

Influence of sodium chloride on wine yeast fermentation performance

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Abstract: This paper concerns research into the influence of salt (sodium chloride) on growth, viability and fermentation performance in a winemaking strain of the yeast, *Saccharomyces cerevisiae*. Experimental fermentations were conducted in both laboratory-scale and industrial-scale experiments. Preculturing yeasts in elevated levels of sodium chloride, or salt “preconditioning” led to improved fermentation performance. This was manifest by preconditioned yeasts having an improved capability to ferment high-sugar containing media with increased cell viability and with elevated levels of produced ethanol. Salt-preconditioning most likely influenced the stress-tolerance of yeasts by inducing the synthesis of key metabolites such as trehalose and glycerol. These compounds may act to improve cells’ ability to withstand osmotic stress and ethanol toxicity during fermentations of grape must. Industrial-scale trials using salt-preconditioned yeasts verified the benefit of this novel physiological cell engineering approach to practical winemaking fermentations.

Keywords: salt, preconditioning, fermentation performance, *Saccharomyces cerevisiae*, wine

Introduction

Modern winemaking has significant demands concerning product consistency and quality and is driving winemakers to make the right choices at every stage of production from grape to glass. It is important to emphasize the importance of yeast genetics and physiology in winemaking since yeast activities during must fermentation impacts directly on: yeast growth, alcohol productivity, stress-tolerance, sugar utilization, volatile (higher alcohol and ester) compound production and off-flavor (carbonyl and sulphur compounds) development.¹⁻³ Various strains of *Saccharomyces cerevisiae* are available as starter cultures to supply distinctive sensory attributes to wine^{4,5} and yeast strain choice can affect the varietal character of aromatically distinctive grape cultivars by influencing the liberation of bound grape congeners. Winemakers rely on fermentations being rapid and producing predictable flavor and aroma characteristics in the finished wine. Wine yeast genetics and cell physiology are therefore of paramount importance in this regard. Desired *S. cerevisiae* characteristics for winemaking include: osmotolerance, relative insensitivity to high acidity, and acceptance of low oxygen concentrations.

Osmotic stress (osmotic stress) represents an environmental insult to yeasts and triggers a series of biological responses as cells strive to maintain viability and cell cycle progress. Many studies of osmotic stress in laboratory strains of *S. cerevisiae* have focused on transcriptional activation, changes in protein synthesis, DNA damage and DNA recovery, gene expression and apoptotic phenomena.^{6,7} Studies of osmotic stress in wine yeasts caused by high sugar have been reported,⁸ but there are few reports specifically investigating effects induced by elevated sodium chloride (NaCl) levels.^{9,10} We therefore aimed to

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further our understanding of cell physiology and fermentation performance in wine yeasts exposed to salt-induced osmotic stress conditions. We focused on the evaluation of salt effects on the wine yeast, *S. cerevisiae* strain VIN 13 (Anchor Ltd, Capetown, South Africa), and subsequent fermentation performance in both laboratory and industrial experiments formulas of salts. Industrial wine fermentations were performed to evaluate the effects of salt preculturing (or preconditioning) of cells regarding alcohol productivity, glycerol production and sugar utilization. Our working hypothesis was that salt stress conditions energized specific genes to enable yeast cells to survive under subsequent stressful conditions during grape must fermentations.

Materials and methods

Laboratory scale experiments

Yeasts cultures and growth conditions

A yeast strain of *S. cerevisiae* (VIN 13) used for experimental fermentations was kindly donated by Anchor Ltd. Yeast cells were grown on defined medium containing (per liter deionized water): 100 g D-glucose, 1 g K₂HPO₄, 1 g K₂H₂PO₄, 0.2 g ZnSO₄, 0.2 g MgSO₄, 2 g yeast extract and 2 g NH₄SO₄. All the media components were purchased from Sigma Chemical Company (Sigma-Aldrich, St Louis, MO, USA).

