New methodology for stuck fermentation management

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ABSTRACT

The influence of salt (sodium chloride) on cell physiology of wine yeast was investigated. Cellular viability and population growth of three winemaking strains of the yeast, *Saccharomyces cerevisiae*, and two non-*Saccharomyces* yeast strains associated with wine must micro flora 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 .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. The results indicate that salt *preconditioning* of yeast seed cultures may have potential benefits for industrial fermentation processes especially in the case of sluggish fermentations.

Key Words: Salt pre-conditioning, Saccharomyces c., non-Saccharomyces, stuck fermentation

INTRODUCTION

During alcoholic fermentation for wine production, yeasts are subjected to several physico-chemical stresses such as: 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, but very few studies of yeast stress responses have been reported in wine strains. In laboratory strains of *Saccharomyces cerevisiae*, many studies have focused on transcriptional activation and gene expression when cells are under stress. Such responses can be distinguished by different stages: cellular changes that occur immediately as direct consequences of the physico-chemical forces; activation of the primary defence 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[8] accumulation of cytosolic glycerol; and triggering of the HOG (Hyper Osmotic Glycerol) signalling pathway[11].

With salt (NaCl) stress, microorganisms such as the yeast *S. cerevisiae* develop systems to counteract the specific effects of sodium chloride. 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, specifically glycerol that are accumulated intracellularly[12,13,15]. Other products synthesized by yeast during osmostress conditions are trehalose and glycogen that may collectively represent 25% of the dry cell mass depending on the environmental conditions. The disaccharide trehalose accumulates not only during salt adaptation[3,16], but also in response to a number of other stress conditions including protection against high temperature 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 increases in water loss. 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 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 could account for this alteration in flux.

In modern wine production processes, winemakers occasionally need to cope with problems due to stuck and sluggish fermentations. *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. The resulting wines are high in residual sugar, making them particularly susceptible to microbial spoilage. Instability is increased further 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 that further retard growth. Osmotic effects of high sugar concentrations can also partially plasmolyze yeast cells, resulting in slow or incomplete fermentation. Researchers[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 provides early indications of potential problems and their possible quick resolution. Once fermentation has stopped, re-initiation is more complicated. When stuck fermentation occurs, successful re-initiation usually requires incremental re-inoculation with special yeast strains (obtained commercially), following racking off from settled lees[4]. The special strains usually possess high ethanol tolerance, as well as the ability to utilize fructose (the sugar whose proportion increases during fermentation). Other approaches include: the addition of supplementary nutrients, yeast “hulls” (cell wall preparations used to remove toxic fatty acids), must aeration, and adjustment of the fermentation temperature (if necessary) usually achieves successful re-fermentation.

Recent research[19,20,21] has shown the benefits of applying a mild osmotic stress to yeasts physiology and fermentation performance. 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 osmotic stress caused by NaCl would improve wine yeast viability due to accumulation of cellular protecting molecules and pre-adapted yeast cells under osmotic stress conditions can be used as inocula for stuck fermentation without any preparation.

2. MATERIALS AND METHODS

2.1 Yeasts cultures and growth conditions

Three different yeast strains of *S. cerevisiae* and two non-*Saccharomyces* strains were used for laboratory experiments. Strains designated as *Chardonnay, KD* and *SCM,* which were produced by Martin Vialatte Epernay France, were kindly gifted by Ampeloiniki S.A. Thessaloniki Greece. *Kluyveromyces thermotelerans* and *Kluyveromyces marxianus* were supplied by the University of Abertay Dundee yeast culture collection.

Yeast cells were grown in defined medium containing (per Lit deionised water): 100g D-glucose , 1g K2HPO4, 1g K2H2PO4, 0.2g ZnSO4, 0.2g MgSO4, 2g yeast extract and 2g NH4SO4. All the media components were purchased from Sigma Chemical Company St Luis USA.

2.2 Inoculum preparation

Dried yeast preparations were rehydrated as follows: 1g dry weight of yeast was diluted in 100 mL of deionised water in an Erlenmeyer flask of 250mL volume at 30-35 ^{o}C, for 30 min. Inocula for experimental fermentations were prepared as follows: after 48h of pre-culturing, 10

mL was collected and centrifuged at 5000rpm for 15min. Cells were resuspended in deionized water and re-centrifuged. This was repeated twice prior to determination of total cell number and cell viability in the final washed inoculum. $5x10^5$ of living cells was used as inoculum to inoculate 250 mL of substrate.

2.3 Fermentation media preparation

The medium for experimental laboratory fermentations consisted of the following: 200 g/L glucose, 1 g/L K2HPO4, 1g/L K2H2PO4, 0.2 g/L ZnSO4, 0.2 g/L MgSO4, 2g/L yeast extract and 2 g/L NH4SO4. Mineral components and the glucose were sterilized separately at 120^{o}C , and 2Atm pressure for 20min. For salt stress induction experiments, 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 300mL volume glass flasks containing 250mL of growth medium without shaking at 25°C. After inoculation 1mL were periodically taken direct 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.

2.4 Yeast growth and viability determination

Yeast cell number was determined using a haemocytometer (Thoma type) and yeast cell viability using the methylene blue method[18]. Yeast cell growth by colony counting was performed as follows: growth medium containing (g/L): 10 glucose, 5 peptone, 4 yeast extract and 15 agar was prepared. After sterilization at 120^{o}C and 2Atm pressure for 20min, approximately 2mL of the medium was added to each Petri dish. Inoculation was made using 0.1mL 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 was used for each measurement.

Cell viability was determined using a haemocytometer (Thoma type) as follows: 1mL of sample medium was taken and diluted in 9 mL of deionized water. 1mL of this solution was dissolved with 1mL of 10% v/v methylene blue solution and left for 10 min. Aliquots of 1μL were placed on the haemocytometer by using a Pasteur pipette. The haemocytometer was then microscopically observed by an optical microscope (Olympus model CHK2-F-GS microscope). Yeast cell viability was calculated and expressed as follow: Viability (%) = a/n x 100

Where a: number of metabolically active cells; n: total cell number. Since cellular viability needed to be determined immediately after hyperosmotic treatments, vital staining with methylene blue, which is rapid and accurate, was used. However, compared to methods that determine yeast reproducibility, methylene blue staining slightly overestimates cell viability[17]. In this regard, comparison studies between methylene blue method and plate counting methods regarding yeast cell viability have been performed. The studies of viability in Petri dishes performed to check the accuracy of the methylene blue method regarding the hypothesis that NaCl effect methylene blue and give fault results. According to Fig A the difference between the two methods was under 5% and was 2.2%.

