POTASSIUM AND CALCIUM ION CONCENTRATION INFLUENCE THE ETHANOL TOLERANCE OF YEAST ISOLATES

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Abstract

Ethanol toxicity in the yeast Saccharomyces cerevisiae reduces titre and productivity in the overall industrial production of ethanol. The study aims to evaluate the effect of potassium chloride and calcium chloride on ethanol production, tolerance of yeast cells and to further optimize the salt concentrations in culture medium. Four commercially available strains of yeast were tested for their ethanol tolerance in absence and presence of 10mM to 50mM concentrations of potassium chloride and calcium chloride.. The isolate Saccharomyces cerevisiae strain OR was selected for further tests based on its ethanol tolerance profile. Potassium chloride at 30mM concentration significantly increased viability of the cells in presence of ethanol. The increased of levels of extracellular potassium and calcium ions strengthens transcellular and intracellular ion gradients, thereby potentiating alcohol tolerance of ethanol producing yeast.

Keywords: Ethanol fermentation, Saccharomyces cerevisiae, Ethanol tolerance, Potassium ion concentration, Calcium ion concentration

Introduction:

Ethanol is a fermentation product utilized in many aspects of human life, viz. Industrial processes, alcoholic beverages and ethanol-based biofuels. However, efficient ethanol production faces a bottle neck in that the yeast *Saccharomyces cerevisiae* most widely used for fermentation dies when ethanol levels exceed a certain concentration.

In general during ethanol fermentation, yeast cells are exposed to various stresses. These stresses include but are not limited to increased ethanol concentration, toxic by-product inhibition, high temperature and osmotic pressure from high concentrations of substrate sugar. Among the factors, ethanol is considered to be the major stress responsible for decreased ethanol production and stuck fermentation (Gibson et al. 2007). High ethanol tolerance capability of yeasts is one of the important factors for ethanol production. The development of such strains is of great economic value to fermentation industries. Ethanol tolerance is a complex phenotype: Studies have shown that no single genetic modification is

capable of eliciting greater resistance at high ethanol levels (van Voorst F. et al., 2006; Swinnen S. et al., 2012; Stanley D. et al., 2010; Alper H. et al, 2006). However most yeast strains are unable to tolerate low concentrations of ethanol (5% v/v) while genetic manipulation and extensive strain improvement programmes have produced strains capable of tolerating up to 20% v/v under exacting (mostly laboratory) conditions (Alcotec 23% Turbo Yeast). Strains of *Saccharomyces cerevisiae* tolerate ~5% ethanol, while *Saccharomyces bayanus* tolerates up to 18%.

Because toxicity may arise from chemical perturbation of the plasma membrane, we surmised that the ionic composition of the culture medium could play a role in exacerbating or ameliorating this destabilization (Madeira, A. et al., 2010; Dickey A. N. et al., 2007; Chanda J. et al., 2006). Therefore, we evaluated effect of salts on ethanol tolerance capacity of yeast.

A team at the Massachusetts Institute of Technology found that adding K^+ and OH^- ions to the fermentation medium can help cells compensate for that membrane damage. By making these changes, they were able to boost yeast's ethanol production by 80%. They also showed that this approach works with other types of alcohols, including propanol and butanol, which are even more toxic to yeast (Lam et al., 2014). However these changes usually do not affect the metabolic pathway used by the yeast to produce ethanol.

Material and Methods

Yeast strains

Four commercially available strains of yeast viz; Lallemand: Lalvin DV 10[Lal], Oenoferm ®Rouge: *Saccharomyces cerevisiae* [OR], Oenoferm ®Rouge: *Saccharomyces cerevisiae* for red wine [ORRw], Institute oenoloique of champagne: Levure yeast culture 153[153] were used for the studies.

Yeast Cultivation

0.02 grams of dry yeast of each strain was inoculated into 5 ml of glucose yeast peptone (GYP) broth (2% glucose, 1% yeast extract, 2% peptone, pH 7.0) and incubated at room temperature for 24 hours. GYP slants of each strain were prepared.

