

# Upregulation of *ALD3* and *GPD1* in *Saccharomyces cerevisiae* during Icewine fermentation

G.M. Pigeau and D.L. Inglis

Department of Biological Sciences, Cool Climate Oenology and Viticulture Institute, Brock University, St Catharines, ON, Canada

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## ABSTRACT

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**Aims:** To determine acetic acid, acetaldehyde and glycerol production by wine yeast throughout Icewine fermentation. The expression of yeast cytosolic aldehyde dehydrogenases (*ALD3* and *ALD6*) and glycerol-3-phosphate dehydrogenase (*GPD1*) were followed to relate metabolites in the wines to expression patterns of these genes.

**Methods and Results:** Icewine juice (38.8°Brix,  $401 \pm 7 \text{ g l}^{-1}$  sugar), diluted Icewine juice (21.3°Brix,  $211 \pm 7 \text{ g l}^{-1}$  sugar) and the diluted juice with sugar levels equal to the original Icewine juice (35.6°Brix,  $402 \pm 6 \text{ g l}^{-1}$  sugar) were fermented in triplicate using the commercial wine yeast K1-V1116. Acetic acid production increased 7.1-fold and glycerol production increased 1.8-fold in the Icewine fermentation over that found in the diluted juice fermentation. *ALD3* showed a 6.2-fold induction and *GPD1* showed a 2.5-fold induction during Icewine *vs* the diluted fermentation. *ALD3* was not glucose repressed when additional sugar was added to diluted juice, but was upregulated 7.0-fold.

**Conclusions:** The NAD<sup>+</sup>-dependant aldehyde dehydrogenase encoded by *ALD3* appears to contribute to acetic acid production during Icewine fermentation. Expression of *GPD1* was upregulated in high sugar fermentations and reflects the elevated levels of glycerol. Solutes in Icewine juice in addition to sugar contribute to the yeast metabolic response.

**Significance and Impact of the Study:** This work represents the first descriptive analysis of the fermentation of Canadian Icewine, the expression patterns of yeast genes involved in metabolite production, and their impact on Icewine quality. A role for *ALD3* in acetic acid production during Icewine fermentation was found.

**Keywords:** acetic acid, aldehyde dehydrogenase, glycerol, glycerol-3-phosphate dehydrogenase, HOG response, hyperosmotic stress, Icewine, *Saccharomyces cerevisiae*.

## INTRODUCTION

Wine yeast display properties which are distinct from laboratory strains of *Saccharomyces cerevisiae* and these differences can impact the organoleptic properties of the finished wine (Bidenne *et al.* 1992; Remize *et al.* 1999; Hauser *et al.* 2001). Analysis of the initial hyperosmotic stress response of wine yeast to fermentable sugars found an

isoform of pyruvate decarboxylase (*PDC6*), a key enzyme in fermentative metabolism, upregulated in the wine yeast (Erasmus *et al.* 2003) but this gene was not upregulated in laboratory yeast strains exposed to salt or sorbitol stress (Rep *et al.* 2000). The adapted stress response of wine yeast to fermentable sugars may also differ from the salt-induced hyperosmotic stress response extensively studied in laboratory strains of *S. cerevisiae* (reviewed in Hohmann 2002) or from the initial osmotic stress response in wine yeast induced by sugars (Erasmus *et al.* 2003).

The fermentation of naturally concentrated Icewine juice places commercial wine yeast under extreme hyperosmotic

Correspondence to: Debra L. Inglis, Department of Biological Sciences, Cool Climate Oenology and Viticulture Institute, Brock University, 500 Glenridge Ave., St Catharines, ON L2S 3A1, Canada (e-mail: dinglis@brocku.ca).

stress as they try to ferment the juice into wine. When grapes naturally frozen on the vine are picked and pressed, ice remains in the berries and the resulting juice is highly concentrated in sugars, acids and nitrogenous compounds. The concentration of soluble solids in Canadian Icewine juice ranges from a minimum of 35°Brix (VQA 1999) to *c.* 42°Brix. Our initial Icewine fermentation trials have shown significant increases in acetic acid and glycerol production by wine yeast. Acetic acid increased from 0.05 to 1.52 g l<sup>-1</sup> and glycerol increased from 6.6 to 11.2 g l<sup>-1</sup> as the juice concentration increased from 20 to 40°Brix respectively (Pigeau *et al.* 2002; Pitkin *et al.* 2002).

Salt-induced osmotic stress causes cells to produce glycerol to serve as an internal osmolyte to balance the osmotic pressure placed on the cell (Blomberg and Adler 1989; Brewster *et al.* 1993; Nevoigt and Stahl 1997; Blomberg 2000). The rate limiting step in osmotically induced glycerol formation is the expression of glycerol-3-phosphate dehydrogenase (*GPD1*) (Remize *et al.* 2001). This gene product catalyzes the formation of glycerol-3-phosphate from dihydroxyacetone phosphate (DHAP). Two glycerol-3-phosphatases (*GPP1* and *GPP2*) then complete the reaction to produce glycerol. It has been shown that the highly homologous *GPD2* gene is not involved in the osmoregulatory response, but plays a role in redox balance during anaerobic conditions (Albertyn *et al.* 1994; Ansell *et al.* 1997; Pahlman *et al.* 2001; Remize *et al.* 2003). The reduction of DHAP to glycerol 3-phosphate during glycerol production oxidizes NADH to NAD<sup>+</sup>.

Due to the lack of a transhydrogenase in yeast to convert reducing equivalents between the NAD<sup>+</sup>/NADH system and the NADP<sup>+</sup>/NADPH system, yeast must rely on metabolite formation to maintain the intracellular redox balance for the coenzyme systems (van Dijken and Scheffers 1986). Acetic acid production has been suggested as a mechanism through which yeast balance excess NAD<sup>+</sup> produced from glycerol formation during the salt-induced hyperosmotic stress response (Blomberg and Adler 1989; Miralles and Serrano 1995; Navarro-Avino *et al.* 1999). This may occur through the action of cytosolic, NAD<sup>+</sup>-dependent aldehyde dehydrogenases, which reduce NAD<sup>+</sup> to NADH while oxidizing acetaldehyde to acetic acid thus restoring internal redox balance. There are three cytosolic aldehyde dehydrogenases known in *S. cerevisiae*, two of which are NAD<sup>+</sup> dependent (encoded by *ALD2* and *ALD3*) (Navarro-Avino *et al.* 1999) and one of which is NADP<sup>+</sup> dependent (encoded by *ALD6*) (Meaden *et al.* 1997). Several studies have shown that Ald6p is the main aldehyde dehydrogenase responsible for acetic acid production in *S. cerevisiae* strains during fermentation of glucose media (Radler 1993; Remize *et al.* 1999; Eglinton *et al.* 2002), although most recently, the NADP<sup>+</sup>-dependent mitochondrial Ald5p was also found to contribute to acetate production

(Saint-Prix *et al.* 2004). Under salt stress, of these two NADP<sup>+</sup>-dependent isoforms, only *ALD6* was found upregulated (Akhtar *et al.* 1997; Rep *et al.* 2000). Due to the high homology between *ALD2* and *ALD3*, it continues to be difficult to independently measure the expression of these genes (Miralles and Serrano 1995; Aranda and del Olmo 2003). However, it is now accepted that *ALD3* and not *ALD2* is responsive to salt stress (Norbeck and Blomberg 2000). Ald2p is reported to have a specialized function in forming β-alanine required for pantothenic acid production by oxidizing 3-amino propanal (White *et al.* 2003). Although Ald3p can partially compensate for Ald2p in *ALD2* deletion mutants, full compensation only occurs when deletion mutants are placed under salt stress, conditions known to stimulate *ALD3* expression (White *et al.* 2003). Although Ald3p has been shown to use acetaldehyde as a substrate (Navarro-Avino *et al.* 1999) and *ALD2/3* expression is upregulated when yeast are subjected to acetaldehyde stress (Aranda and del Olmo 2003), the role of Ald3p has not been investigated during stressful wine fermentations to see if it contributes to acetic acid production. Although many of these past studies have looked at acetic acid production by yeast during fermentation of glucose in a synthetic medium, the medium was not grape juice. Phenolic compounds present in grape juice such as catechin, have been found to reduce acetic acid production during fermentation, possibly by inhibiting aldehyde dehydrogenase activity (Moruno *et al.* 1993; Caridi 2002). Therefore, fermentations carried out in Icewine grape juice where the hyperosmotic stress is induced by solutes naturally concentrated in the juice may result in differing effects from those found by salt-induced stress or hyperosmotic stress from sugar in synthetic media.

