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This is the peer reviewed version of the following article:

Logothetis, S., et al. 2014. Alleviation of stuck wine fermentations using salt-preconditioned yeast. *Journal of the Institute of Brewing*. 120(3): pp.174-182. doi: 10.1002/jib.143

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Journal of The Institute of Brewing 120: 174-182 (2014)

**Alleviation of stuck wine fermentations using salt-preconditioned yeast**

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ABSTRACT

The influence of salt (sodium chloride) on the cell physiology of wine yeast was investigated. Cellular viability and population growth of three winemaking yeast strains of *Saccharomyces cerevisiae*, and two non-*Saccharomyces* yeast strains associated with wine must microflora (*Kluyveromyces thermotolerans* and *K. marxianus*) were evaluated following salt pre-treatments. Yeast cells growing in glucose defined media exposed to different sodium chloride concentrations (4%, 6% and 10% w/v) exhibited enhanced viabilities compared with non-treated cultures in subsequent trial fermentations. Salt "preconditioning" of wine yeast seed cultures was also shown to alleviate stuck and sluggish fermentations at the winery scale, indicating potential benefits for industrial fermentation processes. We hypothesise that salt induces specific osmostress response genes to enable yeast cells to better tolerate the rigours of fermentation, particularly in high sugar and alcohol concentrations.

Key Words: Salt pre-conditioning, wine yeast, *Saccharomyces cerevisiae*, non-*Saccharomyces*, stuck fermentation

INTRODUCTION

During alcoholic fermentations for wine production, yeasts are subjected to several physico-chemical stresses including the following: initially high sugar concentrations and low temperature; and latterly increasing ethanol and carbon dioxide concentrations. Such conditions trigger a series of biological responses in an effort to maintain yeast cell viability and cell cycle progress. However, very few studies of yeast stress responses have been reported in wine strains. In laboratory strains of the yeast *Saccharomyces cerevisiae*, many studies have focused on transcriptional activation and gene expression when the cells are under stress. Such responses can be distinguished by different stages: cellular changes that occur immediately as direct consequences of physico-chemical forces, activation of primary defense processes and changes in cell homeostasis. Concerning osmostress, a number of physiological changes take place, including efflux of intracellular water, with associated rapid reduction in total cell volume, including the vacuole *(1)*, transient increases in glycolytic intermediates *(2),* accumulation of cytosolic glycerol, and triggering of the HOG (Hyper Osmotic Glycerol) signalling pathway *(3,4)*.

With salt (NaCl) stress, microorganisms such as the yeast *S. cerevisiae* develop systems to counteract the specific salt-induced effects. For example, salt-induced stress results in both ion toxicity and osmotic stress and cellular defense responses are based on sodium exclusion and osmolyte synthesis, respectively. The latter includes polyols, notably glycerol that accumulates intracellularly *(12,13,15)*. Other products synthesized by yeast during osmostress conditions are trehalose and glycogen, which may collectively represent 25% of the dry cell mass depending on the environmental conditions. The disaccharide trehalose accumulates not only during salt adaptation *(21,3,16)*, but also in response to a number of other stress conditions including protection against high temperatures, where it acts by stabilizing proteins and maintaining membrane integrity*(5,16)*.

Exposing yeast cells to a hyper-osmotic environment leads to a rapid initial efflux of cellular water into the medium, effectively dehydrating the cell. Intracellular water can also be recruited from the vacuole into the cytoplasm, thus partially compensating for sudden water losses. Additionally, the cytoskeleton collapses leading to depolarization of actin patches. Cell dehydration leads to growth arrest and cellular accumulation of compatible solutes (to balance intracellular osmotic pressure) and represents a major compensatory or adaptation mechanism. Depending on the osmotic stressor, the compatible solutes can be glycerol, trehalose, amino acids, and fatty acids in cell membranes. Hyperosmotic stress caused by sodium chloride leads to increases in intracellular glycerol concentrations, due to elevated biosynthesis,

increased retention by cytoplasmic membranes, or decreased dissimilation or uptake of glycerol from the medium. Glycerol is produced during glycolysis by reduction of dihydroxyacetone phosphate to glycerol 3-phosphate by glycerol 3-phosphate dehydrogenase (GPD) *(2,22,23)*. Under osmotic stress, GPD activity is enhanced and this requires an equimolar amount of cytoplasmatic NADH, resulting in decreased reduction of acetaldehyde to ethanol and increased oxidation to acetate. The observed decrease in the synthesis of alcohol dehydrogenase, as well as the increase of the aldehyde dehydrogenase, accounts for this alteration in flux.

