THE EFFECT OF PRECONDITIONING CELLS UNDER OSMOTIC STRESS ON HIGH ALCOHOL PRODUCTION

ABSTRACT: This paper focuses on the research into the influence of salt on physiology of the yeast, *Saccharomyces cerevisiae*. Specifically, the work focused on how NaCl affected the growth, viability and fermentation performance of this yeast in laboratory-scale experiments. One of the main findings of the research presented involved the influence of salt “preconditioning” of yeasts which represents a method of pre-culturing of cells in the presence of salt in an attempt to improve subsequent fermentation performance. Such an approach resulted in preconditioned yeasts having an improved capability to ferment high-sugar containing media (up to 60% w/v of glucose) with increased cell viability and with increased levels of produced ethanol (higher than 20% in vol.). Salt-preconditioning was most likely influencing the stress-tolerance of yeasts by inducing the synthesis of key metabolites such as trehalose and glycerol which act to improve cells’ ability to withstand osmostress and ethanol toxicity. The industrial-scale trials using salt-preconditioned yeasts verified the benefit of the physiological engineering approach to practical fermentations. Overall, this research has demonstrated that a relatively simple method designed to adapt yeast cells physiologically – by salt-preconditioning – can have distinct advantages for alcohol fermentation processes.

KEY WORDS: alcohol production, osmotic stress, preconditioning, viability, yeast

1. INTRODUCTION

During alcoholic fermentation, yeasts are subjected to several physico-chemical stresses such as: initially, high sugar concentrations and low temperature; and latterly, increasing ethanol 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.
Such responses can be distinguished by different stages: cellular changes that occur immediately as direct consequences of the physic-chemical forces; activation of the primary defensive processes and changes in cell homeostasis. Concerning the osmostress, a number of physiological changes take place, including: efflux of intracellular water, with associated rapid reduction in total cell volume, including the vacuole (Albertyn et al., 1994); transient increases in glycolytic intermediates (Alexandre and Charpentier, 1995); accumulation of cytosolic glycerol; and triggering of the HOG (Hyper Osmotic Glycerol) signaling pathway (Alexandre H. et al., 2001).

With salt 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 (Allison S. D. et al., 1999, Arnegborg N. et al. 1995 Attfield P. V., 1987). Other products synthesized by yeast under 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 (Attfield P. V., 1997, Attfield P. V. and Bell P.J.L., 2003), 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 (Backhus L. E., 2001, Bellingery Y. and Larher F., 1987, Beney L., et al., 2001).

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 the 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) (Bett K. E. and Cappi J. B., 1965, Blomberg A., and Adler L., 1989, Blomberg A., and Adler L., 1992, Brewster J. L. et al., 1993). Under osmotic stress, GPD activity is enhanced and this requires an equimolar amount of cytoplasmic 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.
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 inoculums to run high gravity fermentations and to produce high alcohol content.

Recent research (Logothetis S. et al., 2006, 2007, 2012) 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.

2. MATERIALS AND METHODS

2.1 Yeast cultures

Three different yeast strains of \textit{S. cerevisiae} were used for laboratory experiments. Strains designated as \textit{DV 10}, \textit{Fermol} and \textit{Syrah}, which were produced by Lalvin kindly gifted by EnoChimiki S.A. Athens Greece, Speedal S.A. Thessaloniki Greece and Martin Vialatte, were kindly gifted by Ampeloiniki S.A. Thessaloniki Greece.

2.2 Inoculum preparation (cell preconditioning)

Yeast cells were grown in defined medium containing (per liter deionised water): 100 g D-glucose, 1 g K\textsubscript{2}HPO\textsubscript{4}, 1 g K\textsubscript{2}H\textsubscript{2}PO\textsubscript{4}, 0.2 g ZnSO\textsubscript{4}, 0.2 g MgSO\textsubscript{4}, 2 g yeast extract and 2 g NH\textsubscript{4}SO\textsubscript{4}. All the media components were purchased from Sigma Chemical Company. Medium contained NaCl (commercial NaCl was used) 6% w/v for 24 hours were used for pre conditioning under osmotic stress experiments. The total volume of the medium for each fermentation medium was 250 ml.