With the upper allowable limit of volatile acidity at 2.1 g l<sup>-1</sup> acetic acid for Canadian Icewines, it is crucial to understand the fermenting yeast's adapted response during Icewine fermentation to determine how and why acetic acid is produced. A recent survey of commercial Canadian Icewines found an average acetic acid concentration of 1.3 g l<sup>-1</sup> ranging from 0.49 to 2.29 g l<sup>-1</sup> (Nurgel *et al.* 2004).

To determine if wine yeast's adapted stress response to high concentrations of fermentable sugars is comparable with the salt-induced stress response of laboratory strains leading to increased acetic acid production, we have followed the gene expression of the two salt-induced, cytosolic aldehyde dehydrogenases, *ALD3* and *ALD6*, during the fermentation of Icewine juice and compared these profiles to that found in yeast fermenting diluted Icewine juice and diluted juice containing added sugars. The expression of *GPD1* was followed as a control for the hyperosmotic stress response. Expression patterns of these genes were compared with acetaldehyde, acetic acid and glycerol levels produced in the wines to ascertain the relationship between gene

expression in wine yeast induced by high concentrations of fermentable sugars and metabolite production during Icewine fermentation. The role that aldehyde dehydrogenases encoded by *ALD3* and *ALD6* play in the elevated level of acetic acid found in Icewine was investigated.

## MATERIALS AND METHODS

### Yeast strains

The commercial yeast strain used for wine fermentations, *S. cerevisiae* K1-V1116, was kindly provided by Lallemand Inc., Montreal, QC, Canada. Yeast strains used for the validation of DNA probe specificities were deletion mutants ( $\Delta$ *ALD3*,  $\Delta$ *ALD6*, and  $\Delta$ *GPDI*) of *S. cerevisiae* Hansen BY4742 (mat alpha his3D1 leu2D0 lys2D0 ura3D0) and were obtained from Research Genetics.

### Medium

For probe specificity validation experiments, cells were grown overnight in YPD medium (1% yeast extract, 2% peptone, 2% dextrose) at 30°C and 150 rev min<sup>-1</sup>. Deletion mutants were supplemented with geneticin (G418) at 200 mg l<sup>-1</sup>. YPD supplemented with 0.7 mol l<sup>-1</sup> NaCl was used to induce osmotic stress on yeast cells as described by Rep *et al.* (2000), using a 2.5-h salt exposure time prior to RNA isolation.

### Icewine juice

To investigate osmotic stress on wine yeast, Icewine juice was kindly provided by Inniskillin Wines Inc., Niagara-on-the-Lake, ON, Canada. The juice was sterile-filtered through coarse, medium and fine pore-size pad filters followed by membrane filtration through a 0.22- $\mu$ m membrane cartridge filter (Millipore, Etobicoke, ON, Canada). Three fermentation conditions were investigated using this juice: undiluted Icewine juice at 38.8°Brix (401  $\pm$  7 g l<sup>-1</sup> reducing sugars,  $A_w$  = 0.948), diluted Icewine juice at 21.3°Brix (211  $\pm$  7 g l<sup>-1</sup> reducing sugars,  $A_w$  = 0.975) and chaptalized dilute Icewine juice at 35.6°Brix (402  $\pm$  6 g l<sup>-1</sup> reducing sugars,  $A_w$  = 0.954). To prepare the latter juice, Icewine juice was first diluted to 21.3°Brix using sterile water (RiOs-16; Millipore) and this juice was then chaptalized with an equal mixture of glucose (206  $\pm$  22 g l<sup>-1</sup>) and fructose (215  $\pm$  5 g l<sup>-1</sup>) to match the sugar concentration in the original, undiluted Icewine juice.

### Yeast inoculation procedure for fermentations

A 5.0-g of wine yeast K1-V1116 was rehydrated with 50 ml of sterile, 40°C water (RiOs-16; Millipore) for 15 min, swirling gently every 5 min. An equal volume of diluted

Icewine juice at 21.3°Brix was then added to the rehydrated yeast and this starter culture was held at 25°C for 1 h, gently swirling every 30 min. Icewine juice (50 ml) was then added to the starter culture, achieving a final juice concentration of 21.7°Brix. The starter culture was held at 20°C for 2 h, swirling every 30 min. A 15-ml starter culture was then used to inoculate 1 l each of three different juices to achieve a yeast inoculum rate of 0.5 g dry weight l<sup>-1</sup>. Fermentations were carried out at 17°C and continued until the yeast stopped consuming sugar, determined by no change in the sugar concentration in the fermentations for 3 days. Daily sampling of the fermentations occurred after stirring the fermentation for 5 min to ensure a homogeneous mixture. Fermentations were performed in triplicate.

### Fermentation parameters and biochemical determinations

Soluble solids of the initial juices were determined in duplicate with an ABBE bench top refractometer and water activities ( $A_w$ ) were determined in triplicate with a vapour pressure osmometer (Wescor 5500; Wescor Inc., Logan, UT, USA). Yeast assimilable amino acid nitrogen levels in the initial juice and the final wines were determined in duplicate from each sample following the nitrogen by *O*-phthaldialdehyde (NOPA) assay as outlined by Dukes and Butzke (1998). Ammonia nitrogen in the initial juice was determined in duplicate using the Boehringer Mannheim ammonia enzyme assay kit (Boehringer Mannheim, Darmstadt, Germany). Reducing sugar content of the fermentations were determined in duplicate by the Lane–Eynon titration method as described in Zoecklein *et al.* (1996). Yeast biomass accumulation was determined by a filter retention assay. A 5-ml sample from each fermentation was removed and passed through Whatman 0.22  $\mu$ m cellulose nitrate filters. The membrane was washed three times with MilliQ water. Filters were dried for 2 days at 60°C and biomass was determined by the difference in mass. Fermentation samples (5 ml) for metabolite analysis were removed daily, sterile-filtered through a 0.22- $\mu$ m syringe filter and stored at -30°C until metabolite analysis was performed. Glucose, fructose, acetic acid, acetaldehyde, glycerol and succinate were determined in duplicate for each sample with Boehringer Mannheim enzyme assay kits. Ethanol was determined in duplicate by gas chromatography using a Hewlett-Packard 5890 gas chromatograph with a 30 m  $\times$  0.32-mm, 5% phenyl methyl silicone column. Samples were diluted 10-fold and contained 1% 1-propanol as an internal standard.

### DNA probe design for Northern analysis

DNA probes for *GPDI*, *ALD3* and *ALD6* were designed against regions of low homology to related genes as

determined by BLAST analysis of the *Saccharomyces* Genome Database (<http://www.yeastgenome.org/>). Two primers for each gene were designed by the author to PCR amplify these low homology regions (30 cycles of 94°C for 40 s, 55°C for 60 s and 72°C for 90 s). The forward *GPD1* primer (5'-TACTATTGCCAAGGTGGTTGCCGAAA-3') and reverse *GPD1* primer (5'-ATGTAAGAGGATAGCAATTGGACACC-3') amplify a 311-bp fragment which has 100% identity to *GPD1* and 69% identity to *GPD2*. The forward *ALD3* primer (5'-TGTTTATGGTTCCGTTGTGGGGAAA-3') and reverse *ALD3* primer (5'-AAACTCTTGAGTTTGCAGTGCAGAT-3') amplify a 259-bp fragment which has 100% identity to *ALD3* and 80% identity to *ALD2*. The forward *ALD6* primer (5'-ACTTTGACACTGCTGAACCAGTCAA-3') and reverse *ALD6* primer (5'-CGATTGGCTCTAAGGTGGTGAAGTT-3') amplify a 427-bp fragment which has 100% identity to *ALD6* and 60% identity to the closest related aldehyde dehydrogenase (*ALD4*). The forward *IPPI* primer (5'-ACCTACTACCAGACAAATTGGTG-3') and reverse *IPPI* primer (5'-AGATGAAGAACCCTTGTC AATAGA-3') amplify a 799-bp fragment which has 100% identity to *IPPI* and 60% identity to the closest related gene, *IPP2*. The PCR products were run on 1% agarose gel (w/v) to verify size and subsequently purified with a Qiagen, QIAquick gel extraction kit for use as probes. Probes were labelled for Northern analysis with [ $\alpha$ -<sup>32</sup>P]-dATP using Boehringer Mannheim's random-primed labelling kit following the manufacturer's directions.

