Study of New Yeast Strains as Novel Starter Cultures for Riesling Icewine Production

By

Fei Yang, B.Sc.

A Thesis

submitted to the Department of Biological Sciences

in partial fulfillment of the requirements

for the degree of

Master of Science

November, 2010

Brock University

St. Catharines, Ontario

Abstract

Icewine is a sweet dessert wine fermented from the juice of grapes naturally frozen on the vine. The production of Icewine faces many challenges such as sluggish fermentation, which often yields wines with low ethanol, and an accumulation of high concentration of volatile acidity, mainly in the form of acetic acid. This project investigated three new yeast strains as novel starter cultures for Icewine fermentation with particular emphasis on reducing acetic acid production: a naturally occurring strain of *S. bayanus/S. pastorianus* isolated from Icewine grapes, and two hybrids between *S. cerevisiae* and *S. bayanus*, AWRI 1571 and AWRI 1572. These strains were evaluated for sugar consumption patterns and metabolic production of ethanol, glycerol and acetic acid, and were compared to the performance of a standard commercial wine yeast K1-V1116. The ITS rDNA region of the two AWRI crosses was also analyzed during fermentations to assess their genomic stability. Icewine fermentations were performed in sterile filtered juice, in the absence of indigenous microflora, and also in unfiltered juice in order to mirror commercial wine making practices.

The hybrid AWRI 1572 was found to be a promising candidate as a novel starter culture for Icewine production. It produced 10.3 % v/v of ethanol in sterile Riesling Icewine fermentations and 11.2 % v/v in the unfiltered ones within a reasonable fermentation time (39 days). Its acetic acid production per gram sugar consumed was approximately 30% lower in comparison with commercial wine yeast K1-V1116 under both sterile filtered and unfiltered fermentations. The natural isolate *S.*

bayanus/S. pastorianus and AWRI 1571 did not appear to be suitable for commercial Icewine production. They reached the target ethanol concentration of approximately 10 % v/v in 39 day fermentations and also produced less acetic acid as a function of both time and sugar consumed in sterile fermentations compared to K1-V1116. However, in unfiltered fermentations, both of them failed to produce the target concentration of ethanol and accumulated high concentration of acetic acid. Both AWRI crosses displayed higher loss of or reduced copies in ITS rDNA region from the *S. bayanus* parent compared to the *S. cerevisiae* parent; however, these genomic losses could not be related to the metabolic profile.

Acknowledgement

I would like to sincerely thank my supervisor, Dr. Debra Inglis for the opportunity of pursuing a master's degree under her supervision. I greatly appreciate your guidance and help throughout the years. I would also thank my committee members, Dr. Andy Reynolds and Dr. Stuart Rothstein for your invaluable comments on my studies.

My thanks also go to my colleagues in IH210: Ailin, Stephanie, Qing, Shiri, Eric, Heidi, Lisa, Christina, Wendy, Matilda, Anna and Ryan. Your support, help and friendship made it a great experience for me studying here. I also appreciate the assistant from the faculty and staff of the Cool Climate Oenology and Viticulture Institute and the Department of Biological Sciences.

Special thanks are given to my parents. Your support and encouragement from the other side of the earth warmed my heart and always encouraged me to step further.

Table of contents

List of Tables

Chapter 2

Chapter 3

List of Figures

Chapter 1

Chapter 2

Chapter 3

List of Abbreviations

ACS: acetyl-CoenzymeA synthetase

ADP: adenosine biphosphate

ALD: aldehyde dehydrogenase

ATP: adenosine triphosphate

AWRI: Australian Wine Research Institute

DHAP: dihydroxyacetone phosphate

F-1, 6-BP: fructose-1, 6-bisphosphate

G-3-P: glycerol-3-phosphate

G-6-P: glucose-6-phosphate

GPD: glycerol-3-phosphate dehydrogenase

GPP: glycerol-3-phosphatase

HOG: high osmolarity glycerol

ITS: internal transcribed spacer

MAPK: mitogen-activated protein kinase

MAPKK: mitogen-activated protein kinase kinase

MAPKKK: mitogen-activated protein kinase kinase kinase

NAD^+: nicotinamide adenine dinucleotide

NADH: nicotinamide adenine dinucleotide, reduced

$NADP^+$: nicotinamide adenine dinucleotide phosphate

NADPH: nicotinamide adenine dinucleotide phosphate, reduced

PAK: p21-activated protein kinase

PAN: primary amino nitrogen

PKA: protein kinase A

TA: titratable acidity

VQA: Vintners Quality Alliance

YPD: yeast peptone dextrose

Chapter 1. Introduction and literature review

1.1. Introduction

1.1.1. Introduction of the problem

The production of Icewine is often associated with prolonged fermentation time and high concentration of volatile acidity (mainly in the form of acetic acid) due to the high concentration of sugar in Icewine juice which places yeast cells under extremely hyperosmotic stress (Kontkanen *et al.* 2004). In Canada, the maximum allowed acetic acid concentration in Icewine is 2.1 g l^{-1} according to the Vintners Quality Alliance (VQA 1999). The average acetic acid concentration in Canadian commercial Icewine was found to be 1.30 g l^{-1} with a range from 0.49 to 2.29 g l^{-1} (Nurgel *et al.* 2004). Interestingly this is lower than the sensory threshold of acetic acid in Icewine, which was found to be 3.185 g l^{-1} (Cliff and Pickering 2006). However, during fermentation, acetic acid can esterify with ethanol to produce ethyl acetate, which gives the wine a characteristic solvent or nail polish remover smell (Cliff and Pickering 2006). Nurgel *et al.* (2004) found that the average concentration of ethyl acetate in commercial Canadian Icewine was 0.240 g l^{-1} with a range from 0.086 to 0.369 g l^{-1}. The sensory threshold of ethyl acetate in Icewine is 0.198 g l^{-1} (Cliff and Pickering 2006), which falls into the range of ethyl acetate concentrations in commercial Icewine on the market. Therefore, the generation of acetic acid during Icewine fermentation has been a concern for Icewine producers.

The acetic acid generated during Icewine fermentation may be a by-product of retaining redox balance in yeast. Under hyperosmotic stress, *Saccharomyces cerevisiae* produce glycerol as an intracellular osmolyte to counteract with the high osmolarity (Blomberg and Adler 1989). The oxidized cofactor NAD^+ is produced during glycerol formation and this leads to a potential redox imbalance for the NAD^+/NADH cofactor system. Under hyperosmotic stress, acetic acid was suggested to be generated to correct this redox imbalance by NAD^+-dependent aldehyde dehydrogenases through acetaldehyde oxidation and concomitantly reduce NAD^+ back to NADH (Blomberg and Adler 1989; Miralles and Serrano, 1995; Navarro-Avino *et al.* 1999).

Since acetic acid detracts from Icewine quality in various ways, it is critical to understand the generation of acetic acid in yeasts' adapted response during Icewine fermentations and to develop methods which could decrease the acetic acid concentration in industrial Icewine production. Selecting new yeast strains which produce less acetic acid compared to commercially available wine yeasts during Icewine fermentation as novel starter cultures for Icewine production appears as a good approach to decrease acetic acid concentration in the final wine.

1.1.2. Thesis objective

The objective of this study is to evaluate new yeast strains to be used as novel starter cultures for Icewine production. The first hypothesis was that the following new yeast strains, the natural isolate *S. bayanus/S. pastorianus* and two hybrids,

AWRI 1571 and AWRI 1572, will produce less acetic acid compared to a commercially available wine yeast K1-V1116 during Icewine fermentation with target ethanol production of approximately 10 % v/v. The second hypothesis was that the two hybrids will have a stable genome during the Icewine fermentations and will thus produce consistently metabolites during fermentations.

1.1.3. Experimental design

All yeast strains were inoculated to sterile filtered Riesling Icewine juice to evaluate their true metabolic contribution during fermentations; they were also inoculated to unfiltered Riesling Icewine juice to determine their potential in commercial winery environments. Yeast growth, sugar consumption, metabolites production (acetic acid, glycerol, ethanol and ethyl acetate) and genomic stability of hybrids were monitored during fermentations. The potential of yeast strains to be used as novel starter cultures for Icewine production was assessed based on their ethanol production, acetic acid reduction and genomic stability.

1.2. Literature review

1.2.1. Introduction of Icewine

Icewine is a sweet dessert wine of great significance to the Canadian wine industry since Canada is the largest producer by volume of Icewine, with a reported production of 1.053 million litres in Ontario in 2009 (VQAO 2009). Icewine is fermented from the juice of grapes which have been naturally frozen on the vine.

Because of the low temperature of harvesting and pressing, -7 °C (EU regulations) and -8 °C (Canada), the water crystals in grape berries are separated from the highly concentrated juice (Bowen 2010). This leads to an extremely high concentration of soluble solids in Icewine juice, including sugar, acids and nitrogen compounds.

The minimum concentration of soluble solids in Icewine juice for fermentation must reach the minimum value of 35 °Brix (VQA 1999), but commonly the juice with a soluble solids concentration of 38 to 42 °Brix is used for Icewine production (Ziraldo and Kaiser 2007). This high concentration of sugar in the juice places yeast cells under extreme hyperosmotic stress, coupled with the high acidic concentration causes problems during Icewine fermentation. On one hand, the stressed yeast cells require a longer period to adapt to the fermentative environments and consequently result in sluggish fermentations. On the other hand, yeast cells produce high concentration of volatile acidity, mainly in the form of acetic acid, while combating the high osmolarity (Kontkanen *et al.* 2004).

1.2.2. Hyperosmotic stress response of yeasts and the correlation to acetic acid

When exposed to high osmolarity, yeast cells rapidly lose intracellular water, leading to the loss of membrane turgor and shrinkage of cells (Hohmann 2002). In order to decrease efflux of water, yeast cells immediately activate several pathways that can protect cells against the high osmotic stress. One of these actions is the arrest of cell growth and the immediate closure of Fps1p, the membrane channel which controls the export of glycerol, to allow cells to accumulate glycerol internally to

balance the intracellular and extracellular osmolarity (Sutherland *et al.* 1997). In this way, glycerol acts as an internal osmolyte to prevent further water loss and help restore cellular function. Another aspect of the immediate rescue system when yeast cells are placed under hyperosmotic stress is the rapid activation of the high osmolarity glycerol (HOG) pathway, which is responsible for the production of glycerol as an intracellular osmolyte to balance the pressure on cells.

1.2.2.1. Introduction of the HOG pathway of *S. cerevisiae*

The HOG pathway is a typical mitogen-activated protein kinase (MAPK) pathway. MAPK pathway is a signal transduction pathway activated and regulated by protein phosphorylation and dephosphorylation. It exists in all eukaryotic cells (Waskiewicz and Cooper 1995) and regulates specific biological responses through an extensive range of extracellular stimuli (Winter-Vann and Johnson 2007). Each organism has multiple distinct MAPK cascades that can transduce different signals in order to protect cells from different extracellular stresses.

MAPK cascades are modular signaling units composed of three protein kinases: MAPKKK (MAPK kinase kinase), MAPKK (MAPK kinase) and MAPK (Marshall 1994). The transmembrane sensing proteins transduce signal to MAPKKK when they sense extracellular stimuli. Then a MAPKKK activates a cognate MAPKK by phosphorylation; the MAPKK in turn phosphorylates the MAPK, which initiates the transfer of MAPK from the cytosol to the nucleus. The translocated MAPK phosphorylates its target proteins which function as transcriptional factors that

upstream regulate gene expression beneficial for cells to counteract corresponding stress (reviewed in Hohmann 2002). This is the basic scheme for the HOG MAPK phosphorelay cascade that helps yeast cells to combat the hyperosmotic stress.

1.2.2.1.1 Sensing and signaling

The HOG pathway consists of two transmembrane proteins playing the role as osmosensors, the Sho1p and the Sln1p, which can sense the change in membrane turgor (Ostrander and Gorman 1999; Reiser *et al.* 2000). After these two proteins sense the change of membrane turgor, they relay the signal of hyperosmotic stress respectively by activating HOG MAPK pathway (Fig. 1.1).

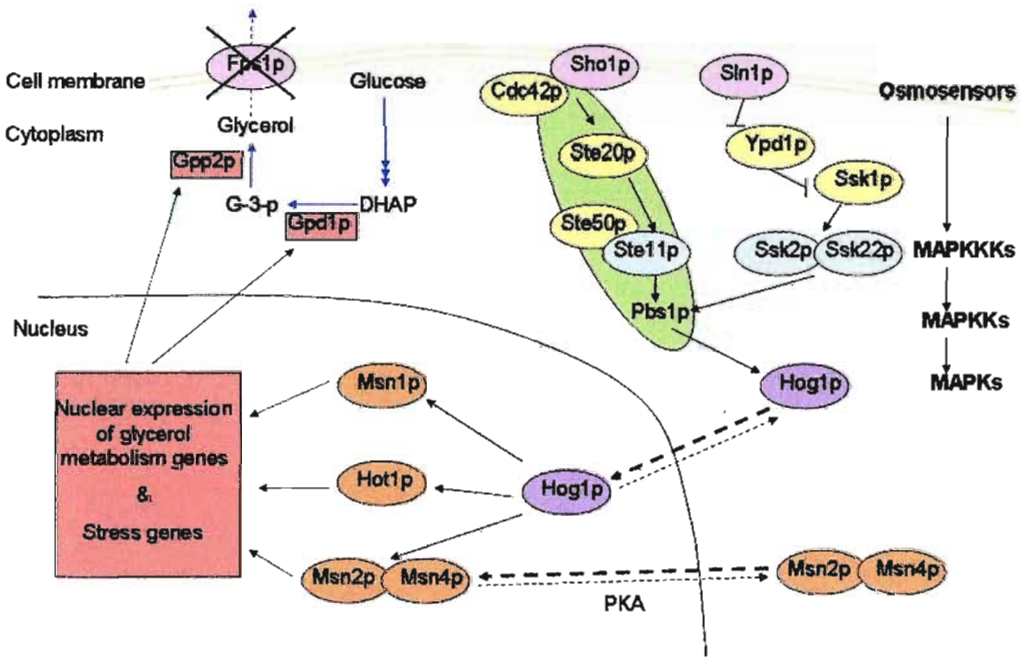


Figure 1.1: Scheme of HOG pathway in *S. cerevisiae* under hyperosmotic stress. Black solid arrows indicate phosphorylation events; green solid arrows indicate association of expression events; blue arrows indicate association of metabolic events; dashed arrows indicate translocation. Adapted from Hohmann (2002).

The Sln1p upstream branches in the HOG pathway (Fig. 1.1) are activated by Sln1p-Ypd1p-Ssk1p phosphate transfer (Posas *et al.* 1996). Sln1p and Ypd1p function

as negative regulators of the HOG pathway, as the study of Maeda *et al.* (1994) showed that the deletion of *SLN1* and *YPD1* would cause lethality of *S. cerevisiae* because of the overactivation of the HOG pathway. Under low osmolarity, Sln1p constantly autophosphorylates itself (Posas *et al.* 1996). The phosphate group is then transferred to Ypd1p and subsequently to Ssk1p (reviewed in Posas *et al.* 1996). When Ssk1p is phosphorylated, it 'turns off' the HOG pathway, whereas the dephosphorylation of Ssk1p under high osmotic stress activates its kinase activity and makes it capable of phosphorylating the MAPKKKs Ssk2p and Ssk22p (Maeda *et al.* 1995; 1994), which 'turns on' the downstream regulation of the HOG pathway. The Ssk2p subsequently phosphorylates and activates MAPKK Pbs2p by the phosphorylation on Ser514 and Thr518 (Posas and Saito 1997).

It is questioned why the phosphorelay system led by Sln1p functions as a negative regulator of the HOG pathway. Hohmann (2002) proposed a reasonable explanation that the HOG pathway was activated until the cell which suffers from low turgor or cell shrinking starts to regain turgor and swells again. Once the cell starts to regain turgor, Sln1p begins to autophosphorylate itself; as such, the Sln1p branch of the HOG pathway was 'turned off'. In this case, the phosphorelay could function as an effective feedback system by itself for the HOG pathway (Hohmann 2002).

Activation of the Sho1p branch of the HOG pathway needs the rapid and transient formation of a protein complex at the cell surface, specifically at places of bud necks (Raitt *et al.* 2000; Reiser *et al.* 2000). This complex consists of at least two components, Sho1p and Pbs2p (Maeda *et al.* 1995; Posas and Saito 1997). In addition,

the complex also contains, although not necessarily at the same time, the p21-activated protein kinase (PAK) Ste20p (or the homolog Cla4p in an *ste20* mutant) (Raitt *et al.* 2000), the rho-like G-protein Cdc42p (Raitt *et al.* 2000), and the MAPKKK Ste11p (O'Rourke and Herskowitz 1998; Posas and Saito 1997), as well as Ste50p, which is required for Ste11p function (O'Rourke and Herskowitz 1998).

The initial signaling event of Sln1p branch in the HOG pathway (Fig. 1.1) may be the activation of Sho1p SH3 domain by an osmotic shock, although the mechanism is not clear (Raitt *et al.* 2000). The Sho1p then binds to Pbs2p, thereby recruiting it to the cell surface, which may mark the generation of the signaling-competent complex (Maeda *et al.* 1995). This complex subsequently recruits Cdc42p with the interacting PAK Ste20p and the MAPKKK Ste11p (Johnson 1999). Cdc42p is known as the monitor of protein complex formation in different contexts, and the assembly of the appropriate signaling complex then leads to activation of the PAK Ste20p (Johnson 1999), which then phosphorylates the MAPKKK Ste11p (Drogen *et al.* 2000). Ste50p might be a cofactor for Ste11p because they form a complex through interaction of their SAM domain during the phosphorylation of Ste11p (Posas *et al.* 1998). The activated Ste11p subsequently phosphorylates the MAPKK Pbs2p on Ser514 and Thr518 (Posas and Saito 1997). Because Pbs2p interacts with multiple proteins, including Sho1p, Ste11p and Hog1p, it has been proposed to act as a scaffold, linking Sho1p to Ste11p activation and, thereby, possibly limiting cross-talk to other Ste11p-dependent MAPK pathways (Posas and Saito 1998).

Once Pbs2p is activated by Ssk2p and Ssk22p of the Sln1p branch or Ste11p of the

Sho1p branch, it immediately activates the MAPK Hog1p by phosphorylation (Fig. 1). It has been proven that the phosphorylation of the Hog1p kinase occurs in the cytosol, because the MAPKK Pbs2p is a cytoplasmic protein and specifically excluded from the nucleus (Reiser *et al.* 1999). Recently, Yang *et al.* (2006) and Hawle *et al.* (2007) proposed that the chaperone Hsp90p and its cochaperone Cdc37p were essential for the stability of Hog1p and thereby were significant for the subsequent Hog1p phosphorylation and downstream function. Hog1p is activated by dual phosphorylation on the conserved Thr174 and Tyr176 (Schüller *et al.* 1994). Coincident with phosphorylation by Pbs2p, Hog1p is rapidly transferred into the nucleus and causes a rapid and marked concentration of Hog1p in the nucleus, while under normal conditions Hog1p appears to be evenly distributed between the cytosol and the nucleus (Reiser *et al.* 1999) (Fig. 1.1).

1.2.2.1.2 Genetic regulation of the HOG pathway

Once entering into the nucleus, Hog1p interacts with several transcriptional factors and hence regulates the expression of numerous genes (Posas *et al.* 2000, Rep *et al.* 2000). The reported numbers of genes that are expression-regulated by osmolarity are different between studies because different growth conditions and thresholds were applied in the analysis. It appears that about 200 to 400 genes are upregulated and approximately 150 to 250 genes are downregulated following a hyperosmotic shock (Hohmann 2002).

Hot1p (Alepuz *et al.* 2003; Rep *et al.* 1999; 2000) is the characterized transcription

factor that is involved in the HOG pathway and plays a significant role in protecting cells from high osmolarity. It is a transcriptional activator which displays Hog1p- and osmotic shock-dependent phosphorylation. Hot1p controls a set of less than 10 genes, including those that encode proteins in glycerol metabolism and uptake, e.g. *GDP1* and *GPP2*. Hot1p binds to the *GPD1* promoter constitutively and recruits Hog1p to the DNA during the HOG response. In addition, Msn1p is also localized at the *GPD1* promoter, but only during hyperosmotic stress (Alepuz *et al.* 2003; Rep *et al.* 1999; 2000). This initiates the upstream expression of *GPD1* which contributes to the formation of the intracellular osmolyte glycerol.

Msn2p and Msn4p are two redundant proteins mediating a general stress response, including osmotic stress (Ruis and Schüller 1995). These two transcriptional factors regulate the stress response elements which are located in the promoter regions of the general stress responsive genes (Martinez-Pastor *et al.* 1996). When yeast cells are placed under hyperosmotic stress, Msn2p and Msn4p are rapidly transferred from the cytosol to the nucleus where they induce gene expression. It has been proven that the nuclear localization of Msn2p/Msn4p is mediated by protein kinase A (PKA) but it functions as a negative control (Görner *et al.* 1998).

1.2.2.1.3 Glycerol production and the redox implication of acetic acid during Icewine fermentation

Glycerol is one of the most crucial products of HOG response. It is produced to serve as an intracellular osmolyte to balance the osmotic pressure placed on the cell (Blomberg and Adler 1992; Blomberg 2000). The rate limiting step in glycerol

formation induced by high osmotic stress is the expression of glycerol-3-phosphate dehydrogenase which is encoded by *GPD1* (Remize *et al.* 2001). This gene product catalyzes the reduction of the glycolytic intermediate dihydroxyacetone phosphate (DHAP) to form glycerol-3-phosphate. The glycerol-3-phosphate is then dephosphorylated by glycerol-3-phosphatase (encoded by *GPP2)* to produce glycerol (Pahlman *et al.* 2001) (Fig. 1.2). Pigeau and Inglis (2005) showed that *GPD1* was upregulated during Icewine fermentation, and reflected the elevated levels of glycerol.

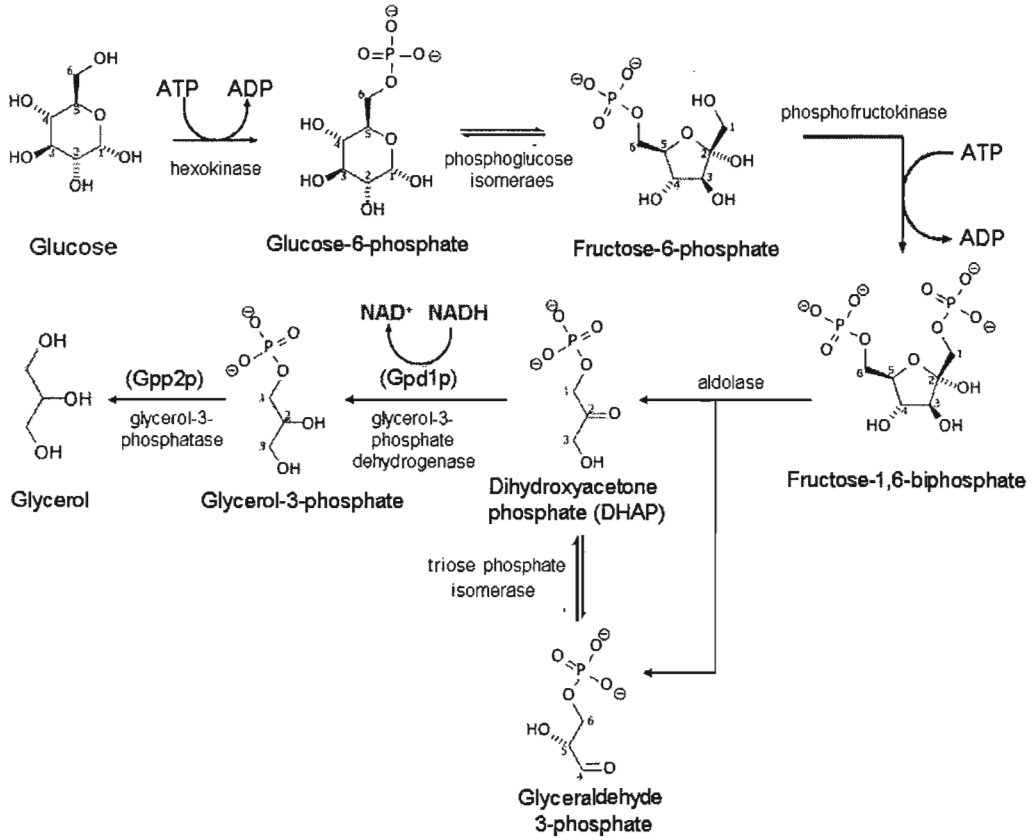


Figure 1.2: Glycerol production induced by high osmolarity

The production of glycerol is an effective strategy to protect yeasts from hyperosmolarity; however, this leads to imbalance of the NAD^+/NADH coenzyme redox system. During glycerol formation, NADH is oxidized to NAD^+ when DHAP is reduced to form glycerol 3-phosphate (Fig. 1.2 and 1.3). Because of the lack of a

transhydrogenase in yeast to convert reducing equivalents between the NAD^+/NADH system and the $NADP^+$/NADPH system (Blomberg and Adler 1989), yeasts must rely on metabolite formation to maintain the redox balance for the coenzyme system (van Dijken and Scheffers 1986). Acetic acid has been considered as such a metabolite by which yeast cells balance the excess NAD^+ produced during glycerol formation under salt-induced osmotic stress (Blomberg and Adler 1989) (Fig. 1.3). This may occur during the oxidation of acetaldehyde to acetic acid, catalyzed by NAD^+-dependent aldehyde dehydrogenases. Certain aldehyde dehydrogenases reduce NAD^+ to NADH while oxidizing acetaldehyde and may be involved in restoring internal redox balance (Blomberg and Adler 1989; Miralles and Serrano 1995; Navarro-Avino *et al.* 1999) (Fig. 1.3).

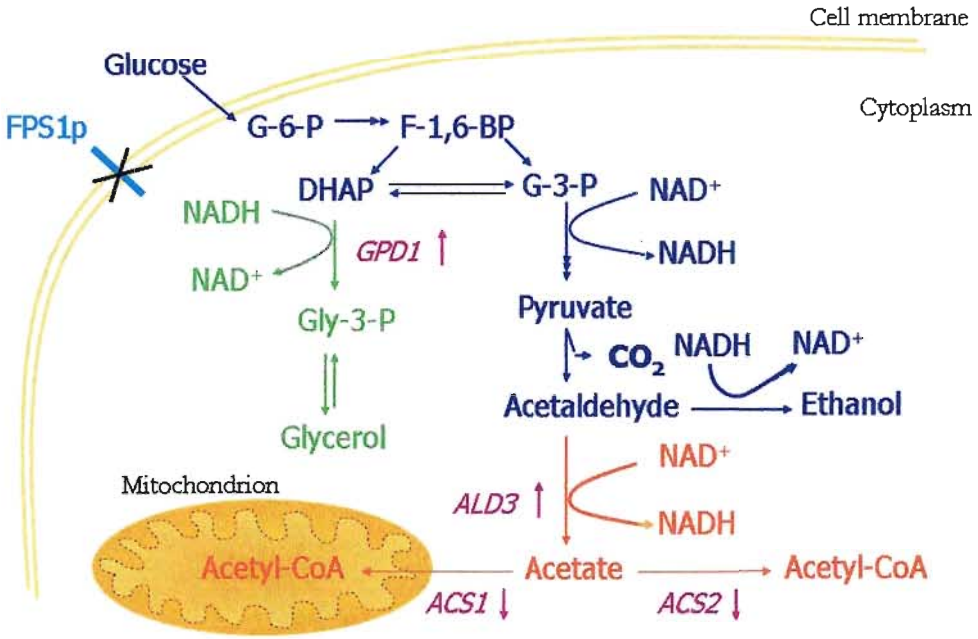


Figure 1.3: Glycerol production and the redox implication of acetic acid during Icewine fermentations in *S. cerevisiae*.