2.6 Industrial scale Fermentations

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

Wine yeast cultures *Vitilevure Chardonnay* (2008) and *Vitilevure Syrah* (2010) (Martin Vialatte Epernay France) kindly provided by Ampeloeniki SA Thessaloniki Greece were used to inoculate grape must using 1250g of dry yeast diluted in 50L water containing 500g of sucrose at 35^{o}C. Yeasts were subjected to salt adaptation by adding 3kgr of crude NaCl into the solution (6% w/v). After 16 hours inocula were added to each fermentation vessel.

The analysis of fermentations was made using Fourier-transformed near infrared spectroscopy with a FOSS Oenos WINE SCAN instrument.

2.7 Statistical analyses

All experiments at laboratory scale were conducted in triplicate and the results are presented as the average of three measurements with min and max standard error. Experiments were designed to examine: effects of various salt concentrations on cell growth/viability; effects of salt concentrations on different yeast species; and effect of salt preconditioning on fermentation performance – sluggish fermentation management. The average of 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. Brachnell, UK using the Basic Describe Statistics package.

RESULTS and DISCUSSION

Experimental fermentations were conducted with three industrial yeast strains: *S. cerevisiae* Chardonnay, *S. cerevisiae* CSM and *S. cerevisiae* KD (which are commercial wine strains) and two non-*Saccharomyces* strains *K. thermotolerans* and *K. marxianus* (which exist as grape must micro flora) to investigate if similar phenomena of growth arrest and increased viability occurred that found on *S.cerevisiae Vin 13*[20] . Such yeasts were chosen since it is well known that during industrial wine making, alcoholic fermentation starts by non-*Saccharomyces* species like *Kloeckera* or *Hansensiaspora.* Then a microbial succession occurs followed by *Saccharomyces* species and species like *Kluyveromyces, Tolulospora*, *Candida* and *Metchenikowia*[6,9,14] *K. thermotolerans* and *K. marxianus* have been already used in industry. For example, *K. thermotolerans* has been employed in mixed culture with *Saccharomyces 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 source[27]. Fig 2 shows that sodium chloride caused similar growth arrest phenomena for the five yeast strains studied. These findings show that NaCl caused a growth arrest in yeast cells 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 x 10^6 cells. When sodium chloride concentration increased total cell number concomitantly decreased. For *K. marxianus,* the growth was almost the same as the *S.cerevisiae* strains. However, *K. thermotolerans* demonstrated a difference in growth especially at 4% and 6% w/v NaCl. For the highest concentration of 10% m/v of sodium chloride, the growth curve appears to very similar for all five species.

The phenomena of growth arrest caused by different sodium chloride concentrations, is represented in Figs 1 and 2. A to E represents the effect of osmotic stress on each yeast strain separately.

These results show that *K. thermotolerans* does not appear unduly affected by the high or low sodium chloride concentrations with regard to viability of the cells. At the beginning and at the end of fermentation cell viability was almost the same for the cells cultivated under osmotic stress conditions and without sodium chloride. The same phenomena also occurred for *K. marxianus* but for salt concentrations of 10% w/v it is apparent that viability was almost the same as that of the inoculum. For concentrations of 4% w/v and 6% w/v, cell viability was higher than the viability of the inoculum. 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 *Saccharomyces* demonstrated similar phenomena. Without NaCl

treatment, cell viability decreased over time for all species. However, at all sodium chloride concentrations employed in pre-cultures, an increased cell viability was demonstrated, even at the highest sodium chloride concentration of 10% w/v. Especially for *S.cerevisiae Chardonnay* and for *S.cerevisiae CSM* strains the viability at the end of the fermentation was the same for all NaCl concentrations and close to 90%. For *S.cerevisiae DK* the highest viability occurred at 10% NaCl treatments.

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[24,25]. Consequently, rapid efflux of water, cytoskeletal collapse, intracellular damage and growth arrest, are the phenomena which follow the 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 HOG MAP kinase pathway[15]. 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 GPD 1and FPS1, which encodes the glycerol transport facilitator and glycerol-3- phosphate dehydrogenase[28]. The temperature (24^{o}C) in these published studies was very close to the temperature that we have employed in our experiments.

It has been reported that osmotic stress caused by 0.3M (1,75 % NaCl w/v) sodium chloride and for a time period of 1h 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 49.2MPa cell viability was close to 94% at a temperature of 23^{o}C, but under higher osmotic pressure of 99MPa the viability decreased dramatically to 25%. It has been reported that under the same conditions of high osmotic pressure of 99MPa, but at a temperature of 5^{o}C, the viability remained at a high level of 81%[3]. Overall, these results show that salt pretreatment of yeast, especially *S.cerevisiae* species, had a positive effect on viability of the cells.

Industrial scale winemaking trials

During the years 2008 and 2010, in industrial scale winemaking procedures conducted at Georgakopoulos Estate, Mendenitsa, Fthiotida, Greece and Thestia Etstate, Kainourgio, Agrinio, Aitoloakarnania, Greece, a stuck fermentation of a 12000L vessel for the year 2008 and a 6000L vessel for the year 2010 containing must from Syrah grape variety occurred. The stuck fermentations were evidenced by the presence of residual sugars (10.6 g/L glucose) persisting for a time period of 5 days. For secondary fermentation and for the re-inoculation we followed the procedure of inoculum preparation described in Materials & Methods. Figures 1 to 5 show that this resulted in an increased alcohol production from 13.6% v/v to 14 % v/v for the year 2008 and from 15.2% v/v to 15.8% v/v for the year 2009. Concomitantly, residual sugar concentration decreased to 3 g/L and 3.5g/L, for 2008 and 2009.The volatile acidity decreased to 0.36 and 0,35 g/L of acetic acid and the total acidity increased to 7 g/L of tartaric acid for both years. The important point to be made here is that in this procedure we have not used any alcohol tolerant yeast strain but the inoculation was made with a preconditioned *Vitilevure Chardonnay* and *Vitilevure Syrah* strain.

The following results (Figure 4) indicate that salt-preconditioning of wine yeast for a specific time can adapt the particular yeast strain to enhanced alcohol tolerance without resulting in problems regarding sugar utilisation and volatile productivity.