Determining inherent ethanol tolerance level of each strain

A suspension matched to 0.5 McFarland standard (corresponding to 1-5 x 10^6 yeast cells/ml) of each strain was prepared in sterile distilled water. The suspension was exposed to increasing concentrations of ethanol (2, 4, 6, 8, 10, 12, 14, 16, 18 and 20% v/v). Cell viability

was determined by streaking a loopful of mixture on GYP agar plates at intervals of 30 minutes up to 5 hours. The experiment was performed in duplicates.

Viability staining and counts

To calculate the proportion of dead and live cells viability staining was performed. Methylene blue (0.1mg/ml) was mixed in equal parts with the cell suspension (Painting et al., 1990). Cell counts were determined using improved Neubauer chamber and a 3.1 MP microscopic image capture device (ToupTek) and software (Toupview).

Determining effect of distilled water on cell viability

Initially all cell suspensions were prepared in sterile distilled water. Ethanol at increasing concentrations was mixed with equal volumes of cell suspension and for 120 minutes. Cell viability was determined at 60 and 120 minutes of exposure time as described above. Proportion of dead and live cells was determined..

Cell counts on exposure to varying concentrations of ethanol

Yeast cells were viable up to 12% (v/v) ethanol concentration after 24 hours. Therefore, 14-20% (v/v) ethanol concentrations were selected to determine cell viability on exposure to the concentration of ethanol that is detrimental to yeast cells. Cell suspensions in sterile saline were exposed to ethanol concentrations 14%-20% (v/v) suspension equal proportion and incubated for 120 minutes. Cell viability and dead to live cell proportion was determined.

Establishing a baseline

A baseline for ethanol tolerance by all tested yeast strains was determined by exposing cell suspensions to different concentrations of ethanol (16- 20% v/v) for 60 and 120 minutes. This procedure was followed for each concentration of ethanol that ranged from 2 to 20 % v/v. Based on the ethanol tolerance profile of strain OR, the test ethanol concentration range was narrowed down to 16-20% (v/v) to establish the baseline as the number of viable cells present at 14 and 15 % (v/v) was still within the range.

Percent reduction in cell viability after exposure to ethanol in presence of salts

To determine the effect of salt on ethanol tolerance of yeast, cells were exposed to increasing concentrations of ethanol (16-20% v/v) in presence of a salt (KCl and $CaCl_2$ at 10mM to 50mM) for 120minutes and cell counts were determined after 60 and 120 min.

The range of salt concentration was 10 mM to 50 mM range since as reported in the literature, 50 mM concentration of KCL was found to be optimum for increasing the cell tolerance to ethanol (F.H. Lam et al., 2014). Therefore 50 mM KCl was tested initially, however it resulted in significant reduction in yeast cell viability. Thus, a lower range was selected for all further salts to be tested; same was followed for $CaCl_2$. All experiments were performed in triplicates.

Results

Determining inherent ethanol tolerance level in each strain

Yeast cells were viable on exposure to ethanol up to 300 minutes, for concentrations up to 12% (v/v) as indicated in the Table 1. Viability was seen up to 24 hours even after exposure to 12 % (v/v). Therefore, 14-20% ethanol concentrations were selected as the test range (to test the effect of ethanol on the cell number) and to find out the exact concentration/s which are detrimental to cells.

Table 1 – Viability of yeast strain over a time period at various concentration of ethanol

Ethanol (% v/v)	Time of Exposure (minutes)																	
	30		60		90		120		150		180		210		240		300	
	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2
2	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
4	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
6	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
8	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
10	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
12	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
14	Y	Y	Y	Y	Y	Y	Y	N	Y	N	N	N	N	N	N	N	N	N
16	Y	Y	Y	Y	Y	Y	Y	N	N	N	N	N	N	N	N	N	N	N
18	N	Y	N	Y	N	Y	N	N	N	N	N	N	N	N	N	N	N	N
20	N	Y	N	Y	N	Y	N	N	N	N	N	N	N	N	N	N	N	N