The traditional wine yeast *Saccharomyces cerevisiae* has five *ALD* isogenes that encode acetaldehyde dehydrogenases involved in the production of acetic acid from

acetaldehyde: *ALD2 ALD3, ALD4, ALD5*, and *ALD6* (Navarro-Avino *et al.* 1999). Ald2p and Ald3p are cytosolic enzymes and NAD^+-dependent; Ald4p and Ald5p are located in mitochondrion and rely on $NAD(P)^+$ and $NADP^+$ respectively; Ald6p is cytosolic and uses $NADP^+$ as coenzyme. Previous studies have correlated acetic acid production to increased glycerol formation in response to hyperosmotic stress (Miralles and Serrano 1995; Navarro-Avino *et al.* 1999). If it is true that acetic acid is produced during Icewine fermentation to correct the redox imbalance of NAD^+/ NADH system by oxidizing acetaldehyde to acetic acid and reduce NADH back to NAD^+ concomitantly by cytosolic, NAD^+-dependent aldehyde dehydrogenases (Miralles and Serrano 1995; Navarro-Avino *et al.* 1999), the two cytosolic, NAD^+-dependent aldehyde dehydrogenases Ald2p and Ald3p are assumed to be mainly responsible for the generation of acetic acid under hyperosmotic stress. Navarro-Avino *et al.* (1999) found that the NAD^+-dependent aldehyde dehydrogenase activity increases in wild-type cells but not in *ald2ald3* mutant cells under NaCl-induced hyperosmotic stress. Pigeau and Inglis (2005; 2007) reported that *ALD3* showed a 6.2-fold upregulation 4 days into the Icewine fermentation compared to the diluted fermentation; whereas the expression pattern of the other *ALD* genes were not affected by this high sugar environment. However, Ald6p was previously reported as the main aldehyde dehydrogenase responsible for acetic acid production in *S. cerevisiae* strains during fermentation of glucose media (Eglinton *et al.* 2002), Ald5p was recently found to contribute to acetic acid production as well (Saint-Prix *et al.* 2004), and both are $NADP^+$-dependent. In contrast, Remize *et al.*

(2000) reported that Ald4p and Ald6p are the major contributors of acetate formation during wine fermentations. In a study of hyperosmotic stress during Icewine fermentation, a wine yeast showed upregulated *ALD2, -3, -4* and *-6* exposed to the highly concentrated sugar after 2 hours (Erasmus *et al.* 2003). These studies indicated that the expression of *ALD* genes varies between yeast strains, media and time of exposure to the hyperosmotic stress, and it is still unclear which isoform is responsible for acetic acid generation during Icewine fermentation. The downregulation of genes involved in acetic acid utilizing pathways, such as *ACS1* and *ACS2* which encode mitochondrial and cytosolic acetyl-CoA synthetases respectively, occurs at the same time as the upregulation of *ALD3*, and may contribute to the elevated acetic acid concentration in Icewine (Fig. 1.3). The down-regulation of *ACS1* and *ACS2* during Icewine fermentation was exhibited by microarray analysis (Martin and Inglis 2006), but the expression in protein level has not been investigated.

1.2.2.2. Strain, solute and time dependency of osmotic stress response

Although the osmotic stress response in *S. cerevisiae* has been well characterized, the majority of these researchers focused on laboratory strains of *S. cerevisiae* under salt-induced osmotic stress for a short period and also under aerobic conditions. However, wine yeast strains are genetically different from laboratory yeast strains and may also differ in their response to hyperosmotic stress under anaerobic fermentation conditions (Bidenne *et al.* 1992, Remize *et al.* 1999). Different solute induced stresses

may affect the cell response as well. Sugar, such as glucose, can eventually be metabolized by the cells, which decreases osmotic stress gradually, but high levels of sodium ion could be toxic to the yeast (Kinclova-Zimmermannova and Sychrova 2006). In addition, the initial response of yeast cells to high osmotic stress differs from the adapted response. The adapted response also contributes to the quality of final wine taking into account that Icewine fermentations require 4-6 weeks to reach the desired ethanol concentration.

Wine yeasts are traditionally selected from the natural environment and selected for positive oenological properties during wine fermentation (Pretorius 2000). They are closely related to *S. cerevisiae* laboratory strains but have distinct physiological properties which make them suitable for wine fermentations (Pretorius 2000). For instance, the commercial wine yeasts were selected with high tolerance of sugar and ethanol. They are capable of efficient fermentations and make contributions to the desired flavour of wine (Pretorius 2000). The different characteristics between wine yeasts and laboratory yeasts affect the organoleptic properties of the finished wines (Bidenne *et al.* 1992; Remize *et al.* 1999). It is suggested that most laboratory yeast strains are either haploid or diploid, whereas commercial yeast strains are possible to be diploid, aneuploid and/or polyploidy (Bidenne *et al.* 1992; Pretorius 2000). The polyploidy might be the reason why the commercial strains can withstand the highly stressed environment during fermentations since they possess a higher dosage of genes essential for fermentation performance (Salmon 1997).

Erasmus *et al.* (2003) proposed that wine yeast exposed to sugar induced osmotic

stress for two hours displayed a different transcriptional response compared to laboratory strains under salt induced stress. In this study, four aldehyde dehydrogenases encoded genes *ALD 2, 3, 4* and *6* were upregulated; whereas only *ALD 3* and *6* were reported to be upregulated in laboratory strains under salt induced osmotic stress (Norbeck and Blomberg 2000). Furthermore, pyruvate decarboxylase *PDC 6,* which encodes a key enzyme in fermentative metabolic pathway, was found upregulated in wine yeast under sugar induced stress (Erasmus *et al.* 2003), but this gene was not affected by salt or sorbitol induced stress (Rep *et al.* 2000).

Nevertheless, the situation of wine yeasts under hyperosmotic stress during Icewine fermentation is also different from the initial sugar induced stress since the juice is not only highly concentrated in sugar but also in acids, nitrogen and other compounds. The adapted response to high osmotic stress during the fermentation differs from the initial response, and the fermentation lasts weeks. For industrial applications of yeast, the changing stress response over time may impact the quality of final wine. Pigeau and Inglis (2005; 2007) suggested that only *ALD3* was differenciately upregulated of all the *ALD*s in wine yeast during Icewine fermentation compared to a dilute juice fermentation, and this increased expression follows increased production of acetaldehyde, which is followed by increased acetic acid production. Interestingly, although the accumulation of acetaldehyde was proven to be the trigger for the upregulation of *ALD3,* the exogenous acetaldehyde alone did not affect the acetic acid concentration during fermentations. It appears that the expression of *ACS* genes varies between yeast strains as well. Erasmus and van Vuuren (2009) compared the

transcripts of *ACS1* between a high acetic acid producing strain VIN7 and a low acetic acid generating strain ST grown in grape musts containing 40% w/v sugar. The transcriptional pattern of *ACS1* in VIN7 was found lower than in ST, while transcription of *ACS1* unchanged in *S. cerevisiae* Vin13 when subjected to high sugar concentrations (Erasmus *et al.* 2003).

1.2.3. Acidic stress response of yeast cells

During Icewine fermentation, yeast cells are not only under hyperosmotic stress caused by the high concentration of sugar, they are also placed under acidic stress due to the high concentration of acids in the juice. Organic acids can enter yeast cells through passive diffusion when they are in the uncharged state outside of cells where the pH value is lower than their pKa's. Once in the cytosol they readily dissociate upon encountering the higher pH environment, generating a proton and acid anion. The anionic form of acid will tend to accumulate intracellularly to a high level, as being charged, they cannot readily diffuse from the cell (reviewed in Piper *et al.* 2001). Therefore, yeast cells have to develop different adaptation mechanisms that will allow them to resist the inhibitory acidic environment. Yeasts' adaptive response to acidic stress includes genetic control, signal transduction, post-translational modifications and protein degradation.

The primary response mechanism to weak acid stress in *S. cerevisiae* is the induction of the plasma membrane H^+ ATPase activation (Pma1p), an ATP driven proton efflux pump (Mollapour *et al.* 2008). The role of Pma1p in yeast cells is to

create the transmembrane electrochemical gradient through the translocation of protons across the plasma membrane. The proton gradient provides the driving force of nutrient uptake. Exposure of *S. cerevisiae* cells to acid stress results in cytosolic acidification due to acid dissociation. To prevent the disruption of cytosolic pH homoeostasis, cells induce the activation of the Pma1 pump (Reviewed in Mollapour *et al.* 2008) (Fig. 1.4). Piper *et al.* (1997) found that the activity of Pma1p increased in response to 2 mM sorbic acid in Hsp30p deletion mutants. This membrane heat shock protein is a negative regulator of Pma1p under sorbic stress. These mutants experienced longer adaptation time as compared to wild type, possibly due to increased use of ATP by the pump, which leads to limited yeast growth (Piper *et al.* 1997).

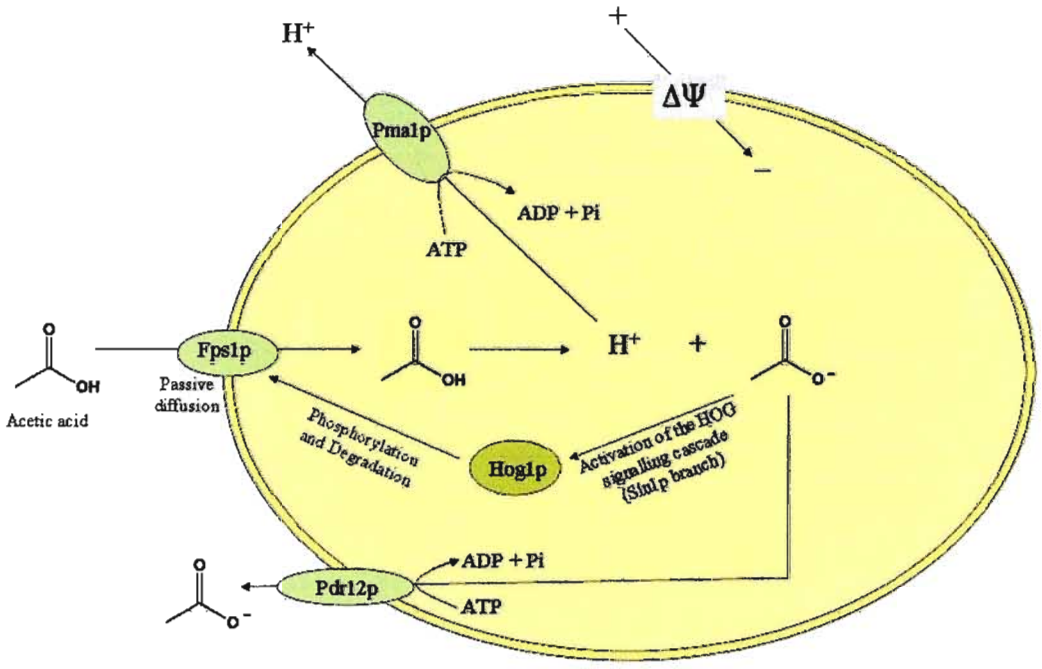


Figure 1.4: The adaptive response of *S. cerevisiae* to acetic acid stress.

Transcriptional profiling of weak acid stress response in *S. cerevisiae* revealed that *PDR12*, the ATP-binding cassette transporter, is one of the most highly induced genes in response to sorbate stress (Schuller *et al.* 2004). It was found that Pdr12p was as an energy dependant carboxylate anion extruder (Piper *et al.* 2008) (Fig. 1.4). *PDR12* gene is induced strongly by 1 mM of sorbate at pH 4.5 by War1p-transcription factor (Kren *et al.* 2003). The extrusion of the acid anion firstly involves the binding of acid anion. Secondly, the energy dependent transporter translocates the anion against its concentration gradient to the periplasmic side of the membrane. This results in reprotonation of the anion due to the encounter with low pH. Removal of the acid anions by Pdr12p is a highly energy demanding process and that is why low biomass is observed under these acid stressors (Reviewed in Mollapour *et al.* 2008).

Mollapour and Piper (2006) demonstrated that when different genes that encode for the proteins of the HOG pathway were deleted, *S. cerevisiae* experienced increased sensitivity to acetic acid stress. Acetate activation of Hog1p is absent in the *ssk*Δ and *pbs*Δ mutants but is present in *sho1*Δ and *ste*Δ, showing that it involves the Sln1p branch of the HOG pathway signaling to Pbs2p (Mollapour and Piper 2006). Interestingly, the induction of the HOG pathway under acetic acid stress conferred yeast resistance to the acid with no increase in the expression of *GPD1* gene or intracellular glycerol concentration, which are both typical events upon activation of the HOG pathway under hyperosmotic stress (Mollapour and Piper 2006).

Thorsen *et al.* (2006) extensively studied the role of Hog1p in response to arsenite toxicity and found the activation of Hog1p did not result in transcriptional regulation

of downstream genes, but rather modulated the activity of Fps1p, a plasma membrane glyceroporin through which arsenite enters the cell. The Hog1p is able to translocate into the plasma membrane and phosphorylate the N-terminal tail of Fps1p, which will induce Fps1p to adopt a closed channel conformation in order to prevent further uptake of arsenite into the cell (Thorsen *et al.* 2006). At low pH, the acetic acid stress generated transient activation of the Hog1p MAP kinase mediated by the Sln1 branch of the HOG pathway (Mollapour and Piper 2007) (Fig. 1.4). Hog1p phosphorylated the Fps1p at two 12 amino acid regions, both located on the cytosolic surface of the membrane. This phosphorylation was the signal for Fps1p to become ubiquitinated, and then was endocytosed to the vacuole for degradation by the 26S proteasome (Mollapour and Piper 2007). Thus, the Hog1p downregulates the entry of arsenite via either the closure of the Fps1p or targeting it for degradation.

Since yeast cells are facing both hyperosmotic stress and acidic stress during Icewine fermentation, it is critical to understand the adaptation response of wine strains to environmental stress under Icewine fermentative condition for the purpose of industrial application. It is also important to select yeast stains well adapted to stressful environments in order to produce the highly qualified and satisfied products.

1.2.4. Introduction of wine yeasts

Traditionally, alcoholic fermentation of grape must is carried out by allowing the microorganisms naturally present on grapes to grow, which is termed spontaneous fermentation. These fermentations were conducted by a broad spectrum of yeast

species. It has been known for a long time that many non-*Saccharomyces* species, especially species of *Hanseniaspora*, *Candida*, *Pichia* and *Metschnikowia*, initiate spontaneous alcoholic fermentation of the juice (reviewed in Fleet and Heard 1993; Fleet 2003). However, they are commonly soon overtaken by the growth of *S. cerevisiae*, the species that dominates the mid to final stages of the process, and most often is the only species found in the fermenting juice at these times (reviewed in Fleet and Heard 1993; Fleet 2003).

Yeast cells of different species metabolize in a variety of ways to produce byproducts from sugar consumption and to counteract the fermentative stress, hence they contribute to different aroma and flavours in the final wine. Being aware of that, pure yeast strains were selected and commercialized to be used as starter cultures in order to produce wines with desired aroma and flavours. The commercial wine yeasts are selected for distinct fermentative properties and high adaptation to the oenological environment, such as high tolerance to alcohol and sugar (Pretorius 2000). In addition, they are able to conduct rapid, complete and efficient conversion of grape sugar to ethanol, carbon dioxide and other minor but critical metabolites without the development of off-flavours (Pretorius 2000).

1.2.4.1. Genus *Saccharomyces* in wine fermentations

The genus *Saccharomyces* plays the central role in wine fermentations. Currently, this genus consists of eight species: *S. cerevisiae*, *S. bayanus*, *S. uvarum*, *S. pastorianus*, *S. paradoxus*, *S. cariocanus*, *S. mikatae* and *S. kudriavzevii* (Kurtzman

2003; Nguyen and Gaillardin 2005). Studies on the genus *Saccharomyces* could date back to 1838, when the first yeast species *Saccharomyces cerevisiae* was named by J. Meyen (reviewed in Rainieri *et al.* 2003). *Saccharomyces* means sugar moulds and *cerevisiae* means beer from the Gaelic word *kerevigia* or old French word *cervoise* (reviewed in Mortimer 2000). Previously the *Saccharomyces sensu stricto* consisted of four species: *S. cerevisiae*, *S. bayanus*, *pastorianus* and *S. paradoxus* (Vaughan-Martini and Martini 1998). *S. bayanus* strains were initially divided into two subgroups: *S. bayanus var. bayanus* and *S. bayanus var. uvarum* by molecular analysis (Nguyen and Gaillardin 1997; Nguyen *et al.* 2000). However, *S. uvarum* was later proven to be a distinct species when Nguyen and Gaillardin (2005) found the nucleotide sequences of several genetic markers from a *S. bayanus* strain diverged from those of the type strain of *S. uvarum*. Three new species were classified to genus *Saccharomyces* in 2000: *S. cariocanus*, *S. mikatae* and *S. kudriavzevii*, on the basis of genetic analysis, molecular karyotyping and sequence analyses of the 18S rRNA and internal transcribed spacer (ITS) region (Naumov *et al.* 2000). A new member recently was also categorized into this genus: *S. arboricolus*, which was isolated from oak trees in China (Wang and Bai 2008), but further identification is required.

S. cerevisiae is the predominant and type species in genus *Saccharomyces* which is responsible for wine, beer, bread and sake fermentations. It has been highly adapted to fermentative environments and most of the commercial wine yeast strains in the market currently belong to this species. *S. bayanus*, which was formerly designated as *S. bayanus var. bayanus* (Nguyen and Gaillardin 1997; Nguyen *et al.* 2000), was

isolated from beer fermentations. Because of its properties of cryotolerance and low acetate generation during wine fermentation, this species recently attracted great attention for investigation in sweet wine making. Eglinton *et al.* (2000) suggested that *S. bayanus* contributes to more glycerol, succinic acid, acetaldehyde and SO_2 than *S. cerevisiae*, but produces less acetic acid, malic acid and ethyl acetate. It is more associated with savoury- and cooked-like aroma attributes; such as 'cooked orange peel', 'honey', 'yeasty', 'nutty' and 'aldehyde' (Eglinton *et al.* 2000). *S. uvarum*, which was formerly designated as *S. bayanus var. uvarum* (Nguyen and Gaillardin 1997; Nguyen *et al.* 2000), has been described as adapted to low temperature fermentations during wine making and cider production (Naumon *et al.* 2001; 2002). *S. pastorianus* is generally known as lager yeast due to its association with beer brewing. *S. pastorianus* is thought to be the natural hybrid between *S. cerevisiae* and *S. bayanus* evolved in a brewing environment. It normally contains multiple copies of parental genomes, either in the form of intact genomes or aneuploid (reviewed in Querol and Bond 2009). *S. paradoxus* has been described as the main yeast species in Croatian vineyards (Redzepovic *et al.* 2002). *S. cariocanus* and *S. mikatae* have never been found and studied in fermentative environments. *S. kudriavzevii*, which was first isolated from decayed leaves and soils in Japan, and from oaks in Portugal (Sampaio and Goncalves 2008), has recently been studied in different fermentative conditions due to its cryotolerant and low acetate-generating characteristics.

The genus *Saccharomyces* consists of hybrids between two or more distinct species. Naturally occurring hybrids have been isolated from different fermentative

environments, such as cider, brewing, and wine fermentations. Many interspecies hybrids were obtained under laboratory conditions, by conjugating (mating) spores, spores with haploid cells, by making use of 'rare mating' occurring between diploid vegetative cells or by fusing protoplasts, in order to study the contribution to wine industry of hybrids with distinct properties from both parental strains (Sipiczki 2008). Apparently, favourable combinations of positive characteristics, including better adaptation, can be accomplished from the mixing of two or more genomes (Sipiczki 2008).

The two major wine yeast species, *S. cerevisiae* and *S. bayanus*, both have characteristically organoleptic contribution to wine, and distinct properties to adapt to fermentative conditions better than the other species (Sipiczki 2008). Therefore, their hybrids, either natural or laboratory-made, which possess combined properties from both parental species, were studied under fermentative environments. Caridi *et al.* (2002) observed low production of acetate and high production of glycerol in a hybrid, two distinct characteristics of the cryotolerant *S. bayanus* parent. The Australian Wine Research Institute (AWRI) hybridized *S. cerevisiae* with additional members of the *Saccharomyces* genus to explore genetic resources for the wine industry (reviewed in Sipiczki 2008). The hybrid AWRI 1503 between *S. cerevisiae* and *S. kudriavzevii*, which was commercialized by Maurivin™, displayed high alcohol tolerance, low volatile acidity, moderate foaming and excellent sedimentation properties after alcoholic fermentation (reviewed in Sipiczki 2008). The hybrid AWRI 1501 between *S. cerevisiae* and *S. paradoxus* is better at building flavour complexity (AWRI 2006).

The natural triple hybrids between *S. cerevisiae, S. bayanus* and *S. kudriavzevii* displayed adaptation to growth under ethanol and temperature stress by inheriting competitive traits from one or another parental species (Belloch *et al.* 2008).

Although the properties of these hybrids in wine making look promising, the genetic analysis of the natural and laboratory-bred hybrids revealed great variability of hybrid genome structures and suggested that the alloploid genome of the zygote can undergo drastic changes during mitotic and meiotic divisions of the hybrid cells (Sipiczki 2008). These variations towards genome-stabilization consist of loss of chromosomes and genes and recombination between the partner genomes (Sipiczki 2008).

In the *Saccharomyces sensu stricto* group of yeasts, the interspecies hybrids can mate, but their hybrid offspring are almost completely sterile, producing less than 1% viable spores (gametes) (Greig *et al.* 2002). The hybrid sterility is thought to arise from the inability of the chromosomes of the heterogeneous genomes to pair in the prophase of meiosis I, which inhibits normal meiotic division (Hawthorne and Philippsen 1994; Sebastiani *et al.* 2002; Antunovics *et al.* 2005). Nevertheless, this postzygotic barrier does not seem to be effective in allotetraploids due to the presence of a matching homologous partner for each chromosome, which makes meiosis possible (Greig *et al.* 2002; Antunovics *et al.* 2005). Since the partner genomes are not isolated in the hybrid cell and can interact, the recombination is manifested, accompanied by extensive reduction of the genome size through the loss of large parts of one or both parental genomes, making the hybrid genome become more stable

(Sipiczki 2008). Although the genetic variation leads to hybrid genomes evolving towards genome-stability, it might cause instability of yeast genomes during wine fermentations and thus results in unpredictable variation in the composition and flavour of the final wine. Therefore, the stability of the hybrids needs to be investigated more thoroughly in order to make it a powerful technique for directed and controllable fermentations.

1.2.4.2. Non-*Saccharomyces* yeast species in wine fermentations

Grape berries and associated processing equipment contain a variety of yeasts that accumulate in the must or juice. Whether or not the juice is inoculated with a starter culture, some yeast strains will grow according to their adaptability to the juice composition and the fermentation conditions. At the same time of building cell concentration, they metabolize grape-derived compounds, to produce a variety of volatile and non-volatile metabolites and carry out transformation reactions. Depending on the vigor of the *Saccharomyces* starter culture and the growth and metabolic activity of the non-*Saccharomyces* yeasts during fermentation, wine composition will be affected to various degrees by the metabolic activities of all yeasts present. Fermentations in which non-*Saccharomyces* species were present in high populations will have greater metabolic impact on wine composition and flavour (Reviewed in Ugliano and Henschke 2009).

The freshly pressed grape juice harbors a diversity of yeast species, principally within the genera *Hanseniaspora* (anamorph *Kloeckera*), *Pichia*, *Candida*,

Metschnikowia, *Kluyveromyces* and *Saccharomyces* (reviewed in Fleet and Heard 1993; Fleet 2003). Occasionally, species in other genera such as *Zygosaccharomyces*, *Saccharomycodes*, *Torulaspora*, *Dekkera* and *Schizosaccharomyces* could be found (reviewed in Fleet and Heard 1993; Fleet 2003). Many of these non-*Saccharomyces* species from the microbial communities of the grape berry and winery environment initiate spontaneous alcoholic fermentation, especially species of *Hanseniaspora*, *Candida*, *Pichia* and *Metschnikowi*a, but are overtaken by *S. cerevisiae* sooner or later during the fermentations (Fleet and Heard 1993; Fleet 2003). A practical problem with non-*Saccharomyces* yeast species is that few strains are able to complete fermentations, and in many cases only a small proportion of grape sugar will be converted to ethanol (Fleet 2008). Two strategies could solve this problem: co-fermentation with a robust *Saccharomyces* strain and sequential fermentation, in which the non-*Saccharomyces* stain and *Saccharomyces* strain are inoculated successively. Thus these non-*Saccharomyces* yeasts could conduct complete fermentations at the same time with the development of desired aroma and flavours in the final wine (Fleet 2008).

Out of all these non-*Saccharomyces* species associated with wine fermentations, two species displayed great potential for Icewine fermentation: *Candida stellata* which displays high osmotolerance (Csoma and Sipiczki 2008; Sipiczki 2008), and *Torulaspora delbrueckii* that reduces volatile acidity in sweet wine fermentations (Ciani and Ferraro 1998; Herraiz *et al.* 1990; Moreno *et al.* 1991).

Yeast strains in the species *Candida stellata* are frequently associated with overripe

and botrytized grapes in sweet wine production and displayed outstanding osmotolerance and cryotolerance (Csoma and Sipiczki 2008; Sipiczki 2008). It possesses several desired properties in wine making: high glycerol and succinic acid production, preference for fructose consumption fermentation, moderate ester and higher alcohol production, and production of novel wine aromas. However, it displayed high variability in acetic acid production amongst strains (Ugliano and Henschke 2009). Csoma and Sipiczki (2008) recently studied 41 strains initially classified as *Candida stellata* but it was found most of them belonged to *Candida zemplinina* and related species. The confusing identification might be the reason for the high variance of acetic acid production within the species. Strain selection of *Candida stellata* and its co- or sequential-fermentation with *Saccharomyces cerevisiae* might be worth re-investigating for Icewine fermentation.

*Torulaspora delbrueckii (*anamorph *Candida colliculosa;* formerly *Saccharomyces rosei)* produces comparatively low concentrations of acetic acid, ethyl acetate, acetaldehyde, and its potential suitability for wine production has been suggested by several studies (Ciani and Ferraro 1998; Herraiz *et al.* 1990; Moreno *et al.* 1991). *Torulaspora delbrueckii* was isolated from high sugar musts, thus its osmotolerant characteristic is investigated in the fermentation of botrytized wines (Henschke and Dixon 1990; Bely *et al.* 2008). Under hyperosmotic stress conditions of sweet fermentations, *Torulaspora delbrueckii* typically retains its distinct properties to produce high glycerol and low acetic acid compared to *Saccharomyces* species, although co-fermentation with *Saccharomyces* is necessary to reach high ethanol

requirements. Based on these characteristics, *Torulaspora delbrueckii* might also be a potential species that would contribute to low volatile acidity during Icewine fermentations. *Torulaspora delbrueckii* has already been developed as a component of a mixed yeast culture with *Saccharomyces cerevisiae* and *Kluyveromces thermotolerans* which was commercialized by Chr. Hansen (Viniflora® HARMONY.nsac and MELODY.nsac) (Ugliano and Henschke 2009). It was also commercialized by Lallemand (LEVEL™ TD) as the world's first sebsequential inoculation kit using two different species of yeast.