Inoculum preparation

Cell rehydration: 1 g dry weight of yeast was diluted in 100 mL of deionized water in an Erlenmeyer flask of 250 mL volume at 30–35°C, for 30 min. Inocula for experimental fermentations were prepared as follows: after 48 hours of preculturing, 10 mL was collected and centrifuged at 5000 rpm for 15 min. Cells were resuspended in deionized water and recentrifuged. This was repeated twice prior to determination of total cell number and cell viability in the final washed inoculum. To inoculate 250 mL of substrate, 5×10^5 of living cells were used as an inoculum.

Fermentation media preparation

The medium for experimental laboratory fermentations consisted of the following: 200 g/L glucose, 1 g/L K₂HPO₄, 1 g/L K₂H₂PO₄, 0.2 g/L ZnSO₄, 0.2 g/L MgSO₄, 2 g/L yeast extract and 2 g/L (NH₄)₂SO₄. Mineral components and the glucose were sterilized separately at 120°C, and 2 atm pressure for 20 min. The pH was adjusted using 1N HCl solution. For salt stress induction experiments, medium contained NaCl (commercial NaCl was used) and the total volume for the medium for each fermentation medium was 250 mL. Batch fermentations were carried out in 300 mL volume

Erlenmeyer flasks containing 250 mL of growth medium without shaking at 25°C. After inoculation 1 mL was periodically taken direct from each flask in order to monitor the differences between stressed and unstressed yeast cells with respect to yeast population growth and cell viability.

Yeast growth and viability determination

Yeast cell number was determined using a hemocytometer (Thoma type) and yeast cell viability using the methylene blue method.¹¹ One mL of sample medium was taken and diluted in 9 mL of deionized water. One mL of this solution was dissolved with 1 mL of 10% v/v methylene blue solution and left for 10 min. Aliquots of 1 µL were placed on the hemocytometer by using a Pasteur pipette. The hemocytometer was then microscopically observed by an optical microscope (Olympus model CHK2-F-GS microscope, Shinjuku, Tokyo, Japan). Yeast cell viability was calculated and expressed as follows:

$$\text{Viability (\%)} = a/n \times 100$$

Where, number of metabolically active cells and n, total cell number. Since cellular viability was determined immediately after hyperosmotic treatments, *in vivo* staining with methylene blue, which is rapid and accurate, was used.

Glucose measurement

Glucose was determined using the dinitrosalicylic acid (DNS) method¹² for lab scale fermentations.

Ethanol measurement

Ethanol was determined using an enzymatic kit from Boehringer Mannheim/R-Biopharm, Darmstadt, Germany, Cat. No. 10 176 290 035.

Glycerol measurement

Glycerol was determined using an enzymatic kit from Boehringer Mannheim/R-Biopharm, Darmstadt, Germany, Cat. No. 0148270.

Industrial scale fermentations

For industrial scale fermentation experiments, a quantity of 10000 L of must from Chardonnay grape varieties was used from a 2005 harvest in Greece. Grapes cultivated from the same area of an altitude of 550 meters came from the same two hectares of vineyard located at the area of Mendenitsa, Fthiotida, Central Greece. Cultivation methods were the same for each year and the grape harvest took place at the same period. A pre-fermentation procedure of cryo-extraction was made at the same temperature and for precisely 12 hours. For the inoculum, 1250 g of dry yeast was diluted in 50 L

water containing 500 g of sucrose at 35°C. Two inocula were made for each tank: one contained NaCl (6% w/v of commercial NaCl in crystal form was used) for yeast osmo-adaptation (or “salt-preconditioning”) and the other without NaCl. After 24 hours inocula were added to each tank. Each fermentation was conducted in stainless steel tanks, 5000 L in volume. Fermentation temperature was maintained at 10°C for Chardonnay fermentations. The analysis of industrial scale fermentations was made by FOSS Oenos Wine Scan™ (FOSS, Copenhagen, Denmark).

Statistical analyses

Firstly, the average of the three values separately was calculated. Secondly, the average value of the three average values was calculated. The column of these results was analyzed with the statistical programme called BioStat Plus 2008 version 5.3.0.6 by AnalySoft Corp (Brachnell, UK) using the Basic Describe Statistics package.

