result is the conversion of malic acid to lactic acid. This application would first require the generation of accurate and robust calibration models for the quantification of malic and lactic acid levels in wines. The creation of these models is expected to be challenging, however, as normal concentrations of both acids in finished wines are often less than the accepted $10 \text{ g l}^{-1}$ FT-NIRS limit of detection for complex systems (Niemoeller & Conzen, 2013). In order to overcome this challenge, a large number of standards representing all possible combinations of malic and lactic acid concentrations will likely be required. In addition, to prevent any false correlations due to colinearity effects, it may be useful to include a small number of semisynthetic standards, as discussed in Chapter 3. Once established, the models could be used to automatically feed finished wine to an active malolactic fermentation at calculated rates such that malic acid concentrations remain low and constant throughout the process. Such an approach might improve lactic acid bacteria viability during malolactic fermentation by maintaining more constant environmental conditions. In addition, it may allow for the malolactic fermentation of a “challenging” wine (i.e. low pH, high alcohol, high SO$_2$, etc) that was previously unsuccessfully inoculated via its
slow and constant addition to a small volume of a successful and active ferment (Davis et al., 1985).

Similarly, calibration models for additional enological parameters like glycerol or titratable acidity, as well as for substrates for other industrial fermentations like acetifications, bio-ethanol fermentations, bacterial or yeast propagations, or those used to generate antibiotics, biopolymers, or other materials could be generated. With the creation and validation of such models, the number of related experiments that could be performed using the engineered system is seemingly limitless.

8.2. General Conclusions

The work presented in this dissertation details the engineering of an automated FT-NIR spectroscopy-based system for the control of sugar levels during fed-batch fermentations. The following discussion aims to summarize the overall scope of the project and important research findings.

Although the vinification of grape juice to yield wine is one of the oldest fermentations known to man, the implementation of process control technology to improve winemaking is still fairly uncommon. In the last few years, a growing number of systems have been offered that allow one to monitor the actual fermentation progress and direct vinification and wine quality. Most currently available systems are based on CO₂ mass flow, must density, or osmotic pressure measurements. Theoretically, these systems would allow the determination of sugar concentrations based on initial Brix values and fermentation progress. However, differences in yeast growth, transformation efficiency, and by-product formation render this impracticable.
Furthermore, even though such technology is becoming increasingly available, many winemakers still don’t implement any form of process control. However, during normal batch fermentations, high initial substrate concentrations can inhibit metabolic activity or cause increased byproduct formation. In the wine industry in particular, problems caused by high substrate concentrations have been exacerbated by the effects of ongoing climate change, which are causing grapes to be harvested with increasingly high sugar concentrations. However, high gravity vinifications may be conducted for stylistic reasons as well, as in the elaboration of ice wines, late harvest wines, or those produced from botrytized grapes. When highly concentrated juice is used to initiate a vinification, there is frequently an incidence of a hyperosmotic stress response in the fermenting yeast, \textit{S. cerevisiae}. In addition to increasing the likelihood of stuck and sluggish formations, the osmotic stress response upregulates the glycolytic and pentose phosphate pathway genes, thereby leading to increased formation of fermentation by-products such as glycerol and acetic acid. In the wine industry, this may be undesirable for sensorial reasons, as an increased concentration of acetic acid may have a negative impact on the aroma and flavor of the finished wine. For all industrial fermentations, however, there are significant economic implications of this metabolic response, too, as an increased incidence of stuck fermentations and a decrease in product yields corresponds to a loss in process productivity and profits. Consequently, the utilization of control strategies to automatically measure fermentation broth sugar concentrations and maintain low sugar levels throughout alcoholic fermentation would be extremely useful and allow the yeast hyperosmotic stress response to be avoided. This, in turn, would necessitate
control of substrate feed rates, which is regularly used for the implementation of various industrial fed-batch fermentations. However, such an approach has not previously been applied to winemaking. Accordingly, this dissertation focused on the development and engineering of an automated system for conducting constant low sugar concentration fed-batch vinifications, and the examination of the effects of such a fed-batch platform on yeast viability and metabolism.

Prior to the development of an automated fed-batch fermentation system, however, an initial experiment was performed in which the fed-batch approach was manually performed by continuously adjusting the output speed of a peristaltic pump to maintain sugar levels at a low level throughout fermentation. Normal and high gravity batch fermentations were also conducted for comparison. While the high gravity and fed-batch fermentations demonstrated similar final ethanol concentrations, the fed-batch treatment exhibited a 30% higher maximum rate of formation. In addition, the fed-batch fermentation demonstrated approximately 5-, 2-, and 2-fold reductions in final acetic acid, glycerol, and acetaldehyde levels, respectively, relative to the high gravity batch fermentation, and significant reductions relative to the normal gravity batch fermentation, too. Live yeast numbers were also highest throughout the fed-batch fermentation.