1.2.4.3. Mixed strains in controlled fermentations – a new fermentative strategy

Yeast strains within and other than *Saccharomyces* species are ecologically and metabolically different in wine fermentation and therefore produces wine with unique and distinctive properties. Thus it would be interesting to exploit the more creative and controlled fermentation of different yeast strains. However, some of the strains with attractive properties are not capable of a fully completed fermentation, limiting in their ability to produce sufficient ethanol or displaying limited cell accumulation which leads to sluggish fermentations.

Under this condition, conducting wine fermentations by controlled inoculation of mixtures of different yeast strains is an effective strategy to harness the unique activity of such yeasts (Fleet 2008). Essentially, there are two ways to introduce mixed strains into controlled fermentation: inoculating the juice with mixed starter culture, and sequential inoculation. In the former, the juice is inoculated with more

than one yeast strain simultaneously, normally *S. cerevisiae* and one or more other strains with relatively weak fermentative ability. In such case, the inoculating ratio of the different yeast stains will affect the completion of fermentation and also flavours of the final wine. A ratio with lower *S. cerevisiae* is essential to allow a slow accumulating rate of this species under fermentative environments. The latter involves inoculating *S. cerevisiae* into grape juice several days after the inoculation of the other strains, thereby permitting the growth of the non-*S. cerevisiae* strain before being overtaken by the more dominant *S. cerevisiae*, and thus allow the non-*S. cerevisiae* yeast to produce important metabolites. Many studies have focused on the mixed inoculation strategy due to the great potential to introduce and combine specific characteristics into wine (reviewed in Fleet 2008). This area of wine technology is very likely to grow in future applications and more study is required to understand the biological mechanisms of how yeasts interact ecologically and metabolically when grown in co-culture and sequential culture under wine making conditions (Fleet 2008).

References

Alepuz, P.M., de Nadal, E., Zapater, M., Ammerer, G. and Posas, F. (2003) Osmostress-induced transcription by Hot1 depends on a Hog1-mediated recruitment of the RNA Pol II. *EMBO J* **22**, 2433-2442.

Antunovics, Z., Nguyen, H.V., Gaillardin, C. and Sipiczki, M. (2005) Gradual genome stabilisation by progressive reduction of the Saccharomyces uvarum genome in an interspecific hybrid with *Saccharomyces cerevisiae*. *FEMS Yeast Res* **5**, 1141-1150.

Australian Wine Research Institute (2006) *Australian Wine Research Institute (AWRI) annual report* pp. 22.

Belloch, C., Orlic, S., Barrio, E. and Querol, A. (2008) Fermentative stress adaptation of hybrids within the Saccharomyces sensu stricto complex. *Int J Food Microbiol* **122**, 188-195.

Bely, M., Stoeckle, P., Masneuf-Pomarede, I. and Dubourdieu, D. (2008) Impact of mixed *Torulaspora delbrueckII-Saccharomyces cerevisiae* culture on high-sugar fermentation. *Int J Food Microbiol* **122**, 312-320.

Bidenne, C., Blondin, B., Dequin, S. and Vezinhet, F. (1992) Analysis of the chromosomal DNA polymorphism of wine strains of *Saccharomyces cerevisiae*. *Curr Genet* **22**, 1-7.

Blomberg, A. (2000) Metabolic surprises in *Saccharomyces cerevisiae* during adaptation to saline conditions: questions, some answers and a model. *FEMS Microbiol Lett* **182**, 1-8.

Blomberg, A. and Adler, L. (1992) Physiology of osmotolerance in fungi. *Adv Microb Physiol* **33**, 145-212.

Blomberg, A. and Adler, L. (1989) Roles of glycerol and glycerol-3-phosphate dehydrogenase (NAD+) in acquired osmotolerance of *Saccharomyces cerevisiae*. *J Bacteriol* **171**, 1087-1092.

Bowen, A. (2010) Managing the quality of icewines. In *Managing wine quality: oenology and wine quality* ed. Reynolds, A.G. pp. 523-552. Cambridge, UK: Woodhead Publishing Ltd.

Caridi, A., Cufari, A. and Ramondino, D. (2002) Winemaking from Gaglioppo grapes with hybrid strains of Saccharomyces. *Folia Microbiol (Praha)* **47**, 407-408.

Cliff, M.A. and Pickering, G.J. (2006) Determination of odour detection thresholds for acetic acid and ethyl acetate in ice wine. *J Wine Res* **17**, 45-52.

Ciani, M. and Ferraro, L. (1998) Combined use of immobilized Candida stellata cells and *Saccharomyces cerevisiae* to improve the quality of wines. *J Appl Microbiol* **85**, 247-254.

Csoma, H. and Sipiczki, M. (2008) Taxonomic reclassification of Candida stellata strains reveals frequent occurrence of Candida zemplinina in wine fermentation. *FEMS Yeast Res* **8**, 328-336.

Drogen, F., O'Rourke, S.M., Stucke, V.M., Jaquenoud, M., Neiman, A.M. and Peter, M. (2000) Phosphorylation of the MEKK Ste11p by the PAK-like kinase Ste20p is required for MAP kinase signaling in vivo. *Curr Biol* **10**, 630-639.

Eglinton, J.M., Heinrich, A.J., Pollnitz, A.P., Langridge, P., Henschke, P.A. and de Barros Lopes, M. (2002) Decreasing acetic acid accumulation by a glycerol overproducing strain of *Saccharomyces cerevisiae* by deleting the ALD6 aldehyde dehydrogenase gene. *Yeast* **19**, 295–301.

Eglinton, J.M., McWilliam, S.J., Fogarty, M.W., Francis, I.L., Kwiatkowski, M.J., Høj, P.B. and Henschke, P.A. (2000) The effect of *Saccharomyces bayanus*-mediated fermentation on the chemical composition and aroma profile of Chardonnay wine. *Aust J Grape Wine Res* **6**, 190–196.

Erasmus, D.J., van der Merwe, G.K. and van Vuuren, H.J. (2003) Genome-wide expression analyses: Metabolic adaptation of *Saccharomyces cerevisiae* to high sugar stress. *FEMS Yeast Res* **3**, 375-399.

Fleet, G.H. (2008) Wine yeasts for the future. *FEMS Yeast Res* **8**, 979-995.

Fleet, G.H. (2003) Yeast interactions and wine flavour. *Int J Food Microbiol* **86**, 11-22.

Fleet, G.H. and Heard, G. (1993) Yeasts-growth During Fermentation. In *Wine Microbiology and Biotechnology* ed. Fleet, G.H. pp. 27-54. Chur, Switzerland: Harwood Academic Publishers.

Gorner, W., Durchschlag, E., Martinez-Pastor, M.T., Estruch, F., Ammerer, G., Hamilton, B., Ruis, H. and Schuller, C. (1998) Nuclear localization of the C2H2 zinc finger protein Msn2p is regulated by stress and protein kinase A activity. *Genes Dev* **12**, 586-597.

Greig, D., Borts, R.H., Louis, E.J. and Travisano, M. (2002) Epistasis and hybrid sterility in Saccharomyces. *Proc Biol Sci* **269**, 1167-1171.

Hawle, P., Horst, D., Bebelman, J.P., Yang, X.X., Siderius, M. and van der Vies, S.M. (2007) Cdc37p is required for stress-induced high-osmolarity glycerol and protein kinase C mitogen-activated protein kinase pathway functionality by interaction with Hog1p and Slt2p (Mpk1p). *Eukaryot Cell* **6**, 521-532.

Hawthorne, D. and Philippsen, P. (1994) Genetic and molecular analysis of hybrids in the genus *Saccharomyces* involving *S. cerevisiae, S. uvarum* and a new species, *S. douglasii*. *Yeast* **10**, 1285-1296.

Henschke, P.A. and Dixon, G. (1990) Effect of yeast strain on acetic acid accumulation during fermentation of Botrytis affected grape juice. In *Proceedings of the seventh Australian wine industry technical conference* ed. Williams, P.J., Davidson, D.M. and Lee, T.H. pp. 242-244 Adelaide, SA: Australian Industrial Publishers.

Herraiz, T., Reglero, G., Herraiz, M., Martinalvarez, P. J. and Cabezudo, M. D. (1990) The influence of the yeast and type of culture on the volatile composition of wines fermented without sulfurdioxide. *Am J Enol Vitic* **41**, 313–318.

Hohmann, S. (2002) Osmotic stress signaling and osmoadaptation in yeasts. *Microbiol Mol Biol Rev* **66**, 300-372.

Hohmann, S. (2002) Osmotic stress signaling and osmoadaptation in yeasts. *Microbiol Mol Biol Rev* **66**, 300-372.

Johnson, D.I. (1999) Cdc42: An essential Rho-type GTPase controlling eukaryotic cell polarity. *Microbiol Mol Biol Rev* **63**, 54-105.

Kinclova-Zimmermannova, O. and Sychrova, H. (2006) Functional study of the Nha1p C-terminus: involvement in cell response to changes in external osmolarity. *Curr Genet* **49**, 229-236.

Kontkanen, D., Inglis, D., Pickering, G. and Reynolds, A. (2004) Effect of yeast inoculation rate, acclimatization, and nutrient addition on Icewine fermentation. *Am J Enol Vitic* **55**, 363-370.

Kren, A., Mamnun, Y.M., Bauer, B.E., Schüller, C., Wolfger, H. Hatzixanthis, K., Mollapour, M., Gregori, C., Piper, P. and Kuchler, K. (2003) War1p, a novel transcription regulator controlling weak acid stress response in yeast. *Mol Cell Biol* **23**, 1775–1785.

Kurtzman, C.P. (2003) Phylogenetic circumscription of Saccharomyces, Kluyveromyces and other members of the Saccharomycetaceae, and the proposal of the new genera Lachancea, Nakaseomyces, Naumovia, Vanderwaltozyma and Zygotorulaspora. *FEMS Yeast Res* **4**, 233-245.

Maeda, T., Takekawa, M. and Saito, H. (1995) Activation of yeast PBS2 MAPKK by MAPKKKs or by binding of an SH3-containing osmosensor. *Science* **269**, 554-558.

Maeda, T., Wurgler-Murphy, S.M. and Saito, H. (1994) A two-component system that regulates an osmosensing MAP kinase cascade in yeast. *Nature* **369**, 242-245.

Marshall, C.J. (1994) MAP kinase kinase kinase, MAP kinase kinase and MAP kinase. *Curr Opin Genet Dev* **4**, 82-89.

Martin, S. and Inglis, D. (2006) Yeast Osmoadaptive response during Icewine fermentation. *Am J Enol Vitic* **57**, 527A.

Martinez-Pastor, M.T., Marchler, G., Schuller, C., Marchler-Bauer, A., Ruis, H. and Estruch, F. (1996) The *Saccharomyces cerevisiae* zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress response element (STRE). *EMBO J* **15**, 2227-2235.

Mollapour, M. and Piper, P.W. (2006) Hog1p mitogen activated kinase determines acetic acid resistance in *Saccharomyces cerevisiae*. *FEMS Yeast Res* **6**, 1274-1280.

Mollapour, M. and Piper, P.W. (2007) Hog1 Mitogen-activated protein kinase phosphorylation targets the yeast *Fps1* aquaglyceroporin for endocytosis, thereby rendering cells resistant to acetic acid. *Mol Cell Biol* **27**, 6466- 6465.

Mollapour, M., Shepherd, A. and Piper, P.W. (2008) Novel stress responses facilitate *Saccharomyces cerevisiae* growth in the presence of the monocarboxylate preservatives. *Yeast* **25**, 169-177.

Miralles, V. and Serrano, R. (1995) A genomic locus in *Saccharomyces cerevisiae* with four genes up-regulated by osmotic stress. *Mol Microbiol* **17**, 653-662.

Moreno, J.J., Millan, C., Ortega, J.M. and Medina, M. (1991) Analytical differentiation of wine fermentations using pure and mixed yeast cultures. *J Ind Microbiol* **7**, 181–189.

Mortimer, R.K. (2000) Evolution and variation of the yeast (*Saccharomyces*) genome. *Genome Res* **10**, 403-409.

Naumov, G.I., Naumova, E.S., Antunovics, Z. and Sipiczki, M. (2002) Saccharomyces bayanus var. uvarum in Tokaj wine-making of Slovakia and Hungary. *Appl Microbiol Biotechnol* **59**, 727-730.

Naumov, G.I., Nguyen, H.V., Naumova, E.S., Michel, A., Aigle, M. and Gaillardin, C. (2001) Genetic identification of Saccharomyces bayanus var. uvarum, a cider-fermenting yeast. *Int J Food Microbiol* **65**, 163-171.

Naumov, G.I., James, S.A., Naumova, E.S., Louis, E.J. and Roberts, I.N. (2000) Three new species in the *Saccharomyces sensu stricto* complex: *Saccharomyces cariocanus, Saccharomyces kudriavzeviiand Saccharomyces mikatae*. *Int J Syst Evol Microbiol* **50**, 1931–1942.

Navarro-Avino, J.P., Prasad, R., Miralles, V.J., Benito, R.M. and Serrano, R. (1999) A proposal for nomenclature of aldehyde dehydrogenases in *Saccharomyces cerevisiae* and characterization of the stress-inducible ALD2 and ALD3 genes. *Yeast* **15**, 829-842.

Nguyen, H.V. and Gaillardin, C. (1997) Two subgroups within the *Saccharomyces bayanus* species evidenced by PCR amplification and restriction polymorphism of the non-transcribed spacer 2 in the ribosomal DNA unit. *Syst Appl Microbiol* **20**, 286–294.

Nguyen, H.V. and Gaillardin, C. (2005) Evolutionary relationships between the former species Saccharomyces uvarum and the hybrids Saccharomyces bayanus and Saccharomyces pastorianus; reinstatement of Saccharomyces uvarum (Beijerinck) as a distinct species. *FEMS Yeast Res* **5**, 471-483.

Nguyen, H.V., Le'pingle, A. and Gaillardin, C. (2000) Molecular typing demonstrates homogeneity of *Saccharomyces uvarum* and reveals the existence of hybrids between *S. uvarum* and *S. cerevisiae*, including the *S. bayanus* type strain CBS 380. *Syst Appl Microbiol* **23**, 71–85.

Norbeck, J. and Blomberg, A. (2000) The level of cAMP-dependent protein kinase A activity strongly affects osmotolerance and osmo-instigated gene expression changes in *Saccharomyces cerevisiae*. *Yeast* **16**, 121-137.

Nurgel, C., Pickering, G.J. and Inglis, D.L. (2004) Sensory and chemical characteristics of Canadian ice wines. *J Sci Food Agric* **84**, 1675-1684

O'Rourke, S.M. and Herskowitz, I. (1998) The Hog1 MAPK prevents cross talk between the HOG and pheromone response MAPK pathways in *Saccharomyces cerevisiae*. *Genes Dev* **12**, 2874-2886.

Ostrander, D.B. and Gorman, J.A. (1999) The extracellular domain of the *Saccharomyces cerevisiae* Sln1p membrane osmolarity sensor is necessary for kinase activity. *J Bacteriol* **181**, 2527-2534.

Pahlman, A.K., Granath, K., Ansell, R., Hohmann, S. and Adler, L. (2001) The yeast glycerol 3-phosphatases Gpp1p and Gpp2p are required for glycerol biosynthesis and differentially involved in the cellular responses to osmotic, anaerobic, and oxidative stress. *J Biol Chem* **276**, 3555-3563.

Pigeau, G.M. and Inglis, D.L. (2007) Response of wine yeast (*Saccharomyces cerevisiae*) aldehyde dehydrogenases to acetaldehyde stress during Icewine fermentation. *J Appl Microbiol* **103**, 1576-1586.

Pigeau, G.M. and Inglis, D.L. (2005) Upregulation of ALD3 and GPD1 in Saccharomyces cerevisiae during Icewine fermentation. *J Appl Microbiol* **99**, 112-125.

Piper, P., Calderon, C.O., Hatzixanthis, K. and Mollapour, M. (2001) Weak acid adaptation: The

response that confers yeast with resistance to organic acid food preservatives. *Microbiol* **147**, 2635-2642.

Piper, P.W., Mahe, Y., Thompson, S., Pandjaitan, R., Holyoak, C., Egner, R., Mühlbauer, M., Coote, P. and Kuchler K. (1998) The *Pdr12* ABC transporter is required for the development of weak organic acid resistance in yeast. *EMBO J* **17**, 4257-4265.

Piper, P.W., Ortiz-Calderon, C., Holyoak, C., Coote, P. and Cole, M. (1997) Hsp30, the integral plasma membrane heat shock protein of *Saccharomyces cerevisiae,* is a stress-inducible regulator of plasma membrane ATPase. *Cell Stress Chap* **2**, 12–24.

Posas, F., Chambers, J.R., Heyman, J.A., Hoeffler, J.P., de Nadal, E. and Arino, J. (2000) The transcriptional response of yeast to saline stress. *J Biol Chem* **275**, 17249-17255.

Posas, F. and Saito, H. (1998) Activation of the yeast SSK2 MAP kinase kinase kinase by the SSK1 two-component response regulator. *EMBO J* **17**, 1385-1394.

Posas, F. and Saito, H. (1997) Osmotic activation of the HOG MAPK pathway via Ste11p MAPKKK: scaffold role of Pbs2p MAPKK. *Science* **276**, 1702-1705.

Posas, F., Witten, E.A. and Saito, H. (1998) Requirement of STE50 for osmostress-induced activation of the STE11 mitogen-activated protein kinase kinase kinase in the high-osmolarity glycerol response pathway. *Mol Cell Biol* **18**, 5788-5796.

Posas, F., Wurgler-Murphy, S.M., Maeda, T., Witten, E.A., Thai, T.C. and Saito, H. (1996) Yeast HOG1 MAP kinase cascade is regulated by a multistep phosphorelay mechanism in the SLN1-YPD1-SSK1 "two-component" osmosensor. *Cell* **86**, 865-875.

Pretorius, I.S. (2000) Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking. *Yeast* **16**, 675-729.

Querol, A. and Bond, U. (2009) The complex and dynamic genomes of industrial yeasts. *FEMS Microbiol Lett* **293**, 1-10.

Radler, F. (1993) Yeast's Metabolism of Organic Acids. In *Wine Microbiology and Biotechnology* ed. Fleet, G.H. pp. 165–182. Chur, Switzerland: Harwood Academic Publishers.

Rainieri, S., Zambonelli, C. and Kaneko Y. (2003) *Saccharomyces sensu stricto*: systematics, genetic diversity and evolution. *J Biosci Bioeng* **96**, 1-9.

Raitt, D.C., Posas, F. and Saito, H. (2000) Yeast Cdc42 GTPase and Ste20 PAK-like kinase regulate Sho1-dependent activation of the Hog1 MAPK pathway. *EMBO J* **19**, 4623-4631.

Redzepovic, S., Orlic, S., Sikora, S., Majdak, A. and Pretorius, I.S. (2002) Identification and characterization of *Saccharomyces cerevislae* and Saccharomyces paradoxus strains isolated from Croatian vineyards. *Lett Appl Microbiol* **35**, 305-310.

Reiser, V., Ruis, H. and Ammerer, G. (1999) Kinase activity-dependent nuclear export opposes stress-induced nuclear accumulation and retention of Hog1 mitogen-activated protein kinase in the budding yeast *Saccharomyces cerevisiae*. *Mol Biol Cell* **10**, 1147-1161.

Reiser, V., Salah, S.M. and Ammerer, G. (2000) Polarized localization of yeast Pbs2 depends on osmostress, the membrane protein Sho1 and Cdc42. *Nat Cell Biol* **2**, 620-627.

Remize, F., Barnavon, L. and Dequin, S. (2001) Glycerol export and glycerol-3-phosphate dehydrogenase, but not glycerol phosphatase, are rate limiting for glycerol production in *Saccharomyces cerevisiae*. *Metab Eng* **3**, 301-312.

Remize, F., Roustan, J.L., Sablayrolles, J.M., Barre, P. and Dequin, S. (1999) Glycerol overproduction by engineered *saccharomyces cerevislae* wine yeast strains leads to substantial changes in By-product formation and to a stimulation of fermentation rate in stationary phase. *Appl Environ Microbiol* **65**, 143-149.

Rep, M., Krantz, M., Thevelein, J.M. and Hohmann, S. (2000) The transcriptional response of *Saccharomyces cerevisiae* to osmotic shock. Hot1p and Msn2p/Msn4p are required for the induction of subsets of high osmolarity glycerol pathway-dependent genes. *J Biol Chem* **275**, 8290-8300.

Rep, M., Reiser, V., Gartner, U., Thevelein, J.M., Hohmann, S., Ammerer, G. and Ruis, H. (1999) Osmotic stress-induced gene expression in *Saccharomyces cerevisiae* requires Msn1p and the novel nuclear factor Hot1p. *Mol Cell Biol* **19**, 5474-5485.

Ruis, H. and Schuller, C. (1995) Stress signaling in yeast. *Bioessays* **17**, 959-965.

Salmon, J.M. (1997) Enological fermentation kinetics of an isogenic ploidy series derived from an industrial *Saccharomyces cerevisiae* strain. *J Ferm Bioeng* **83**,253-260.

Sampaio, J.P. and Goncalves, P. (2008) Natural populations of Saccharomyces kudriavzevii in Portugal are associated with oak bark and are sympatric with *S. cerevisiae* and *S. paradoxus*. *Appl Environ Microbiol* **74**, 2144-2152.

Schuller, C., Brewster, J.L., Alexander, M.R., Gustin, M.C. and Ruis, H. (1994) The HOG pathway controls osmotic regulation of transcription via the stress response element (STRE) of the *Saccharomyces cerevisiae* CTT1 gene. *EMBO J* **13**, 4382-4389.

Schuller, C., Mamnun, M.Y., Mollapour, M., Krapf, G., Schuster, M., Bauer, B.E., Piper, P.W. and Kuchler, K. (2004) Global phenotypic analysis and transcriptional profiling defines the weak acid stress response regulon in *Saccharomyces cerevisiae*. *Mol Biol Cell* **15**, 706-720.

Sebastiani, F., Barberio, C., Casalone, E., Cavalieri, D. and Polsinelli, M. (2002) Crosses between *Saccharomyces cerevisiae* and *Saccharomyces bayanus* generate fertile hybrids. *Res Microbiol* **153**, 53-58.

Sipiczki, M. (2008) Interspecies hybridization and recombination in Saccharomyces wine yeasts. *FEMS Yeast Res* **8**, 996-1007.

Sutherland, F.C., Lages, F., Lucas, C., Luyten, K., Albertyn, J., Hohmann, S., Prior, B.A. and Kilian, S.G. (1997) Characteristics of Fps1-dependent and -independent glycerol transport in *Saccharomyces cerevisiae*. *J Bacteriol* **179**, 7790-7795.

Thorsen, M., Di, Y., Tangemo, C., Morillas, M., Ahmadpour, D., Van der Does, C., Wagner, A., Johansson, E., Boman, J., Posas, F., Wysocki, R. and Tamás, M.J. (2006) The MAPK hog1p modulates Fps1p-dependent arsenite uptake and tolerance in yeast. *Mol Biol Cell* **17**, 4400–4410.

Ugliano, M. and Henschke, P.A. (2009) Yeast and Wine Flavour. In *Wine Chemistry and Biochemistry* ed. Moreno-Arribas, M.V. and Carmen, P.M. pp. 314-392. New York: Springer

Van Dijken, J. and Scheffers, W. (1986) Redox balances in the metabolism of sugars by yeasts. *FEMS Microbiol Rev* **32**, 199-224.

Vaughan-Martini, A. and Martini, A. (1998) *Saccharomyces* Meyen ex Rees, In *The yeasts, a Taxonomic Study* (4th) ed. Kurtzman, C.P. and Fell, J.W. pp.358-371. Amsterdam: Elsevier Science.

Vintners Quality Alliance Act (VQA) (1999) Vintners Quality Alliance Act. Ontario Regulation 406/00.

Vintners Quality Alliance Ontario (2009) *Vintners Quality Alliance (VQA) annual report* pp. 9.

Wang, S.A. and Bai, F.Y. (2008) Saccharomyces arboricolus sp. nov., a yeast species from tree bark. *Int J Syst Evol Microbiol* **58**, 510-514.

Waskiewicz, A.J. and Cooper, J.A. (1995) Mitogen and stress response pathways: MAP kinase cascades and phosphatase regulation in mammals and yeast. *Curr Opin Cell Biol* **7**, 798-805.

Winter-Vann, A.M. and Johnson, G.L. (2007) Integrated activation of MAP3Ks balances cell fate in response to stress. *J Cell Biochem* **102**, 848-858.

Yang, X.X., Maurer, K.C., Molanus, M., Mager, W.H., Siderius, M. and van der Vies, S.M. (2006) The molecular chaperone Hsp90 is required for high osmotic stress response in *Saccharomyces cerevisiae*. *FEMS Yeast Res* **6**, 195-204.

Ziraldo, D. and Kaiser, K. (2007) Science, In *Icewine: Extreme Winemaking* pp. 73-107. Toronto: Key Porter Books.

Chapter 2. Study of new yeast strains as novel starter cultures for Icewine production in sterile Icewine fermentations

F. Yang[1,2], J. R. Bellon[3] and D.L. Inglis[1,2,4]

1 Cool Climate Oenology and Viticulture Institute, Brock University, St Catharines, ON, Canada
2 Centre for Biotechnology, Brock University, St Catharines, ON, Canada
3 The Australian Wine Research Institute, Urrbrae, SA, Australia
4 Department of Biological Sciences, Brock University, St Catharines, ON, Canada

2.1. Abstract

Aims

The objective of this study is to investigate three new yeast strains as novel starter cultures for Icewine fermentation: a naturally occurring strain of *S. bayanus/S. pastorianus* isolated from Icewine grapes, and two hybrids between *S. cerevisiae* and *S. bayanus*, AWRI 1571 and AWRI 1572, compared to commercial wine yeast *S. cerevisiae* K1-V1116.

Methods and Results

All yeast strains were inoculated to sterile filtered Riesling Icewine juice (41.6°Brix, reducing sugar 473 ± 11 g l^{-1}) at 1×10^7 cells ml^{-1}. Fermentations were conducted at 17 °C in triplicate. Both viable and total cell concentration, reducing sugar concentration and acetic acid concentration were monitored during the course of fermentation. Ethanol, glycerol, nitrogen and ethyl acetate production were also measured in final wines to determine the metabolic conversion of sugar during fermentation. The hybrids were tested for genetic stability during fermentation. All three new strains produced less acetic acid as a function of both time and sugar

consumption compared to the commercial yeast K1-V1116. The two hybrids were able to consume as much sugar as K1-V1116, but the isolated strain *S. bayanus/S. pastorianus* displayed lower sugar-consuming ability and correspondingly produced low concentration of ethanol. Both AWRI 1571 and AWRI 1572 showed some genomic loss during sterile fermentations but it was not correlated to metabolic profile.

