

## ORIGINAL ARTICLE

# Response of wine yeast (*Saccharomyces cerevisiae*) aldehyde dehydrogenases to acetaldehyde stress during Icewine fermentation

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**Keywords**

acetaldehyde, acetic acid, aldehyde dehydrogenase, glycerol, glycerol-3-phosphate dehydrogenase, hyperosmotic stress, Icewine, *Saccharomyces cerevisiae*.

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**Abstract**

**Aims:** We previously reported that the aldehyde dehydrogenase encoded by *ALD3* but not *ALD6* was responsible, in part, for the increased acetic acid found in Icewines based on the expression profile of these genes during fermentation. We have now completed the expression profile of the remaining yeast aldehyde dehydrogenase genes *ALD2*, *ALD4* and *ALD5* during these fermentations to determine their contribution to acetic acid production. The contribution of acetaldehyde stress as a signal to stimulate *ALD* expression during these fermentations was investigated for all *ALD* genes. The expression of glycerol-3-phosphate encoded by *GPD2* was also followed during these fermentations to determine its role in addition to the role we already identified for *GPD1* in the elevated glycerol produced during Icewine fermentation.

**Methods and Results:** Icewine juice (38.5°Brix, 398 ± 5 g l<sup>-1</sup> sugar), diluted Icewine juice (20.8°Brix, 196 ± 4 g l<sup>-1</sup> sugar) and the diluted juice with sugar levels equal to the original Icewine juice (36.6°Brix, 395 ± 6 g l<sup>-1</sup> sugar) were fermented in duplicate using the commercial wine yeast K1-V1116. Acetic acid and glycerol production increased 8.4- and 2.7-fold in the Icewine vs the diluted juice fermentation, respectively, accompanied by a fourfold transient increase in acetaldehyde in the Icewine condition during the first week. Both mitochondrial aldehyde dehydrogenases encoded by *ALD4* and *ALD5* were expressed, with *ALD5* expression highest at the start of all fermentations and *ALD4* expression increasing during the first week of each condition. *ALD2*, *ALD4*, *ALD5* and *GPD2* showed no differential expression between the three fermentation conditions indicating their lack of involvement in elevating acetic acid and glycerol in Icewine. When yeast fermenting the diluted fermentation was exposed to exogenous acetaldehyde, the transient spike in acetaldehyde increased the expression of *ALD3* but this response alone was not sufficient to cause an increase in acetic acid. Expression of the other aldehyde dehydrogenases was unaffected by the acetaldehyde addition.

**Conclusions:** The aldehyde dehydrogenases encoded by *ALD2*, *ALD4* and *ALD5* do not contribute to the elevated acetic acid production during Icewine fermentation. Expression of *GPD2* was not upregulated in high sugar fermentations and does not reflect the elevated levels of glycerol found in these wines. Acetaldehyde at a concentration produced during Icewine fermentation stimulates the expression of *ALD3*, but has no impact on the expression of *ALD2*, -4, -5 and -6. Upregulation of *ALD3* alone in the dilute fermentation is not sufficient to increase acetic acid in wine and requires additional responses found in cells under hyperosmotic stress.

**Significance and Impact of the Study:** This work confirms that increased acetic acid and glycerol production during Icewine fermentation follows upregulation of *ALD3* and *GPD1* respectively, but upregulation of *ALD3* alone is not sufficient to increase acetic acid production. Additional responses of cells under osmotic stress are required to increase acetic acid in Icewine.

## Introduction

The fermentation of Icewine by wine yeast is stressful, leading to prolonged fermentation times, slow growth, altered yeast metabolism and the production of high levels of acetic acid and glycerol (Kontkanen *et al.* 2004; Pigeau and Inglis 2005). These responses are because of the concentrated nature of Icewine juice that presents a unique hyperosmotic environment to the fermenting yeast, with high concentrations of glucose and fructose (approximately 200 g l<sup>-1</sup> each) in a low pH matrix of additional concentrated juice solutes such as organic acids and assimilable nitrogenous compounds (Pigeau and Inglis 2005).

Glycerol is the major compatible solute produced when yeast cells are subjected to osmotic stress (Blomberg and Adler 1989; Brewster *et al.* 1993; Nevoigt and Stahl 1997; Blomberg 2000). Expression of glycerol-3-phosphate dehydrogenase (*GPD1*) is the rate-limiting step in the hyperosmotically induced production of glycerol (Remize *et al.* 2001). The other isoform of this enzyme encoded by *GPD2* is reported to have a role in maintaining redox balance under anaerobic conditions (such as fermentation) and is not induced by osmotic stress (Albertyn *et al.* 1994; Ansell *et al.* 1997; Pahlman *et al.* 2001; Remize *et al.* 2003). This enzyme is NADH-dependent, reducing dihydroxyacetone phosphate (DHAP) to glycerol-3-phosphate, which is then dephosphorylated to glycerol by the glycerol-3-phosphatases, encoded by *GPP1* and *GPP2* (Norbeck *et al.* 1996).

As the osmotic induction of *GPD1* does not impede the anaerobic induction of *GPD2* (Ansell *et al.* 1997), the increased production of glycerol-3-phosphate from osmotic and anaerobic stress is controlled by individual isozymes and separate regulatory mechanisms. We have already shown that the expression of *GPD1* is upregulated during Icewine fermentations and corresponds to the increased glycerol production found in these wines (Pigeau and Inglis 2005), but it remains of interest to determine the expression profile of *GPD2* under anaerobic Icewine fermentation conditions.