In modern wine production processes, winemakers occasionally need to cope with problems due to stuck and sluggish fermentations. A stuck fermentation refers to the premature termination of fermentation before all but trace amounts of fermentable sugars have been metabolized. Both stuck and sluggish fermentations have been problematic since the early years of winemaking. In the absence of adequate cooling, fruit harvested and fermented under hot conditions can readily overheat and fermentations become stuck due to thermal stress on the yeast. The resulting wines are high in residual sugar, making them particularly susceptible to microbial spoilage. Instability is further increased if the grapes are low in acidity, high in pH, or both. The extensive use of temperature control during fermentation has essentially eliminated overheating as a significant factor in stuck fermentations. The desire to accentuate the fresh, fruity character of white wines has encouraged the use of cool temperatures. This can limit yeast growth and potentially favour microbial contaminants, which further retard growth. Osmotic effects of high sugar concentrations can also partially plasmolyze yeast cells, resulting in slow or incomplete fermentations. Bisson and Butzke *(4)* have listed the following categories of stuck wine fermentations:

1) Low initiation (eventually becoming normal)
2) Continuously sluggish
3) Typical initiation, but becoming sluggish and
4) Normal initiation but abrupt termination.

Comparison of sugar consumption, temperature, nutrient profiles, and records of procedures from past fermentations often provide an early indication of potential problems and their possible quick resolution. Once fermentation has stopped, re-initiation is more complicated. When a stuck fermentation occurs, successful re-initiation usually requires incremental re-inoculation with special commercial yeast strains, following racking off from the settled lees *(4)*. These special strains usually possess a high ethanol tolerance, as well as the ability to utilize fructose (a sugar whose proportion increases during wine fermentation). Other approaches include the addition of supplementary nutrients such as yeast “hulls” (cell wall preparations used to remove toxic fatty acids), must aeration, or

adjustment of the fermentation temperature (if necessary) to achieve successful re-fermentation.

Recent research *(18, 19, 2)* has shown the benefits to yeast physiology and fermentation performance of applying a mild osmotic stress. For example, preconditioning yeast cells with salt imparts an ability to tolerate subsequent fermentation stresses due to high alcohol content, high sugar concentrations, low pH and fluctuating temperatures. The present work was based on the hypothesis that the osmotic stress caused by NaCl would improve wine yeast viability, due to the accumulation of cellular protectant molecules. Furthermore, the hypothesis was tested on whether yeast cells would pre-adapt under osmotic stress conditions and if these can be used as inocula for alleviating stuck wine fermentations.

## MATERIALS AND METHODS

### *Yeasts cultures and growth conditions*

Three different yeast strains of *S. cerevisiae* and two non-*Saccharomyces* strains were used for laboratory experiments. *S. cerevisiae* winemaking strains designated as *Chardonnay, KD* and *SCM,* produced by Martin Vialatte Epernay France, were a kind gift from Ampeloiniki S.A. Thessaloniki Greece. The strains of *Kluyveromyces thermotolerans* and *K. marxianus* were supplied by the University of Abertay Dundee yeast culture collection.

Yeast cells were grown in a defined medium containing (per L deionised water): 100 g D-glucose , 1 g $K_2HPO_4$, 1 g $K_2H_2PO_4$, 0.2 g $ZnSO_4$, 0.2 g $MgSO_4$, 2 g yeast extract and 2 g $NH_4SO_4$. All the media components were purchased from the Sigma Chemical Company (St Louis, MO USA).

### *Inoculum preparation*

Dried yeast preparations were rehydrated as follows: 1 g dry weight of yeast was diluted in 100 mL of deionised water in an Erlenmeyer flask of 250 mL volume at 30-35$^{o}$C, for 30 min. Inocula for experimental fermentations were prepared as follows: after 48 h of pre-culturing, 10 mL was collected and centrifuged at 5000 rpm for 15 min. Cells were resuspended in deionised water and re-centrifuged. This was repeated twice prior to the determination of total cell number and cell viability in the final washed inoculum. An inoculum of $5x10^5$ of living cells was used to inoculate 250 mL of substrate.

### *Fermentation media preparation*

The medium for experimental laboratory fermentations consisted of the following: 200 g/L glucose, 1 g/L $K_2HPO_4$, 1g/L $K_2H_2PO_4$, 0.2 g/L $ZnSO_4$, 0.2 g/L $MgSO_4$, 2 g/L yeast extract and 2 g/L $NH_4SO_4$. Mineral components and glucose were sterilized separately at 120$^{o}$C, and 2 atm pressure for 20 min.

For salt stress induction experiments, the medium contained NaCl (commercial NaCl was used) from 1 to 10% w/v and the total volume for the medium for each fermentation medium was 250 mL. Batch fermentations were carried out in 300 mL volume flasks, containing 250 mL of growth medium, without shaking at 25°C. After inoculation, 1 mL samples were periodically taken directly from each flask in order to monitor the differences between stressed and un-stressed yeast cells, with respect to yeast population growth and cell viability.

*Yeast growth and viability determination*

The wine yeast strain VIN13, a kind gift from Anchor Ltd, South Africa, used for the experiments for the comparison of the two methods of viability determination.

Yeast cell number was determined using a haemocytometer (Thoma type) and yeast cell viability using the methylene blue method *(17)*.

Microscopic observations were made using an Olympus model CHK2-F-GS microscope. Yeast cell viability was calculated and expressed as follows: Viability (%) = a/n x 100
Where a: number of metabolically active cells; n: total cell number.