These findings indicated that preconditioning cells under salt-induced osmotic stress conferred an ability to ferment small amounts of residual sugars under high alcohol concentrations since the starting concentrations of alcohol for 2008 was 13.2% v/v and for

2010 was 15.2 % v/v. We have previously shown such treatments to have a positive effect on yeast viability in high gravity fermentations[20], and our current findings indicate salt pre-conditioning gives yeast cells the ability to remain metabolically active under the stressful conditions of industrial wine fermentations. No fermentations using non-preconditioned yeast cells were conducted due to economic risk for the participating wineries regarding loss of product and potential deleterious impact on final wine quality. In addition, traditional approaches to manage stuck fermentations are unduly time-consuming and frequently fail. Without any further interventions, these fermentations remained stuck. Our current findings have shown that salt preconditioning confers yeast cells with an ability to remain metabolically active under stress, and that such treatments can prove beneficial in alleviating stuck fermentations in industrial winemaking processes.

Conclusion

It is well known that in the Black Sea and in the areas where salt was collected from the sea, there exists some species of halotolerant yeast and fungi. For example, the yeast *Debaromyces hansenii* is salt-tolerant and has been a subject of research for several years now.

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, all yeast strains surprisingly retained high viability when exposed to high concentrations of NaCl. It is conceivable that under osmotic stress conditions caused by NaCl, cell defense mechanisms are triggered including cell membrane compositional changes together with elevated levels of compatible solutes (glycerol and trehalose) that confer on cells an ability to survive for long time periods under extreme conditions. This may explain why salt-preconditioned yeasts can alleviate stuck fermentations by fermenting the small amounts of residual sugars and at the same time tolerating alcohol toxicity. Further research with additional industrial yeast strains (eg. brewing and bioethanol yeasts) will verify the applicability of NaCl-conditioning in other yeast biotechnologies.

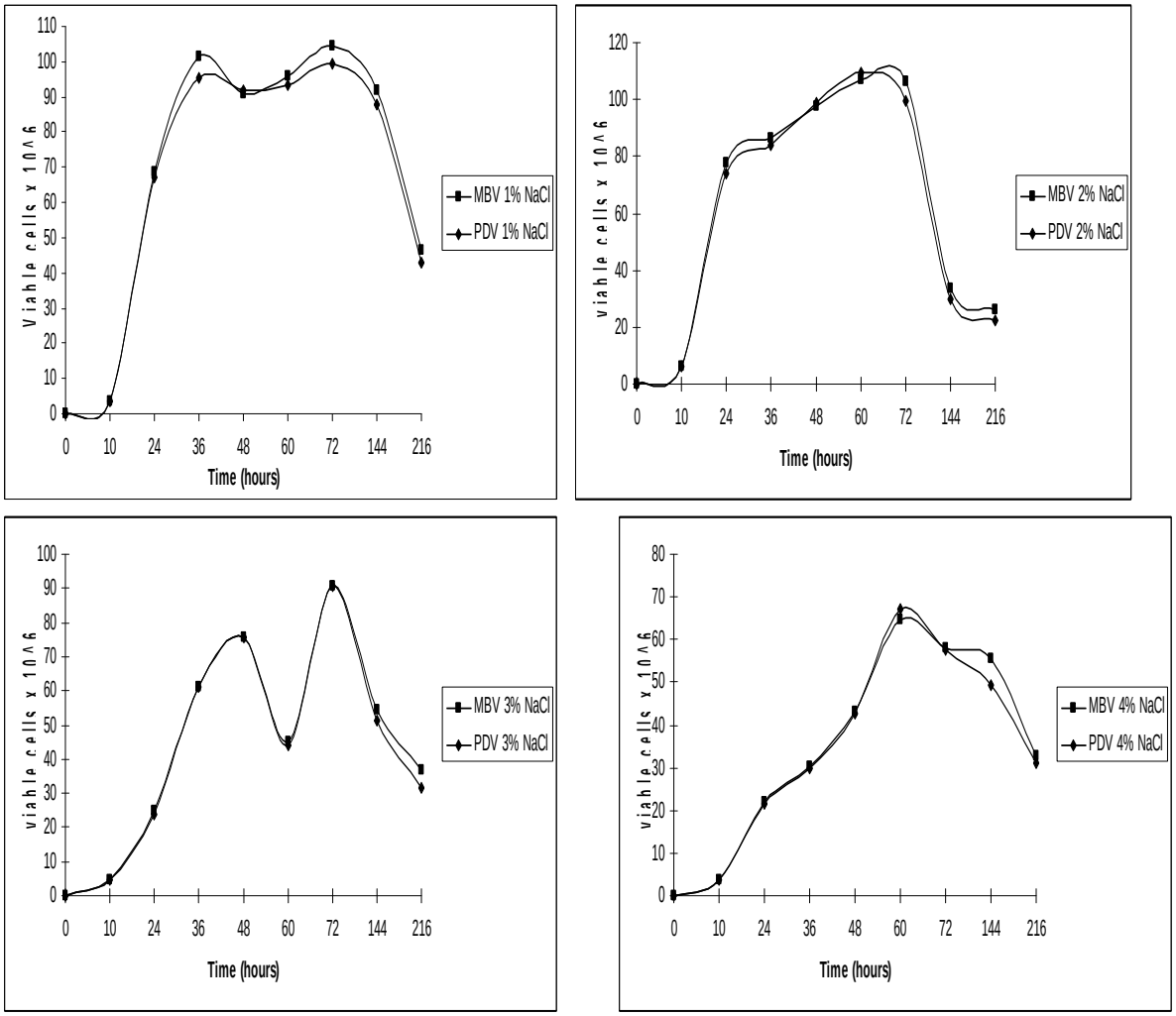
REFERENCES

1. Attfield, P. V. and Bell P.J.L. Genetics and classical genetic manipulations of industrial yeasts. In :Functional Genomics of Industrial Yeasts Springer-Verlag, 2003.
2. Bellinger, Y., and Larher, F. A 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.,* 1987, **34,** 605-612.
3. Beney, L., de Marañón, I. M. , Marechal, P., Moundanga, S., and Gervais, P. Osmotic destruction of Saccharomyces cerevisiae is not related to a high water flow rate across the membrane. *Biochemical Engineering Journal*, 2001, **9(3)**,205-210
4. Bisson, L.F.and. Butzke, C.E. Diagnosis and Rectification of Stuck and Sluggish Fermentations. *Am. J. Enol. Vitic*., 2000, **51**,68-177.
5. Cavazza, A., Versini, G., DallaSerra, A., and Romano, F. Characterization of six Saccharomyces cerevisiae strains on the basis of their volatile compounds production, as found in wines of different aroma profiles. *Yeast*, 1989, **5**,163–167.
6. Ciani, M., and Maccarelli, F. Oenological properties of non-Saccharomyces yeasts associated with wine-making. *World Journal of Microbiology and Biotechnology*, 1998, **14**,199-203
7. Ciani, M,. Comitini, F., Di Pietro, N., Zacchi, L., Mannazzu, I. Kluyveromyces phaffii killer toxin active against wine spoilage yeasts: purification and characterization.

