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ORIGINAL PAPER

Generation and characterisation of stable ethanol-tolerant mutants of *Saccharomyces cerevisiae*

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Abstract *Saccharomyces* spp. are widely used for ethanologenic fermentations, however yeast metabolic rate and viability decrease as ethanol accumulates during fermentation, compromising ethanol yield. Improving ethanol tolerance in yeast should, therefore, reduce the impact of ethanol toxicity on fermentation performance. The purpose of the current work was to generate and characterise ethanol-tolerant yeast mutants by subjecting mutagenised and non-mutagenised populations of *Saccharomyces cerevisiae* W303-1A to adaptive evolution using ethanol stress as a selection pressure. Mutants CM1 (chemically mutagenised) and SM1 (spontaneous) had increased acclimation and growth rates when cultivated in sub-lethal ethanol concentrations, and their survivability in lethal ethanol concentrations was considerably improved compared with the parent strain. The mutants utilised glucose at a higher rate than the parent in the presence of ethanol and an initial glucose concentration of 20 g l^{-1}. At a glucose concentration of 100 g l^{-1}, SM1 had the highest glucose utilisation rate in the presence or absence of ethanol. The mutants produced substantially more glycerol than the parent and, although acetate was only detectable in ethanol-stressed cultures, both mutants produced more acetate than the parent. It is suggested that the increased ethanol tolerance of the mutants is due to their elevated glycerol production rates and the potential of this to increase the ratio of oxidised and reduced forms of nicotinamide adenine dinucleotide (NAD^+/NADH) in an ethanol-compromised cell, stimulating glycolytic activity.

Keywords *Saccharomyces* · Ethanol · Stress · Mutant · NADH

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Introduction

Microbial production of ethanol has become increasingly important due to renewed interest in its use as a biofuel. Many new ethanol plants are being built to increase supply, and researchers are investigating ways of increasing ethanol output [3]. One approach that can be used to achieve this end is improvement of the microbial strains used in fermentation [25]. A considerable amount of research to date has focussed on improving the ethanol tolerance of ethanol-producing organisms, in the belief that such improvement will consequently lead to higher ethanol productivities and yields [10, 29].

The use of genetic engineering to improve the ethanol tolerance of *Saccharomyces cerevisiae* is limited by our lack of knowledge, and the complexity, of the ethanol-stress-related mechanisms that inhibit cell performance [16]. It is perhaps not surprising that research on ethanol tolerance mechanisms in *S. cerevisiae* has mostly involved isolating ethanol-sensitive mutants to identify genes that are necessary for growth under ethanol stress [34]. While this has provided some insight into ethanol tolerance per se, the mechanisms associated with ethanol sensitivity do not necessarily translate into effective strategies for improving

ethanol tolerance [15]. The creation of strains with improved stress tolerance is, however, achievable using approaches such as chemical mutagenesis and adaptive evolution with an appropriate selection pressure to isolate stress-tolerant variants. Adaptive evolution works on the principle that populations of cells adapt to their environment over time by natural selection. A number of studies have used adaptive evolution to create yeast mutants that are tolerant to various stressors, such as freeze–thaw [27], temperature [33], salt concentration [19] and acetic acid concentration [1].

Given the potential of adaptive evolution to generate mutants with improved stress tolerance, it is surprising that there are only two reported studies that used this approach to improve ethanol tolerance in yeast [7, 12]. Brown and Oliver [7] created ethanol-tolerant mutants of *S. uvarum* using continuous culture with frequent, semi-continuous ethanol addition. The 'fitness' of the culture was ascertained by measuring the amount of CO_2 released from the fermenter, and this information determined when to increase the ethanol concentration in the culture as it adapted to its environment. Five mutants were isolated that showed higher CO_2 production rates compared with wild type in the presence and absence of ethanol; however, variant phenotypes were not further characterised. Dinh et al. [12] increased the ethanol concentration of serial batch *S. cerevisiae* cultures from 2.5% to 10% (v/v) ethanol over a period of 28 days (ca. 100 generations). Compared with parent strain cultures, the ethanol-adapted cultures were found to have slightly higher specific growth rates, and cells from the ethanol-adapted cultures were larger and had a lower proportion of $C_{16:0}$ fatty acids in their cytoplasmic membrane [12].

Random mutagenesis is also used to generate genetically diverse phenotypes; however, the subsequent isolation of stable mutants with desired characteristics can be difficult, particularly when dealing with quantitative traits. This process can be more effective when combined with a directed evolution procedure, such as chemostat incubation using an appropriate selection pressure to enrich for variants with desired characteristics [4]. Jimenez and Benitez [17] used a chemostat to select ethanol-tolerant yeast hybrids generated by crossing ethanol-tolerant wine yeast with laboratory yeast strains. The authors created 25 hybrids that were subsequently subjected to a competitive growth selection process comprising semi-continuous culture conditions and a fixed sub-lethal ethanol concentration in the feed. A hybrid strain was isolated that was more ethanol tolerant than the parental wine strains and also showed a slight increase in ethanol yield. Alper et al. [2] used global transcription machinery engineering to generate ethanol-tolerant *S. cerevisiae* strains; mutations in the TATA-binding protein gene *SPT15* were introduced using the polymerase chain reaction, followed by selection for ethanol-tolerant phenotypes using serial subculturing in 6% (v/v) ethanol. The best-performing isolate displayed a prolonged exponential growth phase, faster and more complete glucose utilisation and increased ethanol productivity and yield under a number of different conditions and glucose concentrations [2].

Although the use of directed evolution to enrich ethanol-tolerant mutants offers many advantages over traditional selection methods, it is not always productive. Cakar et al. [9] tested a number of different selection procedures on ethyl methane sulphonate (EMS)-treated *S. cerevisiae* to isolate mutants with improved tolerance to multiple stressors, in particular ethanol, heat, oxidative and freeze–thaw stress. The authors tried various stress conditions in a continuous culture inoculated with the variants but were unable to successfully isolate mutants with improved ethanol stress tolerance [9]. Difficulty in generating and isolating stable ethanol-tolerant phenotypes has meant that very few ethanol-tolerant mutants have been created and, where they have, growth and metabolic product profiles have not been extensively characterised. This is unfortunate since such investigations could provide knowledge on the mechanisms underpinning ethanol tolerance, thereby informing strategies for the development of new, improved strains.