### RNA extraction and Northern analysis

Samples (10 ml) were removed from the fermentations at set time points and were supplemented with 0.01 mg ml<sup>-1</sup> of cycloheximide. The cells were pelleted at 3800 g for 5 min at 4°C using a Sorvall RC 5C plus centrifuge (Sorvall, Newtown, CT, USA). The cells were resuspended in 5 ml of DEPC-treated water and were pelleted as before. Cells were resuspended in 375  $\mu$ l of cold extraction buffer [0.1 mol l<sup>-1</sup> NaCl, 10 mmol l<sup>-1</sup> Tris-Cl (pH 8.0), 1 mmol l<sup>-1</sup> EDTA (pH 8.0) and 5% Triton X-100] and 250  $\mu$ l phenol : chloroform : isoamyl alcohol (PCI) and then mixed with 300 mg of glass beads. This suspension was vortexed at 2500 rev min<sup>-1</sup> for 6 min in order to lyse the yeast cells. After mixing, 6  $\mu$ l of 20% (w/v) SDS was added and the solution was kept on ice for 15 min. This solution was centrifuged in a Sorvall RMC-14 for 10 min at 4°C and 16 000 g. RNA was precipitated from the supernatant with 15  $\mu$ l of 5 mol l<sup>-1</sup> NaCl and 1250  $\mu$ l of 100% (v/v) ethanol. RNA samples from duplicate fermentation trials were quantified at 260 nm and 30  $\mu$ g from each time point was electrophoresed at 95 V for 4.5 h in 18% formaldehyde, 1.25% agarose gels (w/v) and blotted onto

positively charged nylon membranes (Boehringer Mannheim) through upward capillary transfer. After transfer the RNA was cross-linked to the membrane using a Hoefer UVC 500, UV crosslinker (Hoefer Inc., San Francisco, CA, USA). The bound RNA was hybridized to 20 ng of [ $\alpha$ -<sup>32</sup>P] dATP-labelled probes by incubating at 42°C for overnight in 50 ml of hybridization solution (5x SSC, 5x Denhardt's solution, 50% (w/v) formamide, 1% (w/v) SDS and 0.1 mg ml<sup>-1</sup> sheared salmon sperm). The membranes were washed twice with 50 ml of 2x SSC/0.1% SDS for 20 min at room temperature and then twice with 50 ml of 0.2x SSC/0.1% SDS for 15 min at 50°C. The membrane was exposed to Fujifilm phosphorimaging screen for 2 days and the hybridization signal intensities were obtained with a Fujifilm FLA-3000 phosphorimager, using a helium-neon laser at 633 nm, and subsequently quantified with Fujifilm Image Gauge software (version 3.46). Gene expression levels were normalized to 18S rRNA intensities as described by Hocquette and Brandstetter (2002).

### Statistical analysis

SPSS statistical software package release 11.5 (SPSS, Chicago, IL, USA) was used for data analysis. Statistical methods used for data analysis were analysis of variance (ANOVA) with mean separation by Fisher's least significant difference (LSD).

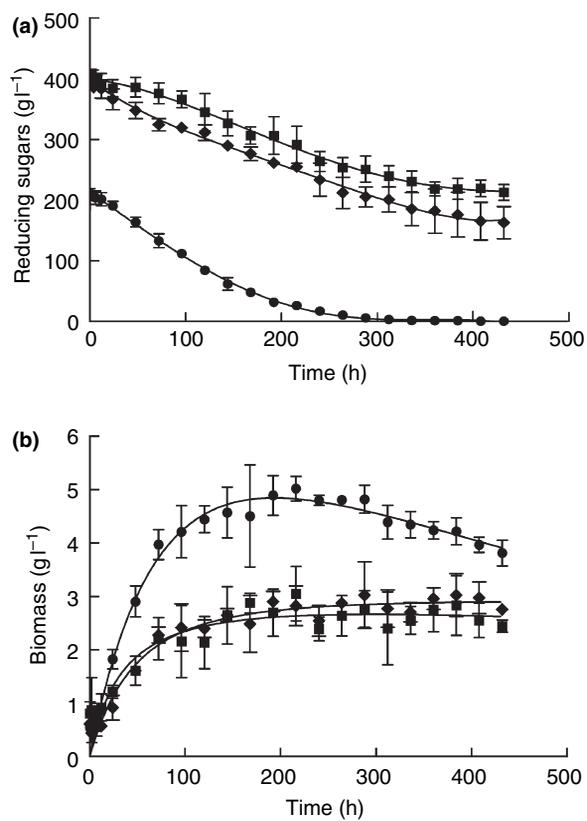
## RESULTS

### Fermentation kinetics

The chemical composition of the Icewine juice is listed in Table 1. Sugar consumption in the Icewine fermentation was slightly delayed in comparison with both the chaptalized juice and the diluted juice fermentations (Fig. 1a). About half of the available fermentable sugars were consumed in both the high sugar fermentations, with 188.2 g l<sup>-1</sup> of reducing sugar consumed in the Icewine fermentation and

**Table 1** Vidal Icewine juice parameters in sterile-filtered juice ( $\pm$ standard deviation)

Parameter	Value
Brix	38.8 g soluble solids 100 g solution <sup>-1</sup>
Reducing sugars	401 $\pm$ 7 g l <sup>-1</sup>
Titratable acidity	5.8 $\pm$ 0.1 g l <sup>-1</sup> Tartaric acid
pH	3.64
Assimilable amino acid nitrogen	358 $\pm$ 9.5 mg N l <sup>-1</sup>
Ammonia nitrogen	31.6 $\pm$ 4.6 mg N l <sup>-1</sup>
Acetic acid	0.03 $\pm$ 0.01 g l <sup>-1</sup>
Glycerol	1.61 $\pm$ 0.1 g l <sup>-1</sup>



**Fig. 1** Sugar consumption and yeast biomass production during fermentation. Icewine juice (■), chaptalized diluted Icewine juice (◆), and diluted Icewine juice (●) were inoculated with the commercial yeast K1-V1116. Reducing sugar consumption (a) and biomass accumulation (b) were measured daily throughout the course of the fermentations. Reducing sugar and biomass values represent the average  $\pm$  standard deviation of the mean of triplicate fermentations

231.5 g l<sup>-1</sup> reducing sugar consumed in the chaptalized fermentation in 432 h (Fig. 1a). This contrasts with the dilute Icewine fermentation, which exhibited a more rapid fermentation rate, consuming all of the fermentable sugars (210.8 g l<sup>-1</sup>) in 312 h (Fig. 1a). Biomass accumulation was faster and to a greater extent in the diluted juice fermentation in comparison with both the high sugar fermentations (Fig. 1b). The high sugar fermentations only accumulated half of the yeast biomass that was accumulated in the dilute fermentation even though nitrogen was not a limiting factor, with 278 mg N l<sup>-1</sup> remaining in the Icewine and 92.6 mg N l<sup>-1</sup> remaining in the wine produced from the chaptalized juice after completion of the fermentations (Table 2).