Yeast cell growth by colony counting was performed as follows: growth medium containing (per L deionised water): 10 g glucose, 5 g peptone, 4 g yeast extract and 15 g agar was prepared. After sterilization at 120$^{o}$C and 2 atm pressure for 20 min, approximately 2 mL of the medium was added to each Petri dish. Inoculation was made using 0.1 mL from each fermentation flask. Serial dilutions for 0% NaCl were $1/10^{-5}$ and for 1, 2, 3, 4, 5% NaCl was $1/10^{-4}$. Three Petri dish spread plate dishes were used for each measurement.

Since cellular viability needed to be determined immediately after hyperosmotic treatments, vital staining with methylene blue, which is simple and rapid, was used. However, compared to methods that determine yeast reproductive capacity, methylene blue staining slightly overestimates cell viability *(17)*. In this regard, comparison studies of yeast cell viability between the methylene blue method and plate counting methods were performed. Figure 1 shows minimal differences between the two methods.

*Industrial scale fermentations*

Experimental wine fermentations were conducted in stainless steel tanks of 12000 L and 6000 L containing wine must from Syrah and Chardonnay variety grapes.

Wine yeast (*S. cerevisiae*) cultures *Vitilevure Chardonnay* (2008) and *Vitilevure Syrah* (2010,2012) (Martin Vialatte Epernay France), kindly provided by Ampeloeniki SA Thessaloniki Greece, were used to inoculate grape must, using 1250 g of dry yeast diluted in 50 L water, containing 500 g of sucrose at 35°C.

Yeasts were subjected to salt adaptation by adding 3 kg of crude NaCl into the solution (6% w/v). After 16 h, yeast inocula were added to each fermentation vessel. Preconditioning of the yeast cells with a concentration of 6% w/v of NaCl was chosen since at this salt concentration, yeast cells retained the highest levels of growth and viability (Fig. 2 and 3).
The analysis of fermentations was made using Fourier-transformed near infrared spectroscopy with a FOSS Oenos WINE SCAN instrument (Foss DK-3400 Hillerod Denmark ).

*Statistical analyses*
All experiments at the laboratory scale were conducted in triplicate and the results are presented as the average of three measurements, with minimum and maximum standard error. Experiments were designed to examine the following: effects of various salt concentrations on cell growth and viability; effects of salt concentrations on different yeast species; and effect of salt preconditioning on fermentation performance – sluggish fermentation management. The average of the three values (separately) was calculated. Secondly, the average value (of the three average values) was calculated. Data was analyzed using the statistical programme BioStat Plus 2008 version 5.3.0.6 by AnalySoft Comp. Bracknel, UK using the Basic Describe Statistics package.

## RESULTS and DISCUSSION

### Laboratory scale fermentations

Experimental fermentations were conducted with three commercial wine yeast strains: *S. cerevisiae* Chardonnay, *S. cerevisiae* CSM and *S. cerevisiae* KD and two non-*Saccharomyces* strains: *K. thermotolerans* and *K. marxianus* (which exist as grape must microflora). These latter yeasts were chosen as it had been previously established that during industrial winemaking, a microbial succession occurs, in which alcoholic fermentation starts with non-*Saccharomyces* species such as *Kloeckera* or *Hanseniaspora,* followed by species of *Kluyveromyces, Torulaspora*, *Candida* and *Metchnikowia (6,9,7)* and finally by *Saccharomyces* species. The strains *K. thermotolerans* and *K.*

*marxianus* have been already used in the industry. For example, *K. thermotolerans* has been employed in mixed cultures with *S. cerevisiae* by C. Hansen Ltd (Copenhagen, Denmark) in wine fermentations and has also been a subject of research as a fermentation multistarter culture for alcoholic fermentations *(7)*. In general, *Kluyveromyces* species have been widely studied for fermentation, using glucose and lactose (from cheese whey) as carbon sources *(26)*. Figure 2 and 3 show that sodium chloride caused a similar growth arrest in the five yeast strains studied and that the difference between untreated cells, and the cells which were treated under the highest salt-induced stress (10% NaCl w/v), was around 2.5 x $10^6$ cells. When the sodium chloride concentration increased, the total cell number decreased concomitantly. For *K. marxianus,* the growth was similar to the *S. cerevisiae* strains, but *K. thermotolerans* demonstrated a difference in growth, particularly at 4% and 6% (w/v) NaCl. For the highest concentration of 10% (w/v) of sodium chloride studied, the growth curves appeared similar for all five species.

For the two species of *Kluyveromyces,* there appeared to be minimal effects of salt induced osmotic stress on yeast cell viability.

The three species of *S. cerevisiae* demonstrated similar responses, but at all sodium chloride concentrations employed in pre-cultures, increased cell viability was demonstrated, even at the highest sodium chloride concentration (10% w/v). For *S. cerevisiae* Chardonnay and for *S. cerevisiae* CSM strains, cell viability at the end of the fermentation was the same for all NaCl concentrations and remained at around 90%. For *S. cerevisiae* KD, the highest viability occurred with the 10% (w/v) NaCl treatments (Fig. 3 and 4).