This paper describes the creation of two ethanol-tolerant mutants of *S. cerevisiae* using adaptive evolution of chemically mutagenised and non-mutagenised *S. cerevisiae* W303-1A; in both cases ethanol stress provided the selection pressure. The phenotypes of the isolates were determined according to their growth and product profiles, which provided insight into the mechanisms responsible for their improved ethanol tolerance.

Materials and methods

Strain, media and culture conditions

The strain used in this study was *Saccharomyces cerevisiae* W303-1A, (MATa leu2-3, 112 ura3-1 trp1-92 his3-11, 15ade2-1 can1-100 GAL SUC mal), a haploid laboratory strain. *S. cerevisiae* cultures were grown in a defined or nutrient-rich yeast extract peptone dextrose (YEPD) medium. YEPD medium comprised per litre: 10 g yeast extract, 20 g bacto-peptone, 20 g D-glucose and, in the case of solid medium, 15 g bacto-agar. The components were dissolved in distilled de-ionised water and autoclaved at 121°C for 20 min. Defined medium contained per litre: 20 g D-glucose, 5 g ammonium sulphate and 1.7 g yeast nitrogen base, without amino acids and ammonium sulphate (Difco). The yeast nitrogen base was prepared according to the manufacturer's instructions as a 10× solution (1.7 g nitrogen base in 100 ml sterile water). This

solution was filter sterilised using a 0.22-μm filter prior to adding 900-ml autoclaved glucose and ammonium sulphate. Amino acids and uracil were prepared as stock solutions, filter sterilised and stored at −4°C; exceptions included uracil and adenine, which were stored at room temperature to prevent precipitation.

Yeast cultures were grown aerobically in YEPD at 30°C/110 rpm in an orbital-shaker incubator, unless otherwise stated. Culture vessels were Erlenmeyer or sidearm flasks (500 ml) with cotton-wool plugs and working volumes of 200 ml. Yeast cultures were stored in 2-ml vials containing 1 ml sterile glycerol storage medium (comprising 20 g l^{-1} yeast extract, 40 g l^{-1} bacto-peptone, 40 g l^{-1} D-glucose and 15% (v/v) glycerol) at −80°C.

Inocula preparation

The preparation of inocula was carefully managed to improve the reproducibility and accuracy of experimental results. In particular, inocula were prepared from late exponential-phase parent cultures, each inoculum was washed with pre-warmed fresh medium, experimental cultures were inoculated to approximately the same initial cell population and all experimental cultures within each experiment were inoculated from the same parent culture. Reproducibility of culture profiles was determined by repeating each experiment a minimum of three times.

A loopful of yeast was taken from YEPD culture plates, aseptically transferred into 200 ml YEPD in a 500-ml sterile Erlenmeyer flask and incubated overnight at 30°C/110 rpm. This culture was then used to inoculate 200 ml fresh YEPD to generate a 'parent culture' which, at an OD_{620} of 1.0 (i.e. late exponential phase), was used to provide inocula for experimental cultures. Parent cultures were collected by centrifugation at about 3,000*g* in a swinging rotor centrifuge for 5 min at 30°C. The supernatant was discarded and the cells washed in pre-warmed (30°C) fresh medium. Following washing, the OD_{620} of the parent culture was used to determine the inoculum size (ca. 20 ml) required to achieve an initial OD_{620} of 0.1 in the experimental cultures.

Fermentation profile experiments

For determination of ethanol stress tolerance, yeast cultures were incubated in the presence or absence of added ethanol and their growth and product profiles determined. For all experiments, fresh YEPD and glassware were pre-warmed to 30°C. Control cultures comprised the same medium and conditions as test cultures but without added ethanol. A calculated volume of parent culture was inoculated to achieve an initial OD_{620} of 0.1 (around 2×10^6 cells ml^{-1}) into experimental flasks containing YEPD. The cultures were immediately transferred to a shaker incubator and grown aerobically at 30°C/110 rpm. Samples were taken at regular intervals of 1–25 h, depending on the experiment, for optical density, viable plate counts and fermentation product analysis using high-performance liquid chromatography (HPLC). For HPLC analysis, culture samples were centrifuged for 5 min at 30°C in a swing rotor centrifuge at 3,000*g*. The supernatant and pellet were separated, snap-frozen in liquid nitrogen and stored at −80°C. The supernatant was used for HPLC analysis.

Adaptive evolution of non-mutagenised cultures

Adaptive evolution of non-mutagenised cultures was performed by exposing W303-1A chemostat cultures to constant ethanol stress. A 1.5-l chemostat bioreactor with working volume of 1 l was inoculated to an OD_{620} of 0.1 and initially cultured batchwise without added ethanol. After reaching late exponential phase, the feed pump was turned on and fresh YEPD containing ethanol (initially at 7% v/v) was fed into the bioreactor at a dilution rate of 0.073 h^{-1}. The ethanol selection pressure was maintained by manually increasing the ethanol concentration in the feed each time the biomass levels in the chemostat stabilised. Biomass levels (OD_{620} and viable cell population) were monitored daily; if the OD_{620} measurements continued to decline over a period of 5–10 days, the feed ethanol concentration was decreased to allow biomass recovery and avoid wash-out.

Chemical mutagenesis and adaptive evolution of mutagenised cultures

Chemical mutagenesis was performed using ethyl methane sulphonate (EMS). Optimisation of mutagenesis conditions was conducted using EMS kill curves, from which it was decided to use 1% (v/v) EMS and an incubation time of 1 h, such conditions resulting in an approximately 50% death rate. EMS mutagenesis was performed as follows. *S. cerevisiae* W303-1A was grown overnight to a population of approximately 1–1.2 × 10^8 cells ml^{-1}. A portion of the culture (50 ml) was transferred into Falcon tubes, the cells were centrifuged and culture broth replaced with 0.01 M phosphate buffer. EMS was added to a concentration of 1% (v/v), and the tubes were wrapped in parafilm and incubated at 30°C for 1 h. Mutagenised cells were harvested by centrifugation (about 3,000*g*) and washed with 5% sodium thiosulphate, then with sterile distilled water and finally with 10% ascorbic acid [8]. The pellet was resuspended in YEPD and inoculated into 1-l Chemostat fermentors [containing YEPD and 7.5% (v/v) ethanol] and grown batchwise. When the batch culture reached stationary phase, the chemostat was started by pumping YEPD

with 7.5% (v/v) ethanol at a dilution rate of 0.073 h^{-1}. The ethanol selection pressure was maintained by manually increasing the ethanol concentration in the feed each time the biomass levels in the chemostat stabilised. Biomass levels (OD_{620} and viable cell population) were monitored daily; if the OD_{620} measurements continued to decline over a period of a few days, the feed ethanol concentration was decreased to allow biomass recovery and avoid wash-out.