Conclusions

AWRI 1571 and AWRI 1572 displayed potential for use as novel starter cultures for Icewine production. They produced target ethanol concentration of approximately 10 % v/v in a reasonable fermentation time and low acetic acid. Their genomic instability during fermentations did not detract from wine composition. *S. bayanus/S. pastorianus* produced less acetic acid but failed to reach the target ethanol concentration.

Significance and Impact

This work investigated the potential of three new yeast strains used as novel starter cultures for Icewine fermentation. The genomic instability of newly formed hybrids during Icewine fermentations was investigated for the first time. Hybrids AWRI 1571 and AWRI 1572 were determined as potential candidates for Riesling Icewine fermentations.

Key words: acetic acid, Icewine, sterile, *S. cerevisiae*, *S. bayanus*, *S. bayanus/S. pastorianus*, hybrids, stability

2.2. Introduction

Icewine is an intensely sweet late harvest wine fermented from the juice of naturally frozen grapes. The grapes are typically harvested and pressed below -7 °C (EU regulations) and -8 °C (Canada) between December and January, making the water crystals in grape berries separated from the highly concentrated juice (Bowen 2010). The removal of water results in extremely high concentration of soluble solids in Icewine juice, including sugar, acids and nitrogen compounds.

The minimum allowed soluble solids concentration in juice for Icewine fermentation is 35 °Brix according to regulations (VQA 1999), but commonly juice with soluble solids concentration between 38 to 42 °Brix is used for Icewine production (Ziraldo and Kaiser 2007). Such highly concentrated sugar in Icewine juice places yeast cells under extreme hyperosmotic stress, coupled with the high acid concentration often results in sluggish fermentation and high concentrations of volatile acidity, mainly in the form of acetic acid (Kontkanen *et al.* 2004). In Canada, any Icewine with acetic acid concentration > 2.1 g l^{-1} can not be designated as an Icewine (VQA 1999). Furthermore, acetic acid formed during fermentation can esterify with ethanol to produce ethyl acetate, which gives wine an undesired solvent or nail polish remover aroma (Cliff and Pickering 2006). Nurgel *et al.* (2004) found that the average concentration of ethyl acetate in commercial Canadian Icewine was 0.240 g l^{-1}, which was lower than the sensory threshold of ethyl acetate in Icewine, 0.198 g l^{-1} (Cliff and Pickering 2006). Therefore, it is of great significant to decrease the acetic acid production during Icewine fermentation.

Since wine yeast strains vary greatly in their ability to produce acetic acid (Delfini and Cervelli 1991), investigating new strains of *Saccharomyces* may offer a solution to overcome the elevated volatile acidity in Icewine production. Erasmus *et al.* (2004) compared seven commercially available wine yeast strains (ST, N96, Vin13, Vin7, EC1118, 71B, V1116) for Icewine production, and found that Vin 7 produced almost twice the amount of acetic acid as ST. Eglinton *et al.* (2000) noted that Chardonnay wine fermented with cryophilic strains of yeast *S. bayanus* produced lower amounts of acetic acid compared with *S. cerevisiae*. Because of its properties of cryotolerance and low acetate generation during wine fermentation, the application of this species may also be favourable for Icewine production.

The genus *Saccharomyces* consists of hybrids between two or more distinct species, including naturally occurring hybrids isolated from different fermentative environments and hybrids obtained under laboratory conditions (Sipiczki 2008). It seems that favourable combinations of positive characteristics from parental species, including better adaptation, can be accomplished from the mixing of two or more genomes, and it could also contribute to wine fermentations (Sipiczki 2008). *S. cerevisiae* plays the most important role at the fermentative stage due to its high adaptation and great fermentative ability, but its high acetic acid generation during Icewine fermentation has been a concern; whereas *S. bayanus* displayed properties of low acetate generation during wine fermentation and cryotolerance which suits Icewine fermentative environment, but lower efficiency in fermentations. Therefore, it would be interesting to investigate the behavior of hybrids between these two species,

which possess combined properties from both parental species, in Icewine fermentation. In fermentations of Gaglioppo red wine must, one hybrid between *S. cerevisiae* and *S. bayanus* showed low production of acetic acid and a high concentration of glycerol, two distinct characteristics of the *S. bayanus* parent (Caridi *et al.* 2002).

In our study, one *S. cerevisiae* strain, one *S. bayanus/S. pastorianus* strain isolated from Icewine grapes and two hybrids between *S. cerevisiae* and *S. bayanus* were compared for Icewine production. Yeast strains were evaluated for fermentation rates, ethanol, glycerol and acetic acid production. The two hybrids were also tested for genomic stability. Fermentations were conducted using sterile-filtered Icewine juice in order to determine the fermentative properties of the yeast strain without the interference of indigenous microflora.

2.3. Materials and methods

2.3.1. Yeast strain

The commercial yeast strain *S. cerevisiae* K1-V1116 was supplied by Lallemand Inc. (Montreal, QB, Canada). The natural yeast strain *S. bayanus/S. pastorianus* was isolated from Icewine grapes. The hybrid yeast strain AWRI 1571 and AWRI 1572 were kindly provided by the Australian Wine Research Institute (AWRI), Glen Osmond, SA, Australia. These strains are hybridized by natural yeast mating techniques. They are hybrids between a *S. cerevisiae* wine strain (AWRI 838-an isolate of EC1118) and a *S. bayanus* strain (AWRI 1176) isolated from grape

juice fermenting slowly at 4 °C.

2.3.2. Icewine juice for fermentation trials

Riesling Icewine juice was kindly provided by Niagara Vintage Harvesters Ltd (Virgil, ON, Canada). To remove the indigenous microorganisms in the juice, the juice was filtered through coarse, medium and fine pore-size pad filters and subsequently through a 0.22 μm membrane cartridge filter (Millipore, Etobicoke, ON, Canada). 500 mg l^{-1} of diammonium phosphate was added into the juice in order to increase the nitrogen. After filtration, the juice was aliquoted into 1 litre sterile bottles and stored at -20 °C until use.

2.3.3. Chemical composition of Icewine juice

Soluble solids of Icewine juice was determined with a bench top refractometer (AO ABBE unit, model 10450, American Optical, Buffalo, NY, USA). Reducing sugar concentration was measured using Lane-Eynon method (Zoecklein *et al.* 1996). Juice pH was measured with a 455 Corning pH meter and titratable acidity (TA; recorded in units of g l^{-1} tartaric acid) was titrated against standardized NaOH (0.1 N) to a pH 8.2 endpoint (Zoecklein *et al.* 1996). Yeast assimilable nitrogenous compounds were determined by measuring primary amino nitrogen (PAN) and ammonia nitrogen using enzymatic kits (K-PANOPA and K-AMIAR, Megazyme International Ireland Ltd). Juice variables were as follows: 41.6± 0 °Brix, reducing sugar 473 ± 11 g l^{-1}, pH 3.48 ± 0, TA 6.1 ± 0.1 g l^{-1} tartaric acid, ammonia nitrogen 143 ± 3 mg N l^{-1}, PAN 216 ± 6 mg N l^{-1}.

2.3.4. Yeast inoculation procedure for fermentations

All four yeast strains were plated out on yeast peptone dextrose (YPD) plates (10 g l^{-1} yeast extract, 20 g l^{-1} peptone, 20 g l^{-1} dextrose, and 20 g l^{-1} agar) before use in fermentations. 100 ml of Riesling Icewine juice was diluted to 10 °Brix and had 2 g l^{-1} of diammonium phosphate was added to the dilute juice to prepare the growth media to build up the yeast cultures. Using a sterile wire loop, a loop-full of each of the four yeast strains were inoculated into the growth media. These cultures were grown aerobically at 25 °C with shaking at 130 rpm until the cell concentrations reached 2×10^8 cells ml^{-1}. 25 ml of these cultures were then added into 25 ml of diluted Riesling Icewine juice (20 °Brix) respectively and held for 1 h without shaking at room temperature. 25 ml of undiluted Riesling Icewine juice (41.6° Brix) were then added into these 50 ml cultures respectively, and the cultures were held for 2 h without shaking at room temperature. Following this acclimatization procedure, the starter cultures (75 ml) were inoculated into 425 ml of Riesling Icewine juice (41.6 °Brix) to achieve a yeast inoculum rate of 1×10^7 cells ml^{-1} in a final volume of 500 ml.

2.3.5. Fermentation monitoring

Fermentations were carried out at 17 °C in triplicate and continued until the yeast stopped consuming sugar, signaled by no further change in sugar concentration for three days. Daily sampling (1.5 ml) of the fermentations occurred after stirring the fermentations for 5 minutes to ensure a homogeneous mixture. Yeast cell

concentrations were measured by cell counting via haemocytometer outlined by Zoecklein *et al.* (1996). Differentiation of viable and dead cells was determined by methylene blue staining. The remaining fermentation samples were centrifuged to remove yeast cells and then the supernatants were frozen at -20 °C for determination of sugar, acetic acid, glycerol, nitrogen, ethanol and ethyl acetate. The sugar concentration in fermentation samples were measured via Lane-Eynon method. Acetic acid, glycerol, ammonia nitrogen and PAN were determined by enzymatic kits (K-ACET, K-GCROL, K-AMIAR and K-PANOPA, Megazyme International Ireland Ltd). Ethanol and ethyl acetate were measured via gas chromatography (Agilent, CA, USA) using an Agilent 6890 system equipped with flame ionization detector and DB Wax (30m × 0.23mm × 0.25μm) column. The carrier gas was helium. For ethanol measurement, samples were diluted 10-fold and 1.0 μl was injected into the injection port heated to 225°C. The column head pressure was set as 24.4 psig and the flow rate of helium gas was 2.5 ml min^{-1}. The oven temperature was programmed to start at 60 °C, increase to 95 °C at 15 °C min^{-1}, and then increase to 225 °C at 75 ml min^{-1} and hold for 1 min. The detector temperature was 225 °C and 2% 1-butanol was used as an internal standard. For ethyl acetate measurement, 1.0 μl of sample was injected and heated to 230 °C. The column head pressure was 15.4 psig and helium flow rate was 1.5 ml min^{-1}. The oven temperature was hold at 35 °C for two min, and then increased to 230 °C at 10 ml min^{-1} and hold for 2 min. The detector temperature was 230 °C and 5% 4-methyl-2-pentanol (2 g l^{-1}) was used as an internal standard.

Significant Difference (LSD, $p < 0.05$).

2.4. Results

2.4.1. Fermentation kinetics

The four different strains consumed similar amount of reducing sugar with analogous consumption rates in the first 10 days, but differences were displayed beyond day 10 of the sterile fermentations (Fig. 2.1A). The Icewine isolate *S. bayanus/S. pastorianus* showed the slowest sugar consumption rate (Fig. 2.1A) and consumed the least amount of reducing sugar (148 g l^{-1}) over the 39 day fermentations (Fig. 2.1B). The sugar consumption of commercial yeast K1-V1116, AWRI 1571 and AWRI 1572 (ranging from 186 to 190 g l^{-1}) were higher compared with *S. bayanus/S. pastorianus* (148 g l^{-1}) (Fig. 2.1B). The cell accumulation was faster and to a greater extent in fermentations by K1-V1116 and *S. bayanus/S. pastorianus* in comparison with fermentations by the two hybrids (Fig. 2.2A and 2.2B), although *S. bayanus/S. pastorianus* consumed less sugar even with the higher cell population (Fig. 2.1B).

2.3.6. Stability analysis of hybrids AWRI 1571 and AWRI 1572

Yeast samples were taken on day 34 of the fermentations, and 0.1 ml of the sample was added into 0.9 ml of sterilized 0.1% peptone buffer to reach a 10-fold dilution. Then 0.1 ml of diluted sample was added into another 0.9 ml of buffer to reach a 100-fold dilution. Such successive dilution of the previously diluted samples was applied to obtain a serial dilution from 10^{-1} to 10^{-4}. Each diluted sample was plated on YPD plates, incubated at 25 °C until colonies formed, and then kept at 4 °C until use. A total number of 74 colonies from AWRI 1571 and 75 colonies from AWRI 1572 were analyzed for stability of the ribosomal DNA region spanning the internal transcribed spacers (ITS 1 and 2) and the 5.8S rRNA gene. Primer pairs used to amplify the ITS region are ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). The PCR thermal cycling parameters were an initial denaturation at 95 °C for 6 min, followed by 35 cycles of denaturation at 95 °C for 30 sec, annealing at 55 °C for 30 sec, and extension at 72 °C for 30 min, with a final extension at 72 °C for 10 min. 4.0 µl of PCR products were digested with the restriction endonuclease *Hae*III. Restriction fragments were electrophoresed on 2% agarose gel.

2.3.7. Statistical analysis

Differences between variables were determined by XLSTAT statistical software package released by Addinsoft (Version 7.1; Paris, France). Statistical methods used were analysis of variance (ANOVA) with mean separation by Fisher's Least

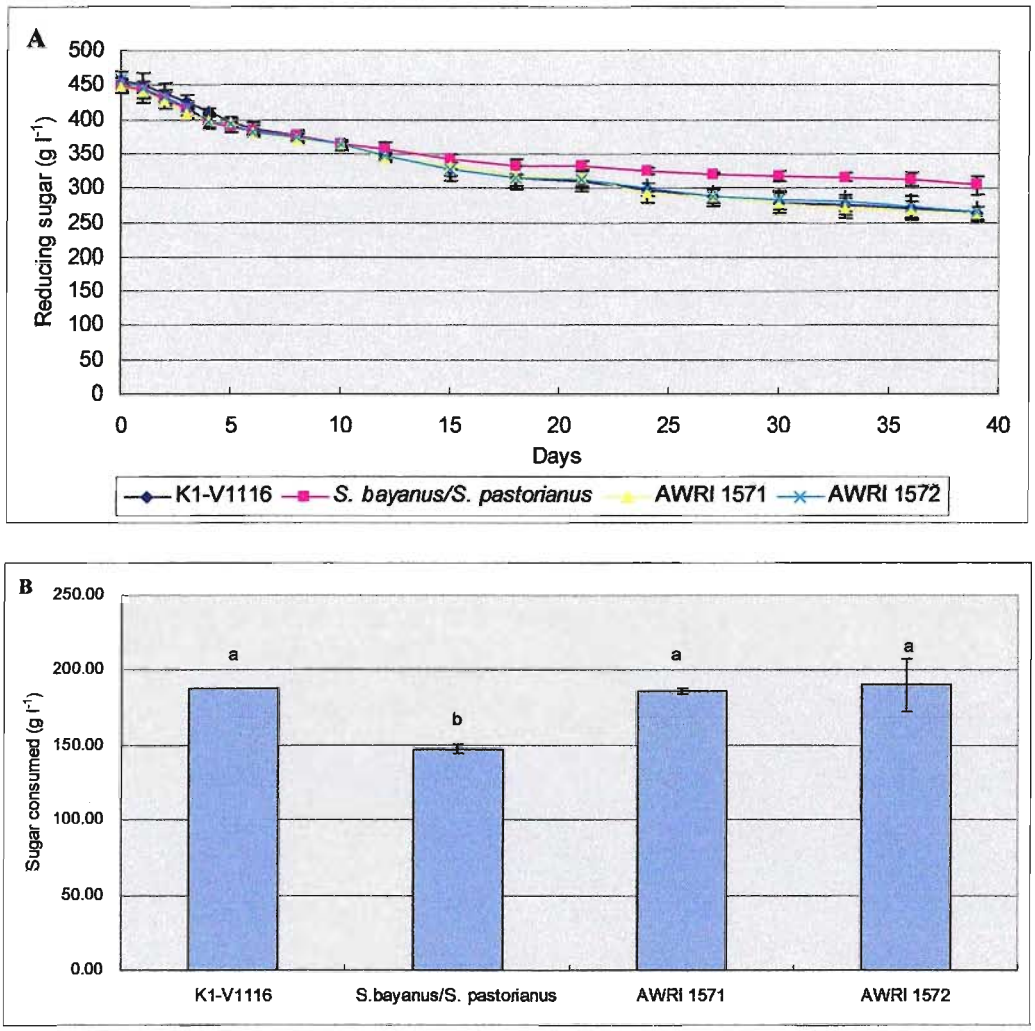


Figure 2.1: Sugar consumption during sterile filtered Riesling Icewine fermentation. Reducing sugar consumption was followed throughout the course of fermentation (A) and the total sugar consumed was compared among yeast strains (B). Fermentations were performed in triplicate and samples from each trial were tested in duplicate. Sugar values represent the average ± standard deviation of the mean of triplicate fermentations. Statistical methods used were analysis of variance (ANOVA) with mean separation by Fisher's Least Significant Difference (LSD; $p<0.05$). Lowercase letters indicate statistical difference in sugar consumption between strains in descending order analyzed by Fisher's LSD ($p<0.05$).

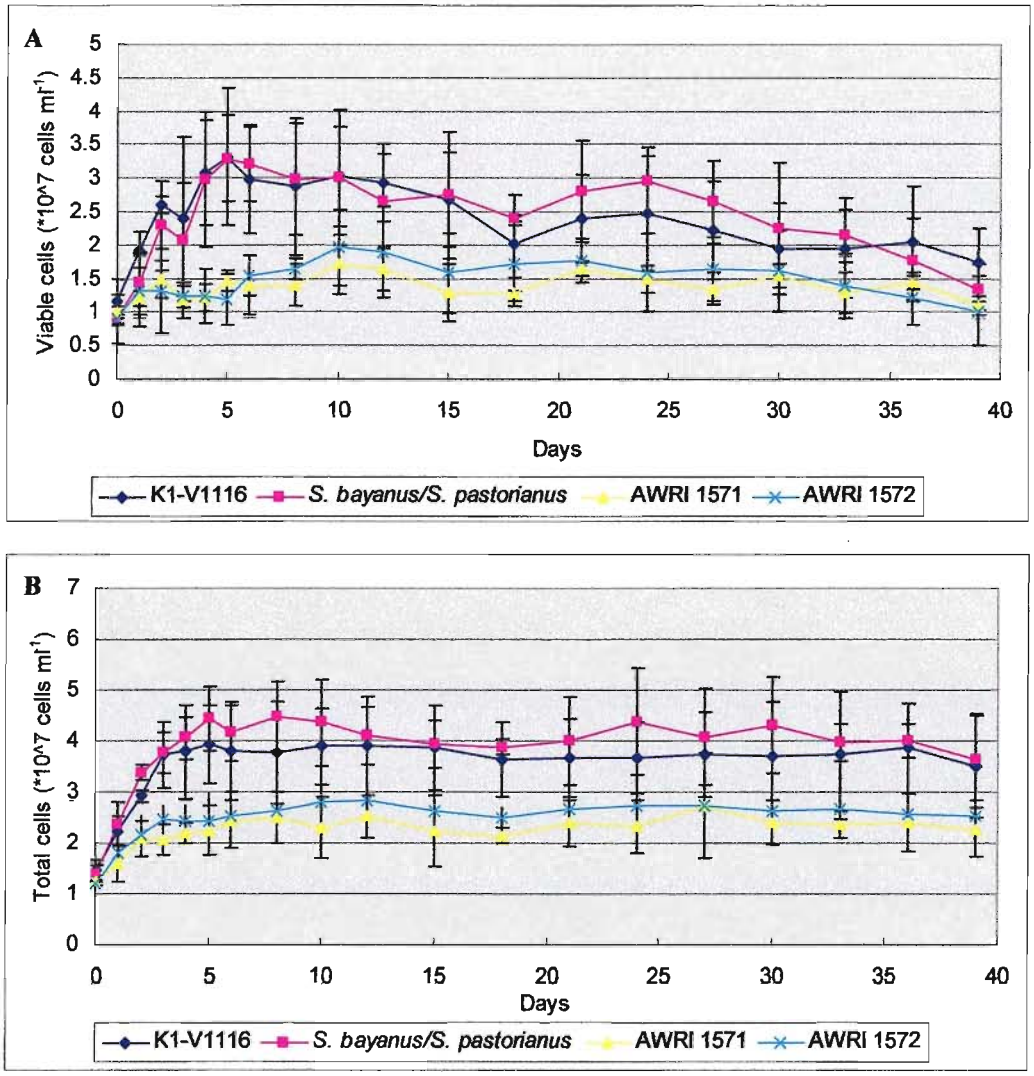


Figure 2.2: Viable and total yeast cell accumulation during sterile filtered Riesling Icewine fermentation. Both viable yeast cell concentrations (A) and total yeast cell concentrations (B) were monitored throughout the course of fermentation. Fermentations were performed in triplicate and samples from each trial were tested in duplicate. Cell values represent the average ± standard deviation of the mean of triplicate fermentations.

2.4.2. Yeast metabolite conversion

2.4.2.1. Acetic acid production during fermentations

All three new strains produced less acetic acid compared to the commercial wine yeast K1-V1116 during 39 days of fermentations (Fig. 2.3A and 2.3B). The fermentation by *S. bayanus/S. pastorianus* displayed the lowest acetic acid production during the course of fermentations and in the final wine, followed by AWRI 1571 and AWRI 1572 (Fig. 2.3A and 2.3B, Table 2.2).

Since acetic acid is a byproduct of sugar consumption, the acetic acid production needed to be normalized to the total amount of sugar consumed for each yeast strain tested in order to compare metabolic conversion across the strains. K1-V1116 produced higher acetic acid as a function of the sugar consumed when compared with the other strains throughout the entire fermentations (Fig. 2.4A). All three new strains, *S. bayanus/S. pastorianus*, AWRI 1571 and AWRI 1572 produced less acetic acid per total amount of sugar consumed as opposed to K1-V1116 (Fig. 2.4B). Although *S. bayanus/S. pastorianus* produced the least acetic acid, there was no difference in ratios of acetic acid production to the sugar consumption between the three new strains (Fig. 2.4B).

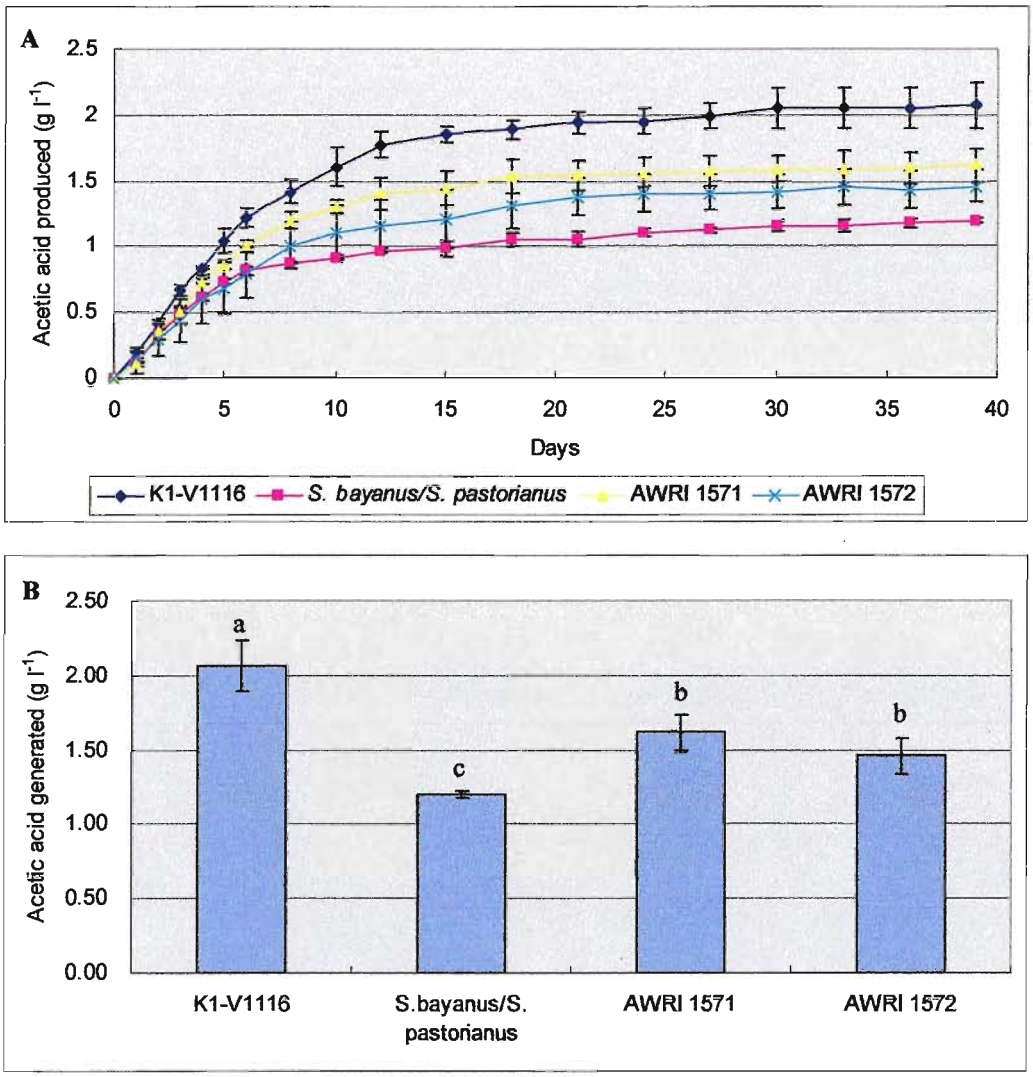
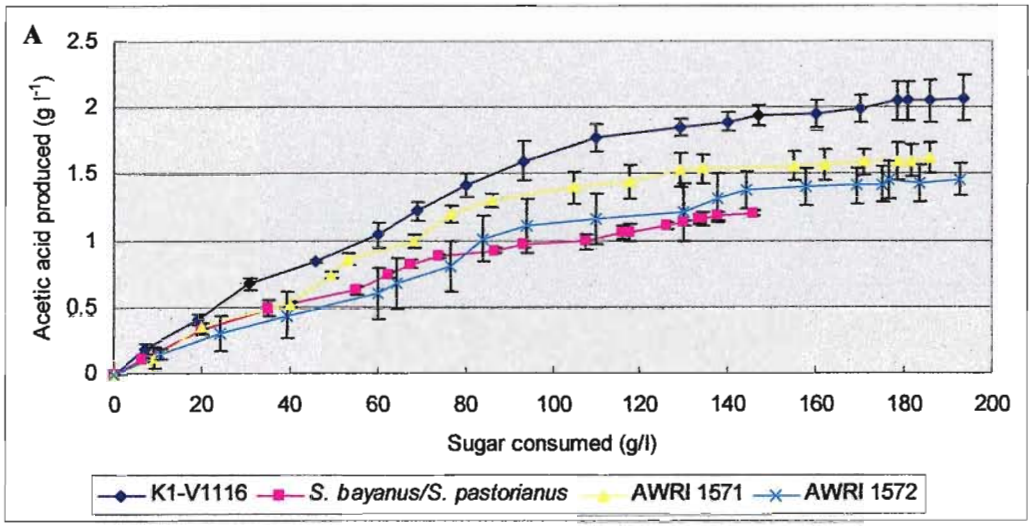


Figure 2.3: Production of acetic acid during sterile filtered Riesling Icewine fermentation. Acetic acid production was followed throughout the course of fermentation (A), and the total acetic acid production was compared among yeast strains (B). Fermentations were performed in triplicate and samples from each trial were tested in duplicate. Acetic values represent the average ± standard deviation of the mean of triplicate fermentations. Statistical methods used were analysis of variance (ANOVA) with mean separation by Fisher's Least Significant Difference (LSD; $p<0.05$). Lowercase letters indicate statistical difference between strains in descending order analyzed by Fisher's LSD ($p<0.05$).