Salt-induced osmotic stress may lead to increased cellular electrolyte levels in yeast and decreased cellular water potential *(24,25)*. Consequently, rapid efflux of water, cytoskeletal collapse, intracellular damage and growth arrest, are physiological phenomena, which follow the salt stress. Yeast cells adapt to these conditions retaining turgor pressure, polarizing the cytoskeleton, repairing cellular damage and resuming growth. It is conceivable that such responses are governed by the HOG MAP (High Osmotic Glycerol Mitogen Activated Protein) kinase pathway *(14, 15, 27)*. 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 over expression of GPD 1and FPS1, which encode the glycerol transport facilitator and glycerol-3- phosphate dehydrogenase, respectively. The temperature (24$^o$C) employed by Wojda et al. (27) was very close to the temperature employed in our experiments.

It has been reported that osmotic stress caused by 0.3 M (1.75 % w/v) sodium chloride for a time period of 1 h may prolong the life span of yeasts *(25)*. The relationship between temperature and

osmotic stress regarding osmotolerance and yeast cell viability has previously been reported *(27)*. For example at 23$^{o}$C, cell viabilities under osmotic pressures of 49.2 MPa and 99 Mpa were 94% and 25%, respectively, but at 99 MPa osmotic pressure at 5$^{o}$C, viability remained at a high level of 81% *(3)*. Overall, these findings suggest that salt pre-treatment of yeast; especially *S. cerevisiae* species impart a positive effect on cell viability.

Industrial scale winemaking trial fermentations

During the years 2008, 2010 and 2012, industrial scale winemaking procedures were conducted at Georgakopoulos Estate (Mendenitsa, Fthiotida, Greece), Thestia Etstate (Kainourgio, Agrinio, Aitoloakarnania, Greece), and Jasmin Art in Wines (Avlida, Halkida Central Greece). Stuck fermentations were observed in a 12000 L vessel for the year 2008; a 6000 L vessel for the year 2010 and a 5000 L vessel for the year 2012. Each vessel contained must from Syrah grape variety and for the last year must from a Chardonnay grape variety. The stuck fermentations were evidenced by the presence of residual sugar (10.6, 7.8, 8.9 g/L), persisting for a time period of 5 days. For secondary fermentation and for the re-inoculation, the procedure of inoculum preparation described in the Materials & Methods was followed. Figure 5 shows that this resulted in an increased alcohol production from 13.6% (v/v) to 14 % (v/v) for the year 2008; from 15.2% (v/v) to 15.8% (v/v) for the year 2009; and from 14.2% (v/v) to 15.3% (v/v) for the year 2012. Concomitantly, residual sugar concentration decreased to 3g/L , 3.5g/L and 3.4g/L for 2008, 2009 and 2012, respectively.

The volatile acidity decreased to 0.36, 0,35 and slightly increased to 0.32 g/L of acetic acid for the years 2008, 2010 and 2012, respectively.

The total acidity increased to 7 g/L of tartaric acid for years 2008 and 2010 and to 6.6 g/L for 2012 (Fig. 5). It is important to note that this procedure was not used with a specific alcohol tolerant yeast strain. The inoculation was solely conducted with salt-preconditioned *Vitilevure Chardonnay* and *Vitilevure Syrah* strains of *S. cerevisiae*.

Results shown in Fig. 5 suggest that salt-preconditioning of wine yeast for a specific time can adapt cells to enhanced alcohol tolerance without resulting in problems regarding sugar utilisation and volatile productivity. Pre-conditioning the cells using a salt-induced osmotic stress conferred the ability to ferment small amounts of residual sugars under high alcohol concentrations (13.2% (v/v), 15.2% (v/v) and 15.3% (v/v) for the 2008, 2010 and 2012 winemaking seasons, respectively). It was previously shown that such treatments had a similar positive effect on yeast viability in high gravity

wine fermentations *(20)*.

No fermentations using non-preconditioned yeast cells were conducted due to the economic risk for the participating wineries, regarding loss of product and a potential deleterious impact on final wine quality.

Traditional approaches to manage stuck fermentations are unduly time-consuming and frequently fail. However, without any further interventions, these fermentations remain stuck. Current findings reported in this paper have shown that salt preconditioning confers yeast cells with an ability to remain metabolically active under stress, and that such treatments could be beneficial in alleviating stuck fermentations in the industrial winemaking process.

## Conclusions

The results of the present work show that *S. cerevisiae* and non-*Saccharomyces* wine yeast cells exhibit limited growth under osmotic stress due to NaCl. However, surprisingly, all yeast strains retained good viability when exposed to high concentrations of NaCl. It is conceivable that under osmotic stress conditions, caused by NaCl, cell defence mechanisms are triggered including cell membrane compositional changes, together with elevated levels of compatible solutes (glycerol and trehalose), which confer on the cells an ability to survive for long time periods under extreme conditions. This could explain why salt-preconditioned yeasts can alleviate stuck fermentations, by fermenting the small amounts of residual sugars while at the same time tolerating alcohol toxicity. Further research with additional industrial yeast strains (e.g. brewing and bioethanol yeasts) will be required to verify the applicability of salt-preconditioning for other yeast biotechnologies.