2.3 Fermentation media preparation

The medium for experimental laboratory fermentations consisted of the following: 550 g/L glucose, 1 g/L K\textsubscript{2}HPO\textsubscript{4}, 1 g/L K\textsubscript{2}H\textsubscript{2}PO\textsubscript{4}, 0.2 g/L ZnSO\textsubscript{4}, 0.2 g/L MgSO\textsubscript{4}, 2 g/L yeast extract and 2 g/L NH\textsubscript{4}SO\textsubscript{4}. Mineral components and the glucose were sterilized separately at 120°C, and 2 Atm pressure for 20min. Batch fermentations were carried out in 300 ml volume of glass flasks containing 250 ml of growth medium without shaking at 25°C. After inoculation, 1 mL was periodically taken directly from each flask in order to monitor the differences between stressed and unstressed yeast cells with respect to yeast population growth and cell viability.
2.4 Yeast growth and viability determination

Yeast cell number was determined by using the Thoma haemocytometer and yeast cell viability by using the methylene blue method of Lee et al., (1981).

Cell viability was determined using the Thoma haemocytometer as follows: 1 mL of sample medium was taken and diluted in 9 mL of deionised water. The sample of 1 mL of this solution was dissolved with 1 mL of 10% v/v methylene blue solution and left for 10 minutes. 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.

2.5 Fermentation analysis

The analysis of glucose, alcohol and glycerol was made by ELITE LaChrom HPLC system comprised of a VWR HITATCH L-2130 pump, a VWR HITATCH L-2200 autosampler fitted with a 20 µL sample loop, and a VWR HITATCH L-2455 Diode Array detector. Peak data were collected with Agilent EZChrom Elite Client/Server Enterprise Data System Aminex HPX-87X.

2.6 Statistical analyses

All experiments were conducted in triplicate. Results were analyzed with the statistical program called BioStat Plus 2008 (version 5.3.0.6 by AnalySoft Comp) using the Basic Describe Statistics package.

3. RESULTS AND DISCUSSION

3.1 The effect of preconditioning under osmotic stress conditions caused by NaCl on growth and viability of the industrial yeast strains

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 (Rodríguez-Navarro and Ortega, 1981). Similarly, Fuping et al. (2005) have shown that treatment of S. cerevisiae cells with KCl of up to 6% causes a great loss of viability. A gradual in-
crease of sodium chloride in a growth medium containing yeast cells can cause a cell growth arrest depending on the sodium chloride concentration (Norberg and Blomberg, 1997). Experiments have shown that the leavening ability of baker yeasts decreases dramatically during cultivation in media containing sodium chloride concentrations between 0 and 3% (oda and Tomura, 1992). Previous research (Morris et al., 1986) showed that the greatest loss in cell viability was caused under hyper osmotic 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 (Morris et al., 1986). Comparative studies on the effect of osmotic stress on \textit{S. cerevisiae} and non-\textit{Saccharomyces} strains showed that non-\textit{Saccharomyces} strains displayed higher salt tolerance (Garsia et al., 1997). In general, previous studies have revealed that osmotic stress and especially sodium chloride-induced stress cause growth arrest and have negative effect on the viability of yeast cells.

In contrast, latest research works have shown that long adaptation to saline stress (up to 10% of NaCl w/v) have positive effect on yeast cell viability (Logothetis S. et al., 2006).

In the following laboratory-scale series of experiments (Figures 1 to 2), the effects of preconditioning to saline stress and saline stress (6% NaCl w/v) on industrial wine yeast cells growth and viability were investigated. The findings indicate that the preconditioning caused a faster growth and the difference between untreated cells and the cells which were pretreated under osmotic stress was significantly high. The results shown in Fig 1 indicate the difference between the three species. If we compare Figures 1 and 2 which show yeast growth and yeast viability, it can be seen that yeast cells exposed to sodium chloride have the highest growth in comparison to the cells which were untreated. Especially, Figure 2 shows the highest difference regarding cell growth for a time period of 88 hours. Previous research supports the concept that when \textit{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 the previous treatment with 0.7 M NaCl (4.09 % NaCl w/v) (Varela et al., 1992). Overall, previous reports describe the osmotic stress of yeast cells for no more than a few hours, but in the current research, cells are treated for 120 hours (Fig. 1) during the fermentation process.

\textbf{3.2 High Gravity Fermentation}

Previous studies have reported that some agents, like sodium chloride, play an important role in minimizing or inhibiting the fermentation process, specifically with regard to glucose utilization for the production of yeast biomass (El-Samary and Zali, 1988). Much earlier studies regarding this subject had been performed. For example, in the 1920s, studies concerned with the fermentability of yeasts during cereal wort fermentation under different sodium chloride concentrations showed that the fermentative ability of
Fig. 1. – Influence of salt-induced osmotic stress (6% w/v NaCl) on yeast cell growth. *Saccharomyces cerevisiae* (strains DV10, Syrah and Fermol) was grown on a glucose-based defined medium, without shaking at 25°C. Yeast cell growth was determined by methylene blue staining using a haemocytometer at the intervals shown and the standard error was between 2.75 and 4.28%.