Acetic acid production has been suggested as a mechanism through which the yeast balances the excess NAD<sup>+</sup> produced from glycerol formation during the hyper-

osmotic stress response (Miralles and Serrano 1995; Navarro-Avino *et al.* 1999). This may occur through the action of NAD<sup>+</sup>-dependent aldehyde dehydrogenase activity which reduces NAD<sup>+</sup> to NADH while oxidizing acetaldehyde to acetic acid, thus restoring internal redox balance. We have previously reported increased expression of a wine yeast cytosolic, NAD<sup>+</sup>-dependent aldehyde dehydrogenase encoded by *ALD3* that corresponded to the increase in acetic acid production during Icewine fermentation (Pigeau and Inglis 2005). Yeast-fermenting Icewine juice showed a sixfold higher expression level for *ALD3* over that found in yeast-fermenting dilute juice, with the expression peaking 4 days into the fermentation. However, the main cytosolic isoform, encoded by *ALD6*, showed the same expression pattern for both environments. The response we reported during fermentation differs significantly from the initial hyperosmotic stress response found in a wine yeast 2 h after sugar exposure, where *ALD2*, -3, -4 and -6 were upregulated (Erasmus *et al.* 2003). Also, wine yeast responses differ from that found in laboratory yeast strains exposed to salt stress, where both *ALD3* and *ALD6* were upregulated (Norbeck and Blomberg 2000). It has previously been reported that Ald6p is the main aldehyde dehydrogenase responsible for acetic acid production in *Saccharomyces cerevisiae* strains during fermentation of glucose media (Eglinton *et al.* 2002), although most recently, mitochondrial Ald5p was also found to contribute (Saint-Prix *et al.* 2004). Clearly, wine yeast strains display properties that are distinct from well-studied laboratory yeast strains, and these differences can impact on the organoleptic properties of wine (Remize *et al.* 1999; Hauser *et al.* 2001; Rossignol *et al.* 2003). These differences are not unexpected as *S. cerevisiae* wine yeast have been selected for desirable fermentation properties (Pretorius 2000; Hauser *et al.* 2001). However, the molecular basis of these differences is largely unknown and require further investigation (Pretorius 2000; Rossignol *et al.* 2003).

During Icewine fermentation, acetaldehyde production precedes upregulation of *ALD3* and subsequent acetic acid production (Pigeau and Inglis 2005). Because of the high level of homology between *ALD2* and *ALD3*, their ability to functionally compensate for each other (White *et al.* 2003) and the induction of *ALD2/3*, *ALD4* and *ALD6* by

acetaldehyde (Aranda and del Olmo 2003), it remains of interest to examine the expression profile of the remaining aldehyde dehydrogenase genes in wine yeast during the course of Icewine fermentation and to determine if acetaldehyde is the stress that induces *ALD* gene expression during these fermentations.

We have followed the expression of glycerol-3-phosphate dehydrogenase encoded by *GPD2* and the three remaining aldehyde dehydrogenase genes not previously tested (encoded by *ALD2*, *ALD4* and *ALD5*) during the fermentation of Icewine juice and compared these profiles with that found in yeast-fermenting diluted Icewine juice and diluted juice containing added sugars. A time course of glycerol, acetaldehyde and acetic acid produced in the wines were compared with the expression patterns of these genes to determine the relationship between gene expression in wine yeast during Icewine fermentation and their relationship to metabolite production during these fermentations. The role that acetaldehyde plays in the induction of aldehyde dehydrogenases (*ALD2*, -3, -4, -5 and -6) was also investigated to determine if acetaldehyde stress induces *ALD* gene expression in wine yeast during fermentation.

## Materials and Methods

### Yeast strains

The commercial yeast strain used for wine fermentations, *S. cerevisiae* K1-V1116, was kindly provided by Lallemand Inc. Yeast strains used for the validation of DNA probe specificities were deletion mutants ( $\Delta$ *ALD2*,  $\Delta$ *ALD4*,  $\Delta$ *ALD5* and  $\Delta$ *GPD2*) of *S. cerevisiae* Hansen BY4742 (mat alpha his3 $\Delta$ 1 leu2 $\Delta$ 0 lys2 $\Delta$ 0 ura3 $\Delta$ 0) and were purchased from Open Biosystems (Huntsville, AL, USA). These commercially available knockout strains were created by Wach *et al.* (1994) using polymerase chain reaction (PCR) and homologous recombination to insert a KanMX cassette, conferring geneticin resistance, at the location of the gene of interest.

### 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>. Media for deletion mutant cultures was supplemented with geneticin (G418) at 200 mg l<sup>-1</sup> to maintain selection pressure for the knockout strains. YPD supplemented with 0.7 mol l<sup>-1</sup> of 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 (Niagara on the Lake, Ontario, 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 Milli RO water (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. In an additional experiment, the amount of acetaldehyde in Icewine juice and diluted Icewine juice fermentations were determined at 48 h with an enzymatic detection kit (Megazyme International Ireland Inc. Bray, Co. Wicklow, Ireland). Acetaldehyde was added to the dilute Icewine fermentation at 48 h, to equal (or double) the level found in the Icewine fermentations and RNA was isolated at 96 h to determine aldehyde dehydrogenase expression levels.