S 1- set 1, S 2- set 2   Y = Growth, N = No Growth

Viability staining

Viable yeast cells contain enzymes that decolourize methylene blue, whereas dead cells do not. When cells from a yeast sample are suspended in the dye, it penetrates into all the cells, but is reduced only in the living cells. Hence, dead cells stain blue while living cells remain unstained (Painting et al., 1990).
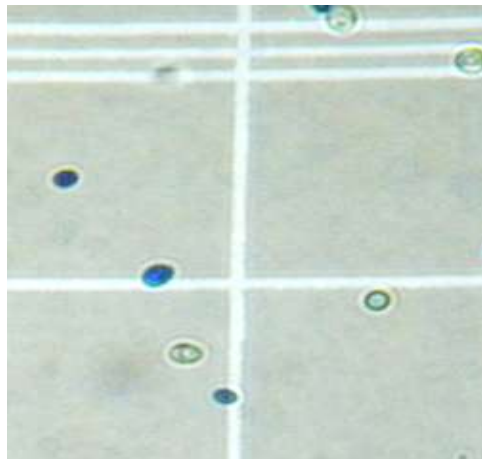
Figure 1: Viability staining with methylene blue (Dead cells are stained blue, while colourless cells viable)

Determining effect of distilled water on cell viability

Only 83.5% of the cells were viable in distilled water at the onset of the assay (0 min). Complete cell death was observed before the assay was completed (120 min). Hence, physiological saline was used to prepare yeast cell suspensions.
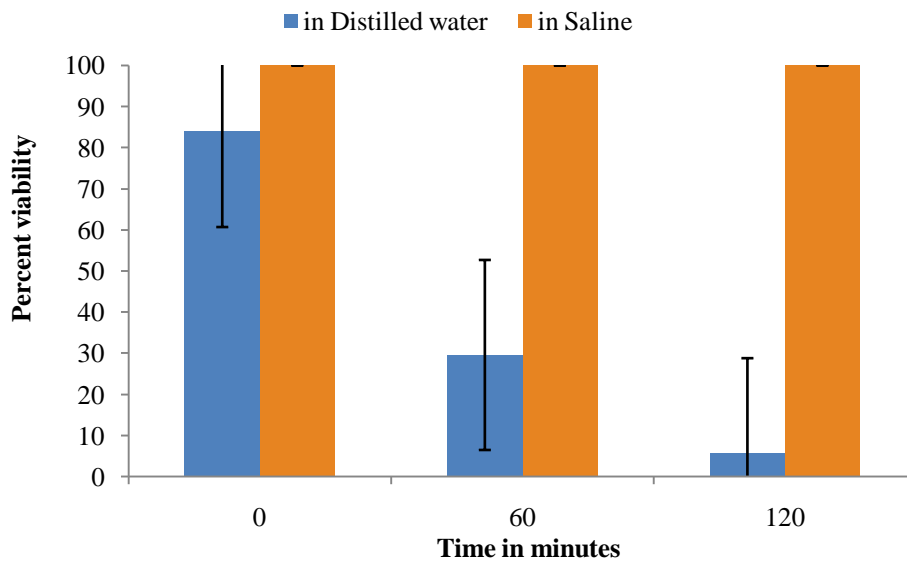

Figure 2: Effect of distilled water and saline on cell viability over time

Determining inherent ethanol tolerance level of each strain

In order to study the effect on tolerance capacity (decrease or increase) to ethanol, the proportion of dead and live cells was estimated and expressed as percent cell reduction in viability.
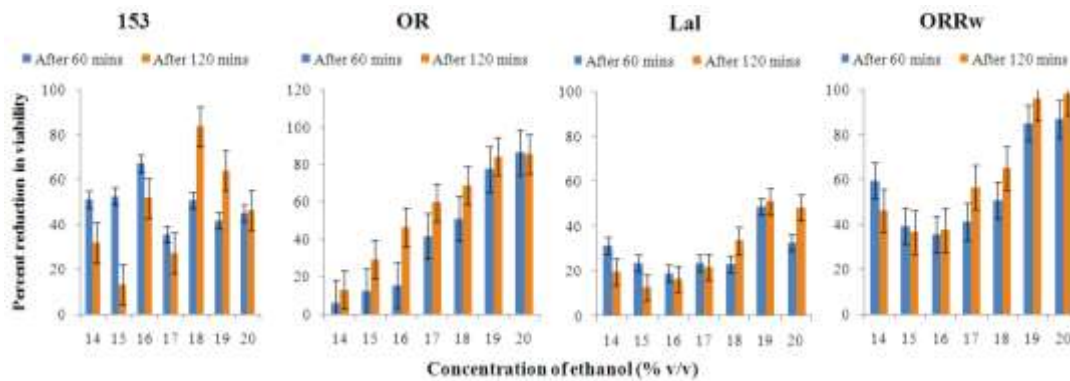
Figure 3: Percent reduction in viability of four yeast strains in presence of ethanol

Based on the ethanol tolerance profiles of the four yeast strains tested, OR strain was selected for further studies; as it exhibited a linear increase in cell death over increasing concentrations of ethanol.