Fig. 2. – Influence of preconditioning under osmotic stress (6% w/v NaCl) on yeast cell growth. *Saccharomyces cerevisiae* (strains DV10, Syrah and Fermol) was pretreated under osmotic stress conditions caused by 6% w/v NaCl for 24 hours and grown on a glucose-based defined medium, without shaking at 25°C. Yeast cell growth was determined by methylene blue staining using a haemocytometer at the intervals shown and the standard error was between 2.03 and 3.88%.
yeast increased when pre-conditioned with 5% w/v NaCl, but for higher concentrations, a gradual reduction of fermentative efficiency was reported (Spemann et al., 1928). In the second part of the presented work, it is clearly shown that preconditioning of the yeast cells under 6% NaCl for 24 hours had a good glucose utilization considering that the starting concentration of glucose was 550 g/L Fig 4.

The main hypothesis of this section was that the preconditioned yeast cells had acquired an increased alcohol tolerance and ethanol production capability. Additionally, osmotic pre-stress due to NaCl-preconditioning enabled yeast cells to ferment sugars at high concentrations and produce 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 the increases in the intracellular concentration of stress-tolerance metabolites such as glycerol and trehalose.

Figure 3 shows that the level of alcohol after 288 hours of fermentation was 23.5% in volume since the highest level that Syrah strain can rich, as specified by Martin Viallate, is 16.5% in volume.

Fig. 3. – Alcohol and glycerol production by salt-preconditioned wine yeasts during fermentation. Fermentations were conducted with preconditioned yeasts (strain Syrah) using 6% NaCl and growing cells for 24 hours prior to inoculation. Standard error was from 3.4 to 4.95%. 
Fig. 4. – Sugar consumption by salt-preconditioned wine yeast during high gravity fermentation. Fermentations were conducted with preconditioned yeasts (strain Syrah) using 6% NaCl and growing cells for 24 hours prior to inoculation. Standard error was between 3.7 and 4.6%.

4. CONCLUSIONS

To summarize, this research work has revealed that osmotic pre-stress due to NaCl-preconditioning enabled yeast cells to ferment sugars at high concentrations and achieve 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 the increases in the intracellular concentration of stress-tolerance metabolites such as glycerol and trehalose. Additional research that has already been made (Logothetis S., et.al., 2012) can verify the applicability of NaCl-conditioning in other yeast biotechnologies like brewing industry or distillery industry.

REFERENCES


УТИЦАЈ ПРЕКОНДИЦИОНИРАЊА ЋЕЛИЈА У УСЛОВИМА ОСМОТСКОГ СТРЕСА НА ПОВИШЕЊЕ ПРИНОСА АЛКОХОЛА

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Резиме

Овај рад се односи на испитивање утицаја соли на физиологију квасца Saccharomyces cerevisiae. Тачније, у раду се испитује утицај NaCl на раст, вијабилност и ферментациону активност овог квасца у лабораторијским условима. Најважније откриће овог истраживања се односи на утицај прекондиционирања ћелија квасца натријум-хлоридом као начина припреме култивације ћелија у присуству соли, на повећање ферментационог учинка овог квасца. Показало се да прекондицијонирани квасци имају побољшану способност ферментације у медијумима са високом концентрацијом шећера (до 60% m/v глукозе) уз повећану вијабилност ћелија и повишен ниво продукције етанола (преко 20% vol). Прекондиционирање у условима повишених концентрација соли је највероватније имало утицај на повећање толеранције стреса код ћелија квасца изазивајући синтезу кључних метаболита као што су трехалоза и глицерол који делују у правцу повећања способности ћелије да се одупре осмотском стресу и токсичном деловању етанола. Пробе у посебним условима у којима је коришћено прекондиционирање ћелија високим солевима у свим условима су потврдиле да приступ физиолошког инжењеринга може да допринесе унапређивању практичних резултата. Ово истраживање је показало да относно једноставан поступак прекондиционирања ћелија квасца у условима повишене концентрације соли може значајно да побољша учинак процеса алкохолне ферментације.

КЉУЧНЕ РЕЧИ: производња алкохола, осмотски стрес, прекондиционирање, вијабилност, квасац