Isolation of ethanol-tolerant mutants from chemostat cultures

Samples (10 ml) were taken from the chemostat cultures on a daily basis and spread on YEPD plates to determine cell population in the chemostat. The remaining portion was inoculated into fresh YEPD (200 ml) and grown for a minimum of five successive serial cultures (approximately 30 generations) without added ethanol. The fifth culture was subjected to a lethal ethanol stress experiment using 18% or 20% (v/v) ethanol; samples were taken hourly and plated. Colonies of yeast that survived for the longest time period were selected, grown for three successive overnight cultures without added ethanol and subjected to growth profile experiments (see above) using a range of ethanol concentrations (0–20% v/v). Cell population profiles were measured and compared with the parent; the best-performing cultures were selected and stored at −80°C.

HPLC analysis

Analyses to determine glucose, glycerol, acetic acid and ethanol concentrations were performed on a Varian Star Chromatography Workstation, using a BIORAD organic acid column HPX-87H and RI detector ERC-7515A by ERMACR.INC. Samples were thawed, filtered (0.22 μm) and diluted in sterile milliQ water to an appropriate dilution. Isovaleric acid (the internal standard) was added to each sample vial to a concentration of 0.25% (v/v) in both sample and standard solutions. Standards were prepared by diluting stock solutions of acetic acid, glycerol, glucose and ethanol to concentrations that covered the range of detection sensitivity for the column. All standards were prepared using HPLC-grade chemicals. The final volume in HPLC vials was 1.0 ml.

The mobile phase comprised filtered (0.45 μm) 5 mM sulphuric acid at a pressure of 50 atm. Helium was used for continuous degassing of the mobile phase. Each sample was injected (20 μl) into the column operating at 60°C and with a mobile-phase flow rate of 0.6 ml min^{-1}. All peaks for standards and samples were well resolved. The results were viewed and analysed using Star 6.41 Chromatography Workstation software.

Results

The *S. cerevisiae* W303-1A ethanol-tolerance phenotype

The growth profile of *S. cerevisiae* W303-1A was determined before commencing chemostat work so that critical dilution rates could be estimated at various ethanol concentrations. W303-1A was inoculated into medium containing ethanol concentrations ranging from 5% to 20% (v/v) ethanol, and the mean specific growth rates (SGR) of the cultures were plotted against the corresponding ethanol concentration (Fig. 1). It was estimated from this data that the limiting ethanol concentration for a chemostat operating at a dilution rate of 0.073 h^{-1} (i.e. a fixed feed flow rate of 0.073 l h^{-1} to a 1-l bioreactor) was 7.3% (v/v) (Fig. 1). In practice it was found that the limiting feed-ethanol concentration for W303-1A, in a chemostat operating at a dilution rate of 0.073 h^{-1}, was 6.8% (v/v).

Adaptive evolution to generate ethanol-tolerant mutants

An adaptive evolution approach was used to generate spontaneously derived and chemically induced ethanol-tolerant yeast mutants. For spontaneous mutants *S. cerevisiae* W303-1A was subjected to increasing ethanol concentrations in a chemostat over an extended time period. Following inoculation, the chemostat bioreactor was operated at a dilution rate of 0.073 h^{-1} with a feed containing 7% (v/v) ethanol (Fig. 2); this ethanol concentration, being slightly higher than the limiting feed ethanol concentration, was chosen to provide a strong selection pressure from the onset. After 14 days the biomass in the culture had substantially decreased and the cell population was close to total wash-out. The culture was revived by reducing the feed-ethanol concentration to 4% (v/v) and, when the cell population had recovered, the ethanol concentration was increased to 5% (v/v). This was the operational procedure for the chemostat over a continuous 6-month period. After 192 days of continuous operation the culture had gone through 486 generations and was able to maintain a high biomass level with 8.5% (v/v) ethanol in the feed, indicating that the population had evolved over this time (Fig. 2).

A chemical mutagen, EMS, was used to increase genetic diversity in the starting population of a parallel adaptive evolution experiment. A suspension of an EMS-treated population was inoculated into a chemostat bioreactor containing YEPD and ethanol (7.5% v/v) and grown batchwise to stationary phase, at which time the feed pump was activated; the initial ethanol concentration in the feed was 7.5% (v/v) and the dilution rate was 0.073 h^{-1}; the initial ethanol concentration in the feed was higher than in the previously described adaptive evolution experiment since the purpose now was to screen for EMS-generated mutants that had

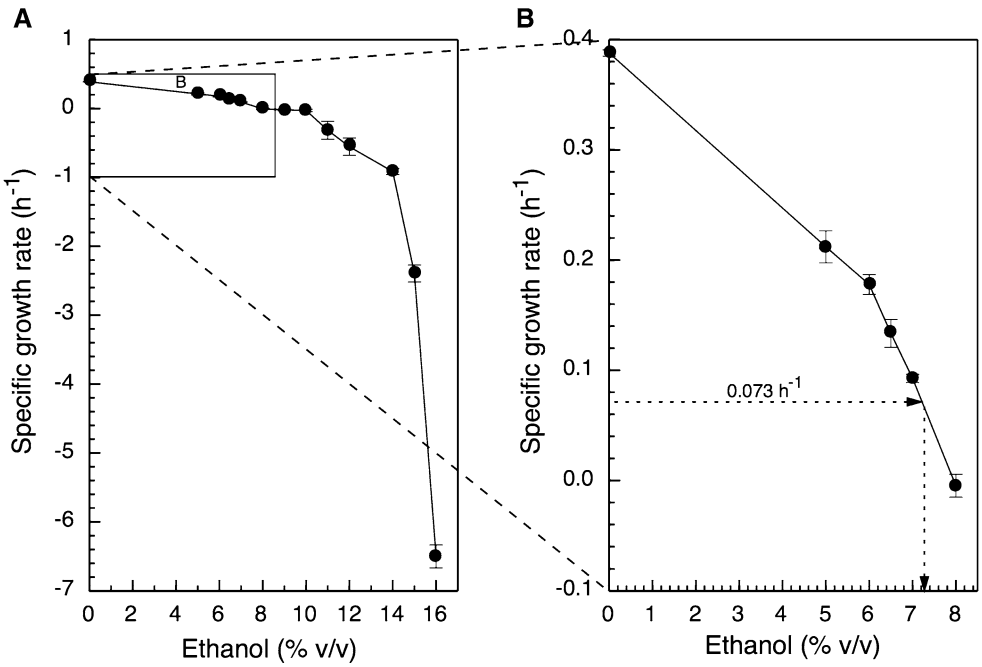

Fig. 1 Specific growth rate of *S. cerevisiae* W303-1A relative to ethanol concentration for batch aerobic cultivations in YEPD medium at 30°C/ 110 rpm. *Panel B* represents an enlargement of *panel A*. *Panel B* demonstrates that the critical ethanol concentration for a dilution rate of 0.073 h^{-1} is 7.3% (v/v). Data are mean values of four replicate cultivations for each ethanol concentration