### Yeast inoculation procedure for fermentations

Five grams of wine yeast K1-V1116 was rehydrated with 50 ml of sterile, 40°C Milli-RO water 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. Fifteen millilitres of the 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). 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- $\mu\text{m}$  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- $\mu\text{m}$  syringe filter and stored at -30°C until metabolite analysis was performed. Acetic acid, acetaldehyde and glycerol were determined in duplicate for each sample with Megazyme enzyme assay kits (Megazyme International Ireland Inc. Bray, Co. Wicklow, Ireland).

#### DNA probe design for northern analysis

DNA probes for *GPD2*, *ALD2*, *ALD4* and *ALD5* 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 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 *GPD2* primer (5'-AAAGTCAATGCGGAAAACACAGAATT-3') and reverse *GPD2* primer (5'-ACATAGGAGGATAGCAATTG-CACACC-3') amplify a 352-bp fragment which has 100% identity to *GPD2* and 69% identity to *GPD1*. The forward *ALD2* primer (5'-GCAGGAACTGTTTGGATCAACT-3') and reverse *ALD2* primer (5'-TTAGTTGTCCAAAGAGAGATTT-3') amplify a 137-bp fragment which has 100% identity to *ALD2* and 77% identity to *ALD3*. The forward *ALD4* primer (5'-TATTTATGAAGGTAGAGAGGACGATG-3') and reverse *ALD4* primer (5'-ATTTTTATCAGCAAAGCCAGCAGAAG-3') amplify a 261-bp fragment which has 100% identity to *ALD4* and 60% identity to the closest related aldehyde dehydrogenase (*ALD6*). The forward *ALD5* primer (5'-GAACATTGCCTTCGGTATTTT-TTACA-3') and reverse *ALD5* primer (5'-CTGTTGGCT-TGACAAAATAACCTTTA-3') amplify a 289-bp fragment which has 100% identity to *ALD5* and 64% identity to the closest related aldehyde dehydrogenase (*ALD4*). The forward *IPP1* primer (5'-ACCTACACTACCAGACAAAT-TGGTG-3') and reverse *IPP1* primer (5'-AGATGAAGAA-CCACTTGTC AATAGA-3') amplify a 799-bp fragment which has 100% identity to *IPP1* and 60% identity to the closest related gene, *IPP2*. Probes for *ALD3* and *ALD6* were as previously described (Pigeau and Inglis 2005). The PCR products were run on 1% agarose gel (w/v) to

verify size and subsequently purified with a Qiagen, QIA-quick 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 (see Figure S2 in Supplementary material).

#### 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. The cells were resuspended in 5 ml of diethyl pyrocarbonate (DEPC)-treated water and were pelleted as before. RNA was isolated from the cells using a phenol : chloroform : isoamyl alcohol and glass bead method previously described (Pigeau and Inglis 2005). RNA samples from duplicate fermentation trials were quantified and 30  $\mu\text{g}$  from each time point was electrophoresed at 95 V for 4.5 h in 18% formaldehyde, 1.25% agarose gels (w/v), blotted onto positively charged nylon membranes (Boehringer Mannheim) and cross-linked using a Hoefer UVC 500, UV cross-linker. The membrane bound RNA was hybridized to 20 ng of [ $\alpha$ -<sup>32</sup>P] dATP-labelled probes and washed under conditions previously described (Pigeau and Inglis 2005). The membrane was exposed to a Fujifilm phosphorimaging screen for 2 days and the hybridization signal intensities were obtained with a Fujifilm FLA-3000 phosphorimager 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). For each gene investigated, expression levels are relative to the highest peak expression found for that gene which was then equated to one.