Results and discussion

The effect of NaCl on growth and viability of the industrial yeast strain, *S. cerevisiae* VIN 13.

It is known that when NaCl is added to a growth medium containing yeast cells the intracellular concentration of Na⁺ increases, and this has a negative effect on yeast growth and viability.¹³ Similarly,¹⁴ studies have shown that treatment of

S. cerevisiae cells with KCl up to 6% caused a great loss of viability. A gradual increase of NaCl in a growth medium containing yeast cells can cause a cell growth arrest depending on the NaCl concentration.¹⁵ Experiments have shown that the leavening ability of baker’s yeast decreases dramatically during cultivation in media containing NaCl concentrations between 0 and 3%.¹⁶ Prior research¹⁷ showed that the greatest loss in cell viability was caused under hyperosmotic conditions between 0 and 1 OSM, (OSM = Osmolarity = 5.85% NaCl w/v). Interestingly, between 1 and 4 OSM, the loss of viability was smaller.¹⁷ Comparative studies on the effect of osmotic stress in *S. cerevisiae* and non-*Saccharomyces* strains have shown that non-*Saccharomyces* strains displayed higher salt tolerance.¹⁸ In general, previous studies have revealed that osmotic stress and especially NaCl-induced stress, caused growth arrest and negatively affected the viability of yeast cells.

In the following laboratory-scale series of experiments (Figures 1 to 3) the effects of salt from concentrations ranging from 0%–10% NaCl w/v on wine yeast (*S. cerevisiae* VIN 13 strain) cell growth and viability were investigated. The findings indicate that NaCl caused a growth arrest in this strain and the difference between untreated cells and the cells which were treated under the highest salt-induced osmotic stress (10% NaCl w/v) was around 2.5×10^6 cells. When NaCl concentration increased the total cell number concomitantly