REFERENCES

1. Attfield, P. V. and Bell, P.J.L. (2003) Genetics and classical genetic manipulations of industrial yeasts. In: Functional Genomics of Industrial Yeasts ( De Winde, J.H., Ed.),pp. 17-55, Springer-Verlag, Heidelberg.
2. Bellinger, Y., and Larher, F. A. (1987) 13C comparative nuclear magnetic resonance study of organic solute production and excretion by the yeast *Hansenula anomala* and *Saccharomyces cerevisiae* in saline media. *Can. J. Microbiol. 34,* 605-612.
3. Beney, L., de Marañón, I. M. , Marechal, P., Moundanga, S., and Gervais, P. (2001) Osmotic destruction of *Saccharomyces cerevisiae* is not related to a high water flow rate across the membrane. *Biochemical Engineering Journal 9(3)*,205-210
4. Bisson, L.F. and. Butzke, C.E. (2000) Diagnosis and Rectification of Stuck and Sluggish Fermentations. *Am. J. Enol. Vitic*. *51*,68-177.
5. Cavazza, A., Versini, G., DallaSerra, A., and Romano, F. (1989) Characterization of six *Saccharomyces cerevisiae* strains on the basis of their volatile compounds production, as found in wines of different aroma profiles. *Yeast 5*,163–167.
6. Ciani, M., and Maccarelli, F. (1998) Oenological properties of non-*Saccharomyces* yeasts associated with wine-making. *World Journal of Microbiology and Biotechnology 14*,199-203
7. Ciani, M,. Comitini, F., Di Pietro, N., Zacchi, L., Mannazzu, I. (2004) *Kluyveromyces phaffii* killer toxin active against wine spoilage yeasts: purification and characterization. *Microbiology 150*, 2535-41
8. Erasmus, D. J. (2003) Genome-wide expression analyses: metabolic adaptation of *Saccharomyces cerevisiae* to high sugar stress. *FEMS Yeast Res. 3*,375–399.
9. Fleet, G.H., Lafon-Lafourcade, S., and Ribéreau-Gayon, P. (1984) Evolution of yeasts and lactic acid bacteria during fermentation and storage of Bordeaux wines. *Applied and Environmental Microbiology 48*, 1034-1038.
10. Fuping, L., Yu Wang, Dongqing, B., and Lianxiang Du. (2005) Adaptive response of *Saccharomyces cerevisiae* to hyperosmotic and oxidative stress. *Process Biochemistry 40*,3614-3618.
11. García, M.R., Ríos, G., Ali, R., Bellés J.M., and Serrano, R. (1997) Comparative physiology of salt tolerance in *Candida tropicalis* and *Saccharomyces cerevisiae*. *Microbiology 143*,1125-1131.
12. Grando, M.S., Versini, G., Nicolini, G., and Mattivi, F. (1993) Selective use of wine yeast strains having different volatile phenols production. *Vitis 32*,43–50.
13. Heard, G.M., and Fleet, G.H. (1986) Evaluation of selective media for enumeration of yeasts

during wine fermentation. *Journal of Applied Bacteriology 60*,477-481.

14. Hohmann, S. (2002) Osmotic Stress Signalling and Osmoadaptation. *Yeasts Microbiology and Molecular Biology Reviews 66*,300-372.
15. Tamás, M.J. and Hohmann, S. (2003) The osmotic stress response of *Saccharomyces cerevisiae*. in Yeast Stress Responses (Hohmann, S. and Mager, W.H. Eds.) pp.121-200.
16. Jones, R.P. (1987) Factors influencing deactivation of yeast cells exposed to ethanol. *J. Appl. Microbiol. 63*,153-164.
17. Lee, S.S., Robinson, F.M., Wang, H.Y. (1981) Rapid determination of yeast viability Biotechnol. Bioeng. Symp.; (United States); Journal Volume: 11; Conference: 3. Symposium on biotechnology in energy production and conservation, Gatlinburg, TN, USA, 1981 Research Org Univ. of Michigan, Ann Arbor.
18. Logothetis S., Walker G., and Nerantzis E.T. (2007) Influence of salt hyper osmotic stress on yeast cells viability *Proc. Nat. Sci., Matica Srpska 113*, 271—284.
19. Logothetis, S. (2009) Influence of sodium chloride on wine yeast physiology and fermentation performance. Thesis, University of Abertay Dundee, Scotland.
20. Logothetis, S, Nerantzis, E, Goulioti, A, Kannelis, T, Tataridis, P and Walker, G. (2010) Influence of sodium chloride on wine yeast fermentation performance. *International Journal of Wine Research 2*,35-42
21. Meikle, A. J., Reed, R.H., Chudek, J.A., and Gadd, G.M. (1991) Natural abundance 13C-nuclear magnetic resonance spectroscopic analysis of acrylic polyol and trehalose accumulation by several yeast species in response to salt stress. *FEMS Microbiol. Let. 66(2)*,163-167.
22. Reed, R. H., Chudek, J.A., Foster, R., and Gadd, G.M. (1987) Osmotic significance of glycerol accumulation in exponentially growing yeasts. *Appl. Env. Microbiol. 53*,2119-2123.
23. Shimizu, H., Hirasawa, T., Ashitani, K., Yoshikawa, K., Nagahisa, K., Furusawa, C., Katakura, Y., and Shioya, S. (2006) Comparison of transcriptional responses to osmotic stress induced by NaCl and sorbitol additions in *Saccharomyces cerevisiae* using DNA microarray. *J. Biosc. Bioeng. 102*,568-571.
24. Soveral, G., Madeira, A., Loureiro-Dias, M.C., and Moura, F.T. (2007) Water transport in intact yeast cells assessed by fluorescence self. *Quenching Appl. Environ. Microbiol. 10*,1-12.
25. Swiecilo, A., Krawiec, Z., Wawryn, J., Bartosz, G., Bilinski, T. (2000) Effect of stress on the life span of the yeast *Saccharomyces cerevisiae*. *Acta Biochim. Polon. 47*, 355-364.
26. Walker, G. M., and O'Neil J.D. (1989) Morphological and metabolic changes in the yeast *Kluyveromyces marxianus* var. marxianus NRRLy2415 during fermentation of lactose. *J. Chem. Tech. Biotechnol. 49*,75-89.
27. Wojda, I., Alonso-Monge , R., Bebelman, J.P., Mager, W., and Siderius, M. (2003) Response