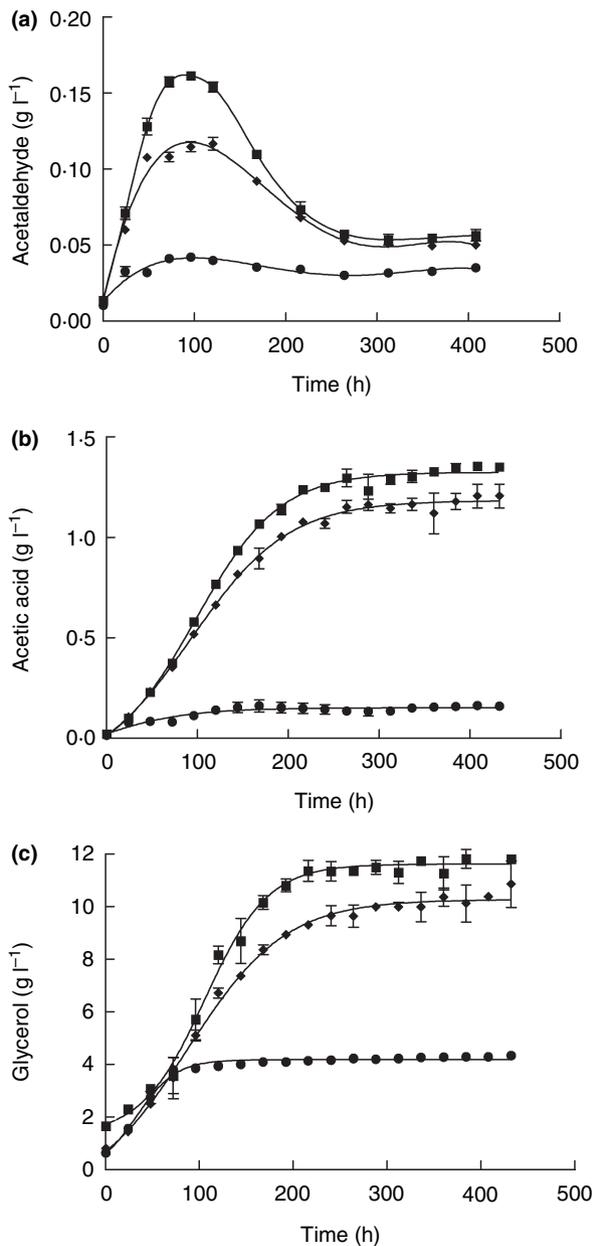
#### Statistical analysis

A two-factor analysis of variance (ANOVA) was used to evaluate the effect of acetaldehyde addition on *ALD* gene expression levels (XLSTAT version 7.1; Addinsoft, New York, USA). Differences between expression levels were determined using Fisher's least significant difference (LSD) test ( $P < 0.05$ ).

## Results

#### Yeast metabolite production

Acetaldehyde production occurred between 24 and 72 h and differed between all three conditions (Fig. 1a). Peak acetaldehyde levels were found at 96 h and were highest in the Icewine fermentation at  $0.160 \pm 0.001 \text{ g l}^{-1}$ . Unlike



**Figure 1** Yeast metabolite production. Icewine juice (■), chaptalized diluted Icewine juice (◆), and diluted Icewine juice (●) were inoculated with the commercial yeast K1-V1116. Acetaldehyde (a), acetic acid (b) and glycerol (c) were measured daily throughout the course of the fermentations. Values represent the average  $\pm$  standard deviation of the mean of duplicate fermentations.

other metabolites measured, acetaldehyde levels rapidly decreased after 120 h, leaving  $0.056 \pm 0.004$  g l<sup>-1</sup>,  $0.050 \pm 0.002$  g l<sup>-1</sup> and  $0.035 \pm 0.001$  g l<sup>-1</sup> in the Icewine, chaptalized juice and diluted juice fermentations, respectively. In agreement with our previous study (Pigeau and Inglis 2005), acetic acid and glycerol production were greater in

the high sugar fermentations compared with the dilute juice fermentation, resulting in an acetic acid increase of 8.4-fold and glycerol increase of 2.7-fold in the Icewine (Fig. 1b,c). However, addition of sugar alone to the dilute juice does not cause the same metabolic response in wine yeast fermenting authentic Icewine juice. Yeast cells in the chaptalized fermentation produce less acetaldehyde, acetic acid and glycerol in comparison with cells in the authentic Icewine fermentation, indicating that additional solutes concentrated in Icewine juice contribute to the yeast response (Fig. 1a–c).

#### Expression of *ALD2*, *ALD4*, *ALD5* and *GPD2* throughout the fermentations

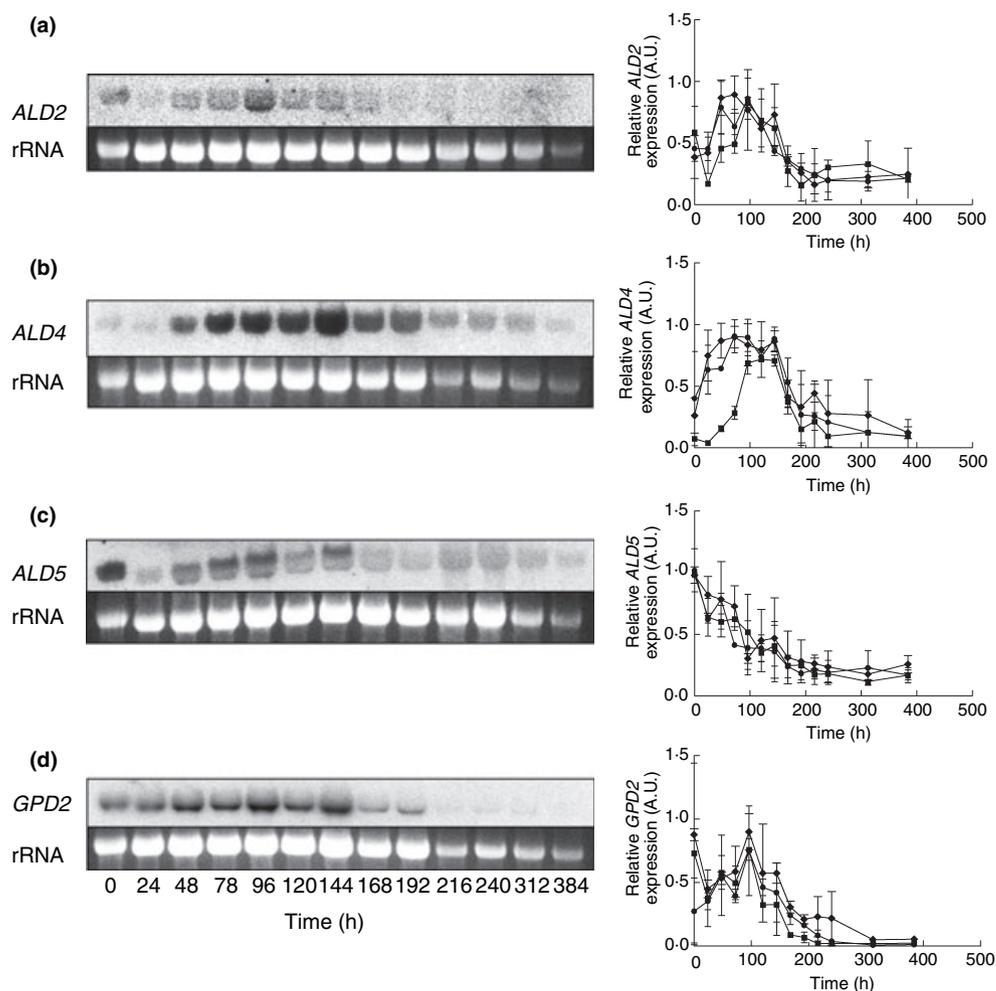
Previously, we identified a higher level of expression of *ALD3* but not *ALD6* in wine yeast fermenting Icewine juice over that found in the yeast fermenting dilute juice, but the remaining aldehyde dehydrogenases (*ALD2*, -4 and -5) had not been tested. To determine the contribution of the remaining *ALD* genes to the elevated acetic acid production in Icewine, the average expression profiles of these genes were determined from duplicate fermentations of Icewine, diluted and chaptalized juice and are illustrated in Fig. 2a–c.