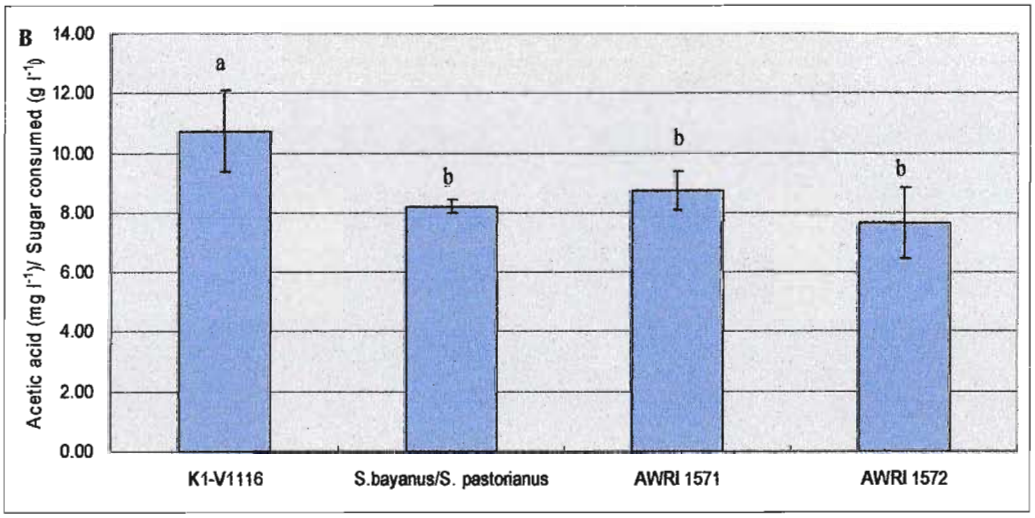


Figure 2.4: Acetic acid produced/Sugar consumed during sterile filtered Riesling Icewine fermentations. Acetic acid production was plotted versus sugar consumed throughout the course of fermentation (A), and the total acetic acid production was normalized to sugar consumed and compared among yeast strains (B). Fermentations were performed in triplicate and samples from each trial were tested in duplicate. Values represent the average ± standard deviation of the mean of triplicate fermentations. Statistical methods used were analysis of variance (ANOVA) with mean separation by Fisher's Least Significant Difference (LSD; $p<0.05$). Lowercase letters indicate statistical difference between strains in descending order analyzed by Fisher's LSD ($p<0.05$).

2.4.2.2. Conversion of other metabolites during fermentations

Compositions of the final Icewine fermentated from sterile filtered Riesling juice were listed in Table 2.1. Icewine fermented by the isolate *S. bayanus/S. pastorianus*

had a higher concentration of residual ammonia and displayed the lowest TA. Ethyl acetate concentration was tested to determine the level of esterification between acetic acid and ethanol across the four strains. Although all three new strains displayed lower acetic acid production compared with K1-V1116, only *S. bayanus/S. pastorianus* showed lower concentration of ethyl acetate than the commercial yeast in the final wine (Table 2.1).

Table 2.1: Final concentration of compounds in sterile fermented Riesling Icewine. Fermentations were performed in triplicate and samples from each trial were tested in duplicate. Values represent the average ± standard deviation of the mean of triplicate fermentations. Statistical methods used were analysis of variance (ANOVA) with mean separation by Fisher's Least Significant Difference (LSD; $p<0.05$). Lowercase letters indicate statistical difference between strains in descending order analyzed by Fisher's LSD ($p<0.05$).

	K1- V1116	*S. bayanus/S. pastorianus*	AWRI 1571	AWRI 1572
Sugar ($g\ l^{-1}$)	264 ± 10 b	305 ± 13 a	263 ± 10 b	267 ± 14 b
Ethanol (% v/v)	10.8 ± 1.4 a	7.7 ± 0.5 b	10.5 ± 0.9 a	10.3 ± 1.2 a
TA ($g\ l^{-1}$ tartaric acid)	7.9 ± 0.3 a	6.9 ± 0.0 c	7.6 ± 0.2 ab	7.5 ± 0.1 b
pH	3.64 ± 0.03	3.67 ± 0.03	3.61 ± 0.04	3.61 ± 0.03
Ammonia ($mg\ N\ l^{-1}$)	93 ± 14 ab	112 ± 6 a	82 ± 11 b	82 ± 9 b
PAN ($mg\ N\ l^{-1}$)	188 ± 7	191 ± 7	188 ± 8	187 ± 12
Glycerol ($g\ l^{-1}$)	9.57 ± 0.39 a	8.12 ± 0.80 b	10.22 ± 0.43 a	10.46 ± 0.95 a
Acetic acid ($g\ l^{-1}$)	2.13 ± 0.16 a	1.29 ± 0.05 c	1.72 ± 0.10 b	1.57 ± 0.19 b
Ethyl acetate ($mg\ l^{-1}$)	79 ± 31 a	29 ± 11 b	79 ± 22 a	63 ± 21 ab

During fermentations, the yeast metabolites measured are derived from sugar

metabolism, so the production of acetic acid, glycerol, ethanol and ethyl acetate during fermentations was normalized to sugar consumption (Table 2.2), by subtracting out the starting concentrations before fermentations from the final concentrations in the wines. The metabolites produced were then divided by the sugar consumed for the purpose of normalizing the data for the comparison across the four yeast strains.

Table 2.2: Conversion of metabolites during sterile filtered Riesling Icewine fermentation. Fermentations were performed in triplicate and samples from each trial were tested in duplicate. Values represent the average ± standard deviation of the mean of triplicate fermentations. Statistical methods used were analysis of variance (ANOVA) with mean separation by Fisher's Least Significant Difference (LSD; $p<0.05$). Lowercase letters indicate statistical difference between strains in descending order analyzed by Fisher's LSD ($p<0.05$).

	K1- V1116	*S. bayanus/S. pastorianus*	AWRI 1571	AWRI 1572
Sugar consumed ($g\ l^{-1}$)	188 ± 0 a	148 ± 4 b	186 ± 2 a	190 ± 18 a
Acetic acid produced ($g\ l^{-1}$)	2.07 ± 0.17 a	1.20 ± 0.02 c	1.62 ± 0.12 b	1.46 ± 0.12 b
Glycerol produced ($g\ l^{-1}$)	9.57 ± 0.39 a	8.12 ± 0.80 b	10.22 ± 0.43 a	10.46 ± 0.95 a
Ethanol produced (% v/v)	10.8 ± 1.4 a	7.7 ± 0.5 b	10.5 ± 0.9 a	10.3 ± 1.2 a
Ethyl acetate production ($mg\ l^{-1}$)	79 ± 31 a	29 ± 11 b	79 ± 22 a	63 ± 21 ab

S. bayanus/S. pastorianus produced less glycerol, ethanol and ethyl acetate compared with the other three strains during the fermentations (Table 2.2). Nevertheless, it also consumed the least amount of sugar (Fig. 2.1B and Table 2.2). When the production of these metabolites was normalized into the amount of sugar consumed for each strain, there was no difference between these strains (Table 2.3).

Table 2.3: Normalized production of metabolites during sterile filtered Riesling Icewine fermentation. Fermentations were performed in triplicate and samples from each trial were tested in duplicate. Values represent the average ± standard deviation of the mean of triplicate fermentations. Statistical methods used were analysis of variance (ANOVA) with mean separation by Fisher's Least Significant Difference (LSD; $p<0.05$). Lowercase letters indicate statistical difference between strains in descending order analyzed by Fisher's LSD ($p<0.05$).

	K1- V1116	*S. bayanus/S. pastorianus*	AWRI 1571	AWRI 1572
Ethanol produced (% v/v) / sugar consumed (g l^{-1})	0.056 ± 0.005	0.053 ± 0.002	0.057 ± 0.005	0.053 ± 0.005
Glycerol produced (g l^{-1}) / sugar consumed (g l^{-1})	0.050 ± 0.002	0.056 ± 0.004	0.055 ± 0.002	0.054 ± 0.005
Ethyl acetate produced (mg l^{-1}) / sugar consumed (g l^{-1})	0.41 ± 0.17	0.20 ± 0.07	0.43 ± 0.12	0.33 ± 0.10
Acetic acid produced (mg l^{-1}) / sugar consumed (g l^{-1})	10.73 ± 1.37 a	8.21 ± 0.24 b	8.74 ± 0.65 b	7.64 ± 1.19 b
Acetic acid produced (g l^{-1}) / ethanol produced (% v/v)	0.20 ± 0.04	0.17 ± 0.01	0.17 ± 0.02	0.15 ± 0.03
Acetic acid produced (g l^{-1}) / glycerol produced (g l^{-1})	0.223 ± 0.021 a	0.159 ± 0.015 b	0.169 ± 0.013 b	0.152 ± 0.028 b

One theory for acetic acid production from acetaldehyde when yeast cells are under hyperosmotic stress is to reduce the NAD^+ generated during glycerol formation in order to maintain intracellular redox balance for the NAD^+/NADH cofactor system. Therefore, the ratios of acetic acid generation to glycerol production were compared among the four strains to determine whether there were any changes in this ratio among yeast strains. The ratio of acetic acid to glycerol was higher for

the commercial yeast strain K1-V1116 in comparison to the isolate and the hybrids (Table 2.3). Acetic acid generation was also normalized to ethanol production to compare the divergence of converting acetaldehyde into either acetic acid or ethanol across the four strains during the Icewine fermentations (Table 2.3). Although all the three new strains displayed lower ratio compared to the commercial yeast K1-V1116, there was no difference between all these treatments (Table 2.3).

2.4.3. Stability analysis of hybrids AWRI 1571 and AWRI 1572

The ribosomal DNA regions of AWRI 1571 and AWRI 1572, which contain sequences from both *S. cerevisiae* and *S. bayanus*, were used to investigate the genomic stability of these heterogeneous strains during Icewine fermentations. After digestion by *Hae*III restriction endonuclease, the ribosomal DNA regions of *S. cerevisiae* and *S. bayanus* displayed different restriction fragments; whereas the hybrids had restriction fragments from both parental species. Therefore, the lost or reduced restriction fragments of this region in hybrids during fermentation may not only suggest an unstable genome of the heterogeneous strains, but also indicate the origin of the lost gene fragments. The ITS region was amplified from hybrids on day 34 which was close to the end of the 39 day fermentations and followed by *Hae*III digestion. Restriction fragments were compared to the rDNA regions of the hybrids before fermentations and the parental species to determine the genomic loss and its origin. Out of 74 colonies from samples of triplicate fermentations, one colony of AWRI 1571 displayed lost or reduced number of the 500 bp rDNA fragment from

the *S. bayanus* parent (AWRI 1176) (lane 11, Fig. 2.5C), but no genomic loss from the *S. cerevisiae* parent (AWRI 838), suggesting a 1.4% genomic instability (Fig. 2.5).

Interestingly, AWRI 1572 displayed a much higher genomic instability during fermentations. Out of 75 colonies, eight colonies displayed lost or reduced copy numbers of the 500 bp rDNA fragment from the *S. bayanus* parent (lane 7 from Fig. 2.6A; lane 2, 8, 9, 12, 19, 23 and 25 from Fig. 2.6B), and another two colonies showed loss of the 320 bp and 180 bp rDNA fragments from the *S. cerevisiae* parent (lane 4 and 21, Fig. 2.6B). This genomic loss was indicative of a genomic instability of AWRI 1572 during Icewine fermentation as high as 13.3%.

2.5. Discussion

Acetic acid has been a concern in Icewine production due to its association with wine spoilage when present at high concentration, because it imparts a vinegar aroma to wines and may contribute to the formation of ethyl acetate. Wine yeasts for Icewine fermentation are desired to conduct efficient fermentation to reach the target ethanol concentration of approximately 10 % v/v in the final wine in a reasonable fermentation time, produce low concentration of acetic acid and ethyl acetate, and display a stable genome during fermentations for consistent organoleptic contribution. We investigated three new yeast strains in sterile Riesling Icewine fermentations compared with a commercial wine yeast K1-V1116 to determine their properties in laboratory conditions in order to assess their potential for being used as

novel starter cultures for Icewine production.

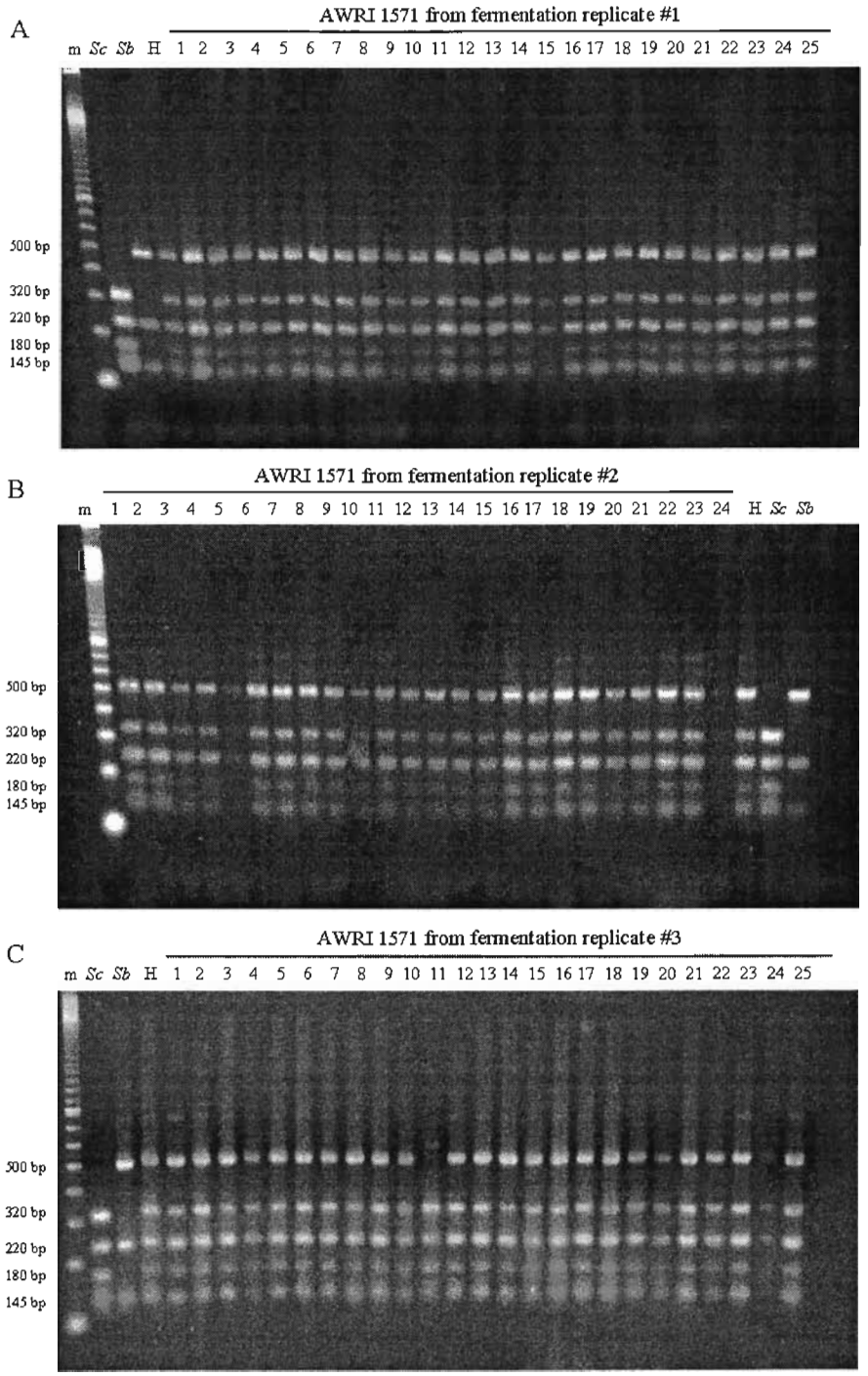


Figure 2.5: ITS restriction fragments (*Hae*III) of hybrid AWRI 1571 during sterile filtered Riesling Icewine fermentation Colonies of hybrid AWRI 1571 isolated on day 34 from fermentation replicate #1 (A), #2 (B), and #3 (C) were analyzed. Lane m represents molecular weight markers; *Sc* represents the *S. cerevisiae* parent (AWRI 838); *Sb* represents the *S. bayanus* parent (AWRI 1176); H represents the original hybrid AWRI 1571 before fermentation. Lanes 1-25 are colonies of hybrid AWRI 1571 isolated on day 34 from triplicate fermentations.

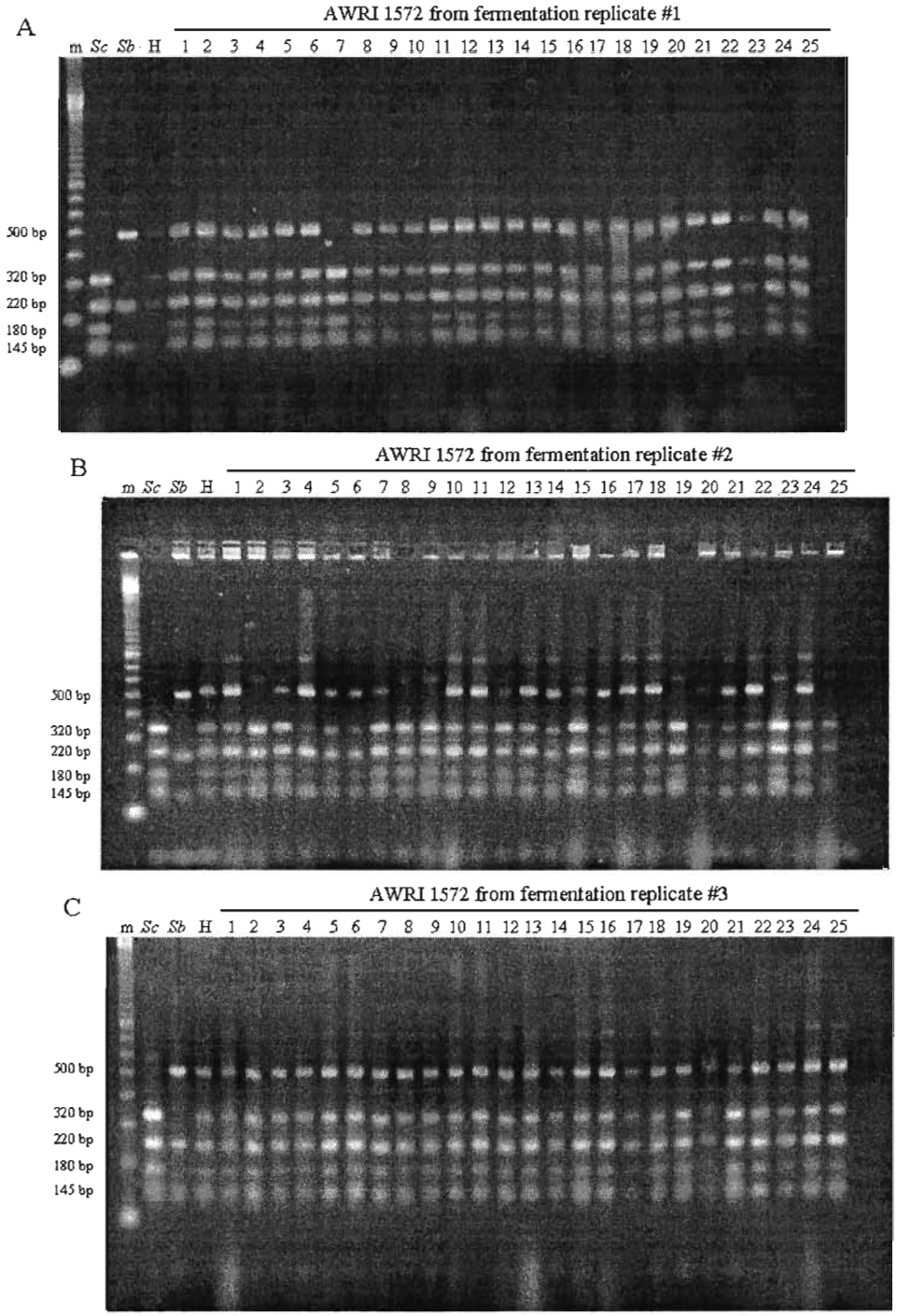


Figure 2.6: ITS restriction fragments (*Hae*III) of hybrid AWRI 1572 during sterile filtered Riesling Icewine fermentation Colonies of hybrid AWRI 1572 isolated on day 34 from fermentation replicate #1 (A), #2 (B), and #3 (C) were analyzed. Lane m represents molecular weight markers; *Sc* represents the *S. cerevisiae* parent (AWRI 838); *Sb* represents the *S. bayanus* parent (AWRI 1176); H represents the original hybrid AWRI 1571 before fermentation. Lanes 1-25 are colonies of hybrid AWRI 1572 isolated on day 34 from triplicate fermentations.

AWRI 1571 and AWRI 1572 produced approximately 10 % v/v of ethanol yields and consumed the same concentration of sugar as K1-V1116 (Fig. 2.1B and Table 2.2) , which suggested that these two hybrids are well adapted to the Icewine fermentation environments and displayed potential to conduct an efficient fermentation alone. In contrast, the natural isolate *S. bayanus/S. pastorianus* does not appear as suitable for such severe fermenting conditions when used as a single starter culture because it consumed 21.3% less sugar compared with K1-V1116 (Fig. 2.1B and Table 2.2) and correspondingly failed to produce the target ethanol concentration (Table 2.2). However, it will be valuable to investigate the co-fermenting property of this strain together with *S. cerevisiae* since it produced 28.8% less acetic acid per final sugar consumed compared to K1-V1116 during fermentations (Table 2.3). Bely *et al.* (2007) studied *Torulaspora delbrueckii*, a low acetic acid producer but with low ethanol production, in mixed and sequential fermentations of high-sugar must (360 g/l of sugar) with *S. cerevisiae*. It was found that a mixed *T. delbrueckii/S. cerevisiae* culture at a 20:1 ratio produced 52.8% less volatile acidity and the sequential fermentation produced 37.1% less compared to a pure culture of *S. cerevisiae*. Therefore, both mixed and sequential fermentations of *S. bayanus/S. pastorianus* with *S. cerevisiae*, which are traditionally used for Icewine production, could be investigated, and a best inoculating ratio for industrial application could be studied.

In our study, all the three new stains displayed lower acetic acid production compared to K1-V1116 as a function of both time and sugar (Fig. 2.3 and 2.4). The

ratios of acetic acid production to glycerol produced by the three new strains were also lower than K1-V116 (Table 2.3). The ethyl acetate production by all yeast strains were well below the sensory threshold, 0.198 g l^{-1} (Cliff and Pickering 2006), but they did not differ as a function of the sugar consumed across the four strains. Under hyperosmotic stress, acetic acid production has been correlated to increased glycerol formation (Miralles and Serrano 1995; Navarro-Avino *et al.* 1999). Yeast cells produce glycerol as a compatible solute when they are placed under hyperosmotic stress in order to keep the membrane turgor and avoid efflux of intracellular water (Blomberg and Adler 1989; Brewster *et al.* 1993; Nevoigt and Stahl 1997; Blomberg 2000). The key enzyme for glycerol formation is NADH-dependent glycerol-3-phosphate dehydrogenases which convert dihydroxyacetone phosphate to glycerol-3-phosphate with the concomitant oxidation of NADH to NAD^+ (Ganccdo *et al.* 1968; Nevoigt and Stahl 1997). The oxidation of cofactor NAD^+ in glycerol formation leads to a potential redox imbalance for this cofactor system. Acetic acid production was suggested as a mechanism to correct this redox shift by reducing NAD^+ back to NADH by cytosolic, NAD^+-dependent aldehyde dehydrogenases when acetaldehyde is oxidized to acetic acid (Miralles and Serrano 1995; Navarro-Avino *et al.* 1999). This might be the mechanism of acetic acid production during Icewine fermentation, in which the high concentration of sugar places yeast cells under an extreme hyperosmotic stress. Pigeau and Inglis (2005) found that the expression of a glycerol-3-phosphate dehydrogenase gene *GPD1* and a NAD^+-dependent aldehyde dehydrogenase gene *ALD3* were both at

higher levels during Icewine fermentation compared to diluted fermentation and displayed a higher increase of acetaldehyde followed by high acetic acid accumulation. In comparing the ratio of acetic acid production to glycerol production among the four strains tested, the natural isolate and the two hybrids showed a lower ratio than the commercial strain K1-V1116, indicating that the these yeasts may either be satisfying redox balance though other metabolites, or the acetic acid generated is being further metabolically converted.

In our study, all three new strains produced less acetic acid compared to K1-V1116. This could be caused by the variation in dissimilation of acetic acid between yeast strains. Acetic acid could be converted into acetyl-CoA by acetyl-CoA synthetase in the pyruvate dehydrogenase bypass, in which acetic acid is an intermediate metabolite that leads to the formation of acetyl-CoA from pyruvate (reviewed in Pronk *et al.* 1996). Erasmus and van Vuuren (2009) found that the transcripts of *ACS1*, an acetyl-CoA synthetase encoding gene, were lower in a high acetic acid producing strain VIN7 than in a low acetic acid generating strain ST grown in grape musts containing 40% w/v sugar, although *ACS1* did not respond to hyperosmotic stress in an industrial strain of *S. cerevisiae* Vin13 subjected to high sugar concentrations (Erasmus *et al.* 2003). Therefore, the variety in expression profile of *ASC* genes between yeast strains might result in various dissimilation of acetic acid and subsequently different concentrations of acetic acid accumulation. This might be the reason of low acetic acid generation by the three new strains. Furthermore, acetic acid might not only be generated to reduce the over-produced

NAD^+ formed during glycerol synthesis catalyzed by a cytosolic, NAD^+-dependent dehydrogenase. The cytosolic, $NADP^+$-dependent aldehyde dehydrogenase Ald6p might contribute to acetic acid generation as well. A recent study suggested that the deletion of *ALD6* and the deletion of its transcription factor, *ZMS1*, in a laboratory wild-type strain BY4741 produced substantially less acetic acid compared to the wild type in media containing 40% w/v glucose. In addition, the expression of *ALD6* was much higher in high acetic acid producing strain grown in grape musts containing 40% w/v sugar (Erasmus and van Vuuren 2009). Nonetheless, this raises the question of how the NAD^+ produced during glycerol formation is reduced back to NADH. Since yeast cells do not have transhydrogenases to convert reducing equivalents between the NAD^+/NADH system and the $NADP^+$/NADPH system (Blomberg and Adler 1989), it must rely on the formation of some other metabolites.

Unstable genomes of yeast strains might result in unpredictable sensory profile in the final wine and might affect the metabolite accumulation, such as acetic acid and glycerol, during the fermentation. The ribosomal DNA regions of AWRI 1571 and AWRI 1572, which contain sequences from both *S. cerevisiae* and *S. bayanus*, were analyzed to determine the genomic stability of these heterogeneous strains during fermentations. This DNA region includes two non-coding regions designated as the internal transcribed spacers (ITS1 and ITS2) and the 5.8S rRNA gene. The ITS regions are much less evolutionarily conserved than the rRNA coding genes (Bruns *et al.* 1991) and have been used for yeast identification (Guillamon *et al.* 1998) and differentiation between species of *Saccharomyces* (Huffman *et al.* 1992; Molina *et al.*

1992). Because the restriction fragments of ITS regions differ between *Saccharomyces* species, they were tested for genomic stability of the hybrids to indicate the parental origin of genomic DNA fragments loss.