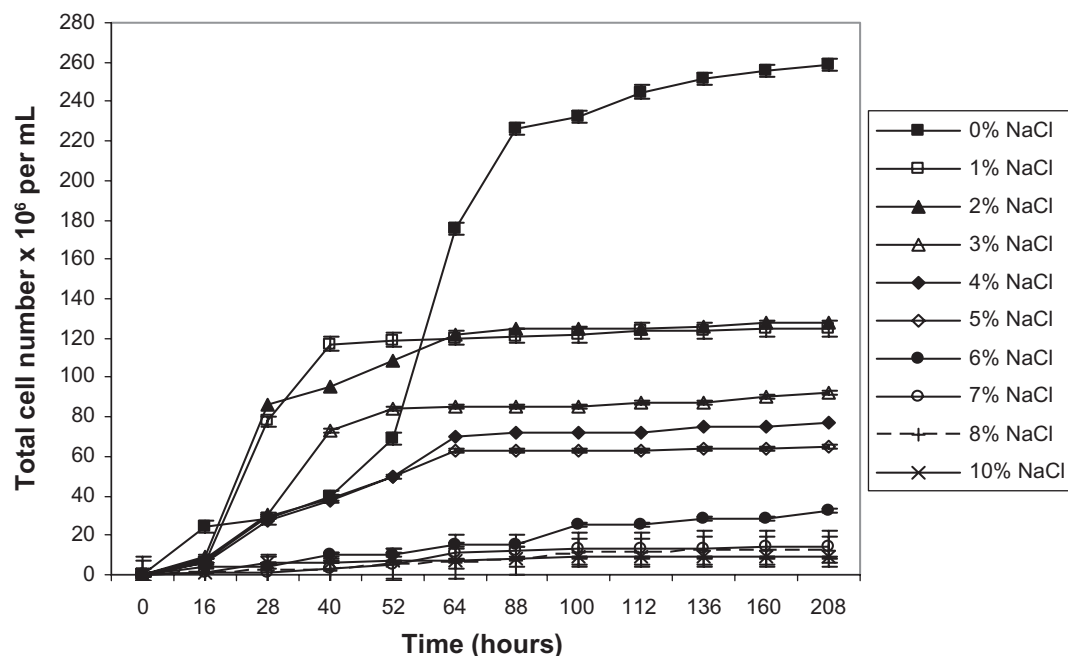


Figure 1 Influence of salt-induced osmotic stress (0%–10% NaCl) on yeast cell growth. *Saccharomyces cerevisiae* (strain VIN 13, Anchor Ltd, Capetown, South Africa) was grown on a glucose-based (200 g/L D-glucose) defined medium, without shaking at 25°C. Yeast cell growth was determined using a hemocytometer at the intervals shown. The standard error was between 1.30 and 8.28%.

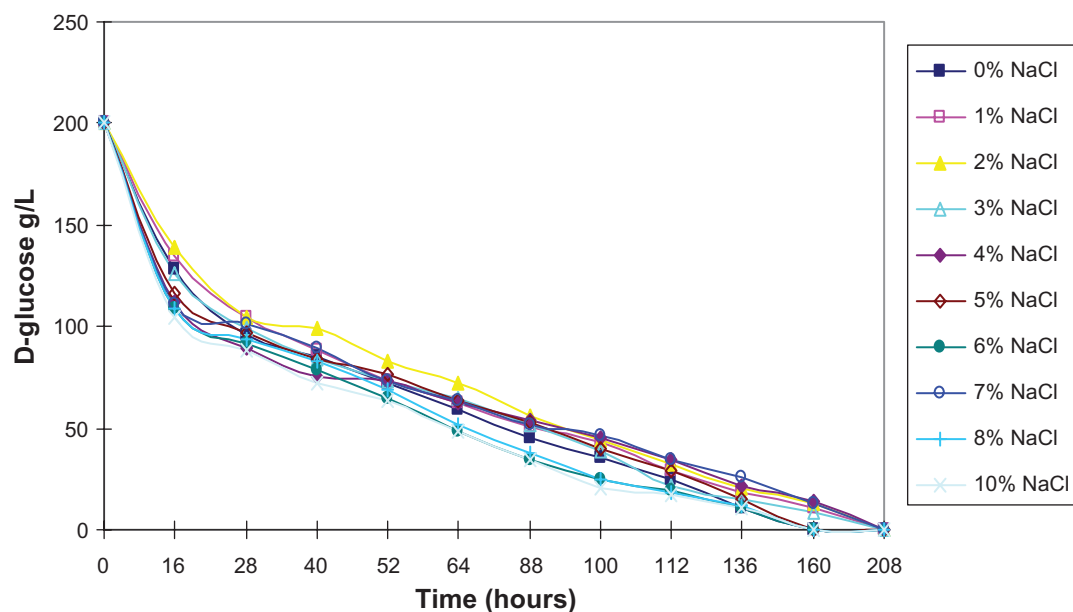


Figure 2 Influence of salt-induced osmotic stress (0%–10% w/v NaCl) on sugar consumption. *Saccharomyces cerevisiae* (strain VIN 13, Anchor Ltd, Capetown, South Africa) was grown on a glucose-based (200 g/L D-glucose) defined medium, without shaking at 25°C containing 200 g/L of D-glucose. Sugar consumption was determined by the DNS method and the standard error was between 1.09 and 3.03%.

decreased. The results shown in Figure 1 are in accordance with previously published findings^{6,19} in that NaCl-induced osmotic stress causes a growth arrest for yeast cells. When osmotic stress (NaCl concentration) is gradually increased, a decrease in yeast growth and the total cell number during time occurred. However, regarding viability, cells treated under higher osmotic shock conditions (>5% w/v NaCl) resulted in cells possessing a higher viability at the end of the fermentation (Figure 3). If we compare Figures 1 and 3 showing yeast growth and yeast viability, it can be seen that yeast cells exposed to higher concentrations of NaCl have the lowest growth but the highest viability compared with cells which were treated under lower osmotic shock conditions. Previous research supports the concept that when *S. cerevisiae* cells are exposed to high concentrations of NaCl, they show reduced viability resulting in cells acquiring tolerance against a severe salt shock (up to 1.4 M NaCl = 8.19% NaCl w/v) following a previous treatment with 0.7 M NaCl (4.09% NaCl w/v).²⁰ Overall, previous reports describe the osmotic stress of yeast cells for no more than a few hours, but in the current research cells were treated for at least 228 hours (Figure 1) during the fermentation process. In Figure 3 the results are contrary to published findings, especially concerning yeast cell viability, presumably due to the longer period of adaptation to the saline environment.