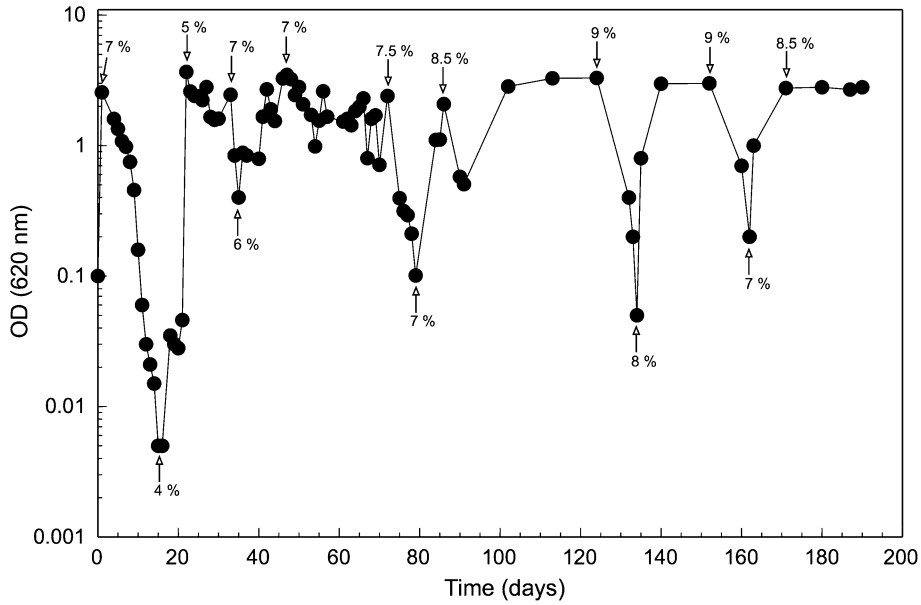

Fig. 2 Biomass profile of the adaptive evolution experiment using non-mutagenised *S. cerevisiae* W303-1A. Ethanol provided the selection pressure in the chemostat; changes in ethanol concentration (% v/v) of the YEPD feed are represented by *arrows*. Ethanol-tolerant mutant SM1 was isolated after 192 days of continuous cultivation (approximately 486 generations). The chemostat was operated at 30°C/110 rpm with a constant dilution rate of 0.073 h^{-1}

already acquired higher ethanol tolerance. On a number of occasions wash-out occurred using an initial feed-ethanol concentration of 7.5% (v/v), noting that the wild type under the same chemostat conditions washed out at ethanol concentrations greater than 6.8% (v/v). Some cultures survived, however, and after eight separate mutagenesis and subsequent chemostat-based selection experiments, three cultures could tolerate a feed-ethanol concentration of 10% (v/v) and two chemostat cultures could tolerate 12% (v/v). This approach required considerably less time (14–28 days) to generate populations carrying ethanol-tolerant mutants than was the case for non-mutagenised cultures (taking 192 days).

Enrichment and isolation of ethanol-tolerant mutants from adaptively evolved populations

Biomass samples from each chemostat were passaged by sub-culturing in YEPD without added ethanol over five successive cultures (around 30 generations) to reduce the population of unstable ethanol-tolerant mutants. Samples of the passaged culture were then inoculated into YEPD containing a lethal ethanol concentration of 18% (v/v) to screen out mutants based on relative ethanol tolerance and, over the next 8–10 h, samples of this culture were plated onto YEPD medium without ethanol to isolate colonies derived

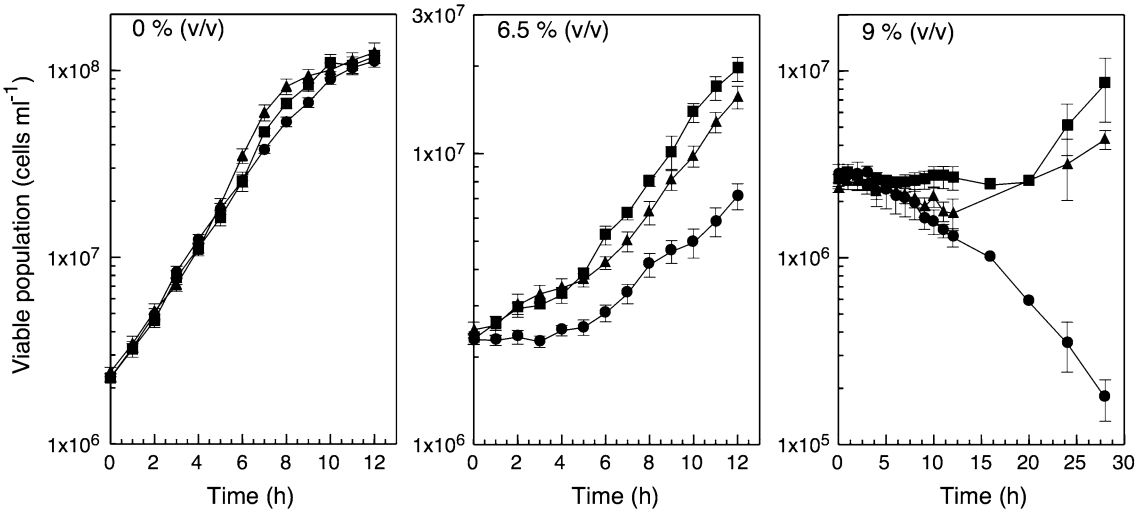

Fig. 3 Viable cell population profiles of the parent (*filled circle*) and mutant strains SM1 (*filled square*) and CM1 (*filled triangle*) under various ethanol stress conditions and incubated at 30°C/110 rpm. The *error bars* represent the standard error for the mean of four replicate experiments. When not visible, *error bars* are smaller than the *symbol*

from the 'longest surviving' cells. A loopful of cells from one of these colonies was passaged in YEPD (without ethanol) for several successive subcultures (approximately 20 generations) and samples were stored at −80°C; these mutants were labelled as either SMX (spontaneously derived mutant) or CMX (chemically derived mutant), where X refers to the colony number on the plate from which the mutant was isolated.