Expression of *ALD2*, the gene encoding the cytosolic NAD<sup>+</sup>-dependent isoform, is low and does not show a differential expression pattern between the three fermentation conditions as observed by northern blot analyses. Peak expression corresponds to the late-logarithmic/early stationary phase of yeast growth at 96 h after which expression in all three conditions declines and becomes barely detectable from 200 h onward (Fig. 2a).

Expression of the mitochondrial *ALD4* follows a similar pattern to that of *ALD2*, except that the magnitude of *ALD4* expression is much greater, as indicated by the intensity of the northern bands (Fig. 2b). In both the diluted and chaptalized juice conditions, *ALD4* expression remained at its peak level from 24 to 144 h and then decreased but remained detectable throughout the remaining time of the fermentation. In Icewine juice, the expression pattern was slightly different in that *ALD4* expression slowly builds, reaching the same peak level as the other conditions at 96 h, and then declined as in the other two conditions (Fig. 2b).

The other mitochondrial isoform, *ALD5*, is only expressed at the beginning of all three fermentations, with no differences in expression between the three conditions. As the fermentations progress, *ALD5* expression levels continually decline, becoming barely detectable after 168 h (Fig. 2c).

*GPD2* is also not differentially expressed under these fermentation conditions. The level of expression gradually



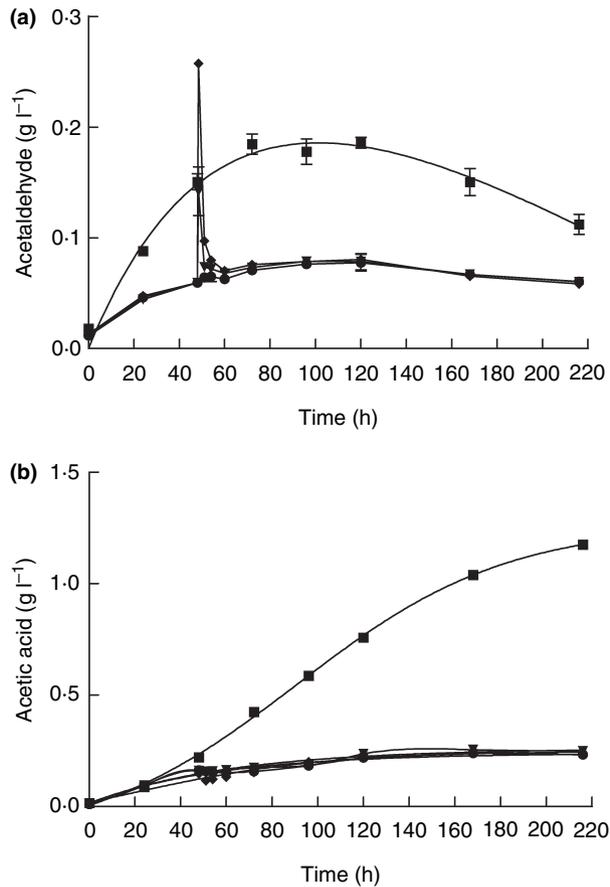
**Figure 2** Gene expression throughout fermentation. Northern blot analyses for expression of: (a) *ALD2*, (b) *ALD4*, (c) *ALD5* and (d) *GPD1* during Icewine fermentation are shown. Expression profiles for these genes during the fermentation of Icewine juice (■), chaptalized diluted Icewine juice (◆) and diluted Icewine juice (●) are shown. Graphs indicate average gene expression profiles from duplicate fermentations. Gene expression levels were normalized to 18S rRNA intensities and peak expression values were equated to one.

builds in all the fermentations, peaking at 96 h, then declines throughout the remainder of the fermentation (Fig. 2d).