to high osmotic conditions and elevated temperature in Saccharomyces cerevisiae is controlled by intracellular glycerol and involves coordinate activity of MAP kinase pathways *Microbiology 149*,1193-1204.

Fig. 1 Comparison between the Methylene Blue Method (MBV) and Petri Dish Method (PDV) for the measurement of yeast cell viability. Cells of yeast strain VIN 13 were exposed to osmotic stress produced by sodium chloride. **(a)** 1% NaCl **(b)** 2% NaCl **(c)** 3% NaCl **(d)** 4% NaCl **(e)** 5% NaCl

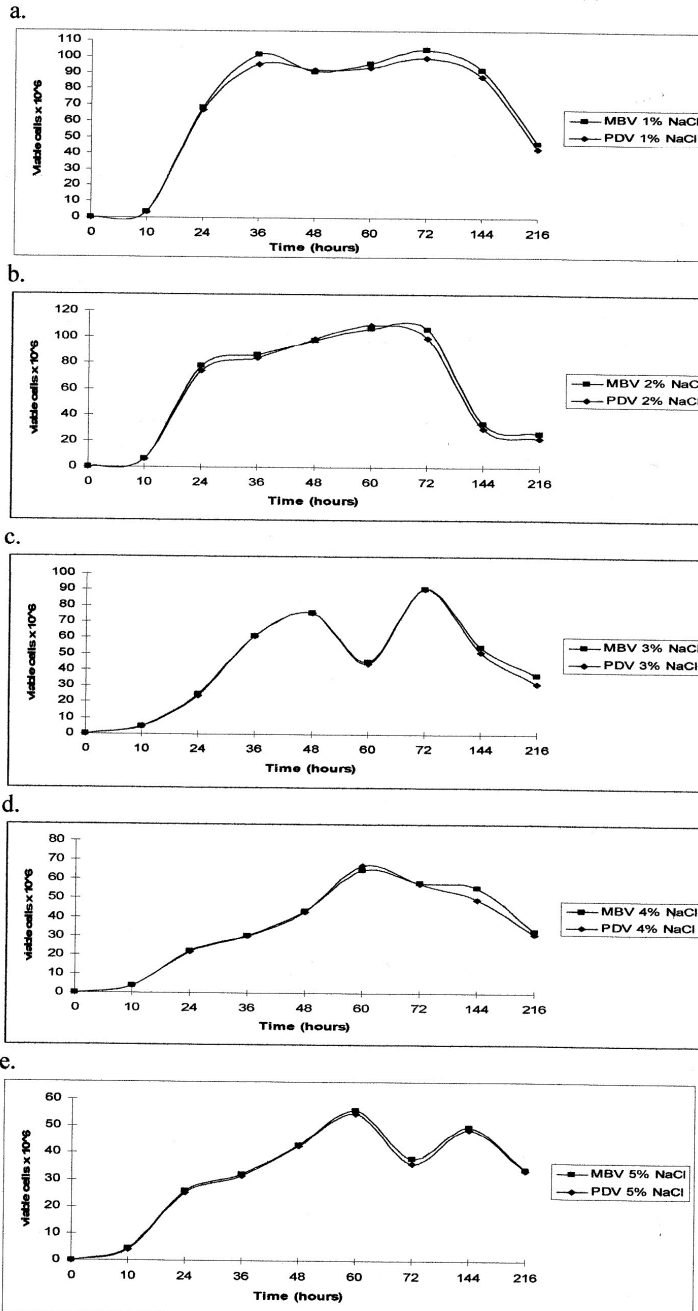
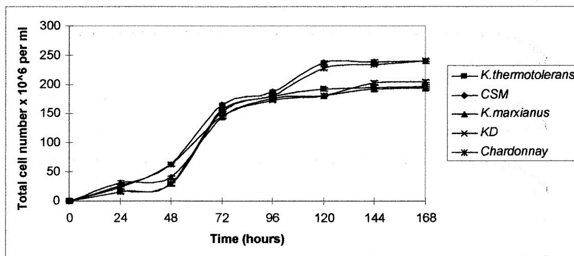