Five isolates (CM1, CM2, CM3, CM4 and SM1) were subjected to survival experiments at 20% (v/v) ethanol to test their ethanol-tolerant phenotype. SM1 and CM1 cultures both had an initial rapid decrease in viability in the first hour, followed by a lower death rate such that a measurable, viable cell population was present after exposure to 20% (v/v) ethanol for 7 h; this level of ethanol tolerance was substantially higher than that of parent strain cultures, which had no viable populations after 1 h (data not shown). The viable population profiles of CM2, CM3 and CM4 cultivations were similar to each other; there was an initial rapid decline in viability, reaching about 1×10^3 cells ml^{-1} during the first 2 h of incubation, and maintaining this population for up to 24 h. Despite their high ethanol tolerance, these isolates were found in subsequent experiments to be unstable and temperature sensitive. Thus it was decided to focus on SM1 and CM1.

Characterisation of SM1 and CM1 growth phenotypes

SM1 and CM1 were shown, using dropout plates, to carry the same auxotrophies as the parent W303-1A, although in both cases the tryptophan requirement was leaky (a feature which is not uncommon for this allele). Further research on these strains, performed at The Australian Wine Research Institute, confirmed the W303-1A parentage of SM1 and CM1 using transposon PCR and contour-clamped homogeneous electric field (CHEF) gels (Tina Tran, The Australian Wine Research Institute, personal communication).

The ability of SM1 and CM1 to acclimate to ethanol stress was assessed by inoculating the mutants into YEPD medium containing added ethanol (6.5% v/v) and determining their growth profiles; control cultures comprised the parental strain. This ethanol concentration was chosen since it was not lethal for the parent strain yet was sufficiently inhibitory to cause an initial lag period of around 4 h before growth commenced. SM1 and CM1 had similar growth profiles to their parent in the absence of ethanol; however, they were able to acclimate more quickly to, and grow more rapidly in, 6.5% (v/v) ethanol compared with the parent (Fig. 3). When exposed to 6.5% (v/v) ethanol, SM1 and CM1 had lag periods of less than 1 h compared with the parent, which had a lag period of around 3.5 h, representing an increase of at least 75% in the acclimation rate by the mutants compared with the parent. Once acclimated, SM1 and CM1 also had higher specific growth rates (0.240 and 0.182 h^{-1}, respectively) than the parent (0.134 h^{-1}) in the presence of 6.5% (v/v) ethanol. In 9% (v/v) ethanol, the minimum concentration that is lethal for the parent, both mutants commenced growth after lengthy lag periods (Fig. 3).

Survival profiles of SM1, CM1 and their parent were determined in the presence of lethal ethanol concentrations. The viable population of the mutant cultures was always higher than for the parent at any point in time and for all lethal ethanol concentrations used (Fig. 4). For example, the viable population (expressed as a percentage of the initial population) of the SM1 and CM1 cultures after 12 h in

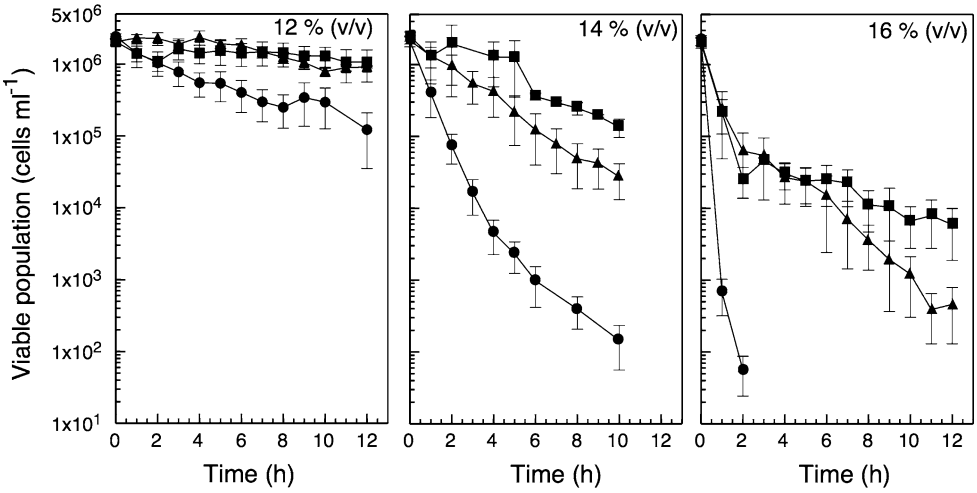

Fig. 4 Viable cell population profiles of the parent (*filled circle*), SM1 (*filled square*) and CM1 (*filled triangle*) strains under lethal ethanol stress conditions and incubated at 30°C/110 rpm. The *values* shown represent the mean of three replicate experiments. *Error bars* represent standard error and, if not visible, are smaller than the *symbol*

12% (v/v) ethanol was 52% and 44%, respectively, compared with 5% for the parent. Also, the time taken for the viable population to decrease by 50% (i.e. halving time) was always higher for the mutant cultures at all ethanol concentrations used (data not shown), demonstrating their enhanced ability to cope with lethal ethanol concentrations. For example, the halving time for the SM1 and CM1 cultures in 16% (v/v) ethanol was 1.60 and 0.94 h, respectively, whereas it was 0.11 h for the parent. These results demonstrate the enhanced survivability of SM1 and CM1 in lethal ethanol concentrations compared with their parent.