### Yeast metabolite production

The rate of acetic acid production differed for the three fermentation conditions (Fig. 2a). Acetic acid was produced

at a slow rate in the dilute juice fermentations during the first 120 h, after which the acetic acid remained constant at only  $0.18 \pm 0.03$  g l<sup>-1</sup> (Fig. 2a) while sugar consumption continued to the 310-h time point. In contrast, in the Icewine juice fermentations, acetic acid was produced at a significantly faster rate, only slowing down after 200 h resulting in  $1.27 \pm 0.09$  g l<sup>-1</sup> acetic acid in the final wines (Fig. 2a). Acetic acid production in the chaptalized fermentation was slower than that in the Icewine fermentation, but also plateaued after 200 h, reaching a lower value than that found in Icewine at  $1.02 \pm 0.04$  g l<sup>-1</sup> (Table 2).

Acetic acid was also plotted as a function of sugar consumed because of the differing sugar consumption rates observed for the three conditions in Fig. 1a. In comparing the acetic acid produced after an equal amount of sugar was consumed for all three fermentation conditions (Fig. 2b and Table 3), sugar alone was not solely responsible for the increased acetic acid in the Icewine. More acetic acid was produced for any given amount of sugar consumed during Icewine fermentation in comparison with the chaptalized and diluted juice fermentations. After 190 g l<sup>-1</sup> of sugar was consumed for each fermentation condition, wine yeast fermenting Icewine juice produced a 7.3-fold increase in acetic acid over that found for the diluted condition but also produced 1.3 times more acetic acid over that found for the chaptalized condition.

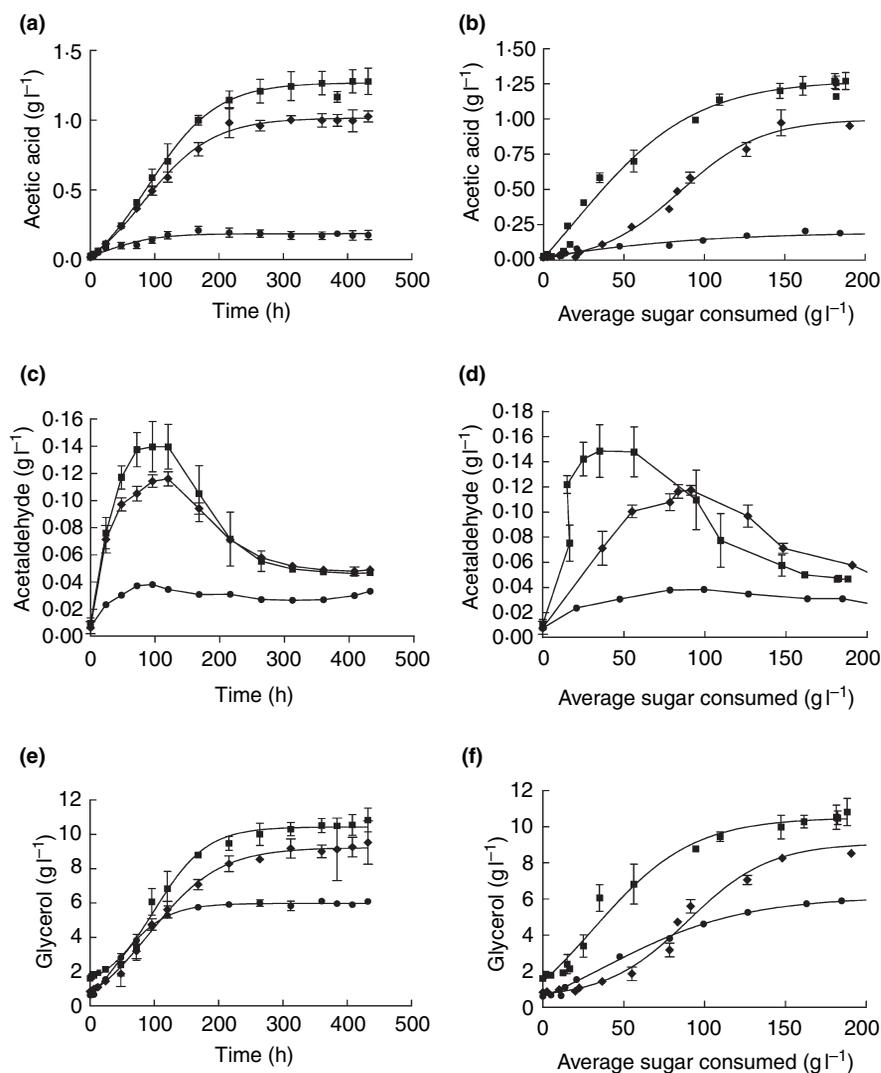
Acetaldehyde production was also found to differ between the three fermentation conditions (Fig. 2c). Similarly to what was found for acetic acid production, acetaldehyde was only produced for a short time in the dilute fermentation. In the high sugar fermentations, Icewine contained the highest peak level of acetaldehyde 120 h into the fermentation ( $0.14$  g l<sup>-1</sup>), but the concentration in the Icewine and chaptalized wine then decreased. A similar trend was observed when acetaldehyde was plotted as a function of sugar consumed.

Glycerol production differed from that found for acetic acid and acetaldehyde where there was no difference in the production rate for all three conditions over the first 120 h. Glycerol then plateaued in the dilute fermentation, resulting in  $6.1$  g l<sup>-1</sup> in the final wine (Fig. 2e). Glycerol production continued to increase in the high sugar fermentations, resulting in final glycerol concentrations in Icewine and the chaptalized wine of  $10.8$  and  $9.3$  g l<sup>-1</sup> respectively (Table 2). Although the Icewine juice had a higher initial concentration of glycerol present in comparison with the other two juices, glycerol production in the Icewine was always higher for the same amount of sugar consumed in comparison with the chaptalized and diluted juice fermentations (Fig. 2f). After subtracting the initial glycerol concentration in the juices, the Icewine fermentation showed a 1.8-fold increase in glycerol over the diluted juice fermentation and a small, but

**Table 2** Residual sugar and yeast metabolites in resulting wines after the yeast stopped fermenting sugar ( $\pm$ standard deviation)

Fermentation	Initial sugar in juice ( $\text{g l}^{-1}$ )	Water activity ( $A_w$ )	Residual sugar ( $\text{g l}^{-1}$ )	Final glycerol ( $\text{g l}^{-1}$ )	Final acetic acid ( $\text{g l}^{-1}$ )	Final nitrogen ( $\text{mg N l}^{-1}$ )
Diluted Icewine juice	$211.2 \pm 6.8$ b	0.975 a	$0.5 \pm 0.6$ c	$6.1 \pm 0.1$ c	$0.18 \pm 0.03$ c	$16.9 \pm 4.3$ c
Chaptalized Icewine juice	$402.3 \pm 5.6$ a	0.954 b	$162.5 \pm 26.3$ b	$9.3 \pm 0.5$ b	$1.02 \pm 0.04$ b	$92.6 \pm 8.3$ b
Icewine juice	$400.7 \pm 6.5$ a	0.948 c	$212.5 \pm 13.3$ a	$10.8 \pm 0.6$ a	$1.27 \pm 0.09$ a	$278.0 \pm 20.1$ a

Average values within the same column followed by the same letter are not statistically different by LSD method ( $P < 0.05$ ).



**Fig. 2** Yeast metabolite production. Acetic acid (a), acetaldehyde (c) and glycerol (e) were measured throughout the course of the fermentations of Icewine juice (■), chaptalized diluted Icewine juice (◆), and diluted Icewine juice (●). Acetic acid (b), acetaldehyde (d) and glycerol (f) were also plotted as a function of sugar consumption. Metabolite values represent the average  $\pm$  standard deviation of the mean from triplicate fermentations

significant 1.2-fold increase over the chaptalized juice fermentation after  $190 \text{ g l}^{-1}$  of sugar was consumed (Table 3).

There was a small, but significant increase in succinic acid in the Icewine ( $0.7 \text{ g l}^{-1}$ ) over that found for the diluted and chaptalized juice fermentations, which both showed

$0.6 \text{ g l}^{-1}$  succinic acid (Table 3). The dilute juice fermentation produced more ethanol from the sugar consumed than either the Icewine or chaptalized juice fermentation (Table 3), which was expected if sugar metabolism was being diverted away from ethanol towards other metabolites to combat the increased osmotic stress.