#### Acetaldehyde stress during fermentation

We determined that acetaldehyde begins to rapidly rise in the Icewine and high sugar fermentations between 24 and 48 h before peaking at 96 h (Fig. 1a). In order to investigate the role of acetaldehyde in stimulating expression of *ALD* genes and increasing acetic acid production, we added acetaldehyde at 48 h to the diluted fermentation, either to the levels found in Icewine at that time, or to twice that level (Fig. 3a), and then measured the acetic acid production and *ALD* gene expression that followed. Results of acetic acid and *ALD*

gene expression were compared between the acetaldehyde spike conditions, the nonspiked dilute juice control and the Icewine condition. There was no significant increase in acetic acid production during the fermentation of the diluted juice that had been exposed to the transient spikes of acetaldehyde (Fig. 3b). However, the *ALD3* expression in the dilute juice condition at 96 h is upregulated 2.5-fold relative to the control in response to exogenous acetaldehyde (Fig. 4). This increase in expression is comparable with the threefold increase in *ALD3* expression found in the Icewine condition at this time point in comparison with the expression level in the control condition. The acetaldehyde-stimulated *ALD3* expression does not seem to be dose-dependent at the acetaldehyde concentrations tested, as there was no difference in expression level when twice the amount of

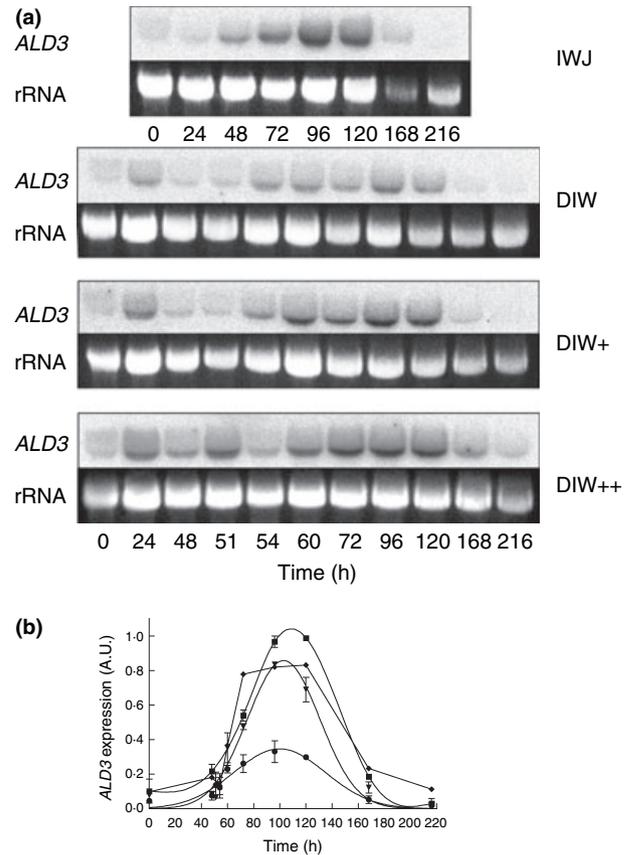


**Figure 3** Effect of acetaldehyde addition on acetic acid production. Icewine juice (■), diluted Icewine juice (●), diluted Icewine juice + 82 mg l<sup>-1</sup> acetaldehyde (▼) and diluted Icewine juice + 197 mg l<sup>-1</sup> acetaldehyde (◆) were monitored daily for: (a) acetaldehyde and (b) acetic acid levels.

acetaldehyde was added (Fig. 4). The expression of *ALD2*, *ALD4*, *ALD5* and *ALD6* in yeast fermenting the dilute juice did not show any increase at the 96-h time point after acetaldehyde addition, staying at the same level as found in the nonspiked control (Fig. 5).

## Discussion

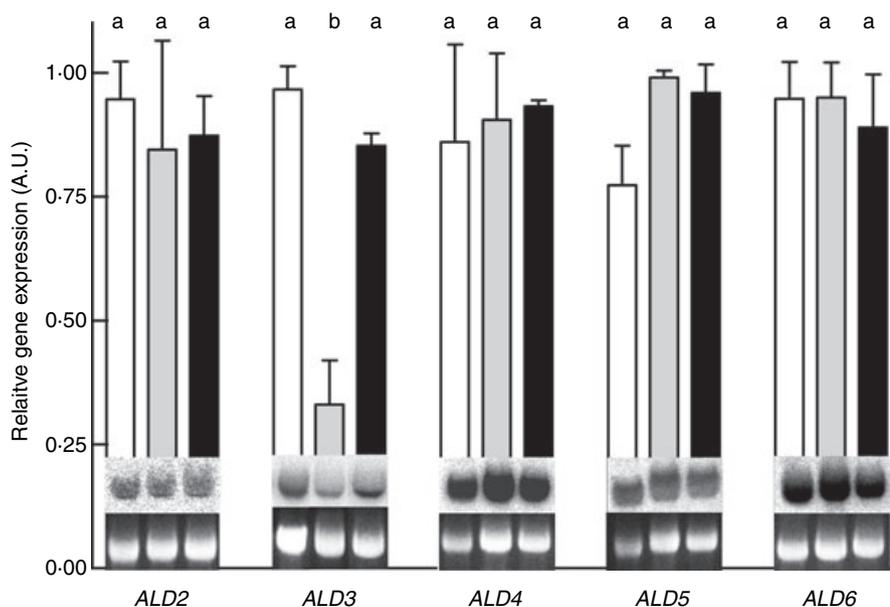
We here report that *ALD2*, *ALD4*, *ALD5* and *GPD2* are not differentially expressed between Icewine and dilute fermentations and do not correlate to the increased acetic acid and glycerol found in the finished Icewines. The responses of all *ALD* genes to acetaldehyde stress were examined and only *ALD3* was upregulated. Acetic acid production is not elevated in a diluted fermentation by upregulation of *ALD3* alone and may require the osmotic stress-induced downregulation of acetate utilizing pathways observed during Icewine fermentations.