Metabolic profiles of SM1, CM1 and the parent in 20 g l^{-1} glucose

Experiments were conducted to measure key end-product and substrate profiles for each strain. There were no significant differences in cell population profiles of all three strains when inoculated into YEPD medium containing 20 g l^{-1} glucose without added ethanol (non-stressed) (Fig. 5). Glucose consumption and ethanol production were similar for all three strains, although SM1 had a slightly lower glucose utilisation rate, and this was reflected in its slightly lower rate of ethanol production. Notably the parent, SM1 and CM1 cultures had respective ethanol productivities (g l^{-1} h^{-1}) of 0.19 ± 0.02, 0.17 ± 0.02 and 0.21 ± 0.02 and ethanol yields (g g^{-1} glucose consumed) of 0.24 ± 0.03, 0.21 ± 0.02 and 0.23 ± 0.02. The low ethanol yields most likely reflect the impact of slight evaporative losses on the small amounts of ethanol produced. There was a substantial difference in glycerol production, with both SM1 and CM1 producing glycerol at a higher rate than the parent; both mutants produced approximately 55% more glycerol than the parent after incubation for 50 h (Fig. 5).

Differences in metabolism were more apparent when the strains were incubated in 6.0% (v/v) ethanol (Fig. 5). As observed previously, SM1 and CM1 cultures had higher specific growth rates, cell yields and shorter lag periods compared with the parent. This was reflected in the glucose consumption rates, which were similar in both mutant cultures and much higher than in parent strain cultures, suggesting that the mutants had acquired the ability to metabolise glucose at a significantly higher rate during sublethal ethanol stress. Ethanol yields and productivities were not determined due to the high concentrations of added ethanol masking the small quantities of ethanol produced. SM1 and CM1 also produced around 60% more glycerol than the parent after 70 h of incubation noting that, for the parent, there was residual glucose of around 6 g l^{-1} at this time point (Fig. 5). Acetic acid production was detectable in ethanol-stressed cultures, with SM1 producing approximately five times more acetic acid than either CM1 or the parent after 70 h cultivation (Fig. 5).

Metabolic profiles of SM1, CM1 and the parent in 100 g l^{-1} glucose

SM1 and CM1 were generated and isolated under aerobic conditions in YEPD medium containing 20 g l^{-1} glucose, and the above metabolic profiles of the strains were determined under the same conditions. Given that most commercial fermentations are conducted using higher substrate concentrations, it was decided to examine how the mutant phenotypes respond to a higher glucose concentration of 100 g l^{-1}, i.e. would the phenotypes be lost. In the absence of ethanol stress, SM1 and the parent grew similarly when inoculated into 100 g l^{-1} glucose in YEPD medium without added ethanol, although SM1 had a higher final cell population; CM1 grew more slowly and had a lower final cell population (Fig. 6). The parent and SM1 had similar rates of glucose consumption and ethanol production, with the same ethanol productivities at 0.36 ± 0.006 g l^{-1} h^{-1}, and ethanol yields of 0.44 ± 0.007 g g^{-1} glucose consumed.

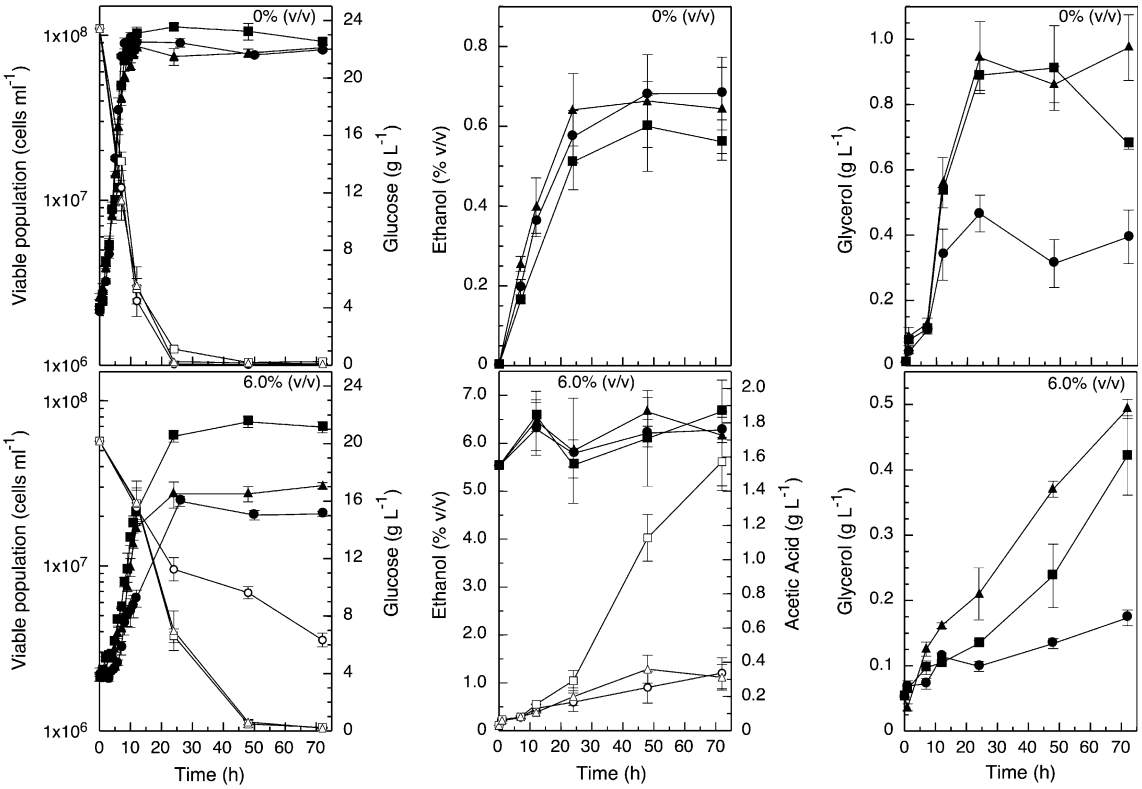

Fig. 5 Cell population and metabolite profiles of the parent (*filled circle*, *open circle*), SM1 (*filled square*, *open square*) and CM1 (*filled triangle*, *open triangle*) strains in the absence and presence of added ethanol (6.0% v/v); *open symbols* represent either glucose or acetate concentrations. The cultures contained 20 g l^{-1} glucose and were incubated at 30°C/110 rpm. Acetic acid concentrations were not detectable in the absence of added ethanol. The values shown are means of three replicate experiments. *Error bars* represent standard error and, if not visible, are smaller than the *symbol*

Compared with the parent and SM1, CM1 incubations had slightly lower ethanol productivities of 0.31 ± 0.005 g l^{-1} h^{-1}; however, the ethanol yields were higher at 0.51 ± 0.008 g g^{-1} glucose consumed, noting that CM1 incubations at this glucose concentration did not go to completion (approximately 25 g l^{-1} glucose remained) and this was reflected in the lower ethanol concentration in the cultures after 160 h. As observed in the 20 g l^{-1} glucose cultures, SM1 and CM1 produced glycerol at a higher rate than the parent, with the mutants producing approximately 3.3 and 2.2 times more glycerol, respectively, after 50 h of incubation (Fig. 6).