**Table 3** Yeast metabolites produced after consumption of 190 g l<sup>-1</sup> of reducing sugars ( $\pm$ standard deviation)

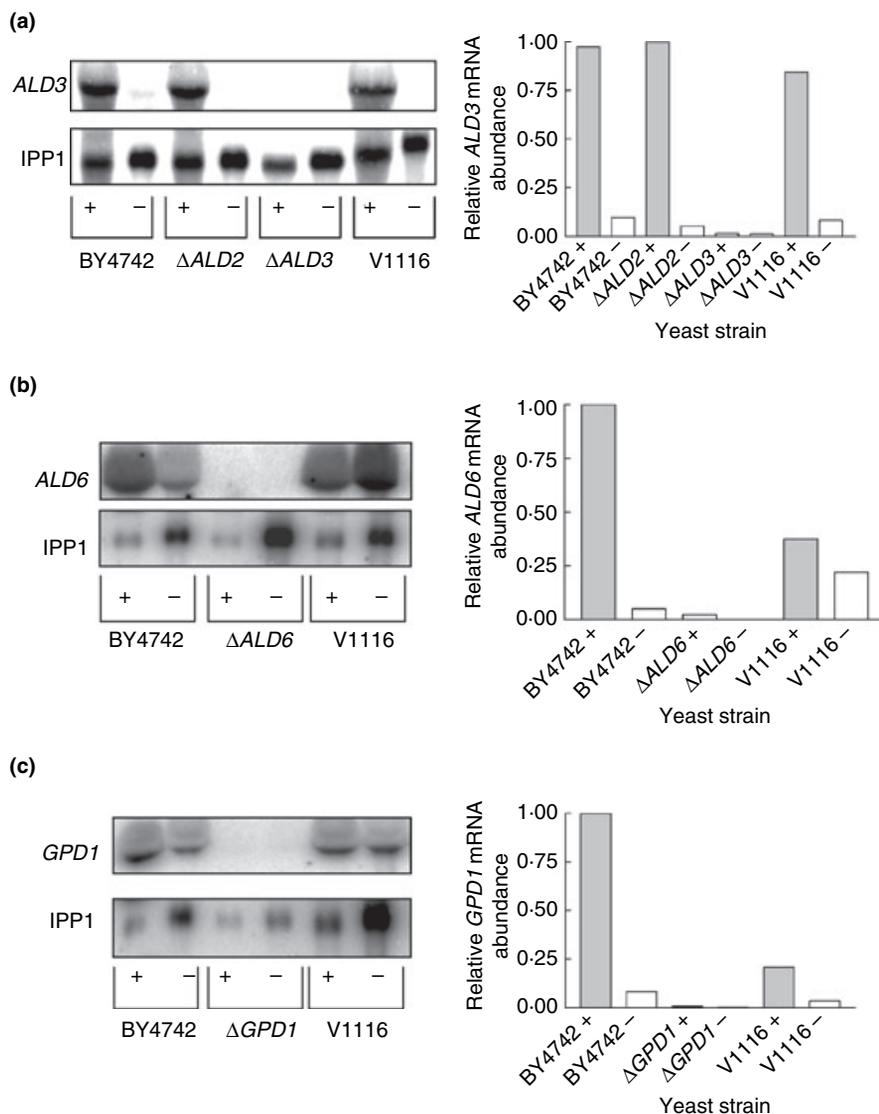
Fermentation	Initial sugar in juice (g l <sup>-1</sup> )	Consumed sugar (g l <sup>-1</sup> )	EtOH% (v/v)	Glycerol (g l <sup>-1</sup> )	Acetic acid (g l <sup>-1</sup> )	Succinate (g l <sup>-1</sup> )
Diluted Icewine juice	211.2 $\pm$ 6.8b	189.5 $\pm$ 7.0 a	12.1 $\pm$ 0.3 a	5.3 $\pm$ 0.1 c	0.17 $\pm$ 0.03 c	0.61 $\pm$ 0.012 b
Chaptalized Icewine juice	402.3 $\pm$ 5.6 a	190.0 $\pm$ 11.1 a	9.7 $\pm$ 0.2 c	7.7 $\pm$ 0.2 b	0.93 $\pm$ 0.03 b	0.60 $\pm$ 0.020 b
Icewine juice	400.7 $\pm$ 6.5 a	188.2 $\pm$ 9.5 a	10.9 $\pm$ 0.8 b	9.3 $\pm$ 0.6 a	1.24 $\pm$ 0.09 a	0.70 $\pm$ 0.004 a

Average values within the same column followed by the same letter are not statistically different by LSD method ( $P < 0.05$ ).

### Probe specificity validation

Salt-induced osmotic stress was used for probe validation as all genes (*GPD1*, *ALD3* and *ALD6*) are upregulated by high salt stress (Rep *et al.* 2000; Remize *et al.* 2001). Inorganic pyrophosphatase (*IPP1*) was used as a loading control as its expression is unaffected by salt-induced osmotic stress

(Norbeck and Blomberg 1997; Rep *et al.* 1999). Probe specificity was important to validate for the aldehyde dehydrogenases as *ALD2* and *ALD3* share 92% sequence identity and often expression from these two genes cannot be differentiated (Miralles and Serrano 1995; Aranda and del Olmo 2003). Figure 3a illustrates that the design of our *ALD3* DNA probe is specific enough to differentiate



**Fig. 3** Probe specificity validation Northern blots. Overnight yeast cultures were induced (+) or not induced (-) with 0.7 M NaCl for 2.5 h prior to RNA isolation. Hybridization of <sup>32</sup>P-labelled DNA probes to RNA isolated from a parent yeast strain (BY4742), knockout strains (a)  $\Delta$ ALD3, (b)  $\Delta$ ALD6 or (c)  $\Delta$ GPD1 and a commercial wine yeast (K1-V1116) are illustrated. Inorganic pyrophosphatase (*IPP1*) was used as an internal control. Relative mRNA abundance is depicted in the adjacent bar graph

between *ALD2* and *ALD3* as the probe binds to RNA of the *ALD2* deletion mutant but not to the RNA of the *ALD3* deletion mutant. It also shows that under salt stress, *ALD3* is upregulated in the commercial wine yeast strain K1-V1116 in a similar fashion as that found in the laboratory strains. The *ALD6* probe was found to be specific to the *ALD6* gene as illustrated in Fig. 3b. Under salt stress, *ALD6* expression was upregulated in the parent and wine yeast strains, but the upregulation was much lower in the wine yeast strain (1.7-fold) compared with the parent strain (20-fold) (Fig. 3b). The *GPD1* probe was also found to be specific to the *GPD1* gene and was also found upregulated by salt stress in both the parent and wine yeast strains (Fig. 3c).

### Expression of *ALD3*, *ALD6* and *GPD1* throughout the fermentation

The relative gene expression profiles for each gene from replicate fermentations are presented in Figs 4b, 5b and 6b for *ALD3*, *ALD6* and *GPD1* respectively. From the Northern blots, it is clear that *ALD3*, which encodes a cytosolic, NAD<sup>+</sup>-dependent aldehyde dehydrogenase, is expressed during all three fermentation conditions. The peak expression of *ALD3* during Icewine and chaptalized juice fermentations occurred 96 h into the fermentations just as the cells were reaching stationary phase, and declined to its lowest expression level by 192 h when acetic acid production levels off in the fermentations. In contrast, *ALD3* expression in cells fermenting the diluted juice remained low throughout the fermentation. The peak gene expression for *ALD3* was 6.2-fold greater in the Icewine fermentation when compared with the peak expression in the dilute Icewine fermentation (Fig. 4c) and is not significantly different from the seven-fold induction observed in the chaptalized dilute Icewine fermentation. Expression of the NADP<sup>+</sup>-dependent aldehyde dehydrogenase *ALD6* did not differ between the three fermentation conditions as shown in Fig. 5b. Although *ALD6* was expressed in all three fermentations, the expression patterns were very similar and there was no significant difference in peak gene expression (Fig. 5c). As with *ALD3*, *ALD6* expression also declined after 96 h and was at a minimal level by 192 h. Expression of *GPD1*, which encodes glycerol-3-phosphate dehydrogenase, peaked in the Icewine and chaptalized juice fermentations between 72 and 96 h (Fig. 6b). The expression of *GPD1* was 2.6-fold greater in the Icewine fermentation and 2.1-fold greater in the chaptalized dilute Icewine fermentation at the 96 h time point when compared with the peak expression in the dilute Icewine fermentation (Fig. 5c). *GPD1* expression for all three conditions declined after 96 h and reached the lowest levels by 216 h.