Microbiology, 2004, **150,** 2535-41
8. Erasmus, D. J. Genome-wide expression analyses: metabolic adaptation of Saccharomyces cerevisiae to high sugar stress. 2003,*FEMS Yeast Res.,* **3,**375–399.
9. Fleet, G.H., Lafon-Lafourcade, S. & Ribéreau-Gayon, P. Evolution of yeasts and lactic acid bacteria during fermentation and storage of Bordeaux wines. *Applied and Environmental Microbiology*, 1984, **48,**1034-1038.
10. Fuping, L., Yu Wang, Dongqing, B., and Lianxiang Du. Adaptive response of Saccharomyces cerevisiae to hyperosmotic and oxidative stress. *Process Biochemistry*, 2005, **40,**3614-3618
11. García, M.R., Ríos, G., Ali, R., Bellés J.M., and Serrano, R. Comparative physiology of salt tolerance in Candida tropicalis and Saccharomyces cerevisiae. *Microbiology*, 1997 **143,**1125-1131
12. Grando, M.S., Versini, G., Nicolini, G., and Mattivi, F. Selective use of wine yeast strains having different volatile phenols production. *Vitis*, 1993, **32,**43–50
13. Heard, G.M., & Fleet, G.H. Evaluation of selective media for enumeration of yeasts during wine fermentation. *Journal of Applied Bacteriology,* 1986,**60,**477-481
14. Hohmann, S. Osmotic Stress Signaling and Osmoadaptation. *Yeasts Microbiology and Molecular Biology Reviews,* 2002,**66,**300-372
15. Hohmann,S. and Mager,W.H. The osmotic stress response of Saccahromyces cerevisiae. *In* Yeast stress response Chapter 4, 2005, pp.133-162
16. Jones, R.P. Factors influencing deactivation of yeast cells exposed to ethanol. *J. Appl. Microbiol.*, 1987. **63,**153-164
17. Lee, S.S., Robinson, F.M., Wang, H.Y. 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 OrgUniv. of Michigan, Ann Arbor
18. Logothetis S., Walker G., Nerantzis E.T. Influence of salt hyper osmotic stress on yeast cells viability *Proc. Nat. Sci., Matica Srpska*, 2007, **113,** 271—284.
19. Logothetis, S. Influence of sodium chloride on wine yeast physiology and fermentation performance. 2009 Thesis, University of Abertay Dundee, Scotland.
20. Logothetis, S, Nerantzis, E, Goulioti, A, Kannelis, T, Tataridis, P and Walker, G. Influence of sodium chloride on wine yeast fermentation performance. International *Journal of Wine Research*., 2010, **2,**35-42
21. Meikle, A. J., Reed, R.H.,Chudek, J.A., and Gadd, G.M. 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*., 1991, **66(2),**163-7
22. Reed, R. H., Chudek, J.A., Foster, R.,and Gadd, G.M. Osmotic significance of glycerol accumulation in exponentially growing yeasts. *Appl. Envirom. Microbial*., 1987, **53,**2119-2123.
23. Shimizu, H., Hirasawa, T., Ashitani, K., Yoshikawa, K., Nagahisa, K., Furusawa, C., Katakura, Y., Shioya, S. Comparison of transcriptional responses to osmotic stress induced by NaCl and sorbitol additions in Saccharomyces cerevisiae using DNA microarray. *J. Biosc. Bioen*., 2006, **102,**568-571.
24. Soveral G., Madeira A., Loureiro-Dias M.C., Moura F.T. Water transport in intact yeast cells assessed by fluorescence self. *Quenching Appl. Environ. Micriob*., 2007, **10,**1-12
25. Swiecilo, A., Krawiec, Z., Wawryn, J., Bartosz, G., Bilinski, T. Effect of stress on the life span of the yeast Saccharomyces cerevisiae. *Acta Biochim Polon.*, 2000, **47,**355-364.
26. Walker, G. M. & O'Neil J.D. Morphological and metabolic changes in the yeast Kluyveromyces marxianus var. marxianus NRRLy2415 during fermentation of lactose.

J. Chem. Tech. Biotechnol., 1989, **49,**75-89
27. Wojda, I., Alonso-Monge , R., Bebelman, J.P., Mager, W., and Siderius, M. 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*, 2003, **149,**1193-1204

Figure A: Comparison between methylene blue method (MBM) and Petri dishes method (PDM) for the measurement of yeast cell viability. Cells of yeast strain VIN 13 (kindly gifted by Anchor south Africa was exposed to osmotic stress produced by 1%-5% of NaCl.



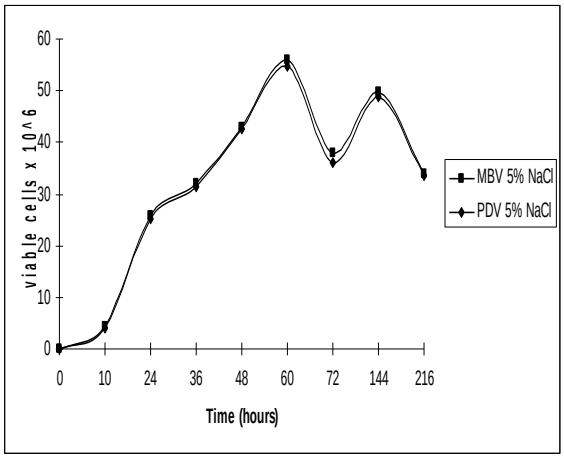
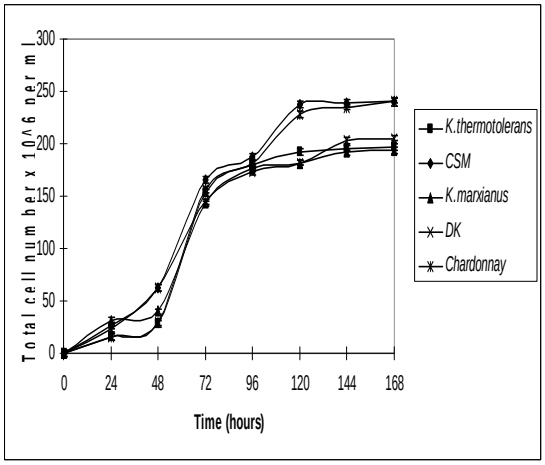
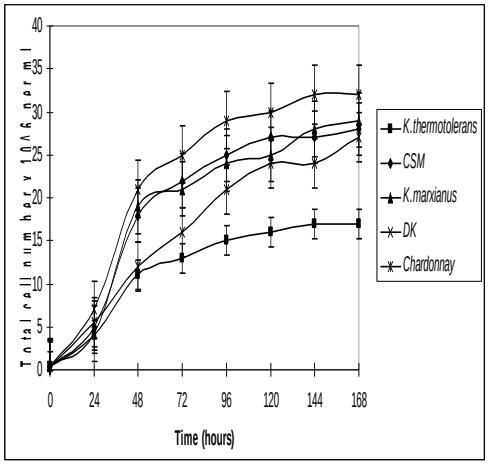