In our study, both hybrids displayed a higher proportion of genomic loss from the *S. bayanus* parent during the Icewine fermentations (Fig. 2.5 and 2.6). These genomic losses might be caused by the extremely stressful environment during Icewine fermentations. James *et al.* (2008) found that the lager yeast *S. pastorianus* (generally believed hybrids between *S. cerevisiae* and *S. bayanus*) were dynamic and can undergo rearrangements, gene amplification and general genome instability in response to exposure to environmental stress during brewing. The genomic instability of hybrid genomes might be the molecular mechanisms for adaptive evolution of hybrid strains driven by environmental stresses (Hittinger and Carroll 2007; Coyle and Kroll 2008). Querol and Bond (2009) proposed that the large variations of *S. cerevisiae* DNA content in lager strains and the relative stablility of the cryotolerant *S. bayanus* DNA content may reflect adaptive pressures to cold fermentations and other environmental stresses experienced during the brewing process. In our study, the opposite result may explain the genome-stabilizing evolution of AWRI 1571 and AWRI 1572 by deleting genomic contents from the parent *S. bayanus* which was less adapted to Icewine fermenting conditions.

However, the genomic instability in our study could not be correlated to metabolic profiles of the fermentation. The loss or reduced expression of parental rDNA occurred in some, but not all of the triplicate fermentations (Fermentation replicate

#3 of AWRI 1571 and fermentation replicate #1 and #2 of AWRI 1572), but it did not make these fermentations differ from the rest of the fermentations in either sugar consumption or acetic acid generation (data not shown). Whether or not the genomic instability of hybrids would affect the wine composition depended on what the actual genomic changes were and to which extent, and also at what timepoint of the fermentations these changes occurred. Currently, there are few studies into the genomic stability of newly-formed yeast interspecific hybrids. Antunovics *et al.* (2005) oberserved the genomic changes of a hybrid between *S. cerevisiae* and *S. uvarum* over four filial generations of viable spores in several gene markers, including *YCL008c*, *HIS4*, *MET10*, *ITS* regions, and also in chromosomal patterns, but this study was not conducted under fermentation coditions. In our study, the 1.4% changes of AWRI 1571 and the 13.3% of AWRI 1572 in their ITS regions on day 34 of fermentations did indicate the genomic instability of these hybrids close to the end of fermentations, but more frequent sampling during the course of fermenations and a study of their chromosomal patterns and more gene markers are required to understand their global genomic stability during fermentations and its relevance to wine composition. It appears that, in this study, the instability of AWRI 1571 and AWRI 1572 did not affect their metabolic conversion and thus would not detract from their potential to be used as starter cultures for Icewine production.

2.6. Conclusions

This study investigated three new yeast strains as novel starter cultures for

Icewine production in fermenting sterile filtered Riesling Icewine juice with focus on the effect of yeast strains on acetic acid production. The two hybrids AWRI 1571 and AWRI 1572 were designated as potential strains used as novel starter cultures for Icewine production since they reached the desired ethanol concentration of approximately 10 % v/v in 39 days fermentations and produced lower acetic acid as a function of both time and sugar compared to commercial wine yeast K1-V1116. They displayed reduced or lost rDNA fragments during fermentations but this genomic instability did not detract from wine composition. The isolate *S. bayanus/S. pastorianus* did not appear suitable for Icewine production due to its failure to reach the target ethanol concentration although it produced less acetic acid in comparison with K1-V1116.

References

Antunovics Z, Nguyen H-V, Gaillardin C and Sipiczki M (2005) Gradual genome stability by progressive reduction of the *Saccharomyces uvasum* genome in an interspecific hybrid with *Saccharomyces cerevisiae FEMS Yeast Res* **5**: 1141-1151.

Bely, M., Stoeckle, P., Masneuf-Pomarede, I. and Dubourdieu, D. (2008) Impact of mixed *Torulaspora delbrueckii-Saccharomyces cerevisiae* culture on high-sugar fermentation. *Int J Food Microbiol* **122**, 312-320.

Blomberg, A. (2000) Metabolic surprises in *Saccharomyces cerevisiae* during adaptation to saline conditions: questions, some answers and a model. *FEMS Microbiol Lett* **182**, 1–8.

Blomberg, A. and Adler, L. (1989) Roles of glycerol and glycerol-3-phosphate dehydrogenase (NAD+) in acquired osmotolerance of *Saccharomyces cerevisiae*. *J Bacteriol* **171**, 1087-1092.

Bowen, A. (2010) Managing the quality of icewines. In *Managing wine quality: oenology and wine quality* ed. Reynolds, A.G. pp. 523-552. Cambridge, UK: Woodhead Publishing Ltd.

Brewster, J.L., de Valoir, T., Dwyer, N.D., Winter, E. and Gustin, M.C. (1993) An osmosensing signal transduction pathway in yeast. *Science* **259**, 1760–1763.

Bruns, T.D., White, T.J. and Taylor, J.W. (1991) Fungal molecular systematics. *Annu Rev Ecol Syst* **22**,524-564.

Caridi, A., Cufari, A. and Ramondino, D. (2002) Winemaking from Gaglioppo grapes with hybrid strains of Saccharomyces. *Folia Microbiol (Praha)* **47**, 407-408.

Cliff, M.A. and Pickering, G.J. (2006) Determination of odour detection thresholds for acetic acid and ethyl acetate in ice wine. *J Wine Res* **17**, 45-52.

Coyle, S. and Kroll, E (2008) Starvation induces genomic rearrangements and starvation-resilient phenotypes in yeast. *Mol Biol Evol* **25**, 310-318.

Delfini, C., and Cervetti. F. (1991) Metabolic and technological factors affecting acetic acid production by yeasts during alcoholic fermentation.*Vitic Enol Sci* **46**,142-150.

Eglinton, J.M., McWilliam, S.J., Fogarty, M.W., Francis, I.L., Kwiatkowski, M.J., Høj, P.B. and Henschke, P.A. (2000) The effect of *Saccharomyces bayanus*-mediated fermentation on the chemical composition and aroma profile of Chardonnay wine. *Aust J Grape Wine Res* **6**, 190–196.

Erasmus, D.J., Cliff, M. and van Vuuren, H.J.J. (2004) Impact of yeast strain on the production of acetic acid, glycerol, and the sensory attributes of Icewine. *Am J Enol Vitic* **55**, 371-378.

Erasmus, D.J., van der Merwe, G.K. and van Vuuren, H.J.J. (2003) Genome-wide expression analyses: metabolic adaptation of *Saccharomyces cerevisiae* to high sugar stress. *FEMS Yeast Res* **3**, 375-399.

Erasmus, D.J. and van Vuuren H.J.J. (2009) Genetic basis for osmosensitivity and genetic instability of the wine yeast *Saccharomyces cerevisiae* VIN7. *Am J Enol Vitic* **60**, 145-154.

Gancedo, C., Gancedo, J.M. and Sols, A. (1968) Glycerol metabolism in yeasts. *Eur J Biochem* **5**, 165-172.

Guillamon, J.M., Sabate, J., Barrio, E., Cano, J. and Querol, A. (1998) Rapid identification of wine yeast species based on RFLP analysis of the ribosomal internal transcribed spacer (ITS) region. *Arch Microbiol* **169**, 387-392.

Hittinger, C.T. and Carroll, S.B. (2007) Gene duplication and the adaptive evolution of a classic genetic switch. *Nature* **449**, 233-244.

Huffman, J.L., Molina, F.I. and Jong, S.C. (1992) Authentication of the ATCC strains in the *Saccharomyces cerevisiae* complex by PCR fingerprinting. *Exp Mycol* **16**,316-319.

James, T.C., Usher, J., Campbell, S. and Bond, U. (2008) Lager yeasts possess dynamic genomes that undergo rearrangements and gene amplification in response to stress. *Curr Genet* **53**, 139-152.

Kontkanen, D., Inglis, D., Pickering, G. and Reynolds, A. (2004) Effect of yeast inoculation rate, acclimatization, and nutrient addition on Icewine fermentation. *Am J Enol Vitic* **55**, 363-370.

Miralles, V.J. and Serrano, R. (1995) A genomic locus in Saccharomyces cerevisiae with four genes up-regulated by osmotic stress. *Mol Microbiol* **17**, 653-662.

Molina, F.I., Inoue, T. and Jong, S.C. (1992) Restriction polymorphisms in the internal transcribed spacers and 5.8S Rdna of *Saccharomyces*. *Curr Microbiol* **25**, 251-255.

Navarro-Avino, J.P., Prasad, R., Miralles, V.J., Benito, R.M. and Serrano, R. (1999) A proposal for nomenclature of aldehyde dehydrogenases in *Saccharomyces cerevisiae* and characterization of the stress-inducible ALD2 and ALD3 genes. *Yeast* **15**, 829-842.

Nurgel, C., Pickering, G.J. and Inglis, D.L. (2004) Sensory and chemical characteristics of Canadian ice wines. *J Sci Food Agric* **84**, 1675-1684

Nevoigt, E. and Stahl, U. (1997) Osmoregulation and glycerolmetabolism in the yeast

Saccharomyces cerevisiae. *FEMS Microbiol Rev* **21**, 231–241.

Pigeau, G.M. and Inglis, D.L. (2005) Upregulation of ALD3 and GPD1 in Saccharomyces cerevisiae during Icewine fermentation. *J Appl Microbiol* **99**, 112-125.

Pronk, J.T., Steensmays, H.Y.and van Dijken J.P. (1996) Pyruvate metabolism in *Saccharomyces cerevisiae*. *Yeast* **12**, 1607-1633.

Querol, A. and Bond, U. (2009) The complex and dynamic genomes of industrial yeasts. *FEMS Microbiol Lett* **293**, 1-10.

Sipiczki, M. (2008) Interspecies hybridization and recombination in Saccharomyces wine yeasts. *FEMS Yeast Res* **8**, 996-1007.

Vintners Quality Alliance Act (VQA) (1999) Vintners Quality Alliance Act. Ontario Regulation 406/00.

Ziraldo, D. and Kaiser, K. (2007) Science, In *Icewine: Extreme Winemaking* pp. 73-107. Toronto: Key Porter Books.

Zoecklein, B.W., Fugelsang K.C. and Gump B.H. (1996) *Wine Analysis and Production* New York: Chapman and Hall.

Chapter 3. Study of new yeast strains as novel starter cultures for Icewine production in unfiltered Icewine fermentations

F. Yang[1,2], J. R. Bellon[3] and D.L. Inglis[1,2,4]

1 Cool Climate Oenology and Viticulture Institute, Brock University, St Catharines, ON, Canada
2 Centre for Biotechnology, Brock University, St Catharines, ON, Canada
3 The Australian Wine Research Institute, Urrbrae, SA, Australia
4 Department of Biological Sciences, Brock University, St Catharines, ON, Canada

3.1. Abstract

Aims

This study investigated three new yeast strains: *S. bayanus/S. pastorianus*, AWRI 1571 and AWRI 1572, in unfiltered Icewine juice which mirrors the winery condition to determine their potential to be used as novel starter cultures for Icewine fermentation. The commercial wine yeast K1-V1116 was used as a control.

Methods and Results

All yeast strains were inoculated into unfiltered Riesling Icewine juice (39.2°Brix, reducing sugar 422 ± 11 g l^{-1}) at 1 × 10^7 cells ml^{-1}. Fermentations were conducted in triplicate at 25 °C for four days and then transferred to 17 °C for the rest of fermentation. Both viable and total cell concentration, reducing sugar concentration and acetic acid concentration were monitored during the course of fermentations. Ethanol, ethyl acetate and glycerol production, and D-glucose, D-fructose and nitrogen consumption were measured to investigate the metabolic conversion of

sugar during fermentations. The hybrids were tested for genetic stability during fermentation. AWRI 1572 consumed higher concentration of sugar compared to K1-V1116, but AWRI 1571 and *S. bayanus/S. pastorianus* showed lower sugar-consuming ability and correspondingly produced low ethanol. *S. bayanus/S. pastorianus* and AWRI 1572 produced less acetic acid as a function of sugar consumed compared with the commercial yeast K1-V1116, but AWRI 1571 displayed a higher ratio. Both AWRI 1571 and AWRI 1572 showed genomic loss during unfiltered fermentations but it was not correlated to metabolic profile.

Conclusions

AWRI 1572 displayed potential for being used as a starter culture for Icewine production with target ethanol production in a reasonable fermentation time and low acetic acid production. *S. bayanus/S. pastorianus* produced less acetic acid but failed to reach the target ethanol concentration. AWRI 1571 had high acetic acid production and low ethanol production compared with K1-V1116. Both hybrids displayed genomic instability during unfiltered Riesling Icewine fermentations but it did not detract from wine composition.

Significance and Impact

This work investigated three new yeast strains as novel starter cultures for unfiltered Icewine fermentation to mirror the winery wine making condition. The genomic instability of hybrids during Icewine fermentations was found and the hybrid AWRI 1572 was determined as a promising candidate for Riesling Icewine production.

Key words: acetic acid, Icewine, unfiltered, *S. cerevisiae*, *S. bayanus*, *S. bayanus/S.*

pastorianus, hybrids, stability

3.2. Introduction

Icewine currently plays an important role in the stage of the Canadian wine industry since Canada produces millions of cases of Icewine every year, with a reported production of 1.053 million litres in Ontario in 2009 (VQAO 2009). Because of the high concentration of sugar in a low pH matrix, the production of Icewine is often associated with prolonged fermentation times and the generation of high concentration of volatile acidity, mainly in the form of acetic acid (Kontkanen *et al.* 2004). In Canada, the concentration of acetic acid in Icewine is strictly regulated and is not permitted above 2.1 g l^{-1} (VQA 1999). Therefore, it is important to understand the mechanism through which acetic acid is produced during Icewine fermentation and develop methods to decrease acetic acid generation in Icewine production.

Selecting new yeast strains as novel starter cultures for Icewine production appears as a good approach to reduce acetic acid production since wine yeast strains display a variety of ability in acetic acid generation (Delfini and Cervelli 1991). The traditional wine yeasts belong to the genus *Saccharomyces* with *S. cerevisiae* being the prominent species that has been highly adapted to fermentative environments. Most of the commercial wine yeast strains in the market currently belong to this species. *S. bayanus*, which was formerly designated as *S. bayanus var. bayanus* (Nguyen and Gaillardin 1997; Nguyen *et al.* 2000), recently attracted great attention for investigation in wine making due to its property of cryotolerance and low acetate

generation during wine fermentation. It was found that *S. bayanus* contributes to more glycerol, succinic acid, acetaldehyde and SO_2 than *S. cerevisiae*, but produces less acetic acid, malic acid and ethyl acetate in Chardonnay wine fermentation (Eglinton *et al.* 2000). The wine produced by *S. bayanus* is more associated with savoury- and cooked-like aroma attributes; such as 'cooked orange peel', 'honey', 'yeasty', 'nutty' and 'aldehyde' (Eglinton *et al.* 2000). Recently, it was noted that hybrid strains have the potential to combine oenological favourable characteristics from both parental species, including better adaptation (Reviewed in Sipiczki 2008). Thus it would also be valuable to investigate hybrid strains between *S. cerevisiae* and *S. bayanus*, with anticipation of low acetic acid production from merits contributed by the *S. bayanus* parent and high fermentation efficiency from the *S. cerevisiae* parent.

We previously investigated three new yeast strains, one natural isolate *S. bayanus/S. pastorianus* and two hybrids between *S. cerevisiae* and *S. bayanus*, AWRI 1571 and AWRI 1572, in fermentations of sterile filtered Riesling Icewine juice in comparison with a commercially available starter *S. cerevisiae* K1-V1116. *S. bayanus/S. pastarianus* displayed lower acetic acid generation but was not able to consume as much sugar as K1-V1116. However, AWRI 1571 and AWRI 1572, reached desired ethanol concentration of approximately 10 % v/v as K1-V1116 but showed much lower acetic acid generation either as a function of time or of sugar consumed, with the lowest ratio of acetic acid produced to sugar consumed displayed by AWRI 1572. Nevertheless, AWRI 1571 displayed 1.4% lost or reduced ITS rDNA fragments, and

AWRI 1571 showed a higher proportion of 13.3%. These results are indicative of the fermentative ability and acetic acid production of these strains under aseptic conditions without the interaction and interference with other micro flora; whereas commercial Icewine production in a winery is conducted in a nonsterile environment using juice that has not been sterile filtered. The freshly pressed grape juice is commonly composed of a diversity of yeasts; such as species in the genera *Hanseniaspora* (anamorph *Kloeckera*), *Pichia*, *Candida*, *Metschnikowia*, *Kluyveromyces* and *Saccharomyces* (reviewed in Fleet and Heard 1993; Fleet 2003). Therefore, it is also important to investigate the performance of these strains under commercial conditions for metabolite production and genomic stability.

In this study, the three strains, *S. bayanus/S. pastorianus*, AWRI 1571 and AWRI 1572, were investigated in unfiltered Riesling Icewine fermentations, designed to mimic the commercial wine making environment. Yeast strains were evaluated for fermentation kinetics, ethanol, glycerol, acetic acid and ethyl acetate production with comparison to a commercial wine yeast *S. cerevisiae* K1-V1116. The two hybrids were also tested for genomic stability during the fermentations.

3.3. Materials and methods

3.3.1. Yeast strain and Icewine juice for fermentation trials

Yeast strains K1-V1116, *S. bayanus/S. pastorianus*, AWRI 1571 and AWRI 1572 were the same as those in Chapter 2, page 43. Riesling Icewine juice was provided by Niagara Vintage Harvesters Ltd (Virgil, ON, Canada). The juice from four

different vintages was blended together, and 500 mg l^{-1} of diammonium phosphate was added into the juice during mixture. The juice was aliquoted into 20 L buckets and stored at -40 °C until required.

3.3.2. Chemical composition of Icewine juice

Soluble solids, reducing sugar concentration, juice pH, titratable acidity (TA), primary amino nitrogen (PAN) and ammonia nitrogen were measured following the methods described in Chapter 2, page 44. Glycerol, acetic acid, and L-malic acid in Icewine juice were measured by enzymatic kits (K-GCROL, K-ACET and K-LMALL, Megazyme International Ireland Ltd). Juice variables were as follows: 39.2 ± 0 °Brix, reducing sugar 423 ± 11 g l^{-1}, pH 3.22 ± 0, TA 8.5 ± 0.1 g l^{-1} tartaric acid, ammonia nitrogen 169 ± 0 mg N l^{-1}, PAN 206 ± 4 mg N l^{-1}, acetic acid 0.004 ± 0.001 g l^{-1}, malic acid 3.8 ± 0.1 g l^{-1}, glycerol 4.31 ± 0.05 g l^{-1}.

3.3.3. Yeast inoculation procedure for fermentations

All four yeast strains were plated out on yeast peptone dextrose (YPD) plates before use in fermentations. Icewine juice was diluted to 10 °Brix and had 2 g l^{-1} of diammonium phosphate added to 525 ml of the dilute juice to prepare the growth media to build up the yeast cultures. Using a sterile wire loop, a loop-full of each of the four yeast strains were inoculated into the media. These cultures were grown aerobically at 25 °C with shaking at 130 rpm until the cell concentrations reached 2 × 10^8 cells ml^{-1}. These cultures were then added into 525 ml of diluted juice (20 °Brix) respectively and held for 1 h without shaking at room temperature. Then 525

ml of undiluted Icewine juice (39.2° Brix) were added, and the cultures were held for 2 h without shaking at room temperature. Following this acclimatization procedure, the starter cultures were then divided into three aliquots (525 ml for each fermentation) and each was inoculated into 2,975 ml of Icewine juice (39.2 °Brix) respectively to achieve a yeast inoculum rate of 1×10^7 cells ml^{-1} in a final volume of 3.5 L.

3.3.4. Fermentation monitoring and stability analysis of hybrids

Fermentations were carried out in triplicate, at 25 °C for four days and then transferred to 17 °C until the yeast stopped consuming sugar, indicated by no further sugar consumption for three days. Daily sampling (3 ml) of the fermentations, measurements of both total and viable yeast cell concentrations and the determination of sugar, acetic acid, glycerol, ammonia nitrogen, PAN, ethanol and ethyl acetate followed the methods described in Chapter 2, page 45-46. D-fructose and D-glucose were measured by enzymatic kits (K-FRUGL, Megazyme International Ireland Ltd). Stability analysis of hybrids followed the method described in Chapter 2, page 47. From each fermentation sample of AWRI 1571 and AWRI 1572, 25 colonies were analyzed.

3.3.5. Statistical analysis

Differences between variables were determined by XLSTAT statistical software package released by Addinsoft (Version 7.1; Paris, France) and method was same as it was applied in Chapter 2.

3.4. Results

3.4.1. Fermentation kinetics

The four yeast strains displayed different sugar consumption during the course and at the end of unfiltered fermentations, ranging from 118 g l^{-1} to 227 g l^{-1} (Fig. 3.1A and 3.1B). AWRI 1572 consumed the highest amount of sugar (227 g l^{-1}), followed by K1-V1116 (169 g l^{-1}) (Fig. 3.1B). AWRI 1571 and *S. bayanus/S. pastorianus* consumed less sugar compared to the commercial wine yeast K1-V1116, with sugar consumption values of 118 g l^{-1} and 169 g l^{-1}, respectively (Fig. 3.1B). D-glucose and D-fructose consumption were also measured to compare the difference of yeast strains on sugar preference (Table 3.1). All four strains were gluco-philic and *S. bayanus/S. pastorianus* consumed a higher proportion of glucose compared to the other three strains (Table 3.1B).

The ammonia concentration in the final wine showed an opposite trend to sugar consumption (Table 3.2 and 3.1A), and correspondingly the consumption of ammonia nitrogen displayed the same trend as sugar consumption (Table 3.1A). PAN was not different in the final wines (Table 3.2) from that measured in the starting unfermented juice, 206 ± 4mg N l^{-1}, suggesting that it was not consumed during fermentations.

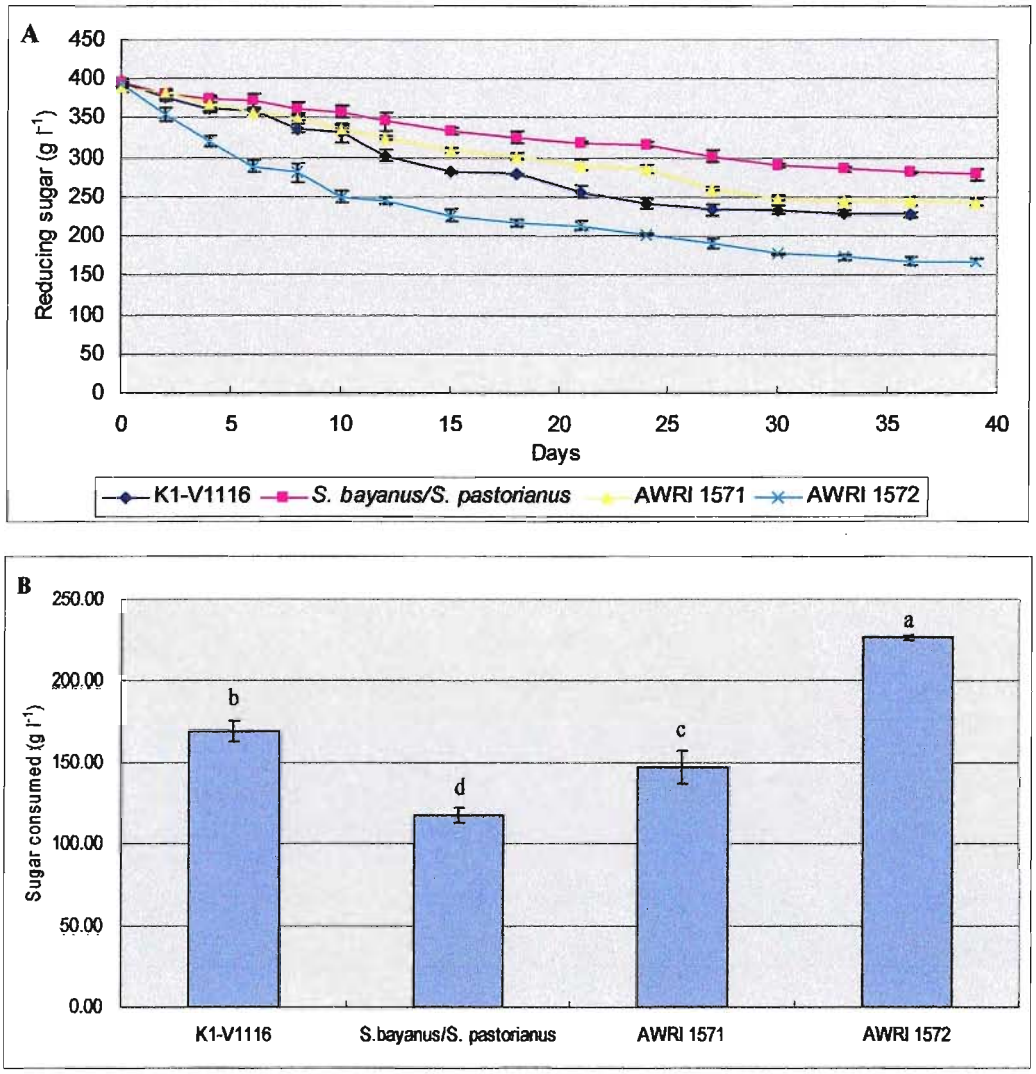


Figure 3.1: Sugar consumption during unfiltered Riesling Icewine fermentation. Reducing sugar consumption was followed throughout the course of fermentation (A) and the total sugar consumed was compared among yeast strains (B). Fermentations were performed in triplicate and samples from each trial were tested in duplicate. Sugar values represent the average ± standard deviation of the mean of triplicate fermentations. Statistical methods used were analysis of variance (ANOVA) with mean separation by Fisher's Least Significant Difference (LSD; $p<0.05$). Lowercase letters indicate statistical difference between strains in descending order analyzed by Fisher's LSD ($p<0.05$).

Table 3.1(A): Sugar and nitrogen consumption in unfiltered Riesling Icewine fermentation. Fermentations were performed in triplicate and samples from each trial were tested in duplicate. Values represent the average ± standard deviation of the mean of triplicate fermentations. Lowercase letters indicate statistical difference between strains in descending order analyzed by Fisher's LSD ($p<0.05$).

	K1-V1116	*S. bayanus/ S. pastorianus*	AWRI 1571	AWRI 1572
D-glucose + D-fructose consumed ($g\ l^{-1}$)	172 ± 5 b	108 ± 4 d	153 ± 9 c	224 ± 3 a
D-glucose consumed ($g\ l^{-1}$)	107 ±2 b	74 ± 2 d	98 ± 4 c	140 ± 1 a
D-fructose consumed ($g\ l^{-1}$)	64 ± 4 b	34 ± 2 d	55 ± 5 c	84 ± 2 a
Ammonia nitrogen consumption ($mg\ N\ l^{-1}$)	48 ± 4 b	14 ±1 d	42 ± 1 c	58 ± 2 a

Table 3.1 (B) Percentage of D-glucose consumption and D-fructose consumption of the total sugar consumed Fermentations were performed in triplicate and samples from each trial were tested in duplicate. Values represent the average ± standard deviation of the mean of triplicate fermentations. Lowercase letters indicate statistical difference in descending order between strains analyzed by Fisher's LSD ($p<0.05$).