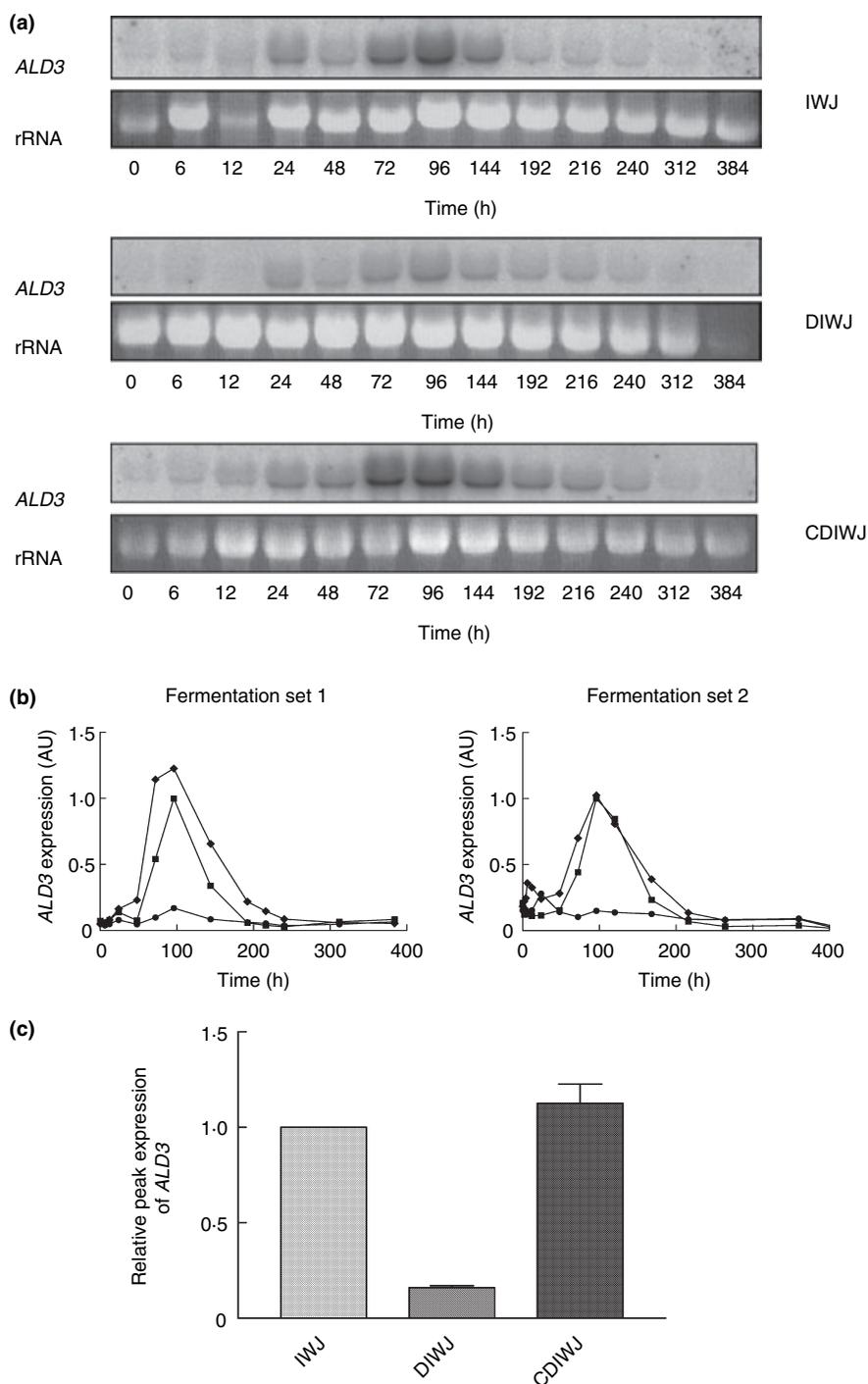
## DISCUSSION

Acetic acid is an important component in wine because of its association with wine spoilage when present at high levels because it imparts a vinegar aroma to wines. Therefore, countries legislate an upper allowable limit of acetic acid in wines. In Canada, the maximum allowable content in Icewine is 2.1 g l<sup>-1</sup> (VQA 1999). Our recent survey of 51 commercial Canadian Icewines showed a large range of acetic acid concentration, with an average value of 1.30 ± 0.48 g l<sup>-1</sup>, ranging from 0.49 to 2.29 g l<sup>-1</sup> (Nurgel *et al.* 2004). Although we had previously discovered the acetic acid in Icewine is from the yeast fermenting this juice as opposed to bacterial contamination (Mottiar 2000), the yeast metabolic reaction used for its production and the reason for its production in Icewine had not been investigated prior to our current study.

### Solutes in addition to fermentable sugars contribute to high glycerol and acetic acid production in Icewine

To explore the stress response of wine yeast to Icewine juice, we designed three fermentation conditions. The dilute Icewine juice represented our reference condition, containing fermentable sugars in the same range as found in juice for table wine production. In contrast, Icewine juice is concentrated in all juice components and enabled us to measure the yeast's metabolic response to the concentrated juice in comparison with the diluted juice. The third condition was the chaptalized, diluted Icewine juice, which had an equal amount of fermentable sugar as the Icewine juice, but all other juice components were at the diluted concentration giving rise to lower °Brix and *A<sub>w</sub>* values for this juice in comparison with the Icewine juice. Using this condition we could determine if the wine yeast's response in the Icewine fermentation was strictly because of the amount of sugar in the starting juice, or if the other concentrated solutes contributed some effect.

While the two high sugar juices tested in this work are equal in fermentable sugars, they differ in osmotic strength. This difference between Icewine juice and the chaptalized juice confers more osmotic stress on the yeast cells in the Icewine juice and is reflected in the longer lag phase at the start of the fermentation, and higher glycerol and acetic acid production. The concentrated acids of Icewine juice may contribute to additional stress on the cells leading to higher glycerol and acetic acid. Yeast in the high sugar conditions did not accumulate the same amount of biomass as in the dilute Icewine fermentations. The reduced biomass in the high sugar fermentations was not because of limited nitrogen availability for the cells as there were residual nitrogenous compounds in both the Icewine and the wine produced from