Previous studies have reported that some agents, like NaCl, play an important role in minimizing or inhibiting the fermentation process, specifically with regard to glucose

utilization for the production of yeast biomass.²¹ Much earlier studies regarding this subject have been performed. For example, in the 1920s, studies concerned with the fermentability of yeasts during cereal wort fermentation under different NaCl concentrations showed that the fermentative ability of yeast increased when preconditioned with 5% w/v NaCl, but for higher concentrations, a gradual reduction of fermentative efficiency was reported.²² In the present work, it is clearly shown that for concentrations from 1 to 10% w/v NaCl, no difference in glucose utilization occurred and the fermentability of the yeast cells was approximately the same for all salt concentrations tested (Figure 2).

From Figure 1 it can be seen that higher NaCl concentrations have a suppressive effect on yeast growth. The difference between total cell number of stressed cells and nonstressed cells is almost 2.5×10^6 cells/mL after 208 hours.

Regarding cell viability, high resistance of the cells to salt stress conditions were observed following a range of NaCl treatments. Results showed that after 64 hours of fermentation, yeast viability for untreated cells and for low NaCl concentrations started to decrease but the viability for stressed cells remained high. After a period of 208 hours, viability for untreated cells fell to around 50% and cells under highest osmotic conditions retained high viability. This may be due to the fact that under osmotic stress, and especially under salinity conditions, an increase in cellular electrolytes and a decrease in cellular water potential occurred.^{23,24} Consequently, rapid efflux of water, cytoskeletal collapse, intracellular damage