Establishing a baseline for ethanol tolerance of yeast suspension in physiological saline

In order to compare whether the added potassium and calcium salts was influencing the ethanol tolerance of the yeast cells, a control was devised where the test parameters were kept constant i.e. inoculum size, volumes, of ethanol and other growth conditions; to establish a baseline.
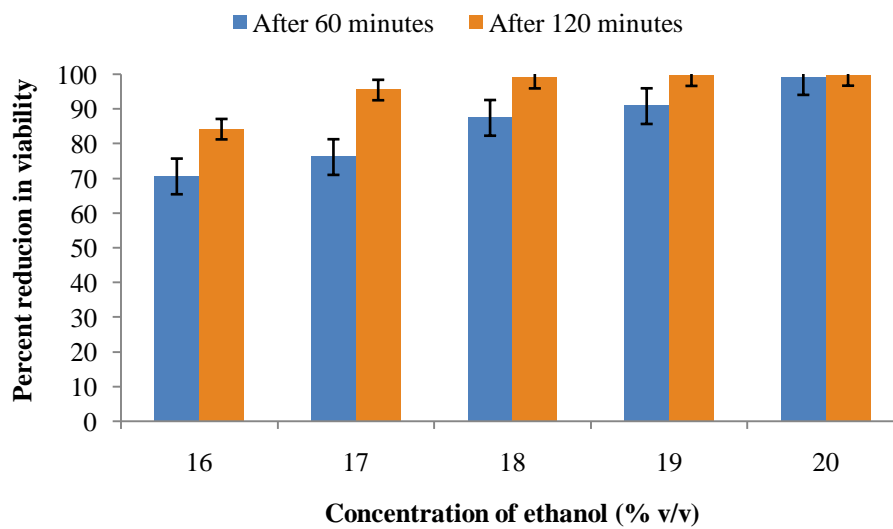

Figure 4: Ethanol tolerance of yeast cells in control conditions

Percent reduction in yeast cell viability on exposure to ethanol in presence of salts

Effect of KCl

Incorporation of 30 mM of KCl in the test system showed a significant decrease in cell death on exposure time of 60 and 120 minutes. Thus, it was concluded that presence of

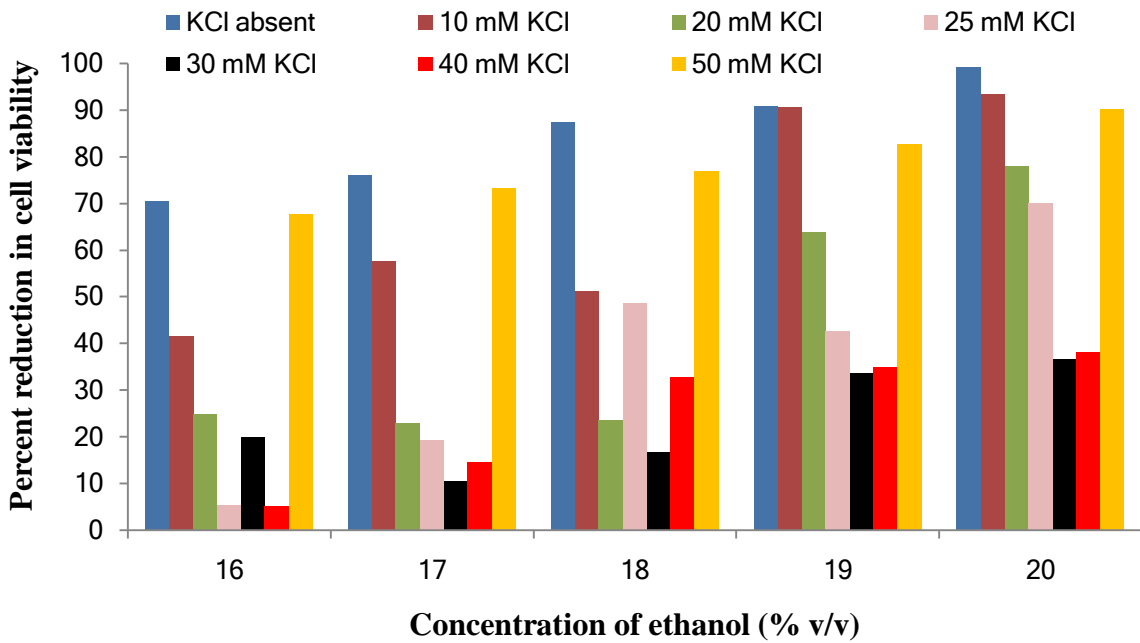KCl at 30mM concentration leads to increase in tolerance of yeast cells from 14 %(v/v) up to 20 % (v/v) of ethanol.