Figure 1 **Influence of sodium chloride on wine yeast growth**

Three strains of *Saccharomyces cerevisiae* (strains Chardonnay, CSM and KD from Martin Vialate, France kindly provided by Ampeloiniki S.A. Thessaloniki Greece) and two non-*Saccharomyces* (*K. thermotolerans* and *K. marxianus* (from the yeast culture collection of University of Abertay Dundee) were grown in glucose-based defined medium, without shaking at 25°C. Yeast cell growth was determined using haemocytometer (Thoma type) and yeast viability was assessed using the methylene blue method at the intervals shown. Standard error was from 1.36 to 3.6 %. Where a. 0% NaCl , b. 4% NaCl, c.6% NaCl, d.10% NaCl w/v

A. B.





C. D.

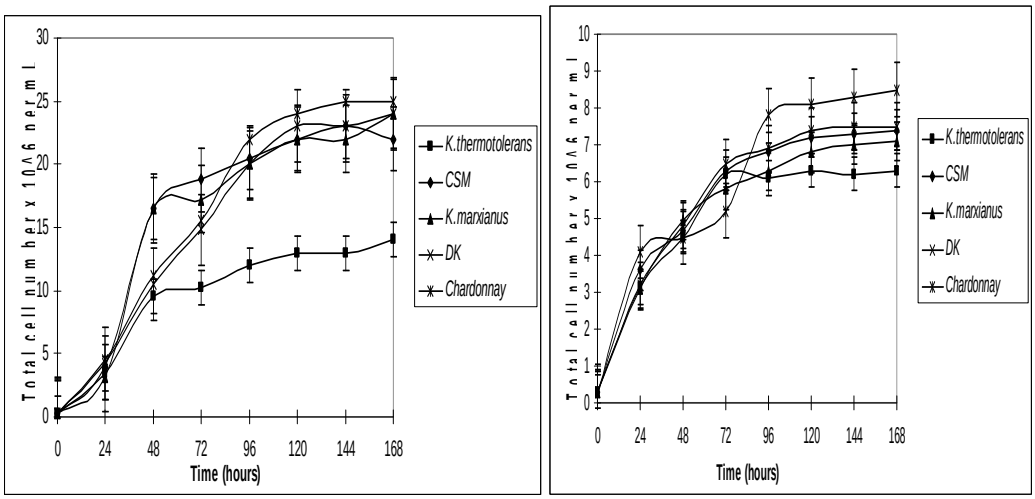
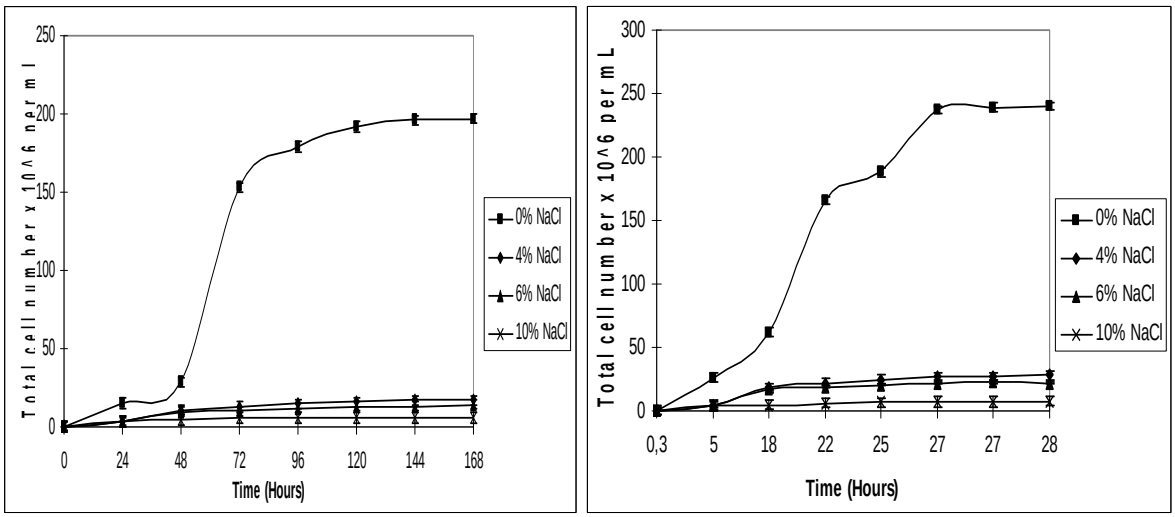


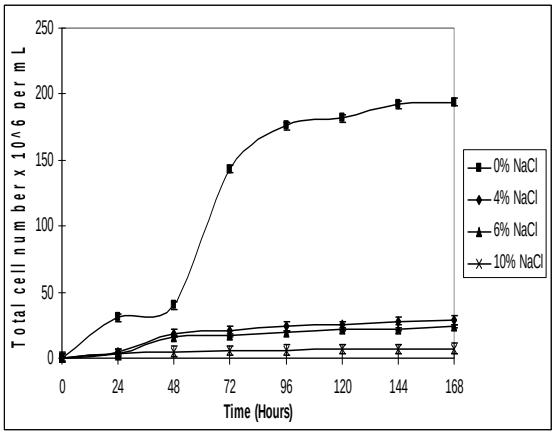
Figure 2 **Influence of sodium chloride on *Saccharomyces* and *non-Saccharomyces* cell growth**