**Figure 4** *ALD3* expression throughout fermentation. (a) Northern blot analyses of *ALD3* expression during fermentation trials. (b) Average gene expression profiles from duplicate fermentations of Icewine juice (■) (IWJ), diluted Icewine juice (●) (DIW), diluted Icewine juice + 82 mg l<sup>-1</sup> acetaldehyde (▼) (DIW+) and a single trial of diluted Icewine juice + 197 mg l<sup>-1</sup> acetaldehyde (◆) (DIW++). Gene expression levels were normalized to 18S rRNA intensities and peak expression levels were equated to one.

## *GPD* expression and glycerol production during Icewine fermentation

It is well known that *S. cerevisiae* cells produce glycerol to serve as an internal osmolyte when subjected to osmotic stress (Blomberg and Adler 1989; Brewster *et al.* 1993; Nevoigt and Stahl 1997; Blomberg 2000). We have previously shown that *GPD1*, encoding a glycerol-3-phosphate dehydrogenase, is upregulated during Icewine fermentation (Pigeau and Inglis 2005). The isoform encoded by *GPD2* is reported to serve a role in maintaining redox balance under anaerobic conditions (Ansell *et al.* 1997). Under optimal growth conditions, glycerol is produced as a redox balancing metabolite, oxidizing the excess NADH generated during biomass formation (Nordstrom 1968; van Dijken and Scheffers 1986). The fermentation of



**Figure 5** Expression of aldehyde dehydrogenases at 96 h of fermentation. Bar graphs show average gene expression levels from duplicate fermentations of Icewine juice (clear bars), diluted Icewine juice (grey bars), diluted Icewine juice + 82 mg l<sup>-1</sup> acetaldehyde (black bars). Average values followed by the same letter are not statistically different by Fisher's Protected LSD<sub>0.05</sub>. Examples of northern blot analyses from duplicate fermentation trials are shown. RNA was extracted from each fermentation condition at 96 h, electrophoresed, transferred to nylon membranes and probed for *ALD2*, *ALD3*, *ALD4*, *ALD5* and *ALD6* expression. Gene expression levels were normalized to 18S rRNA intensities and peak expression levels for each gene were equated to one.

Icewine is unique in that the yeast cells are presented with high osmolarity and an anaerobic environment. Our results show that the expression pattern of *GPD2* did not differ between the low and high sugar environments, and hence *Gpd2p* would not appear to contribute to the elevated glycerol found in the Icewine or chaptalized fermentation. During the fermentation of Icewine, yeast biomass is reduced and growth is retarded, but glycerol production is elevated (Pigeau and Inglis 2005). It then follows that *GPD2* expression would not be required for glycerol production to balance NADH production as biomass formation is curtailed. The increased glycerol during Icewine fermentation appears only because of the upregulation of *GPD1* in response to hyperosmotic stress. However, the contribution of *Gpd2p* to glycerol found in the dilute juice fermentation cannot be ruled out.

#### ***ALD* expression and acetic acid production during Icewine fermentation**

Several aldehyde dehydrogenases are involved in acetic acid production during table wine fermentation but the elevated level of acetic acid in Icewine appears to be due, in part, to the elevated expression of *ALD3*. Taken together with our previous work (Pigeau and Inglis 2005), we have shown that *ALD4*, encoding a mitochon-

dria isoform, and *ALD6*, encoding the main cytosolic isoform, are the most highly expressed aldehyde dehydrogenases in wine yeast and together, along with the comparatively minor contributions of *ALD2* and *ALD5*, produce the acetic acid found in the dilute juice fermentation. Of all the isoforms, only *ALD3*, encoding an NAD<sup>+</sup>-dependent cytosolic isoform, shows an increase in expression between low and high sugar fermentations which could account for the elevated acetic acid found in Icewine. Furthermore, acetaldehyde stress only increased the expression of *ALD3*, but had no effect on the expression of the remaining *ALD*.

It has been reported that *Ald6p*, *Ald5p* and *Ald4p* contribute to acetic acid formation during fermentation of media containing 20% w/v sugar (Remize *et al.* 2000; Saint-Prix *et al.* 2004). Involvement of these proteins was demonstrated with knockout mutants derived from a commercial champagne strain. While these strains had been engineered, they maintained fermentation characteristics similar to commercial wine yeast strains. These experiments are comparable with our diluted Icewine trials but higher levels of sugar that match those found in Icewine juice were not tested to determine the contributions of enzymes encoded by these *ALD* to acetic acid production under elevated osmotic stress. Erasmus *et al.* (2003) reported an increase in expression of *ALD2*, *ALD3*,

*ALD4* and *ALD6* 2 hs after exposure of a wine yeast to 40% w/v sugar stress and related this initial response to the elevated acetic acid measured in the final wine almost 3 weeks later (470 h). The initial increase in expression of these four *ALD* genes may have been transient, similar to the transient responses reported for yeast under salt stress (Blomberg 1995; Norbeck and Blomberg 1996) and may have little in common with the adapted response we observed when elevated acetic acid was being generated throughout the course of the high sugar fermentations.