Figure 5: Effect of KCl on percent reduction in yeast cell viability on 60 minutes exposure time
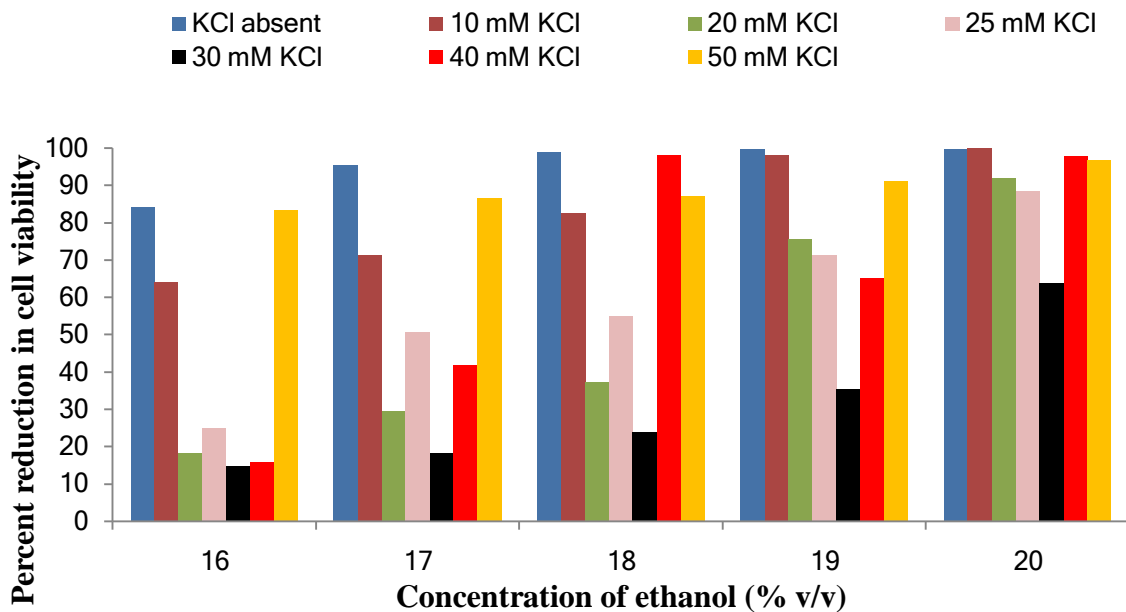

Figure 6: Effect of KCl on percent reduction in yeast cell viability on 120 minutes exposure time