As previously observed for cultures containing 20 g l^{-1} glucose, differences in metabolism at 100 g l^{-1} glucose were more apparent when the strains were incubated in 6.0% (v/v) ethanol (Fig. 5). In the presence of added ethanol, SM1 had a higher final cell population than either the parent strain or CM1. This was reflected in the glucose consumption rates, which were retarded in CM1 and the parent; around 45–50% of the initial glucose was still available after 160 h of incubation. Furthermore, both CM1 and the parent significantly decreased their glucose utilisation rate when around half of the glucose had been consumed. On the other hand, SM1 maintained a relatively high glucose consumption rate and had consumed all of the glucose within 160 h, noting that the glucose consumption rate by SM1 was similar in the absence or presence of 6.0% (v/v) ethanol stress. SM1 cultures had higher ethanol productivities of around 0.18 ± 0.015 g l^{-1} h^{-1} compared with either parent strain (0.10 ± 0.008 g l^{-1} h^{-1}) or CM1 (0.08 ± 0.007 g l^{-1} h^{-1}) cultures and, although the parent strain cultures had higher ethanol yields (0.31 ± 0.03 g g^{-1} glucose consumed) compared with SM1 (0.27 ± 0.02 g g^{-1} glucose consumed) and CM1 (0.23 ± 0.02 g g^{-1} glucose consumed), complete glucose utilisation by SM1 meant that ethanol concentrations in the SM1 cultures after 150 h were substantially higher than in the parent or CM1 cultures. SM1 and CM1 produced around 2.3 times more glycerol than the parent over 160 h of incubation, and SM1 produced considerably more acetic acid during ethanol stress than either CM1 or the parent (Fig. 6).

Discussion

There are, to our knowledge, only two studies that describe the creation of ethanol-tolerant *S. cerevisiae* mutants using

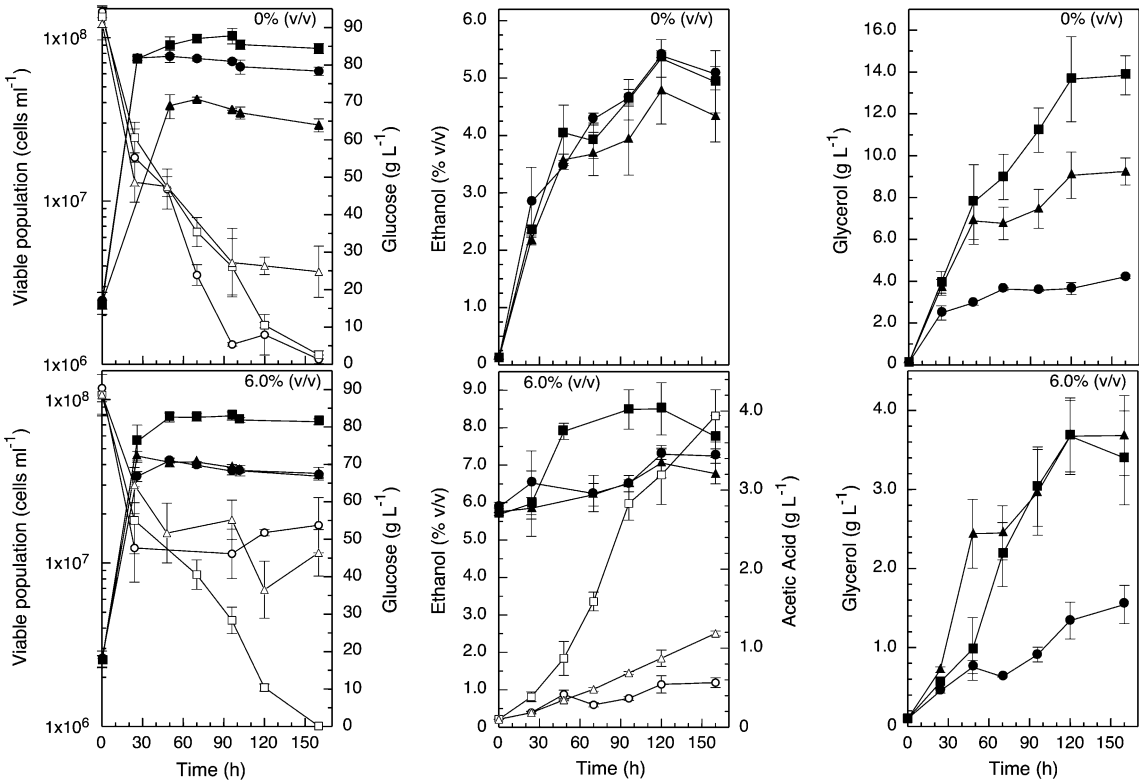

Fig. 6 Cell population and metabolite profiles of the parent (*filled circle*, *open circle*), SM1 (*filled square*, *open square*) and CM1 (*filled triangle*, *open triangle*) strains in the absence and presence of added ethanol (6.0% v/v); *open symbols* represent either glucose or acetate concentrations. The cultures contained 100 g l^{-1} glucose and were incubated at 30°C/110 rpm. Acetic acid concentrations were not detectable in the absence of added ethanol. The values shown are means of three replicate experiments. *Error bars* represent standard error and, if not visible, are smaller than the *symbol*

adaptive evolution of non-mutagenised cells and ethanol stress as the selection pressure [7, 12]. The purpose of the Brown and Oliver [7] study was to demonstrate the principles of using a chemostat and ethanol as a selection pressure to improve the ethanol tolerance of *S. uvarum*; a comprehensive analysis of the physiology of ethanol-tolerant isolates was not undertaken. Dinh et al. [12] used stepwise increases in the ethanol concentration (from 2.5% to 10% v/v) of serial batch *S. cerevisiae* cultivations to obtain ethanol-adapted cultures with higher specific growth rates than cultures of the parent strain. Although metabolite analyses of the cultures were not performed, it was found that cells from the ethanol-adapted cultures were larger and had a lower proportion of $C_{16:0}$ fatty acids in the cytoplasmic membrane compared with parent strain cells.