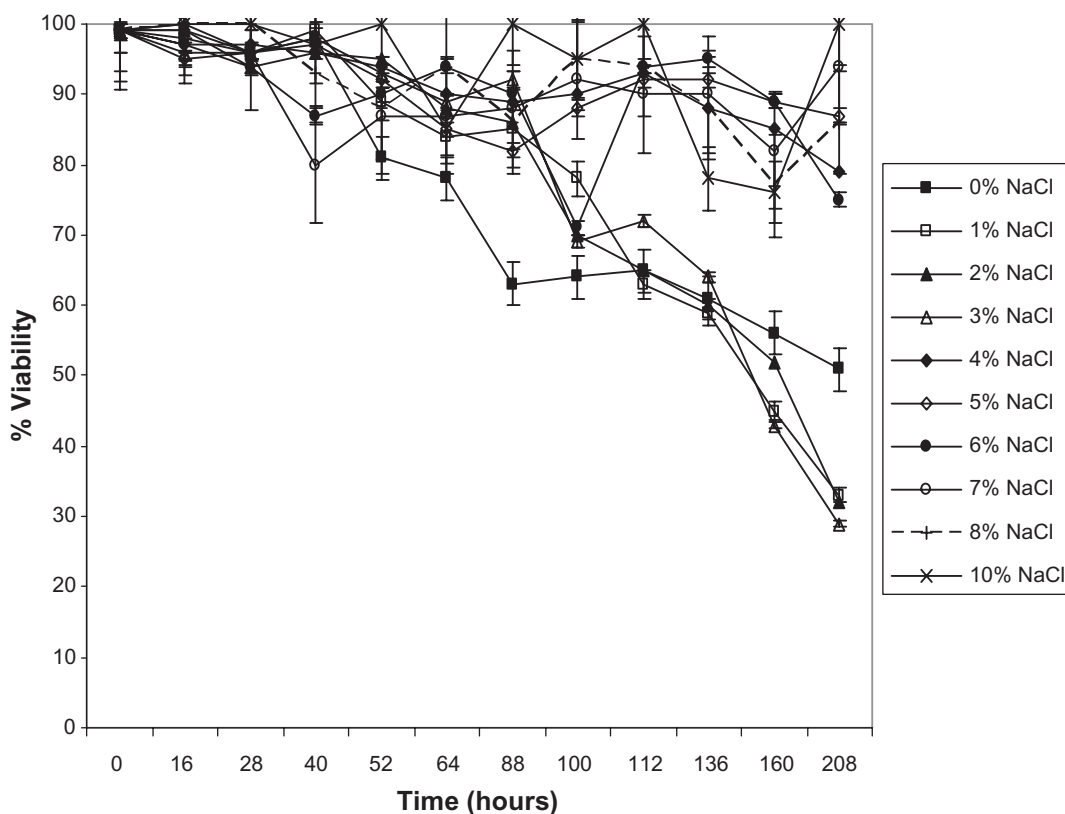


Figure 3 Influence of salt-induced osmotic stress (0%–10% w/v NaCl) on yeast cell viability. *Saccharomyces cerevisiae* (strain VIN 13, Anchor Ltd, Capetown, South Africa) was grown on a glucose-based defined medium, without shaking at 25°C. Yeast cell viability was determined by methylene blue staining using a hemocytometer at the intervals shown and the standard error was between 1.75 and 5.28%.

and growth arrest, are typical phenomena following saline stress. Adaptation to these conditions by yeast cells include: retainment of turgor, polarized cytoskeleton, cellular damage repair, and resumption of growth. It is conceivable that the response is controlled by the high-osmolarity glycerol (HOG) mitogen-activated protein (MAP) kinase pathway.⁶ It has been reported that accumulation of glycerol, which is the main compatible solute that cells produce intracellularly to adapt to the differential extra and intracellular osmotic pressure, is strongly affected by growth temperature and causes the over expression of GPD1 and FPS1 genes, which encode the glycerol transport facilitator and glycerol-3-phosphate dehydrogenase.²⁵ The temperature (24°C) in these published studies was very close to the temperature that this experiment was running.

It has been reported that osmotic stress caused by 0.3 M (1.75% NaCl w/v) NaCl and for a time period of 1 hour may prolong the life span of yeasts.²⁶ The relationship between temperature and osmotic stress regarding osmotic tolerance of cells and viability has previously been reported. Under osmotic pressure of 49.2 MPa cell viability was close to 94% at a temperature of 23°C, but

under higher osmotic pressure of 99 MPa the viability decreased dramatically to 25%.²⁷ It was reported that under the same conditions of high osmotic pressure of 99 MPa, but at a temperature of 5°C, cell viability remained at a high level of 81%.

Fermentation performance in industrial scale experiments

During this part of the research, experiments were conducted on an industrial (Georgakopoulos Estate Winery) scale using the same four industrial yeast strains as per lab-based fermentations. The strain *S. cerevisiae* VIN 13 was employed for the 2005 winemaking season using must from Chardonnay variety grapes. For this 2005 grape harvest we used NaCl preconditioned and unconditioned yeast cells for inoculation of 5000 L stainless steel tanks. Preconditioning of the cells was conducted by treating cells with 6% w/v NaCl for 16 hours in the inoculum volume (200 L). This concentration of NaCl was chosen following promising data from the lab scale experiments (see Figures 1 and 3) that showed better yeast viability and maximum growth compared with higher salt conditions.