### Response of the *ALD* gene family to acetaldehyde

Upon addition of exogenous acetaldehyde to diluted Icewine fermentations, only *ALD3* showed an increase in transcript abundance. A previous study has indicated that in commercial wine yeast, *ALD2/3* were upregulated in response to acetaldehyde stress; as were *ALD4* and *ALD6* in flor yeasts used for sherry production (Aranda and del Olmo 2003). This study could not differentiate the highly homologous *ALD2/3* isoforms and we have shown that *ALD3* and not *ALD2* is responsive to this stressor. Interestingly, the transcriptional response of *ALD3* does not seem to be dependent on the doses of acetaldehyde used in this study and does not result in increased levels of acetic acid in the dilute fermentation condition. Our *ALD5* measurements are in accordance with Aranda and del Olmo (2003) as expression has been shown to be relatively low and unaffected by acetaldehyde. In a strain comparison study, expression of *ALD4* and *ALD6* were upregulated in flor yeast and not in commercial wine yeasts (Aranda *et al.* 2002). Our observations concur with these results, in that *ALD4* and *ALD6* were not upregulated in the commercial wine yeast tested in response to acetaldehyde stress.

The reason for acetaldehyde's ability to induce *ALD3* gene expression in wine yeast fermenting the dilute juice without increasing acetic acid levels in the wine is unclear. It is unlikely because of its lack of entry into the cells as the acetaldehyde stress was sufficient to induce *ALD3* expression. It may be because of the intracellular conversion of acetaldehyde to ethanol making it unavailable for acetate formation or yeast in the dilute juice condition may have converted any acetate formed immediately to acetyl-CoA.

The reaction of acetate and coenzyme A is catalysed by acetyl-CoA synthetase (Acsp). The isoenzyme encoded by *ACS2* is reported to be present in the yeast cytosol under anaerobic conditions and required for viable growth on glucose (van den Berg and Steensma 1995). Acetyl-CoA is required in the cytosol for lipid and sterol biosynthesis and yeast utilize the cytosolic pyruvate dehydrogenase bypass to generate cytosolic acetyl-CoA (Pronk *et al.*

1996). In this pathway, pyruvate is decarboxylated by Pdcp to acetaldehyde, which is then oxidized to acetate via aldehyde dehydrogenase activity. Acetyl-CoA is then formed from acetate in an ATP-dependent reaction catalysed by Acsp.

The formation of acetyl-CoA may be responsible for eliminating acetic acid produced via Ald3p in the dilute fermentation as acetaldehyde was added during the exponential growth phase of the yeast. In support of this hypothesis, wine yeast cells in the dilute juice produce double the biomass and viable cells as compared with the cells growing in Icewine juice (Figure S1) and hence, would have an increased requirement for lipid and sterol biosynthesis.

We have recently reported that in addition to increased expression of *ALD3*, both *ACS1* and *ACS2* are downregulated in Icewine fermentations when compared with diluted juice fermentations (Martin and Inglis 2006) and may be a contributing factor to the elevated acetic acid released into Icewine. Higher expression of *ACS2* in the diluted juice fermentation could facilitate removal of any acetic acid produced through acetaldehyde-induced expression of *ALD3*. The isoenzyme encoded by *ACS1* is present during respiratory growth and is glucose repressed (van den Berg *et al.* 1996), so most likely would not have an impact during Icewine fermentation. In support of this claim, Akamatsu *et al.* (2000) have reported that elevated *ALD2/3* expression in the early stages of sake fermentation correlates to elevated acetic acid production by a *S. cerevisiae* sake yeast. They also report that acetic acid was reduced fourfold, from 104 mg l<sup>-1</sup> to 26 mg l<sup>-1</sup>, by overexpressing *ACS2* in the sake yeast.

The presence of high levels of acetic acid in wines is deleterious to quality because of the vinegar aroma it imparts. This organic acid is associated with wine spoilage and microbial contamination. We have previously reported that the high concentrations of acetic acid found in Icewines is because of the metabolic response of wine yeast during fermentation and is correlated with an increase in glycerol (Pigeau and Inglis 2005). Aldehyde dehydrogenases catalyse the conversion of acetaldehyde to acetic acid (Llorente and Castro 1977) and only *ALD3*, encoding an NAD<sup>+</sup>-dependent cytosolic isoform, shows an increase in expression between low and high sugar fermentations which could account for the elevated acetic acid found in Icewine. Furthermore, acetaldehyde stress only increases the expression of *ALD3*, but has no effect on the expression of the remaining *ALD* genes. Increased expression of *ALD3* appears to be necessary but not sufficient to cause elevated acetic acid during wine fermentations. The reduced expression of enzymes that utilize acetate along with the increased expression of *ALD3* may

both be required for the increased production of acetic acid in Icewine.

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### Supplementary material

The following supplementary material is available for this article online:

**Figure S1** Yeast fermentation kinetics. Icewine juice (■), chaptalized diluted Icewine juice (◆), and diluted Icewine juice (●) were inoculated with the commercial yeast K1-V1116. Total cell production (a), biomass accumulation (b) and reducing sugar consumption (c) were measured daily throughout the course of the ferments. Values represent the average  $\pm$  standard deviation of the mean of duplicate fermentations.

**Figure S2** Probe specificity validation of northern blots. Overnight yeast cultures were induced (+) or not induced (–) with  $0.7 \text{ mol l}^{-1}$  NaCl for 2.5 h prior to RNA isolation. Hybridization of  $^{32}\text{P}$ -labelled DNA probes to RNA isolated from a parent yeast strain (BY4742), knockout strains (a)  $\Delta\text{ALD2}$  and  $\Delta\text{ALD3}$ , (b)  $\Delta\text{ALD4}$ , (c)  $\Delta\text{ALD5}$  or (d)  $\Delta\text{GPD2}$  and a commercial wine yeast (K1-V1116) are illustrated. Inorganic pyrophosphatase (*IPPI*) was used as an internal control.

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