While this initial experiment provided strong preliminary results, performing fed-batch fermentations by manually measuring substrate levels and continuously adjusting feed rates is not a practical option for industry. Consequently, effort was then focused on translating the fed-batch approach into a fully automated system with in-line measurement of fermentation broth sugar levels. While several different
analytical technologies were investigated, including HPLC, segmented flow analysis, various bio- and chemosensors, and ultrasound, ultimately it was determined that the most suitable technique, and indeed the only one capable of providing absolute sugar data in real time in actively fermenting and therefore highly turbid samples, was FT-NIR spectroscopy.

Using a Bruker Optics MPA FT-NIRS, calibrations for glucose, fructose, total sugars, and ethanol in fermenting wine were created using a set of 240 natural and semisynthetic standards. These standards were obtained during various batch, discontinuous fed-batch, and continuous and automated fed-batch fermentations, and contained varying concentrations of yeast and yeast nutrient (GO-FERM®). Given the inherent complexity of the sample matrix, the created and validated models demonstrated highly acceptable statistical parameters, with R² values > 0.93 and RMSEP values of 12.3 g l⁻¹, 10.2 g l⁻¹, 11.6 g l⁻¹, and 0.328 % v/v, respectively. Once calibration models were established, the FT-NIRS was combined with process control software, a PLC, and PID controllers to create an automated fed-batch fermentation system. Specifically, the FT-NIRS continuously measures fermentation sugar levels via an installed transfectance probe, and a PID controller compares the predicted process value to the setpoint value. The output speed of a peristaltic pump delivering additional high gravity must to the active fermentation is then adjusted accordingly, so as to maintain fermentation broth sugar levels at the target value. A second PID controller combined with a RTD probe is used for temperature control and regulates the flow of chilled water through a stainless steel immersion coil submerged in the fermentation volume. When used to maintain a test fed-batch fermentation at a target
sugar concentration of 45 g l\(^{-1}\), the automated system performed very well, keeping sugar levels within 5 g l\(^{-1}\) of the setpoint. Temperature was also effectively maintained within 0.2 °C of the 20 °C target.

The automated FT-NIRS-controlled system was then used to conduct a fed-batch fermentation of high gravity (286 g l\(^{-1}\)) Cabernet Franc juice, where sugar concentration was maintained at 45 g l\(^{-1}\) throughout. When compared to batch fermentation produced wine, the fed-batch wine exhibited significant reductions in the final concentrations of glycerol (-20 %), acetic acid (>86 %), acetaldehyde (-67 %), acetoin (-67 %), and several medium chain saturated fatty acid esters. In particular acetic acid levels in the fed-batch wine were below the HPLC limit of detection (0.05 g l\(^{-1}\)), whereas the concentration in the finished batch wine was 0.36 g l\(^{-1}\).

Simultaneously, the fed-batch wine demonstrated higher final concentrations of lactic acid and α-ketoglutarate, whose final level of 146.5 g l\(^{-1}\) was 3.4 times higher than that found in the batch wine. As with the manually enacted fed-batch fermentation, the FT-NIRS controlled fed-batch fermentation also exhibited higher yeast viability numbers and faster (+13 %) ethanol formation kinetics.

Lastly, effort was placed on the development of a more affordable offline method for enacting fed-batch vinifications. Specifically, an experiment was designed to test the metabolic effects of an offline and discontinuous fed-batch method, where additional juice is added to an active fermentation in discrete steps at specific density measurements. Two automated fed-batch fermentations were also conducted for comparison. While the discontinuous fed-batch fermentation resulted in slightly higher final glycerol and acetic acid concentrations, levels were relatively low across all
fermentations, thereby suggesting that such an offline approach may partly reduce osmotic stress response-related metabolism. The fed-batch fermentations, however, exhibited significantly higher (3.3 – 14x) final concentrations of pyruvate, acetoin, and α-ketoglutarate, which may have implications for wine stability requirements. Final ethanol concentrations were also 8 – 11 % higher in the fed-batch fermentations. The other primary difference observed between the discontinuous and continuous fed-batch fermentations was the kinetics of ethanol formation. Whereas the discontinuous approach causes ethanol levels to remain fairly low throughout the majority of the fermentation, the continuous method causes the ethanol concentration to rapidly rise to a fairly high value, and then to remain relatively constant until the feeding phase ends and the remaining residual sugar is consumed. The enactment of continuous, automated fed-batch fermentations may therefore decrease susceptibility to contamination by spoilage bacteria.

In summation, the enactment of FT-NIRS-automated, constant, low sugar concentration fed-batch fermentations allows for a significant lessening of the yeast osmotic stress response during the vinification of high gravity musts. Using such a strategy, it is possible to ferment highly concentrated juices, as are becoming increasingly common for stylistic reasons and due to the combined effects of ongoing climate change and modified viticultural and harvest decisions, while simultaneously achieving greatly reduced concentrations of glycerol and acetic acid in the finished wines, and enhanced ethanol formation rates. Applied to other industrial fermentations, the fed-batch platform may result in improved product yields and formation kinetics. Furthermore, utilization of an offline method, whereby sugar
concentrations oscillate due to frequent additions of must, may allow for some of the metabolic benefits associated with the automated and constant concentration approach, including a decrease in osmolyte production. The effects of any fed-batch vinification technique on overall yeast metabolism and wine aroma and taste, however, require further investigation.
Reference List