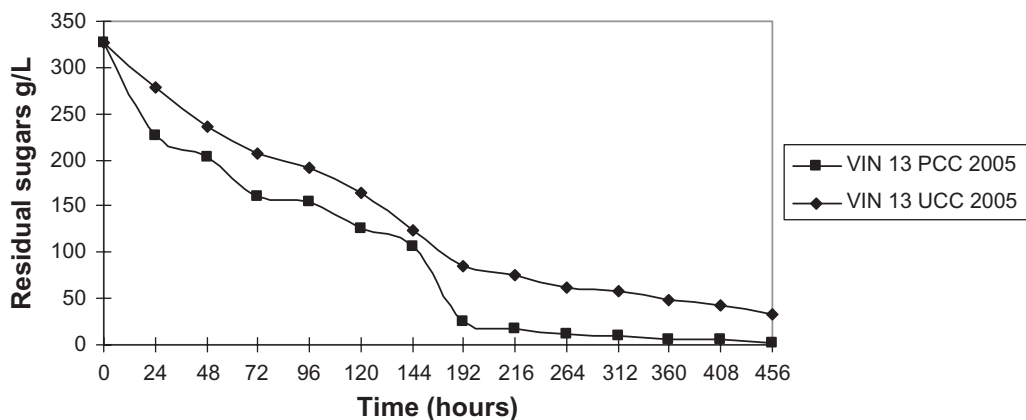


Figure 4 Sugar consumption by salt-preconditioned and unconditioned wine yeast during fermentation of 2005 Chardonnay must. Standard error was 2.3. Fermentations were conducted with preconditioned and unconditioned yeasts (strain VIN 13, Anchor Ltd, Capetown, South Africa) using 6% NaCl and growing cells for 24 hours prior to inoculation of 5000 L stainless steel tanks containing Chardonnay must. Must was clarified by pectynolitic enzymes and filtered before inoculation.

For all winemaking years, results were compared with the “standard” given by the yeast-producing companies (Anchor and Martin Vialate) for each yeast strain. We aimed to show that preconditioning under salt stress conditions for cells had a positive effect regarding: alcohol productivity, fermentation yield (regarding sugar consumption) and sugar and temperature stress tolerance. The latter conditions involved cells withstanding fermentation temperatures for every year of 9–10°C and sugar concentrations higher than 300 g/L.

Figures 4–6 demonstrate the fermentative behavior of the VIN 13 yeast strain regarding sugar consumption, alcohol production and glycerol production. The results compare the behavior of the same strain with cells which were preconditioned under salt stress conditions of 6% NaCl w/v for 24 hours before inoculation and those that were not

preconditioned. From Figure 4 the pattern of sugar consumption for both cases was the same but residual sugars at the end of the fermentation the difference was around 30 g/L of D-glucose. Additionally, alcohol productivity and glycerol production were affected by salt-preconditioning. For example, the alcohol content at the end of the fermentations differed by 3% v/v and the glycerol concentration by 10 g/L. These results show that salt-preconditioned yeast cells are able to ferment high sugar concentrations with low residual sugars at the end of the fermentation and can produce higher yields of alcohol in comparison with the un-preconditioned cells. The higher levels of glycerol produced following preconditioning is significant in winemaking, especially for white wines since glycerol is related with the textural (mouthfeel, body) characteristics of such wines.