	K1-V1116	*S. bayanus/ S. pastorianus*	AWRI 1571	AWRI 1572
D-glucose consumption/ (D-glucose + D-fructose consumption) (%)	62 ± 1 c	69 ± 1 a	64 ± 1 b	63 ± 0 c
D-fructose consumption/ (D-glucose + D-fructose consumption) (%)	38 ± 1 a	31 ± 1 c	36 ± 1 b	37 ± 0 ab

The cell accumulation was faster and to a greater extent in fermentations by K1-V1116 and AWRI 1572 in comparison with fermentations by AWRI 1571 and *S. bayanus/S. pastorianus* (Fig. 3.2A and 3.2B), which agrees with the sugar and nitrogen consumption (Fig. 3.1B and Table 3.1A). Interestingly, although both K1-V1116 and AWRI 1572 displayed higher viable cell concentrations, AWRI 1572 reached the peak earlier and retained in high viable cell values for a longer period

compared to K1-V1116 (Fig. 3.2A). In addition, the peak of viable cells of AWRI 1572 was only slightly lower than its peak of total cells, indicating a high proportion of viable cells (Fig. 3.2A and 3.2B).

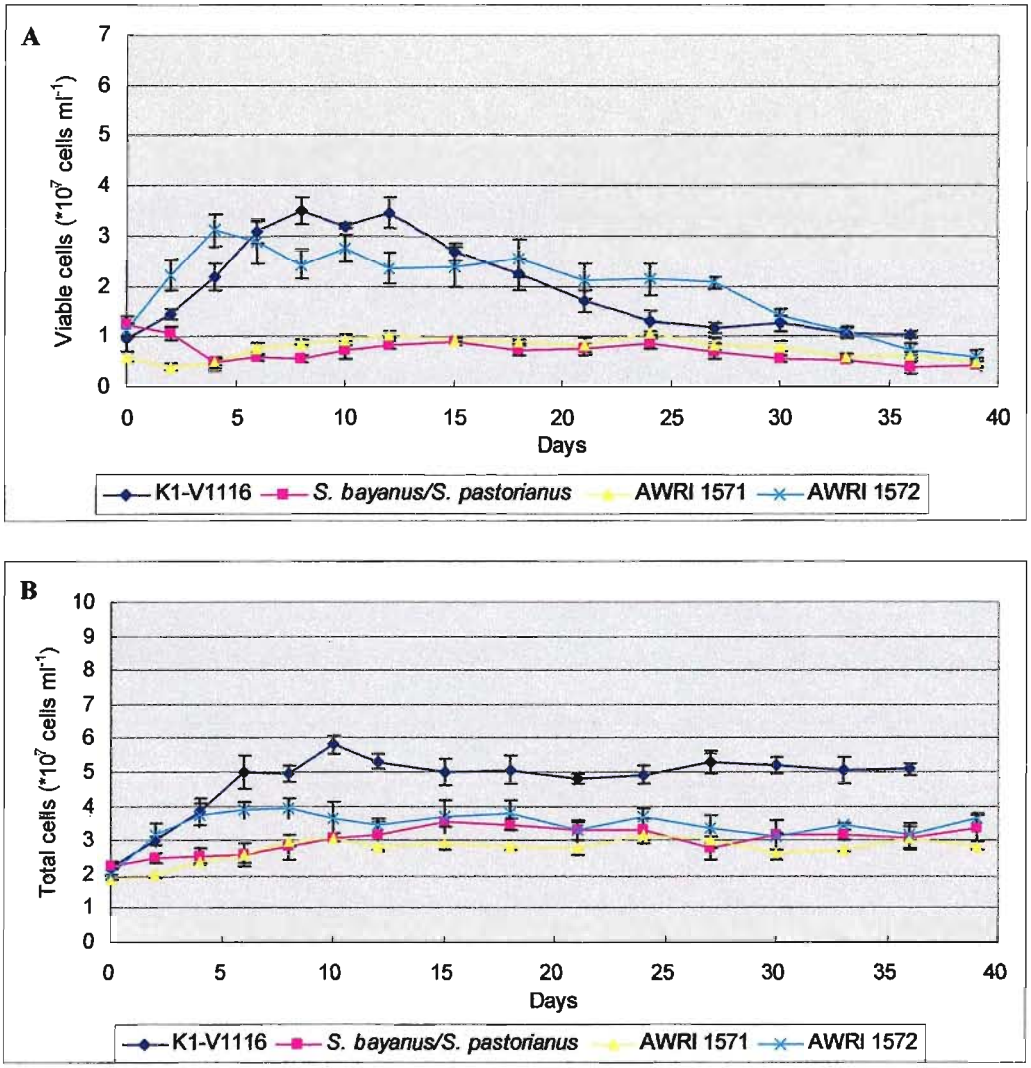


Figure 3.2: Viable and total yeast cell accumulation during unfiltered Riesling Icewine fermentation. Both viable yeast cell concentrations (A) and total yeast cell concentrations (B) were monitored throughout the course of fermentation. Fermentations were performed in triplicate at 25 °C for four days and then transferred to 17 °C for the rest of the fermentations. Samples from each trial were tested in duplicate. Cell values represent the average ± standard deviation of the mean of triplicate fermentations.

3.4.2. Yeast metabolite production

3.4.2.1. Acetic acid production during fermentations

S. bayanus/S. pastorianus displayed lower acetic acid production during the entire course of fermentations. In contrast, AWRI 1572 produced a high concentration of acetic acid from the beginning of the fermentation, which plateaued earlier (on day 10) and maintained this stable concentration until the end of the fermentations (Fig. 3.3A). All three new yeast strains produced less acetic acid compared with the commercial yeast K1-V1116 at the end of the fermentations (Fig. 3.3A and 3.3B, Table 3.3), but only AWRI 1572 produced less acetic acid per gram sugar consumed as opposed to K1-V1116 (Fig. 3.4B). *S. bayanus/S. pastorianus* produced comparable concentration of acetic acid per gram sugar consumed as K1-V1116 (Fig. 3.4B), with both low sugar consumption (Fig. 3.1B) and acetic acid production (Fig. 3.3B). AWRI 1571 produced higher acetic acid when the same amount of sugar was consumed throughout the entire fermentations (Fig. 3.4A and 3.4B).

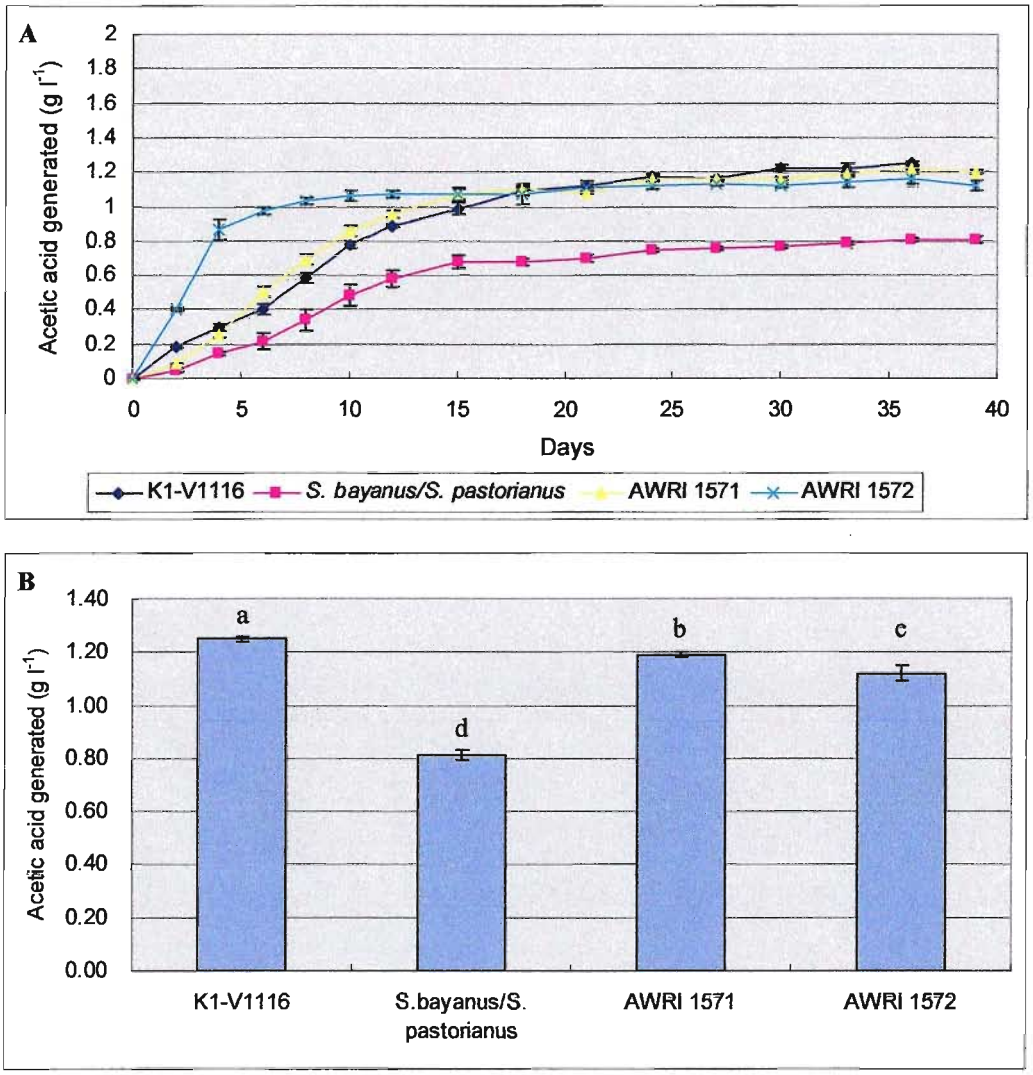
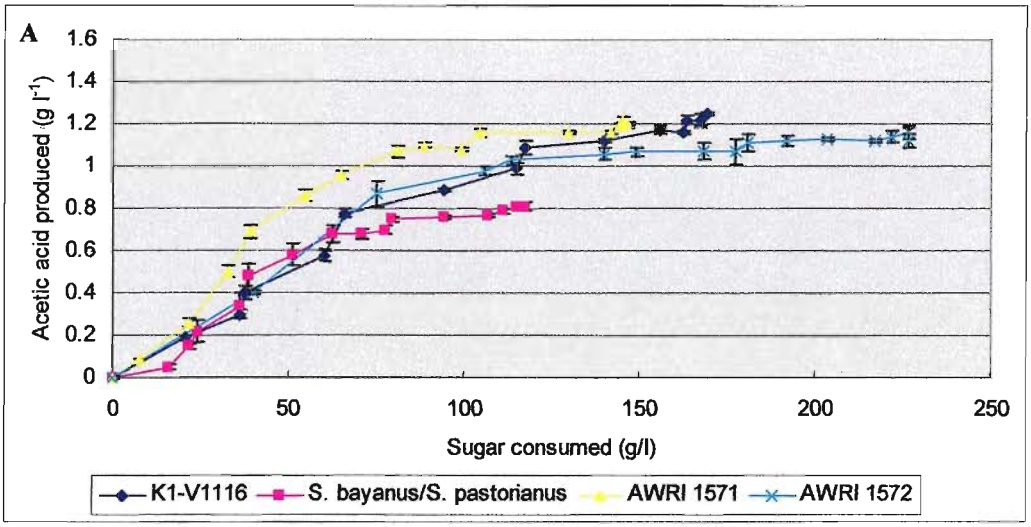


Figure 3.3: Production of acetic acid during unfiltered Riesling Icewine fermentation. Acetic acid production was followed throughout the course of fermentation (A), and the total acetic acid production was compared among yeast strains (B). Fermentations were performed in triplicate and samples from each trial were tested in duplicate. Acetic values represent the average ± standard deviation of the mean of triplicate fermentations. Statistical methods used were analysis of variance (ANOVA) with mean separation by Fisher's Least Significant Difference (LSD; $p<0.05$). Lowercase letters indicate statistical difference between strains in descending order analyzed by Fisher's LSD ($p<0.05$).



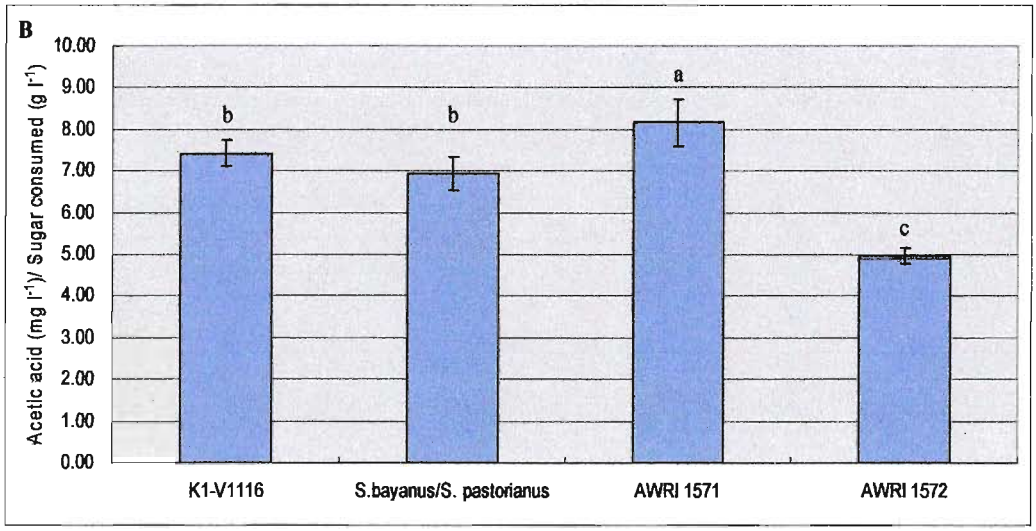


Figure 3.4: Acetic acid produced/Sugar consumed during unfiltered Riesling Icewine fermentations. Acetic acid production was plotted versus sugar consumed throughout the course of fermentation (A), and the total acetic acid production was normalized to sugar consumed and compared among yeast strains (B). Fermentations were performed in triplicate and samples from each trial were tested in duplicate. Values represent the average ± standard deviation of the mean of triplicate fermentations. Statistical methods used were analysis of variance (ANOVA) with mean separation by Fisher's Least Significant Difference (LSD; $p<0.05$). Lowercase letters indicate statistical difference between strains in descending order analyzed by Fisher's LSD ($p<0.05$).

3.4.3.2. Conversion of other metabolites during fermentations

The final Icewines did not differ in TA (Table 3.2). The concentration of ethyl acetate was measured to indicate the levels of esterification of ethanol and acetic

acid across the four yeast strains. *S. bayanus/S. pastorianus* and AWRI 1571 which produced both less acetic acid and ethanol than K1-V1116 also produced less ethyl acetate in the final wine (Table 3.2 and Fig. 3.4B). AWRI 1572 which showed the highest ethanol production produced the same concentration of ethyl acetate as K1-V1116, although it generated less acetic acid (Table 3.3 and Fig. 3.4B).

Table 3.2: Final concentration of compounds in Riesling Icewine fermented from unfiltered juice. Fermentations were performed in triplicate and samples from each trial were tested in duplicate. Values represent the average ± standard deviation of the mean of triplicate fermentations. Statistical methods used were analysis of variance (ANOVA) with mean separation by Fisher's Least Significant Difference (LSD; $p<0.05$). Lowercase letters indicate statistical difference between strains in descending order analyzed by Fisher's LSD ($p<0.05$).

	K1- V1116	*S. bayanus/S. pastorianus*	AWRI 1571	AWRI 1572
Sugar ($g\ l^{-1}$)	228 ± 3 c	279 ±7 a	243 ± 4 b	168 ± 3 d
Ethanol (% v/v)	10.0 ± 0.1 b	5.1 ± 0.1 d	7.6 ± 0.4 c	11.2 ± 0.2 a
TA ($g\ l^{-1}$ tartaric acid)	9.2 ± 0.5	9.1 ± 0.2	9.5 ± 0.3	9.5 ± 0.0
pH	3.37 ± 0.07	3.33 ± 0.05	3.30 ± 0.06	3.33 ± 0.04
Ammonia nitrogen ($mg\ N\ l^{-1}$)	109 ± 4 b	131 ± 2 a	97 ± 1 c	85 ± 2 d
PAN ($mg\ N\ l^{-1}$)	198 ± 8	202 ± 3	206 ± 4	193 ± 3
Glycerol ($g\ l^{-1}$)	15.05 ± 0.02 b	12.68 ± 0.05 d	14.07 ± 0.44 c	15.80 ± 0.22 a
Acetic acid ($g\ l^{-1}$)	1.30 ± 0.01 a	0.87 ± 0.02 d	1.26 ± 0.01 b	1.18 ± 0.03 c
Ethyl acetate ($mg\ l^{-1}$)	64 ± 11 a	21 ± 5 c	46 ± 2 b	69 ± 10 a

Since the four yeast strains consumed various concentration of sugar during the Icewine fermentations, and the yeast metabolites measured are derived from sugar metabolism, the yeast metabolites produced (Table 3.3) were normalized to gram per sugar consumed to compare metabolic conversions across the four yeast strains

(Table 3.4). After normalization, all three new strains produced less ethanol than K1-V1116, indicating K1-V1116 has the most efficient conversion of sugar to ethanol but the *S. bayanus/S. pastorianus* was the least efficient. However, *S. bayanus/S. pastorianus* produced less ethyl acetate per gram sugar consumed in comparison to the other strains even though it was not the lowest producer of acetic acid (Table 3.4). AWRI 1571 produced less glycerol as a function of the sugar consumed compared to the other strains (Table 3.4).

Table 3.3: Production of metabolites during unfiltered Riesling Icewine fermentation. Fermentations were performed in triplicate and samples from each trial were tested in duplicate. Values represent the average ± standard deviation of the mean of triplicate fermentations. Statistical methods used were analysis of variance (ANOVA) with mean separation by Fisher's Least Significant Difference (LSD; $p<0.05$). Lowercase letters indicate statistical difference between strains in descending order analyzed by Fisher's LSD ($p<0.05$).

	K1- V1116	*S. bayanus/S. pastorianus*	AWRI 1571	AWRI 1572
Acetic acid produced ($g\ l^{-1}$)	1.25 ± 0.01 a	0.81 ± 0.02 d	1.19 ± 0.01 b	1.12 ± 0.03 c
Glycerol produced ($g\ l^{-1}$)	10.82 ± 0.04 a	7.67 ± 0.13 c	8.99 ± 0.52 b	10.97 ± 0.40 a
Ethanol produced (% v/v)	10.0 ± 0.1 b	5.1 ± 0.1 d	7.6 ± 0.4 c	11.2 ± 0.2 a
Ethyl acetate production ($mg\ l^{-1}$)	64 ± 11 a	21 ± 5 c	46 ± 2 b	69 ± 10 a

Acetic acid was suggested to be produced under hyperosmotic stress to reduce the excess NAD^+ generated during glycerol formation in order to maintain intracellular redox balance for the NAD^+/NADH cofactor system (Miralles and Serrano 1995; Navarro-Avino *et al.* 1999). Therefore, the ratios of acetic acid generation to glycerol production were compared to determine if this ratio remained consistent among the four strains during fermentations. *S. bayanus/S. pastorianus* and AWRI 1572 generated less acetic acid when the same amount of glycerol was produced as

opposed to K1-V1116; whereas AWRI 1571 displayed the highest ratio (Table 3.4). In addition, acetic acid generation was normalized to ethanol production to compare the conversion of acetaldehyde to either acetic acid or ethanol across the four strains during the fermentations (Table 3.4). Ratios of acetic acid production to ethanol production displayed a different trend. AWRI 1572 remained with the lowest concentration but both *S. bayanus/S. pastorianus* and AWRI 1571 generated higher acetic acid when the same amount of ethanol was produced (Table 3.4).

Table 3.4: Normalized production of metabolites during unfiltered Riesling Icewine fermentation. Fermentations were performed in triplicate and samples from each trial were tested in duplicate. Values represent the average ± standard deviation of the mean of triplicate fermentations. Statistical methods used were analysis of variance (ANOVA) with mean separation by Fisher's Least Significant Difference (LSD; $p<0.05$). Lowercase letters indicate statistical difference between strains in descending order analyzed by Fisher's LSD ($p<0.05$).

	K1- V1116	*S. bayanus/S. pastorianus*	AWRI 1571	AWRI 1572
Ethanol produced (% v/v) / sugar consumed (g l^{-1})	0.059 ± 0.002 a	0.044 ± 0.001 c	0.052 ± 0.004 b	0.050 ± 0.001 b
Glycerol produced (g l^{-1}) / sugar consumed (g l^{-1})	0.064 ± 0.003 a	0.065 ± 0.002 a	0.061 ± 0.003 a	0.048 ± 0.002 b
Ethyl acetate (mg l^{-1}) / sugar consumed (g l^{-1})	0.38 ± 0.07 a	0.18 ± 0.03 b	0.32 ± 0.04 a	0.31 ± 0.04 a
Acetic acid produced (mg l^{-1}) / sugar consumed (g l^{-1})	7.41 ± 0.31 b	6.93 ± 0.39 b	8.16 ± 0.56 a	4.96 ± 0.17 c
Acetic acid produced (g l^{-1}) / ethanol produced (v/v %)	0.13 ± 0.00 b	0.16 ± 0.01 a	0.16 ± 0.01 a	0.10 ± 0.00 c
Acetic acid produced (g l^{-1}) / glycerol produced (g l^{-1})	0.116 ± 0.001 b	0.106 ± 0.003 c	0.133 ± 0.008 a	0.102 ± 0.003 c

3.4.3. Stability analysis of hybrids AWRI 1571 and AWRI 1572

The ribosomal DNA regions of AWRI 1571 and AWRI 1572 were used to investigate the genomic stability of these hybrids during fermentation. The ribosomal DNA region was amplified from hybrids on day 34 of the fermentations and followed by *Hae*III digestion. Restriction fragments were compared to ribosomal DNA regions of hybrids before fermentations and the parental species to determine the genomic loss and its origin. Out of 75 colonies from samples of triplicate fermentations, one colony of AWRI 1571 displayed lost or reduced number of the 500 bp rDNA fragment from the *S. bayanus* parent (AWRI 1176) (lane 57, Fig. 3.5), but no genomic loss from the *S. cerevisiae* parent (AWRI 838), suggesting a 1.3% genomic instability (Fig. 3.5).

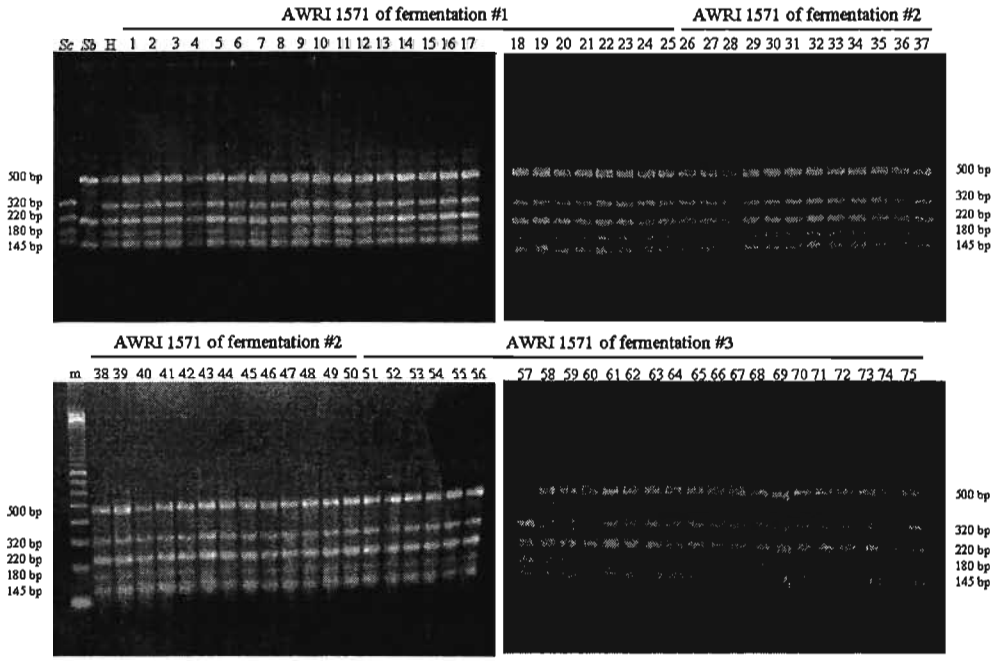


Figure 3.5: ITS restriction fragments (*Hae*III) of hybrid AWRI 1571 during unfiltered Riesling Icewine fermentation 75 colonies of hybrid AWRI 1571 isolated on day 34 of triplicate fermentations were analyzed. Lane m represents molecular weight markers; *Sc* represents the *S. cerevisiae* parent (AWRI 838); *Sb* represents the *S. bayanus* parent (AWRI 1176); H represents the hybrid AWRI 1571 before fermentation. Lanes 1-25 are colonies of

hybrid AWRI 1571 isolated on day 34 from fermentation replicate #1, 26-50 are from replicate #2, and 51-75 are from replicate #3.

Out of of 75 colonies of AWRI 1572 isolated during the fermentations, two colonies displayed loss of the 500 bp rDNA fragment from the *S. bayanus* parent (lane 42 and 48, Fig. 3.6), but no genomic loss from the *S. cerevisiae* parent, which was indicative of a 2.7% genomic instability (Fig. 3.6).

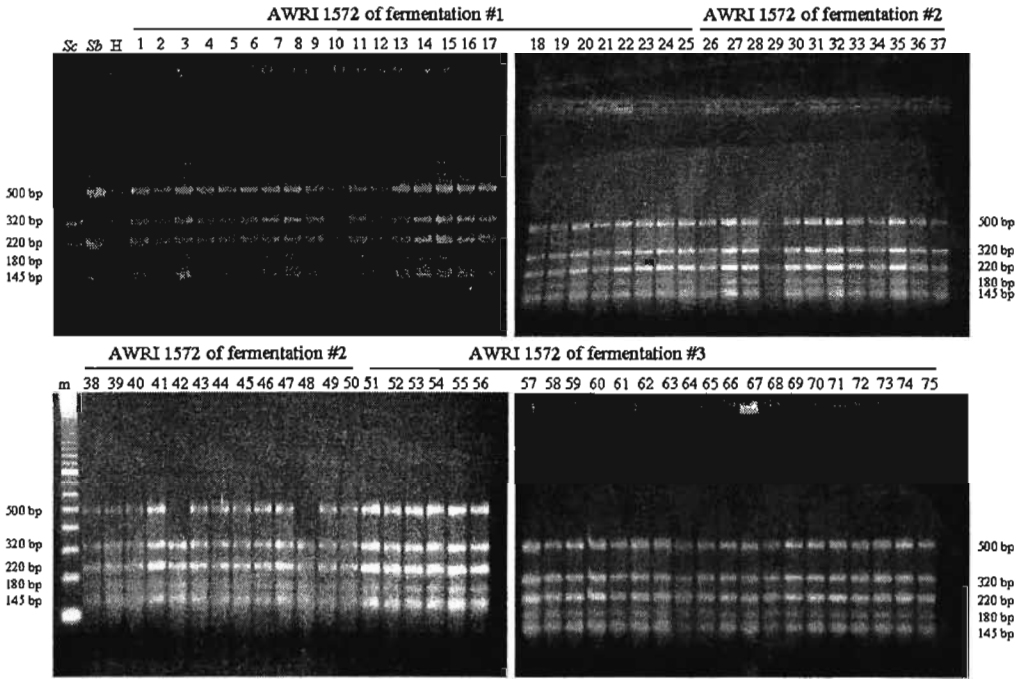


Figure 3.6: ITS restriction fragments (*Hae*III) of hybrid AWRI 1572 during unfiltered Riesling Icewine fermentation 75 colonies of hybrid AWRI 1572 isolated on day 34 of triplicate fermentations were analyzed. Lane m represents molecular weight markers; *Sc* represents the *S. cerevisiae* parent (AWRI 838); *Sb* represents the *S. bayanus* parent (AWRI 1176); H represents hybrid AWRI 1572 before fermentation. Lanes 1-25 are colonies of hybrid AWRI 1572 isolated on day 34 from fermentation replicate #1, 26-50 are from replicate #2, and 51-75 are from replicate #3.