Effect of CaCl₂

Incorporation of 25 mM $CaCl_2$ in test system resulted in significant decrease in cell death on exposure time of 60 and 120 minutes. Thus, it was concluded that presence of $CaCl_2$ at 25mM concentration leads to increase in tolerance of yeast cells from 14 % (v/v) up to 20% of ethanol.
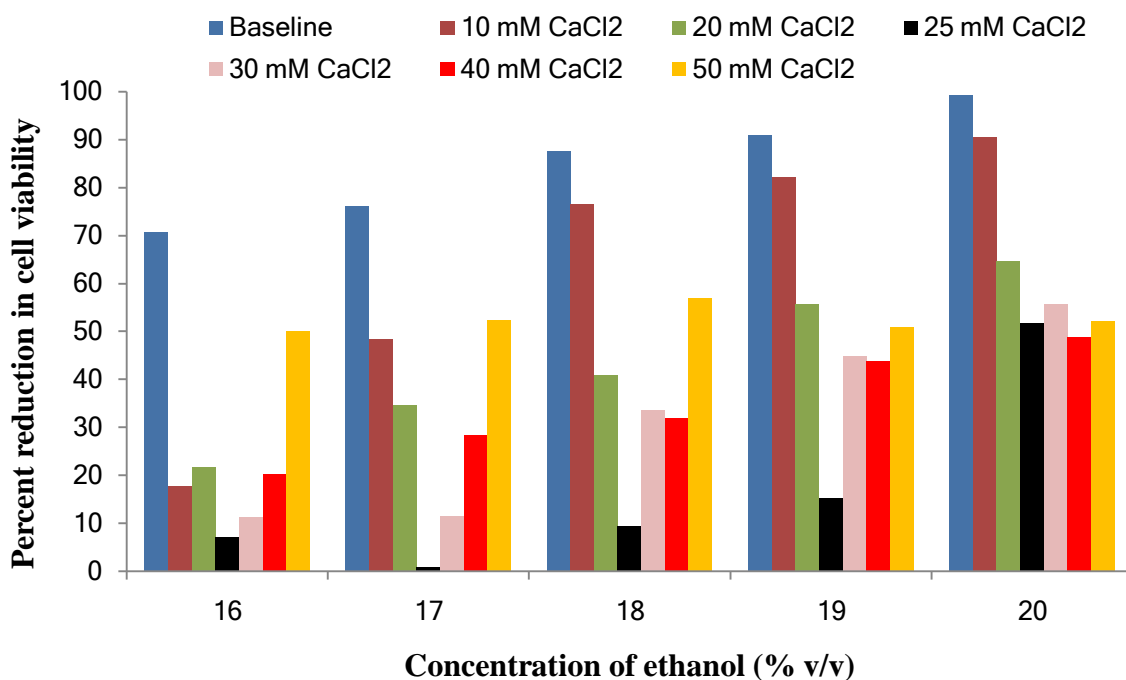

Figure 7: Effect of CaCl₂ on percent reduction in yeast cell viability on 60 minutes exposure time
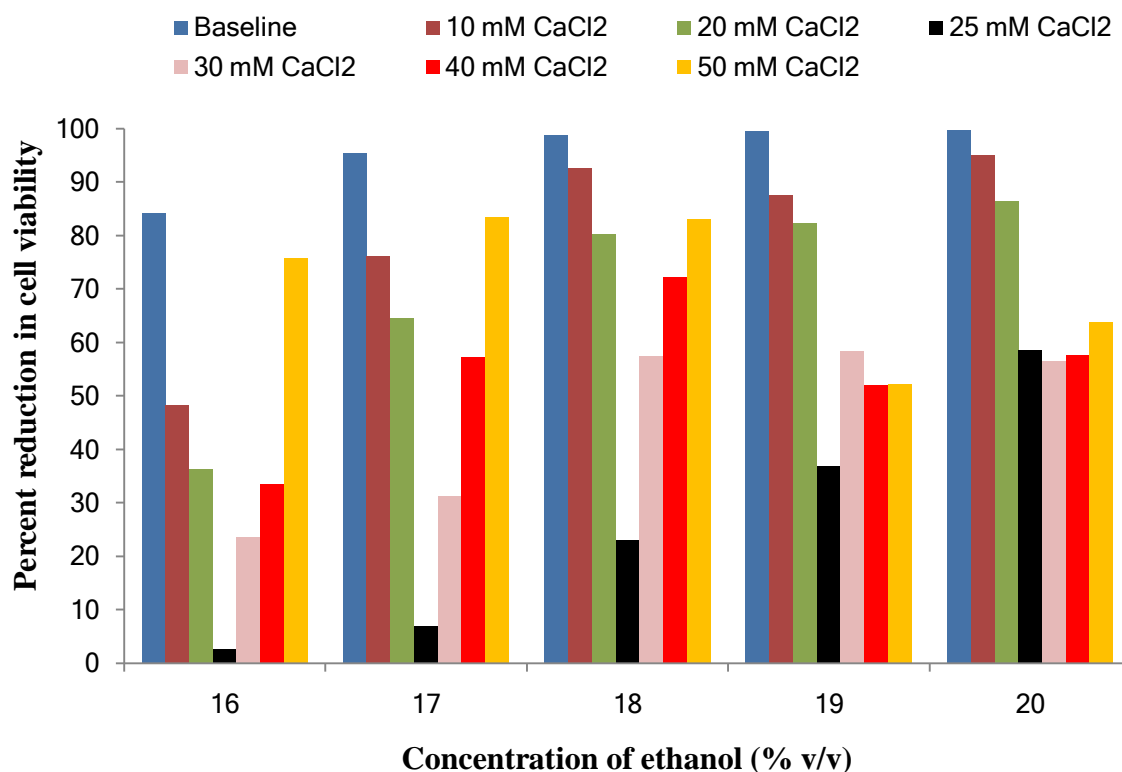
Figure 8: Effect of CaCl$_2$ on percent reduction in yeast cell viability on 120 minutes exposure time