Fig. 2. Influence of sodium chloride on wine yeast growth. Three strains of *Saccharomyces cerevisiae* (strains Chardonnay, CSM and KD )) and two non-*Saccharomyces* (*K. thermotolerans* and *K. marxianus* strains were grown in glucose-based defined medium, without shaking at 25°C. Yeast cell growth and yeast viability was assessed. Sodium chloride levels (w/v) were : (a) 0% NaCl , (b) 4% NaCl, (c) 6% NaCl, (d) 10% NaCl w/v . Standard error was from 1.36 to 3.6 %.

a.

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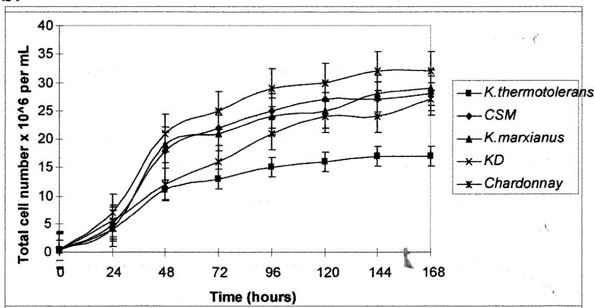


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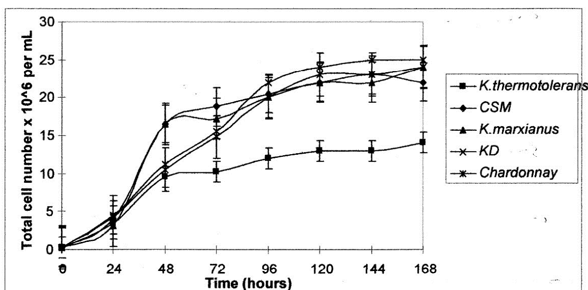


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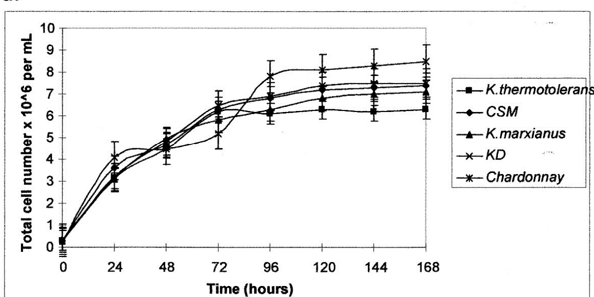
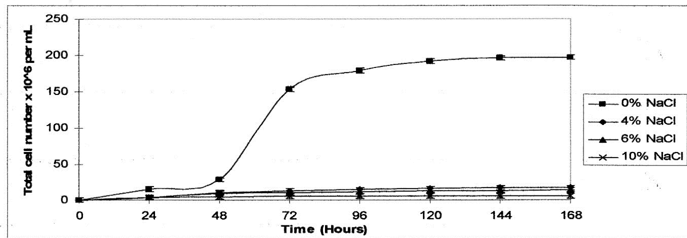


Fig. 3 Influence of sodium chloride on Saccharomyces and non-Saccharomyces cell growth.
Five yeast strains were grown in glucose-based defined medium, without shaking at 25°C. Yeast cell growth was determined using four different concentrations of sodium chloride. Yeast was: (a) *K. thermotolerans*, (b) *S. cerevisiae* CSM, (c) *K. marxianus*, (d) *S. cerevisiae* KD and (e) *S. cerevisiae* Chardonnay. Standard error was 0.88 to 3.78 %.

a.

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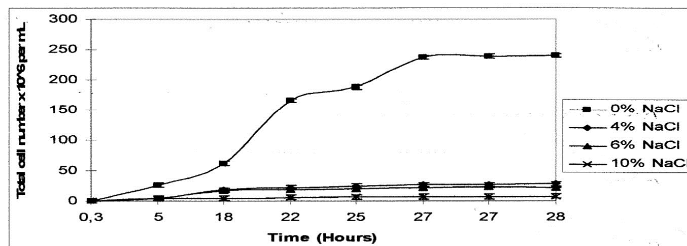
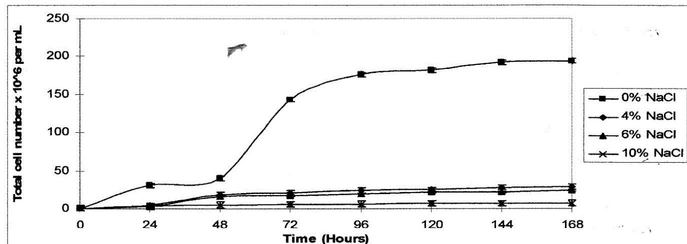



c.