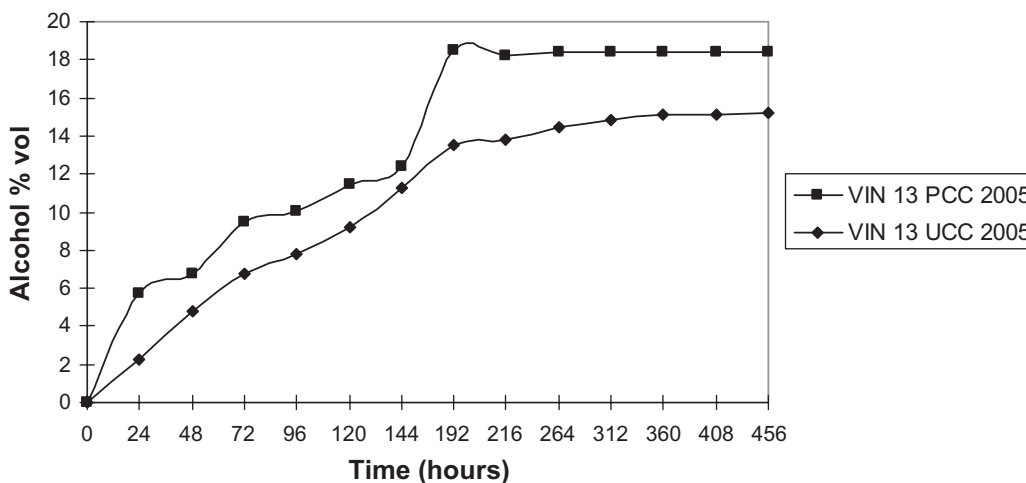


Figure 5 Alcohol production by salt-preconditioned and unconditioned wine yeast during fermentation of 2005 Chardonnay must. Standard error was 1.23. Fermentations were conducted with preconditioned and unconditioned yeasts (strain VIN 13, Anchor Ltd, Capetown, South Africa) using 6% NaCl and growing cells for 24 hours prior to inoculation of 5000 L stainless steel tanks containing Chardonnay must. Must was clarified by pectynolitic enzymes and filtered before inoculation.

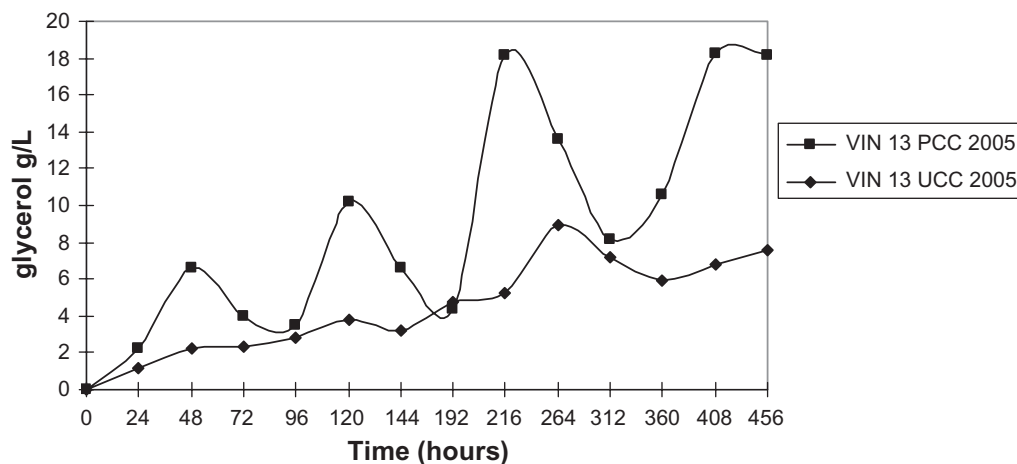


Figure 6 Glycerol production by salt-preconditioned and unconditioned wine yeast during fermentation of 2005 Chardonnay must. Standard error was 1.62. Fermentations were conducted with preconditioned and unconditioned yeasts (strain VIN 13, Anchor Ltd, Capetown, South Africa) using 6% NaCl and growing cells for 24 hours prior to inoculation of 5000 L stainless steel tanks containing Chardonnay must. Must was clarified by pectinolytic enzymes and filtered before inoculation.

Both the fermentation ability and the sugar consumption appeared unaffected and during the first 24 hours sugar consumption was at its highest (Figure 4) – contradiction. Even the low pH (3.23) had little effect on both the fermentability and ethanol productivity. The total sugar concentration at the end of fermentation was low with concomitantly high alcohol levels. This indicates that preconditioned yeast cells had acquired an increased alcohol tolerance and ethanol production capability. Additionally, yeasts displayed: enhanced tolerance to high sugar concentrations, better adaptation to low pH levels and increased sugar consumption (especially for the first 24 hours of fermentation).

In summary, industrial scale fermentation experiments have revealed that osmotic prestress due to NaCl-preconditioning enabled yeast cells to ferment sugars at high concentrations and produced high yields of alcohol. We hypothesize that the likely mechanism for this phenomenon lies at the level of elevated stress gene expression, especially linked to increases in the intracellular concentration of stress-tolerance metabolites such as glycerol and trehalose.

Disclosure

The authors report no conflicts of interest in this work.

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