3.5. Discussion

Acetic acid is an undesired byproduct during Icewine fermentations because it gives wine a vinegar aroma when present at high concentration and can esterify with ethanol to form ethyl acetate, detracting from wine quality. Thus it is important to

develop methods to decrease acetic acid and ethyl acetate concentration in Icewine production. Selecting new yeast strains to be used as novel starter cultures appears to be a good approach to solve this problem. The desired yeast strains for Icewine production are able to conduct efficient fermentation with target ethanol concentration of approximately 10 % v/v in a reasonable fermentation time, produce a low concentration of acetic acid. Ideally, the new strains should also display stable genome during fermentations to produce a consistent and predictable metabolic profile. In this study, three new yeast strains were investigated in comparison with a commercially available wine yeast K1-V1116 in Icewine fermentations. Fermentations were conducted using unfiltered Icewine juice which mimics the industrial environment in order to determine the applicable value of these stains to be used as novel starter cultures in commercial wine making circumstance.

The high sugar consumption of AWRI 1572 (Fig. 3.1B) and correspondingly high ethanol production (Table 3.3), together with high viable cell concentrations during most of the fermentation time (Fig. 3.2A) and low metabolic conversion of sugar to acetic acid (Table 3.4), suggested that this strain is well adapted to the Icewine fermentation environments. It displayed the potential to conduct an efficient fermentation even in the likely presence of competing microflora in the juice. AWRI 1571 and the isolate *S. bayanus/S. pastorianus* did not appear as suitable for Icewine production due to their failure in reaching the target ethanol production with the lowest concentration produced by *S. bayanus/S. pastorianus* (Table 3.3). These two strains consumed much less sugar compared to K1-V1116 during the fermentations

(Fig. 3.1B) and their viable cell concentrations were maintained at low concentration for almost the whole course of fermentations (Fig. 3.2A).

Interestingly, although AWRI 1572 produced the highest concentration of ethanol, its ability to convert sugar to ethanol was lower than K1-V1116 as indicated by the ratios of ethanol produced to sugar consumed (Table 3.2). All three new strains displayed lower ratios compared to K1-V1116 (Table 3.2).

During fermentations, the acetic acid production per sugar consumed varied between yeast strains (Fig. 3.4B and Table 3.2) Acetic acid production under hyperosmotic stress has been suggested as a mechanism to reduce NAD^+ produced during glycerol formation back to NADH by cytosolic, NAD^+-dependent aldehyde dehydrogenases when acetaldehyde is oxidized to acetic acid (Miralles and Serrano 1995; Navarro-Avino *et al.* 1999). This might be the mechanism of acetic acid production during Icewine fermentation as it was discussed in Chapter 2, and thus the production of acetic acid correlated to the production of glycerol during Icewine fermentation. In our study, the ratios of acetic acid produced to glycerol production in unfiltered fermentations varied between yeast strains. *S. bayanus/S. pastorianus* and AWRI 1572 displayed lower ratios compared to K1-V1116; whereas the ratio of AWRI 1571 was higher (Table 3.5). This indicated that acetic acid was produced at different concentrations across the four strains when the same concentration of glycerol was produced. The reason for the different ratios may be several fold: it may be due to metabolites other than acetic acid satisfying the redox balance requirement during glycerol production; acetic acid may be synthesized for various reasons, using

both $NADP^+$- and NAD^+-dependent aldehyde dehydrogenases; acetic acid may be further metabolized in some strains evidenced by the fact we already see difference in ethyl acetate production among the four strains; or glycerol is not only made in response to osmotic stress.

S. bayanus/S. pastorianus and AWRI 1572 accumulated lower acetic acid in response to the same concentration of glycerol production than K1-V1116 (Table 3.2) might be due to a higher level of dissimilation of acetic acid. As discussed in Chapter 2, acetic acid could be converted into acetyl-CoA by acetyl-CoA synthetases in the pyruvate dehydrogenase bypass (reviewed in Pronk *et al.* 1996) and it was found that an acetyl-CoA synthetase encoding gene *ACS1* was expressed at a higher level in a high acetic acid producer VIN7 than in a low acetic acid generating strain ST in fermenting grape must containing 40% w/v sugar (Erasmus and van Vuuren 2009). The reason that AWRI 1571 accumulated higher concentration of acetic acid per glycerol produced than K1-V1116 (Table 3.2) might be caused by higher contribution of $NADP^+$-dependent aldehyde dehydrogenases to acetic acid production. As mentioned in Chapter 2, the cytosolic, $NADP^+$-dependent aldehyde dehydrogenase Ald6p might contribute to acetic acid generation as well (Eglinton *et al.* 2002; Erasmus and van Vuuren 2009) and the expression of *ALD6* was much higher in a high acetic acid producing strain grown in grape musts containing 40% w/v sugar (Erasmus and van Vuuren 2009). In addition, glycerol is made in fermenting yeasts not only by serving as an osmolyte under hyperosmotic stress, but also produced as a redox balancing metabolite, oxidizing the excess NADH generated during biomass

formation (Nordstrom 1968; van Dijken and Scheffers 1986). The higher production of glycerol (Table 3.3) by K1-V1116 and AWRI 1572 might be partially caused by their faster and greater extent of accumulation of cell concentrations (Fig. 3.2), which could be further proved by their higher ammonia nitrogen consumption (Table 3.1A). The isolate *S. bayanus/S. pastorianus* and AWRI 1571 which showed a relatively lower extent of cell accumulation (Fig. 3.2) also produced less glycerol (Table 3.3) and displayed lower ammonia consumption (Table 3.1A).

Unstable genome of yeast strains during fermentation could lead to an unpredictable organoleptic profile in the final wine and might affect the wine composition, such as sugar consumption and acetic acid production. The DNA restriction fragments of rDNA regions comprising the ITS and the 5.8S rRNA gene were analyzed to determine the genomic stability of AWRI 1571 and AWRI 1572 during fermentations. In our study, both hybrids showed a higher portion of genomic instability from the *S. bayanus* parent as opposed to the *S. cerevisiae* parent during unfiltered Icewine fermentations (Fig. 3.5 and 3.6). These genomic losses might be caused by the extremely stressful environment during Icewine fermentations as discussed in Chapter 2 since environmental stresses could drive hybrids evolving towards adaptation (Hittinger and Carroll 2007; Coyle and Kroll 2008). In our study, the higher proportion of DNA variation from the *S. bayanus* parent and relatively stable DNA content from the *S. cerevisiae* parent of the hybrids may reflect that AWRI 1571 and AWRI 1572 were evolving towards adaptation to Icewine production by deleting genomic contents from the parent *S. bayanus* which was less

adapted to Icewine fermenting circumstance.

The loss or reduced expression of parental rDNA occurred in only one out of the triplicate unfiltered fermentations (fermentation replicate #3 of AWRI 1571 and fermentation replicate #2 of AWRI 1572, Fig. 3.5 and 3.6), but there was no difference in either sugar consumption or acetic acid generation among the triplicate fermentations of both hybrid strains (data not shown). This means that the unstable rDNA regions of AWRI 1571 and AWRI 1572 could not be correlated to the metabolic profiles during Icewine fermentations and therefore it would not detract from the composition of the final wine. A global scan of hybrid genomes is required to correlate the genomic instability with the metabolic change during unfiltered Icewine fermentations. It could be either that the genes involved in the metabolism of sugar and acetic acid were not affected by the stressful environment during unfiltered Icewine fermentations or a small extent of genomic instability did not affect the metabolism.

3.6. Conclusions

This study investigated three new yeast strains as novel starter cultures for unfiltered Icewine fermentation and emphasized the effect of yeast strains on acetic acid production. AWRI 1572 was identified as a potential strain used as a novel starter culture for Icewine production since it reached the target ethanol concentration in a reasonable fermentation time with lower acetic acid production as a function of both time and sugar compared to commercial wine yeast K1-V1116.

AWRI 1571 and *S. bayanus/S. pastorianus* do not appear suitable for Icewine production due to their failure in desired ethanol production and high acetic acid production. Both hybrids displayed genomic instability of the rDNA regions during fermentations but it did not affect wine composition.

References

Coyle, S. and Kroll, E (2008) Starvation induces genomic rearrangements and starvation-resilient phenotypes in yeast. *Mol Biol Evol* **25**, 310-318.

Delfini, C., and Cervetti. F. (1991) Metabolic and technological factors affecting acetic acid production by yeasts during alcoholic fermentation.*Vitic Enol Sci* **46**,142-150.

van Dijken, J. and Scheffers, W. (1986) Redox balances in the metabolism of sugars by yeasts. *FEMS Microbiol Rev* **32.** 199-224.

Eglinton, J.M., Heinrich, A.J., Pollnitz, A.P., Langridge, P., Henschke, P.A. and de Barros Lopes, M. (2002) Decreasing acetic acid accumulation by a glycerol overproducing strain of *Saccharomyces cerevisiae* by deleting the ALD6 aldehyde dehydrogenase gene. *Yeast* **19**, 295–301.

Eglinton, J.M., McWilliam, S.J., Fogarty, M.W., Francis, I.L., Kwiatkowski, M.J., Høj, P.B. and Henschke, P.A. (2000) The effect of *Saccharomyces bayanus*-mediated fermentation on the chemical composition and aroma profile of Chardonnay wine. *Aust J Grape Wine Res* **6**, 190–196.

Erasmus, D.J. and van Vuuren H.J.J. (2009) Genetic basis for osmosensitivity and genetic instability of the wine yeast *Saccharomyces cerevisiae* VIN7. *Am J Enol Vitic* **60**, 145-154.

Fleet, G.H. (2003) Yeast interactions and wine flavour. *Int J Food Microbiol* **86**, 11-22.

Fleet, G.H. and Heard, G. (1993) Yeasts-growth during Fermentation. In *Wine Microbiology and Biotechnology* ed. Fleet, G.H. pp. 27-54. Chur, Switzerland: Harwood Academic Publishers.

Kontkanen, D., Inglis, D., Pickering, G. and Reynolds, A. (2004) Effect of yeast inoculation rate, acclimatization, and nutrient addition on Icewine fermentation. *Am J Enol Vitic* **55**, 363-370.

Hittinger, C.T. and Carroll, S.B. (2007) Gene duplication and the adaptive evolution of a classic genetic switch. *Nature* **449**, 233-244.

Miralles, V.J. and Serrano, R. (1995) A genomic locus in Saccharomyces cerevisiae with four genes up-regulated by osmotic stress. *Mol Microbiol* **17**, 653-662.

Navarro-Avino, J.P., Prasad, R., Miralles, V.J., Benito, R.M. and Serrano, R. (1999) A proposal for nomenclature of aldehyde dehydrogenases in *Saccharomyces cerevisiae* and characterization of the stress-inducible ALD2 and ALD3 genes. *Yeast* **15**, 829-842.

Nguyen, H.V. and Gaillardin, C. (1997) Two subgroups within the *Saccharomyces bayanus* species evidenced by PCR amplification and restriction polymorphism of the non-transcribed spacer 2 in the ribosomal DNA unit. *Syst Appl Microbiol* **20**, 286–294.

Nguyen, H.V., Le'pingle, A. and Gaillardin, C. (2000) Molecular typing demonstrates homogeneity of *Saccharomyces uvarum* and reveals the existence of hybrids between *S. uvarum* and *S. cerevisiae*, including the *S. bayanus* type strain CBS 380. *Syst Appl Microbiol* **23**, 71–85.

Nordstrom, K. (1968) Yeast growth and glycerol formation : carbon and redox balances. *J Inst Brew* **74**, 429-432.

Pronk, J.T., Steensmays, H.Y.and van Dijken J.P. (1996) Pyruvate metabolism in *Saccharomyces cerevisiae. Yeast* **12**, 1607-1633.

Sipiczki, M. (2008) Interspecies hybridization and recombination in Saccharomyces wine yeasts. *FEMS Yeast Res* **8**, 996-1007.

Vintners Quality Alliance Act (VQA) (1999) Vintners Quality Alliance Act. Ontario Regulation 406/00.

Vintners Quality Alliance Ontario (2009) *Vintners Quality Alliance (VQA) annual report* pp. 9.

Chapter 4. Discussion and conclusions

4.1. Discussion

Icewine fermentation is often correlated to high concentration of acetic acid production (Kontkanen *et al.* 2004), which has been a concern for Icewine producer because it gives wine an unwanted vinegar aroma and any Icewine in Canada with an acetic acid concentration higher than 2.1 g l^{-1} cannot be designated an Icewine according to the Vintners Quality Alliance (VQA 1999). This study investigated three new yeast strains in Riesling Icewine fermentations to determine their potential to be used as novel starter cultures for Icewine production with highlight on acetic acid production in comparison to a commercially available wine strain K1-V1116. Fermentations were firstly conducted in sterile filtered Riesling Icewine juice to evaluate the authentic characteristics of these yeast strains under aseptic, laboratory condition, and then conducted in unfiltered Riesling Icewine juice, which mimics the winery environment, to assess their applicable potential to be used for industrial Icewine production. It should be noticed that, in this study, the sterile Riesling Icewine juice used for sterile fermentations was not obtained by sterile filtering the same Riesling Icewine juice which was used for unfiltered fermentations. They were different batches of juice from different vintages. This could explain the high variance in compositions between sterile and unfiltered juice listed in Chapter 2 and 3, respectively.

In our study, the commercial wine yeast K1-V1116 produced 1.25 g l^{-1} of acetic

acid in unfiltered fermentations which simulates the winery conditions. This value was in good agreement with the average acetic acid concentration in Canadian commercial Icewine, 1.3 g l^{-1}, reported by Nurgel *et al.* (2004) and the mean volatile acidity concentration of 348 Canadian commercial Icewines, 1.2 g l^{-1}, found by Soleas and Pickering (2007). However, K1-V1116 produced a much higher concentration of acetic acid in our sterile Icewine fermentations (2.07 g l^{-1}) compared to the unfiltered, and the same result can be observed in fermentations conducted by the other yeast strains (Table 2.2 and 3.4). The higher acetic acid production in sterile fermentations could be caused by the higher juice soluble solids and reducing sugar in sterile filtered juice (41.6 ± 0 °Brix, 473 ± 11 g l^{-1} of sugar in sterile juice versus 39.2 ± 0 °Brix, 423 ± 11 g l^{-1} of sugar in unfiltered juice). In compositional analysis of Ontario commercial Icewine, it was reported that volatile acidity was positively correlated with juice soluble solids (Soleas and Pickering 2007). The higher ratios of acetic acid production to sugar consumption in sterile fermentations in our study (Table 2.1 and 3.2) also agreed with the study of Pigeau *et al.* (2007), which showed that the acetic acid production per sugar consumed was positively correlated to the sugar concentration of Icewine juice. In addition, the clarification of Icewine juice for sterile fermentations might lead to an extra stress on yeasts during fermentation. Clarification of juice does not only deplete must microflora but also removes suspended solids present in must, which can supply yeasts with nutritional elements and adsorb certain metabolic inhibitors (Ribereau-Gayon *et al.* 2006). The depletion of such suspended solids might place

yeast cells under a more stressful environment and give rise to difference in the production of metabolites such as acetic acid.

In our study, AWRI 1572 appears as a potential yeast strain to be used as a novel starter culture for Icewine production. It consumed the same concentration of sugar as K1-V1116 in sterile fermentations and produced 28.8% less acetic acid; in unfiltered conditions it displayed even better adaption, with 34.3% higher sugar consumption than K1-V1116 and 33.1% lower acetic acid production. Although it showed lost or reduced copies of ITS rDNA fragments under both fermentation conditions, this genomic instability could not be correlated with the metabolic profile, and therefore may not affect the composition of the final wine. The higher sugar consumption and less acetic acid production by AWRI 1572 under unfiltered fermentations indicated that this strain was able to maintain its metabolic profile displayed in sterile fermentations, and adapted even better in the unfiltered condition similar to commercial conditions. This better adaption might result from the presence of suspended solids in unfiltered Icewine juice, which provided nucleation sites to eliminate CO_2, promoted yeast multiplication (Fig. 2.2 and 3.2) as a support and also supplied yeasts with nutrition and adsorbed certain metabolic inhibitors (Ribereau-Gayon *et al.* 2006).

The isolate *S. bayanus/S. pastorianus* and the other hybrid AWRI 1571 appear to be unsuitable for Icewine production using unfiltered juice, due to their low sugar consumption and corresponding failure in reaching target ethanol concentration in unfiltered fermentations (Table 2.1 and 2.2). The low sugar consumption and

correspondingly low ethanol production of *S. bayanus/S. pastorianus* in both sterile and unfiltered fermentations (Table 2.2, 3.1A and 3.3) indicated that this isolate appears less adaptive to the severe stressful environment of Icewine fermentations. However, its low production of ethyl acetate and relatively low conversion of sugar to acetic acid indicate this yeast may be suitable for other wine styles, such as wines made from grapes concentrated by drying (*appassimento*) where the starting soluble solids concentration is not quite as high as that was found in Icewine juice. The less sugar consumption of AWRI 1571 compared to K1-V1116 in unfiltered fermentations but not in sterile fermentations suggested that this strain appears less suitable in fermenting unfiltered juice in which other microflora and suspended solids are present. Both strains displayed low acetic acid production as a function of both time and sugar in sterile fermentations under aseptic, laboratory conditions (Table 2.2 and 2.3), but they failed to maintain this property in fermenting unfiltered Icewine juice which was the condition for Icewine production in the winery (Table 3.4). It seems that the presence of natural microflora or/and the suspended solids in the unfiltered juice affected yeasts' metabolism of acetic acid during Icewine fermentations.

The variation of acetic acid production of these yeast strains between sterile Icewine fermentations and unfiltered might also be caused by the difference in juice composition since the juice used was from different vintages. Paraggio and Fiore (2004) tested 15 yeast strains, differing in acetic acid production, in fermentation of grape musts of different varieties and found that the strain behavior was dependent

on various grape must. Romano *et al.* (2003) inoculated *S. cerevisiae* strains to grape musts of different varieties and found that the acetic acid production is the result of the interaction between the yeast and the grape must composition. However, Soleas and Pickering (2007) reported that there was no difference in volatile acidity between Icewines produced from 2000 to 2004 in a study of 348 Canadian commercial Icewines. Whether or not the acetic acid producing property of these yeast strains are affected by vintage and variety of juice is required to be further investigated.

The stability analysis of the two hybrids, AWRI 1571 and AWRI 1572, indicated that both strains showed lost or reduced copies of parental rDNA freagments during both sterile and unfiltered Riesling Icewine fermentation. However, the extent of genomic instability of AWRI 1572 is much higher in sterile filtered fermentation compared to the unfiltered (13.3% versus 2.7%) (Fig. 2.6 and 3.6). AWRI 1571 only displayed a slightly higher genomic instability in sterile fermentations (1.4%) compared to that in unfiltered condition (1.3%) (Fig. 2.5 and 3.5).

We previously discussed that the natural and laboratory-bred hybrids could undergo dramatic genomic changes during mitotic and meiotic divisions to evolve towards genome-stabilization, which consists of loss of chromosomes and genes and recombination between the partner genomes (Sipiczki 2008). This evolution could be accelerated by an extremely stressful environment during Icewine fermentation; such as high ethanol, strong acidity and extremely high sugar. The gene loss or reduced expression of hybrid genomes during Icewine fermentation might be the

molecular mechanisms for adaptive evolution of hybrid strains driven by environmental stress (Hittinger and Carroll 2007; Coyle and Kroll 2008). In our study, the juice used for sterile fermentation had much higher sugar concentration and therefore placed yeast cells under a more stressful circumstance in comparison to the unfiltered fermentation. In addition, the depletion of suspended solids could lead to extra stress on yeast cells (Ribereau-Gayon *et al.* 2006). The more stressful environment for yeast cells under sterile fermentations might be the reason for the higher genomic instability of AWRI 1572 during sterile fermentations, since environmental stress could drive genomic modification of hybrids (Hittinger and Carroll 2007; Coyle and Kroll 2008). Furthermore, we previously mentioned that, in this study, the sterile Riesling Icewine juice used for sterile fermentations and the unfiltered Riesling Icewine juice which was used for unfiltered fermentations were from different vintages. The variation of juice compositions might also contribute to the different level of genomic instability of AWRI 1572.

AWRI 1571 displayed lower genomic instability compared to AWRI 1572 and was only affected slightly by different stressful environments under sterile or unfiltered fermentations. This indicated that the genome of AWRI 1571 is relatively stable even under more stressful environments compared to AWRI 1572. Nevertheless, the unstable genome of both hybrids seem not detracting from the limited measurements of wine composition performed in this body of work since it could not be correlated to the metabolic profile.

Since AWRI 1572 produced much less acetic acid in both fermentation conditions,

it would be interesting to investigate the molecular mechanism for its acetic acid production. Analysis of gene expression profile of glycerol and acetic acid metabolism-associated genes is valuable. Microarray or Northern analysis focusing on *GPD*, *ALD* and *ACS* genes are potential investigating methods. In addition, the activity of these related enzymes can be investigated to compare enzyme activity between different strains.

The sensory profile of Icewine produced by these yeast strains should be investigated. The sensory characteristics of yeast strains are another important standard to assess whether or not a yeast strain is suitable to be used as a starter culture for wine production because starter cultures play predominant role in contribution to aroma and sensory profile of the final wine. It was suggested that *S. bayanus* is more associated with savoury- and cooked-like aroma attributes; such as 'cooked orange peel', 'honey', 'yeasty', 'nutty' and 'aldehyde' (Eglinton *et al.* 2000). The sensory evaluation of Icewine produced by these strains could further prove whether these yeasts are qualified to be used as novel starter cultures for Icewine production.

The naturally isolated strain *S. bayanus/S. pastorianus* failed to reach the target ethanol concentration of approximately 10 % v/v in both sterile and unfiltered Icewine fermentations, but it produced less acetic acid and ethyl acetate in the fermentations which are desirable qualities for these and other wine styles. AWRI 1571 failed to produce the desired ethanol concentration in unfiltered fermentations but it also produced less acetic acid in sterile condition. Since acetic acid production

is the result of interaction between yeast strains and juice composition (Romano *et al.* 2003), study of these strains in additional varieties used for Icewine production such as Vidal or Cabernet Franc might provide a more thorough description of characteristics of these yeast strains. It would also be interesting to assess the performance of these strains in *appassimento*-style wines where grapes are ripened off-vine. Ripening the fruit off-vine post harvest could concentrate flavours and sugars. However, desiccation of grapes through the drying process (*appassimento*) provokes physical and biochemical changes resulting from both endogenous grape metabolism and mould development. The loss of water in grapes through this drying process formed high concentrations of ethyl acetate and volatile acidity (Costantini *et al.* 2006). In addition, the mould *Botrytis cinerea* often develops on the grapes during the drying process and contributes to the production of organic acids including acetic acid (Ribereau-Gayon *et al.* 2000). Therefore, yeast strains that produce reduced concentrations of acetic acid and ethyl acetate, such as those investigated in this study, may be favourable in the fermentation of *appassimento*-style wines.

4.2. Conclusions

This study investigated three new yeast strains as novel starter cultures for Icewine production. AWRI 1572 was designated as a potential strain used as a novel starter culture for Icewine production because it reached the target ethanol concentration in a reasonable fermentation time with lower acetic acid production in

comparison with commercial wine yeast K1-V1116 under both sterile filtered and unfiltered Riesling Icewine fermentations. Although it displayed genomic instability of rDNA region, it did not affect the composition of the final wine. AWRI 1571 and *S. bayanus/S. pastorianus* do not appear suitable for Icewine production due to their failure in the desired ethanol production and high acetic acid production in unfiltered fermentations, although both strains produced less acetic acid as a function of both time and sugar consumed in sterile fermentations compared to K1-V1116.

References

Costantini, V., Bellincontro, D., De Santis, D., Botondi, R. and Mencarelli, F. (2006) Metabolic changes of Malvasia grapes for wine production during postharvest drying. *J Agric Food Chem* **54**(9), 3334-3340.

Coyle, S. and Kroll, E (2008) Starvation induces genomic rearrangements and starvation-resilient phenotypes in yeast. *Mol Biol Evol* **25**, 310-318.

Eglinton, J.M., McWilliam, S.J., Fogarty, M.W., Francis, I.L., Kwiatkowski, M.J., Høj, P.B. and Henschke, P.A. (2000) The effect of *Saccharomyces bayanus*-mediated fermentation on the chemical composition and aroma profile of Chardonnay wine. *Aust J Grape Wine Res* **6**, 190–196.

Hittinger, C.T. and Carroll, S.B. (2007) Gene duplication and the adaptive evolution of a classic genetic switch. *Nature* **449**, 233-244.

Kontkanen, D., Inglis, D., Pickering, G. and Reynolds, A. (2004) Effect of yeast inoculation rate, acclimatization, and nutrient addition on Icewine fermentation. *Am J Enol Vitic* **55**, 363-370.

Nurgel, C., Pickering, G.J. and Debra, D.L. (2004) Sensory and chemical characteristics of Canadian ice wines. *J Sci Food Agric* **84**, 1675-1684.

Paraggio, M. and Fiore, C. (2004) Screening of *Saccharomyces cerevisiae* wine strains for the production of acetic acid. *J Appl Microbiol Biotech* **20**, 743-747.

Pigeau, G.M., Bozza, E., Kaiser, K. and Inglis. D.L. (2007) Concentration effect of Riesling Icewine juice on yeast performance and wine acidity. *J Appl Microbiol* **103**, 1691-1698.

Ribereau-Gayon, P., Dubourdieu, D., Doneche, B. and Lonvaud, A. (2006) White Winemaking. In *Handbook of Enology (Volume I): the Microbiology of Wine and Vinifications* pp. 397-444. Wiley: John Wiley & Sons, Ltd.

Romano, P., Fiore, C., Paraggio, M. Caruso, M. and Capece A. (2003) Function of yeast species and strains in wine flavour *Int J Food Microbiol* ***86***, 169-180.

Sipiczki, M. (2008) Interspecies hybridization and recombination in Saccharomyces wine yeasts. *FEMS Yeast Res* **8**, 996-1007.

Soleas, G.J. and Pickering, G.J. (2007) Influence of variety, wine style, vintage and viticultural area on selected chemical parameters of Canadian Icewine. *J Sci Food Agric* **5**, 97-101.

Vintners Quality Alliance Act (VQA) (1999) Vintners Quality Alliance Act. Ontario Regulation 406/00.