The current work describes the generation and isolation of two ethanol-tolerant mutants of *S. cerevisiae* using adaptive evolution of chemically mutagenised (isolate CM1) and non-mutagenised (isolate SM1) populations of strain W303-1A; both approaches used ethanol in the chemostat feed as selection pressure. The creation of these two mutants provided the opportunity to explore differences in metabolism that may account for their improved ethanol tolerance.

SM1 and CM1 were shown to be significantly more growth competitive than the parent strain in the presence of sub-lethal ethanol concentrations. Both mutants had substantially shorter lag periods and higher specific growth rates than the parent when inoculated into medium containing sub-lethal ethanol concentrations, demonstrating improved acclimation to ethanol stress by the mutants. SM1 and CM1 were also able to survive higher ethanol concentrations than the parent strain, with an ethanol concentration of 9% (v/v) being lethal to the parent but not to the two mutants. The mutants also demonstrated considerably higher tolerance than the parent strain to lethal ethanol concentrations. SM1 and CM1 had higher survival rates, suggesting that their ability to resist the damaging and inhibitory effects of lethal ethanol concentrations is markedly improved compared with the parent. Overall, despite the differences in approach used to create SM1 and CM1, their ethanol-tolerant growth phenotypes were remarkably similar across a range of ethanol stress conditions. The ethanol-tolerant phenotypes of the two mutant strains were also stable, being retained after several successive sub-cultures in the absence of stress and after storage in glycerol at −20°C and −80°C; this is most likely attributable to using a chemostat-based approach for strain enrichment, since

unstable mutants are unlikely to survive in the evolving population [13].

Glucose utilisation in the presence of sub-lethal ethanol stress was considerably more compromised for the parent than either SM1 or CM1 in medium containing 20 g l^{-1} glucose. In the presence of 100 g l^{-1} glucose and sub-lethal ethanol concentrations, SM1 showed little change in its glucose utilisation rate compared with a no-ethanol control. In contrast, there was no obvious difference in glucose utilisation between the parent and CM1 in 100 g l^{-1} glucose and sub-lethal ethanol stress, both strains having substantially lower glucose utilisation rates than SM1. This is supported by the higher final cell population achieved by SM1 in all ethanol-stressed cultures, suggesting a greater efficiency in glucose metabolism in this strain when exposed to ethanol stress. Although glucose utilisation by CM1 was similar to SM1 at low glucose concentrations, it had a lower glucose utilisation rate at 100 g l^{-1}, in both the presence and absence of ethanol. This may be due to collateral damage to the genome of this strain arising from EMS mutagenesis and impacting on genes other than those associated with ethanol tolerance.

The improved ethanol tolerance of SM1 and CM1 might be associated with their higher glycerol production rates, a common feature for both strains. Glycerol production has an important role in maintaining redox balance of the cell by oxidising NADH to NAD^+, with the ratio of these two cofactors being influential regulators of central metabolism [6, 21, 30]. There is evidence to suggest that glycolytic flux is inhibited during ethanol stress, resulting in compromised energetics in the cell [10, 14]. It has been suggested that one bottleneck in glycolytic metabolism during ethanol stress is lowered activity of glyceraldehyde-3-phosphate dehydrogenase, which uses NAD^+ as a cofactor and for which supply is thought to be limited due to the loss of intracellular acetaldehyde across an ethanol-compromised plasma membrane [5, 26]. It has been proposed that a loss of intracellular acetaldehyde during ethanol stress reduces the rate of NADH oxidation by alcohol dehydrogenase activity, leading to an imbalance in the NAD^+/NADH ratio [5, 26]; it has been shown that adding acetaldehyde to ethanol-stressed *S. cerevisiae* cultures improves their acclimation to, and growth rate in, non-lethal ethanol concentrations [5, 24, 31, 32]. The higher activity of the glycerol metabolism in SM1 and CM1 could, in part, compensate for the loss of NAD^+ production during ethanol stress by improving the NADH oxidation rate, subsequently increasing the availability of NAD^+ for glyceraldehyde-3-phosphate dehydrogenase activity, which may lead to improved glycolytic flux and cellular energetics.

Both mutants produced more acetate than the parent strain during ethanol stress, with SM1 producing considerably more acetate than the other two. This could be a response to the higher glycerol production in the mutants, which increases NAD^+ supply and/or reduces NADH levels in the cell, which in turn may stimulate aldehyde dehydrogenase activity. This phenomenon has been observed in glycerol overproduction studies where increased glycerol production coincided with higher acetate production, noting that such studies did not involve investigations using ethanol stress [11, 20, 22]. This speculation is, however, inconsistent with the observation in the current study that acetate could not be detected in the absence of ethanol stress, despite the two mutant strains producing much higher amounts of glycerol than the parent strain under these conditions.

The above observation suggests that acetate production was a direct response to ethanol stress, in which case a couple of scenarios could account for this effect. One possibility is that increased acetate metabolism may reflect a need during acclimation, when protein turnover is high, to increase the amount of acetyl-CoA entering the citric acid cycle; this would be facilitated by mitochondrial-based Ald5 activity [18, 23]. Alternatively, it may be due to increased activity of Ald6, which uses $NADP^+$ as a cofactor, increasing the cytosolic supply of NADPH. It has been suggested that an increase in acetate production in strains overproducing glycerol could be a way of providing additional NADPH, since 1 mol of acetate from glucose leads to the production of 2 mol of NADPH [22]. This is plausible given that an ethanol-stressed cell acclimates to the stress by changing the fatty acid profile of its membrane lipids, and fatty acid metabolism requires NADPH as a cofactor [28]. Further work on the acetate metabolism would be required to determine which of these mechanisms is responsible for the acetate production observed in the current studies.

This paper described the generation and isolation of two ethanol-tolerant mutants of *S. cerevisiae* W303-1A using two different adaptive evolution approaches. Compared with their parent, the mutants had increased acclimation and growth rates when cultivated in medium with sub-lethal concentrations of ethanol, and their ability to survive lethal ethanol concentrations was considerably improved. Metabolite analysis revealed that both mutants produce considerably more glycerol than the parent, possibly as a means of increasing NAD^+ supply in an ethanol-stress-compromised cell. Acetate was only detectable in ethanol-stressed cultures, and both mutants produced more of this metabolite than their parent. Although a number of possible mechanisms may account for this, further work is required to determine the underlying mechanism(s).

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