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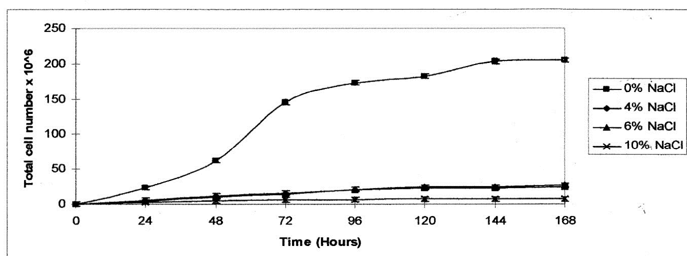


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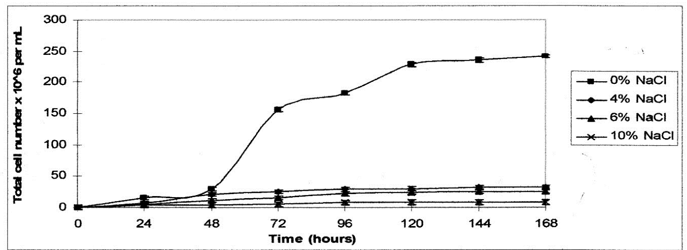
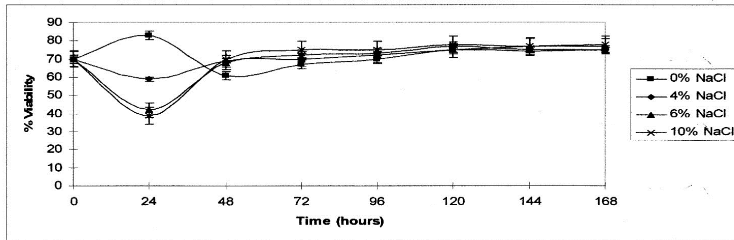


Fig.4 Influence of NaCl on yeast cell viability .Five strains of yeast were grown in glucose-based defined medium, without shaking at 25°C. Yeast cell viability was determined using four different concentrations of sodium chloride ( w/v 0%, 4%, 6%, 10% ). Yeast were: (a) *K. thermotolerans*, (b) *S. cerevisiae* CSM, (c) *K. marxianus*, (d) *S. cerevisiae* KD and (e) *S. cerevisiae* Chardonnay .Standard error was from 1.08 to 5.17 %.

**a.**

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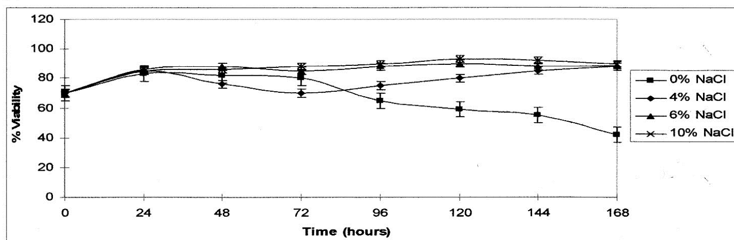


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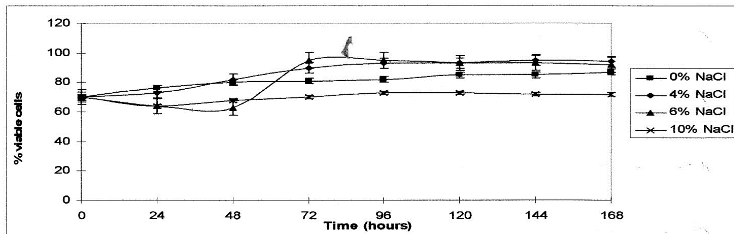


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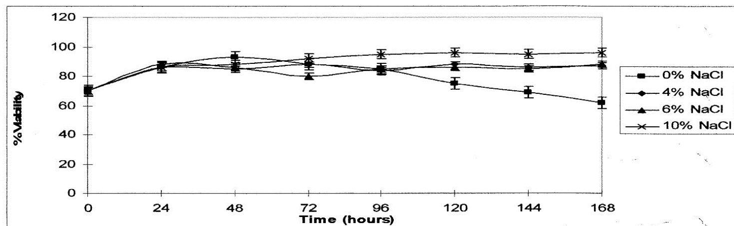


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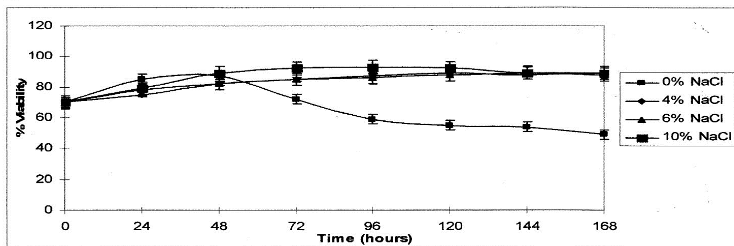

Fig. 5 Alleviation of stuck wine fermentation using salt preconditioned yeast ( 6% w/v NaCl and growing cells for 16h) . Yeast strains *Vitilevure Chardonnay* and *Vitilevure Syrah* were used for re-inoculation of 12000L, 6000L and 5000L stainless steel tanks containing Syrah and Chardonnay wine. Wine was filtered before inoculation. Solid arrow indicates the time of secondary inoculation. Analyse s : (a) alcohol (b) total acidity (c) residual sugars (d) glycerol and (e) acetic acid