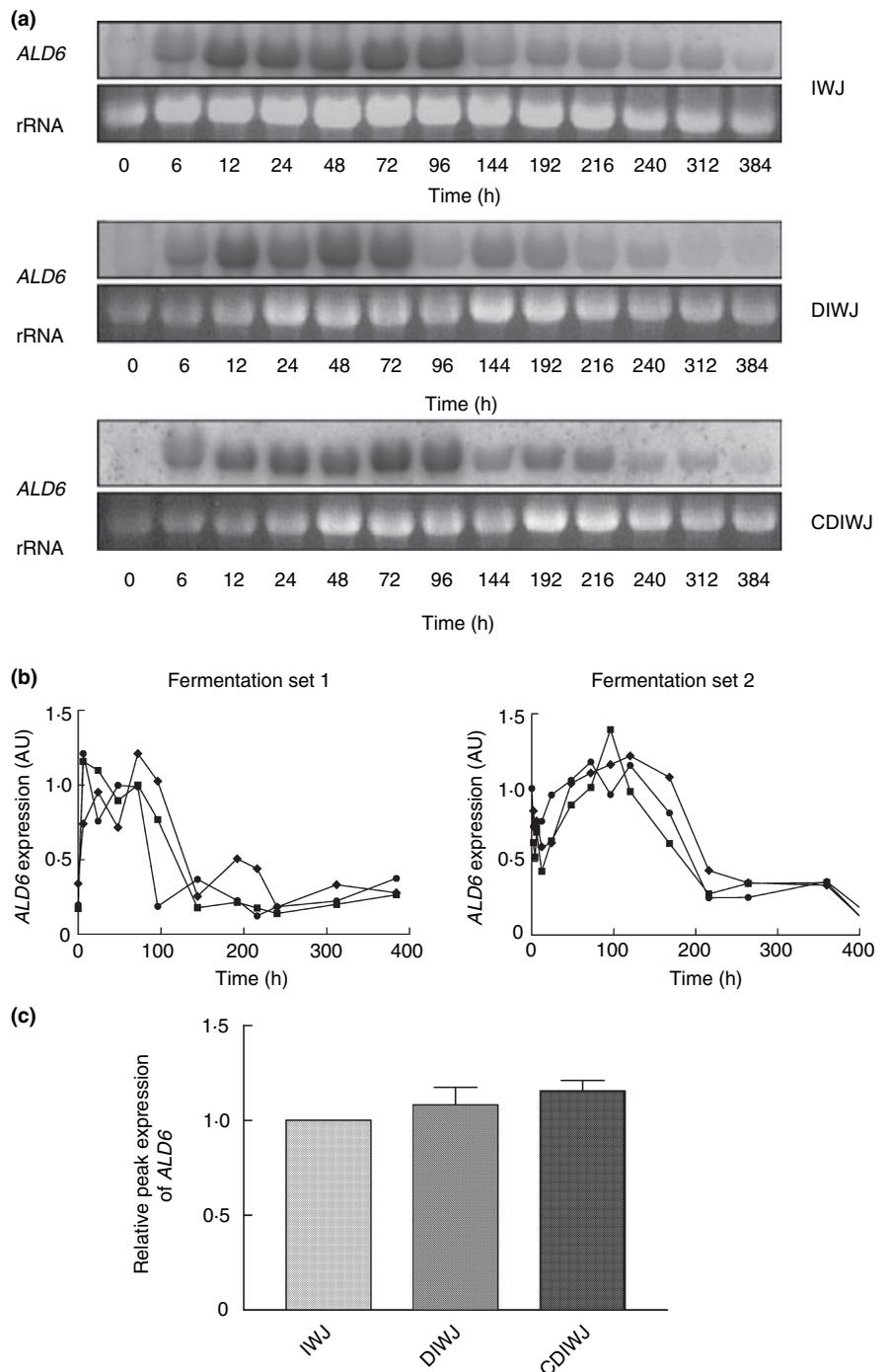


**Fig. 4** *ALD3* expression throughout fermentation. (a) Example Northern blot analyses from duplicate fermentation trials. RNA was extracted from each fermentation condition, electrophoresed, transferred to nylon membranes and probed for *ALD3* expression. 18S ribosomal RNA was used as a loading control. (b) Gene expression profiles from fermentations of Icewine juice (■), chaptalized diluted Icewine juice (◆), and diluted Icewine juice (●) from fermentation sets 1 and 2. Expression of *ALD3* was followed throughout the course of the fermentations. (c) Comparison of relative peak expression levels of *ALD3* from Icewine juice (IWJ), diluted Icewine juice (DIWJ) and chaptalized diluted Icewine juice (CDIWJ) fermentations. Peak expression of *ALD3* occurred 96 h into the fermentations

the chaptalized juice. The reduced biomass in these high sugar fermentations may be due to the dedication of metabolic resources in adapting to the high osmotic strength environment, diverting sugar metabolism towards glycerol and acetic acid production, while yeast in the lower sugar fermentation can use these resources for cell division and growth.

#### Redox balance during Icewine fermentation and the role of Ald3p

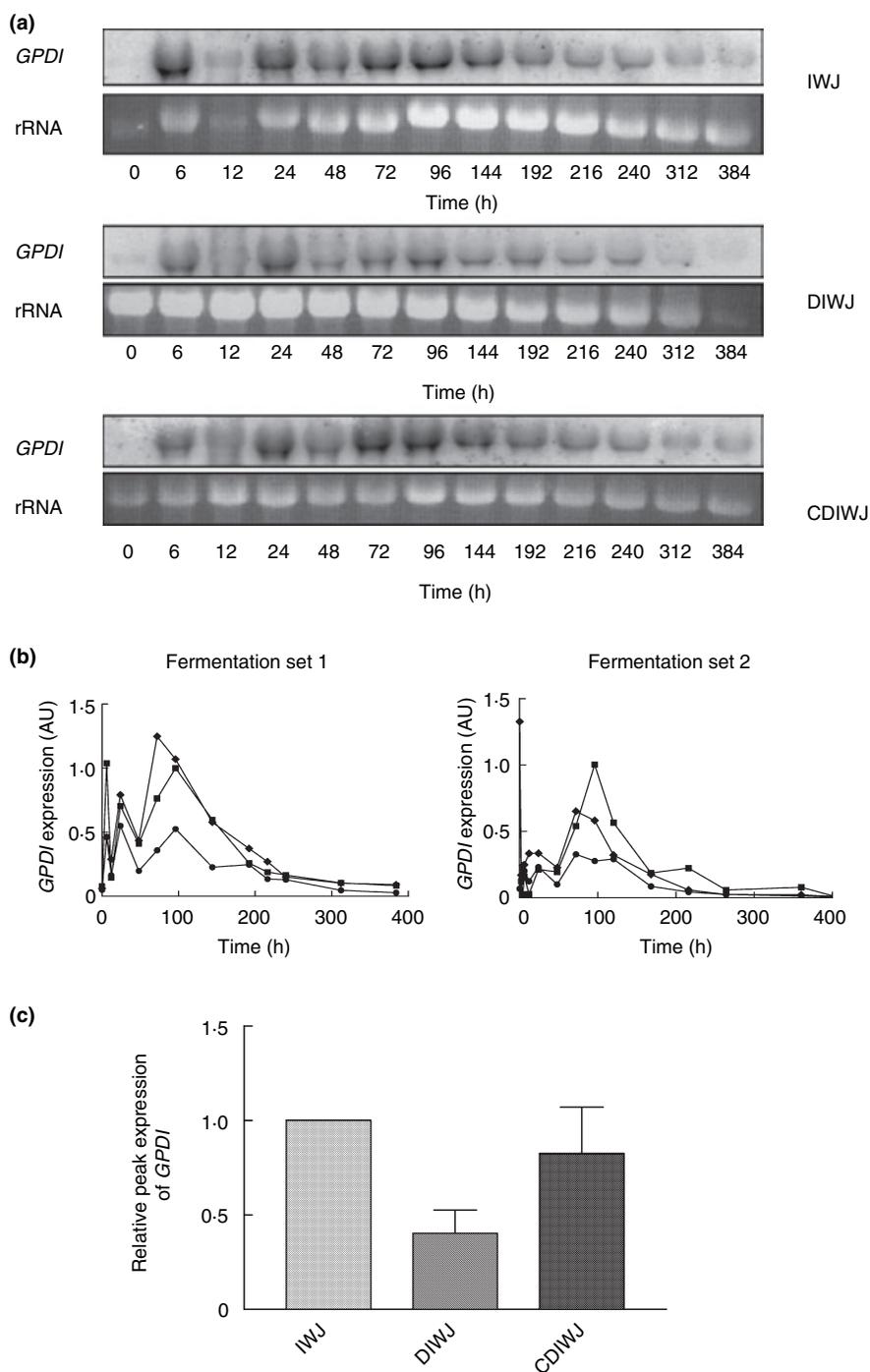
Past studies have concluded that the upregulation of *ALD3* and *ALD6* to produce acetic acid under hyperosmotic stress helps maintain redox balance within the cells during the



**Fig. 5** *ALD6* expression throughout fermentation. (a) Example Northern blot analyses from duplicate fermentation trials. RNA was extracted from each fermentation condition, electrophoresed, transferred to nylon membranes and probed for *ALD6* expression. 18S ribosomal RNA was used as a loading control. (b) Gene expression profiles from fermentations of Icewine juice (■), chaptalized diluted Icewine juice (◆), and diluted Icewine juice (●) from fermentation sets 1 and 2. Expression of *ALD6* was followed throughout the course of the fermentations. (c) Comparison of relative peak expression levels of *ALD6* from Icewine juice (IWJ), diluted Icewine juice (DIWJ) and chaptalized diluted Icewine juice (CDIWJ) fermentations. Peak expression of *ALD6* occurred 72 h into the fermentations