Three strains of *Saccharomyces cerevisiae* (strains Chardonnay, CSM and KD from Martin Vialate, France kindly provided by Ampeloiniki S.A. Thessaloniki Greece) and two non-S*accharomyces* (*K. thermotolerans* and *K. marxianus* (from the yeast culture collection of University of Abertay Dundee) were grown in glucose-based defined medium, without shaking at 25°C. Yeast cell growth was determined using haemocytometer (Thoma type) Four different concentrations of sodium chloride were used 0%, 4%, 6%, 10% NaCl w/v. Standard error was from 0.88 to 3.78 %. Where a.K Thermotolerance, b. S.cerevisiae CSM, c.K Marxianus, d.S.cerevisiae KD and e. S cerevisiae Chardonnay

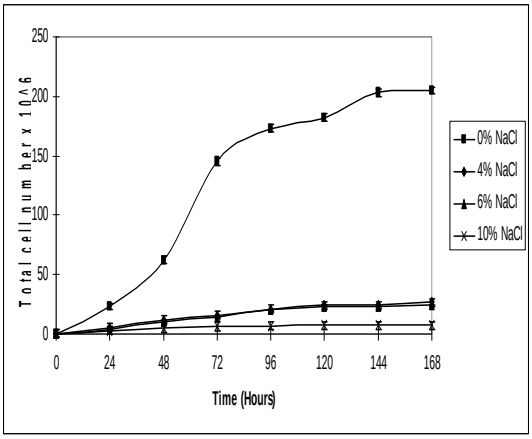
A. B.



C.



D



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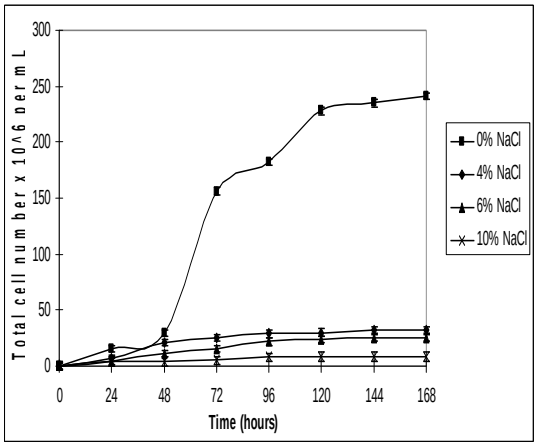
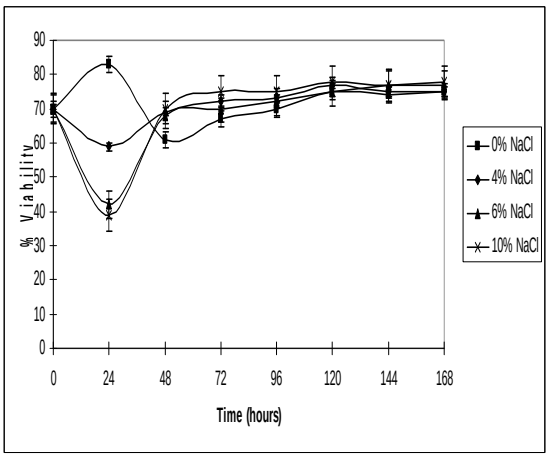


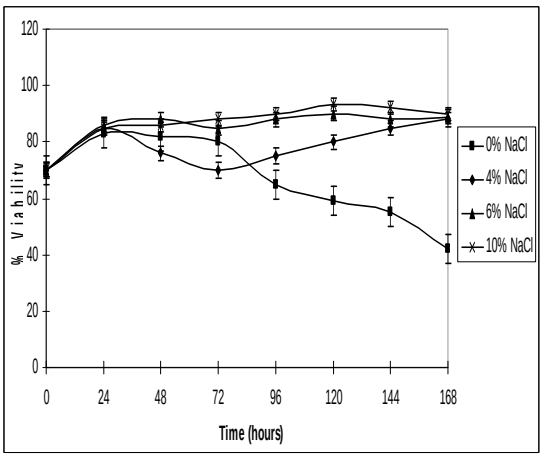
Figure 3 **Influence of NaCl on yeast cell viability**

Three strains of *Saccharomyces cerevisiae* (strains Chardonnay, CSM and KD from Martin Vialate, France kindly provided by Ampeloiniki S.A. Thessaloniki Greece) and two non-S*accharomyces* (*K. thermotolerans* and *K. marxianus* (from the yeast culture collection of University of Abertay Dundee) were grown in glucose-based defined medium, without shaking at 25°C. Yeast cell viability was determined using haemocytometer (Thoma type) Four different concentrations of sodium chloride were used 0%, 4%, 6%, 10% NaCl w/v. Standard error was from 1.08 to 5.17 %. Where A. K Thermotolerance, B. *S.cerevisiae* CSM, C. *K Marxianus*, D. *S.cerevisiae* KD and E. *S cerevisiae* Chardonnay

A.



B.



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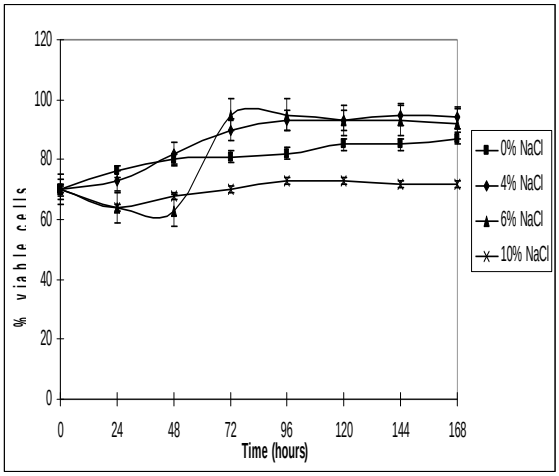

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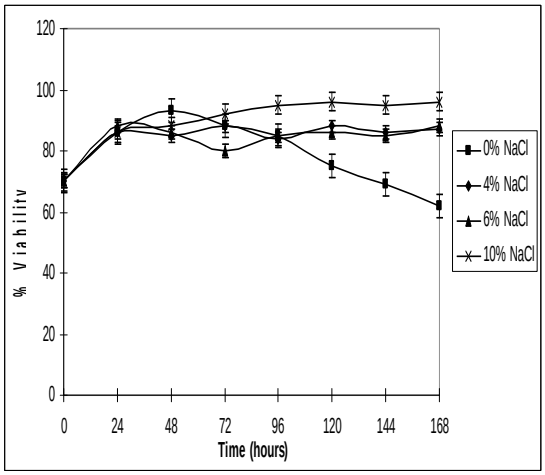