HOG response (Blomberg and Adler 1989; Miralles and Serrano 1995; Navarro-Avino *et al.* 1999; Blomberg 2000; Norbeck and Blomberg 2000). However, Ald3p is NAD<sup>+</sup> dependent (Navarro-Avino *et al.* 1999) and Ald6p is NADP<sup>+</sup> dependent (Meaden *et al.* 1997). The role that each dehydrogenase plays is not clear as yeast lack a transhydrogenase to convert between reducing equivalents

(van Dijken and Scheffers 1986). We report that only *ALD3* and not *ALD6* is differentially upregulated during Icewine and the chaptalized fermentation in comparison with the diluted juice fermentation. The upregulation of *ALD3* can help explain the large fold increase in acetic acid production throughout the fermentations. The osmotic stress, which is primarily induced by the fermentable sugars of the juice,



**Fig. 6** *GPD1* expression throughout fermentation. (a) Example Northern blot analyses from duplicate fermentation trials. RNA was extracted from each fermentation condition, electrophoresed, transferred to nylon membranes and probed for *GPD1* expression. 18S ribosomal RNA was used as a loading control. (b) Gene expression profiles from fermentations of Icewine juice (■), chaptalized diluted Icewine juice (◆), and diluted Icewine juice (●) from fermentation sets 1 and 2. Expression of *GPD1* was followed throughout the course of the fermentations. (c) Comparison of relative peak expression levels of *GPD1* from Icewine juice (IWJ), diluted Icewine juice (DIWJ) and chaptalized diluted Icewine juice (CDIWJ) fermentations. Peak expression of *GPD1* occurred 96 h into the fermentations

induces the increased expression of *GPD1*, and this results in the increased production of glycerol to counteract the external osmotic pressure. Producing glycerol requires NADH and the production of acetic acid by Ald3p can supply that NADH as this aldehyde dehydrogenase is NAD<sup>+</sup> dependent (Navarro-Avino *et al.* 1999). Increased acetic acid production during high sugar fermentations may

occur in part through the upregulation of *ALD3* in order to resupply the NADH requirement, maintaining redox balance.

Although Erasmus *et al.* (2003) showed an initial upregulation of *ALD2*, *ALD3*, *ALD4* and *ALD6* in wine yeast 2 h after cells were placed under high sugar stress, expression of these aldehyde dehydrogenases were not followed to monitor

their importance throughout the entire fermentation. We cannot rule out contributions from *ALD2* and *ALD4* throughout our high sugar Icewine fermentations, but the wine yeast in our study did not show an extended differential expression of *ALD6* between the low and high sugar fermentations. Only *ALD3* showed a differential expression pattern that correlates to the increased acetic acid production during the fermentations. Of importance to note is that salt-induced stress also only caused a marginal upregulation of *ALD6* in the wine yeast strain whereas *ALD3* was highly upregulated. Recently, the mitochondrial Ald5p was identified as contributing to acetate production during glucose fermentation in synthetic medium (Saint-Prix *et al.* 2004) but this isoform has not been reported as upregulated during hyperosmotic stress.

As acetaldehyde has been shown to be a substrate for *ALD3* (Navarro-Avino *et al.* 1999), and acetaldehyde levels increased early in the high sugar fermentation, it appears that the aldehyde dehydrogenase encoded by *ALD3* was a contributor to the elevated acetic acid in the high sugar fermentations. In support of this, Aranda and del Olmo (2003) showed that glucose-repressed *ALD3* expression in *S. cerevisiae* was upregulated with addition of acetaldehyde to the media. In our high sugar fermentations, acetaldehyde production preceded acetic acid production and *ALD3* expression, in agreement with the above observations. In addition, yeast fermenting the diluted juice that contained about half the sugar of the Icewine juice still showed expression of *ALD3*, albeit at a much lower level. The addition of glucose and fructose to this diluted juice caused a significant increase in *ALD3* expression, indicating that hyperosmotic stress induced by fermentable sugars can override glucose repression of *ALD3* in K1-V1116.

Earlier work on relating acetic acid production during table wine fermentation to aldehyde dehydrogenase activity focused on the other cytosolic isozyme encoded by *ALD6*.

Several studies have shown that Ald6p is the aldehyde dehydrogenase responsible for acetic acid production during fermentation of glucose media containing sugar concentrations in the range needed for table wine production (Radler 1993; Remize *et al.* 1999; Eglinton *et al.* 2002). Our studies also show that *ALD6* was expressed under all three fermentation conditions, but did not show a differential expression pattern that could explain the increase in acetic acid production in the Icewine and wine produced from the chaptalized juice. It stands to reason that *ALD6* plays a larger role in acetic acid production in the diluted juice fermentation whereas *ALD3* contributes to acetic acid in the more concentrated juice fermentations, where yeast are placed under additional osmotic stress.

### ***GPD1* expression is more highly upregulated in wine yeast fermenting Icewine juice**

The pattern of *GPD1* expression during the dilute juice fermentation matched the pattern recently reported for *GDP1* in a wine yeast-derived strain fermenting synthetic grape juice medium containing 20% glucose (Remize *et al.* 2003). In both cases, *GPD1* expression showed an increase during the growth phase of the yeast, peaked as the cells entered stationary phase and declined during stationary phase. We found a similar expression pattern in the high sugar fermentations, but the expression levels were consistently higher than those shown for the diluted juice fermentation. As the expression of *GPD1* is at least partially indicative of the HOG response of wine yeast (Remize *et al.* 2003), it follows that *GPD1* would be expressed at an elevated level in fermentations carried out in Icewine juice and the chaptalized juice over that found for the diluted juice. The Icewine fermentations were twice as concentrated as the dilute fermentations and this appeared to be reflected in the increased amount of glycerol produced in these fermentations and induction of *GPD1*. The increased glycerol production in the high sugar fermentations is due to the increased expression of *GDP1*. Remize *et al.* (2003) also found that their *gpd1Δ* mutant did not show the increase in glycerol production as a function of sugar concentration that the parent strain showed. The role of *GPD1* and glycerol production in the hyperosmotic response of bakers' yeast was also reported by Atfield and Kletsas (2000) where strains with more efficient fermentative ability in a high sugar environment were the same strains with higher *GPD1* expression and glycerol production.

### **Succinate production during Icewine fermentation**

There are three pathways in *S. cerevisiae* that can lead to succinate production, a reductive TCA pathway from pyruvate, an oxidative TCA pathway from glutamate and an oxidative glutamate pathway that forms  $\gamma$ -amino butyric acid (GABA) as an intermediate (Radler 1993; Coleman *et al.* 2001). Previous work by Erasmus *et al.* (2003) showed upregulation of the GABA pathway 2 h after wine yeast were exposed to 40% (w/v) sugar indicating that this was the pathway for succinate production during the high sugar fermentation. However, succinate was not measured in their wines to determine if the sugar stress caused an increase in succinate in the wine because of the upregulation of this glutamate catabolic pathway. In our results, there was no significant difference in succinate concentration in wines produced from the diluted and chaptalized juices, conditions comparable with the fermentation conditions tested by Erasmus *et al.* (2003). The initial expression response to high sugar may not represent the adapted response

throughout fermentation, which leads to metabolites in the wine. We cannot rule out that different succinate pathways may be utilized as osmotic pressure and ethanol concentrations change in the fermenting medium without leading to different succinate levels in the wines. Further investigation is required before conclusions are drawn about pathways leading to succinate production and the role succinate plays under varying fermentation conditions.

Further research is needed to examine the expression patterns of *ALD2*, *ALD4*, *ALD5* and *GPD2* during the fermentation of Icewine juice to further our understanding of genes encoding enzymes that may affect acetic acid and glycerol production during fermentation. Measurement of enzyme activity during fermentation will bridge the gap between gene expression and metabolite production. Microarray analysis will help identify entire metabolic pathways affected during the fermentation of Icewine juice that can affect the chemical composition of the wine and overall wine quality.

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