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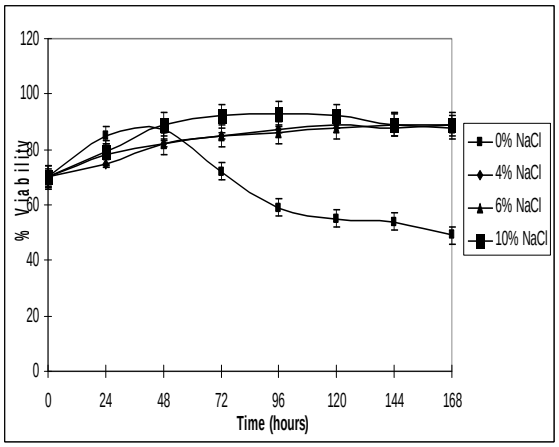
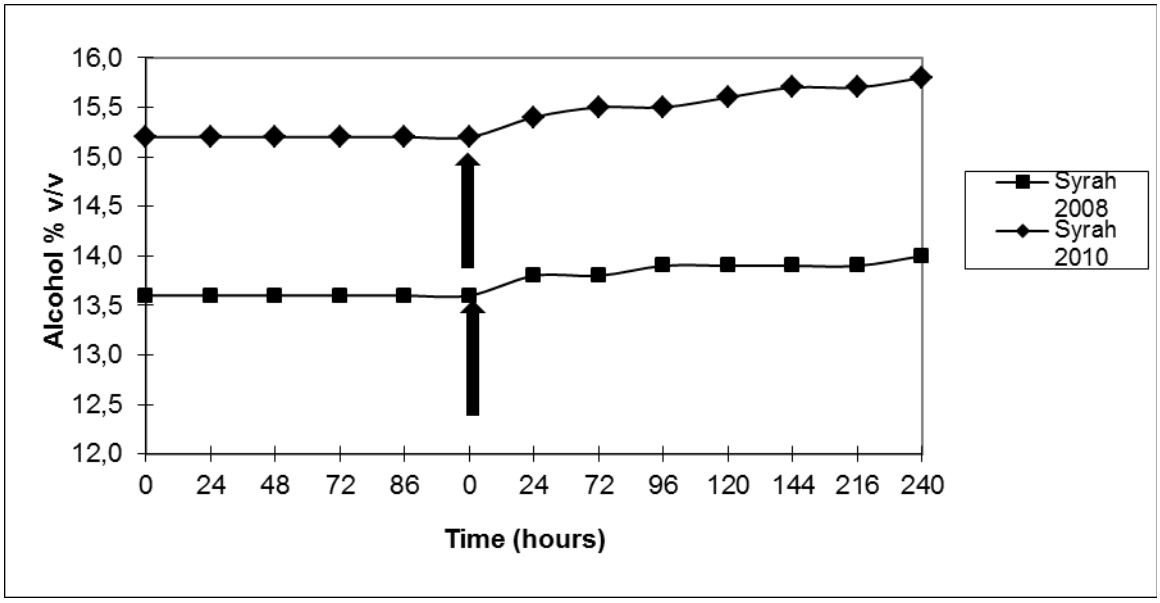


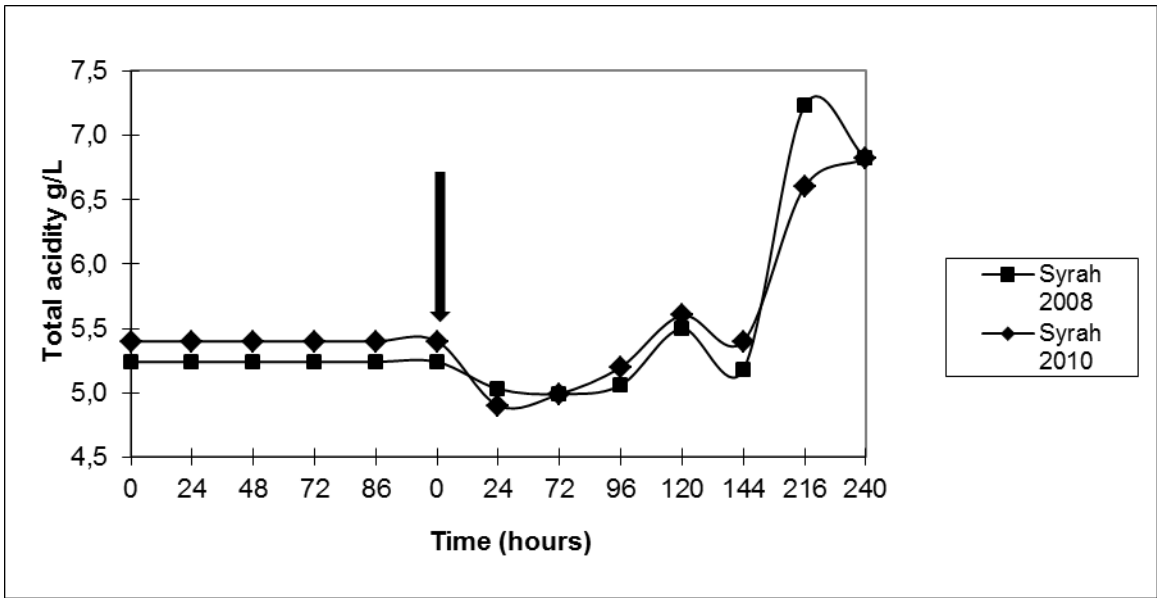
Figure 4 Alleviation of stuck wine fermentation using salt preconditioned yeast: a) alcohol b) Total acidity c) Residuals sugars d) glycerol and e) acetic acid

Salt preconditioning was achieved using 6% w/v NaCl and growing cells for 16 hours. Yeast strains *Vitilevure Chardonnay* and *Vitilevure Syrah* was used for re-inoculation of 12000L and 6000L stainless steel tanks containing Syrah wine. Wine was filtered before inoculation. Solid arrow indicates the time of secondary inoculation

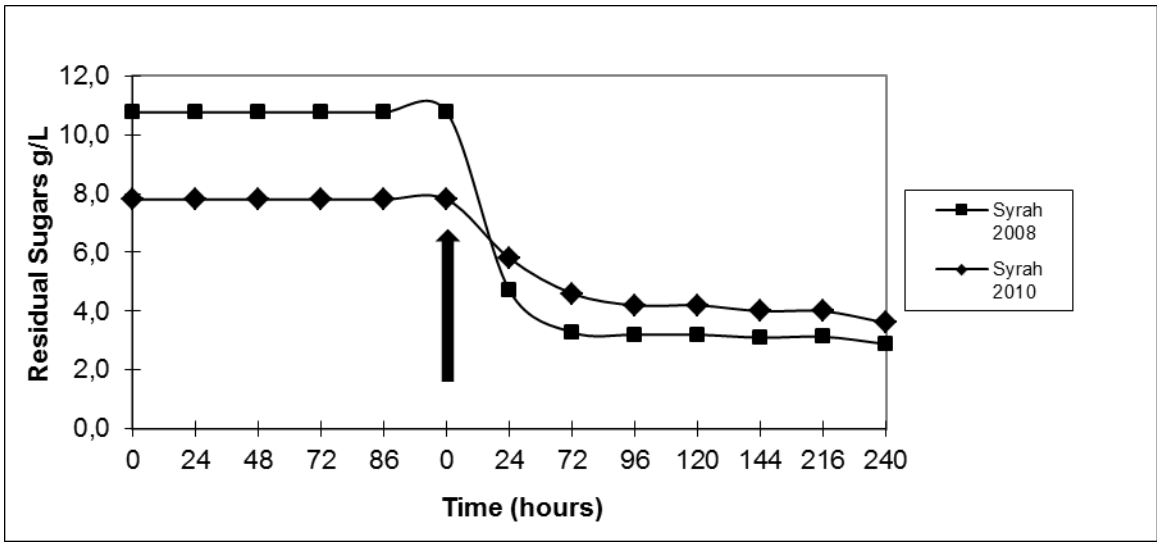
a.


16,0
15,5
15,0
14,5
14,0
13,5
13,0
12,5
12,0
Alcohol % v/v
0 24 48 72 86 0 24 72 96 120 144 216 240
Time (hours)
Syrah 2008
Syrah 2010

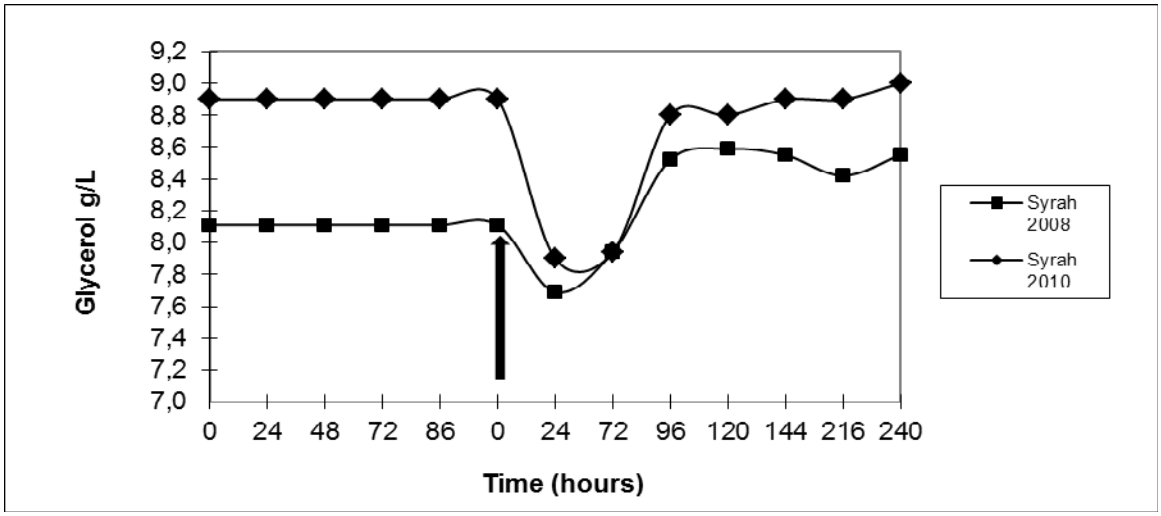
b.


7,5
7,0
6,5
6,0
5,5
5,0
4,5
Total acidity g/L
0 24 48 72 86 0 24 72 96 120 144 216 240
Time (hours)
Syrah 2008
Syrah 2010

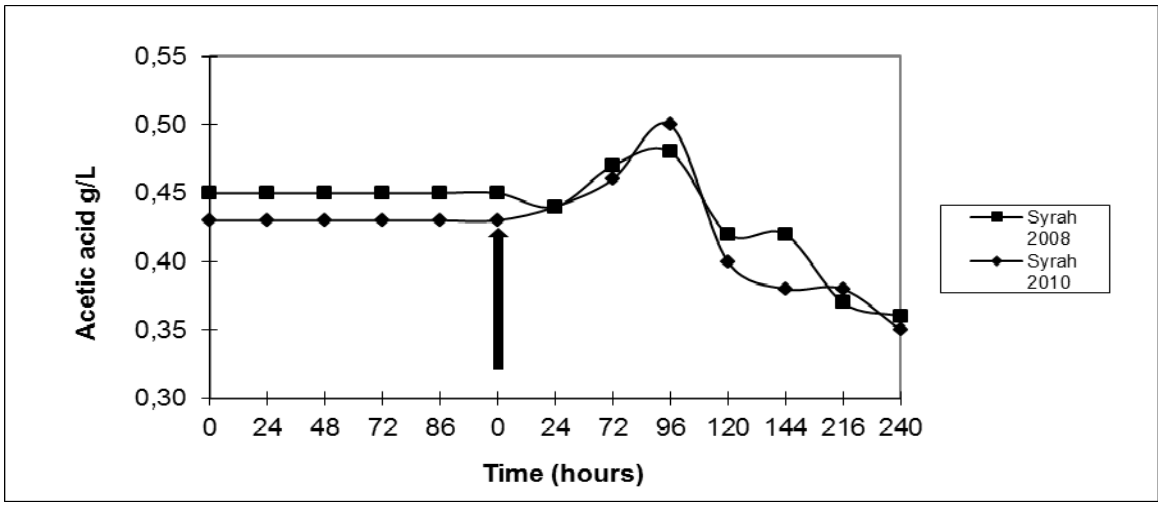
c.


12,0
10,0
8,0
6,0
4,0
2,0
0,0
Residual Sugars g/L
0 24 48 72 86 0 24 72 96 120 144 216 240
Time (hours)
Syrah 2008
Syrah 2010

d.


Glycerol g/L
9,2
9,0
8,8
8,6
8,4
8,2
8,0
7,8
7,6
7,4
7,2
7,0
0 24 48 72 86 0 24 72 96 120 144 216 240
Time (hours)
Syrah 2008
Syrah 2010

e.


Acetic acid g/L
0,55
0,50
0,45
0,40
0,35
0,30
0 24 48 72 86 0 24 72 96 120 144 216 240
Time (hours)
Syrah 2008
Syrah 2010