Statistical analysis

Student's t-test was applied to analyse data and determine significance of the results. It was observed that incorporation of KCl or CaCl$_2$ at 10mM concentrations in the test system did not have significant effect on tolerance of yeast cells to ethanol when exposed for 60 and 120 minutes. However, significant protection from yeast cell death due to ethanol was observed at concentrations 20 to 50mM ($p<0.05$), when exposed for 60 and 120 minutes. Most significant tolerance of yeast cells was seen at 30 mM and 25 mM of KCl and CaCl$_2$ respectively on exposure for 60 and 120 minutes.

Discussion

Ethanol tolerance can be defined in terms of amount of ethanol produced and also in terms of cell growth and viability (Casey et al., 1986). Our work relates ethanol tolerance to cell viability hence the results are expressed in percent reduction in cell viability on exposure to varying concentrations of ethanol (in presence and absence of salt).

The presence of optimal concentrations of potassium and calcium in saline dosed with varying ethanol concentrations (16-20 % v/v) led to a decrease in percentage cell viability. This experimental setup reflected the fermentation stage wherein extracellular ethanol has attained threshold levels. The optimal concentration for the OR strain tested was 30 mM of KCl and 25 mM of CaCl$_2$. Moreover, both calcium and potassium had a dose dependant effect on viability of *S. cereviseae* cells, which has so far been reported only for calcium in *S. bayanus* (Aranha H. et al, 1986; Jones, R. P., 1986; Jones, R. P. et al., 1984).

The increase in the ethanol tolerance of *S. cereviseae* cells incubated with exogenously added ethanol and potassium chloride (30 mM) or calcium chloride (25 mM) proved to underline the positive effect of both of these salts in the final stages of an alcoholic fermentation. This tolerance was characterized by quantifying the percent cell reduction over increasing concentrations of ethanol.

The intrinsic ability of cells to adapt to a wide range of environmental conditions is a fundamental process required for survival. Potassium is an essential cation required for many cellular processes including the regulation of cell volume, intracellular pH, protein synthesis, activation of enzymes, and maintenance of the plasma membrane potential (Blatt M et al., 1987; Rodriguez-Navarro A., 2000; Arino J et al., 2010; Merchan S. et al., 2011). In their natural environment, most cell types have to accumulate intracellular potassium against a strong concentration gradient. Yeast cells utilize the energy stored in ATP to directly pump potassium ions into the cell via the Na$^+$/K$^+$ ATPase. *S. cerevisiae* cells can grow in media with a potassium concentration ranging from 10 mM to 2.5 M. Despite extensive knowledge about the identity and function of most potassium transporters in this organism (Arino J et al., 2010), a system level understanding of the interplay and regulation of the various transport pathways is still lacking.

In *S. cereviseae*, uptake of potassium across the plasma membrane is driven by the membrane potential, which itself is generated by proton pumping via the H$^+$-ATPase, Pma1 (Buch-Pedersen MJ et al., 2006; Serrano R, 1983). Efflux of potassium is strongly pH-dependent and coupled to sodium toxicity. (Ban͂uelos M et al., 2002; Navarrete C et al., 2010). Besides protons, a number of other ions, namely bicarbonate anions and ammonium ions are associated with the transport of potassium.

Among the important roles played by the calcium ion in yeasts, it plays a major part in the maintenance of the membrane permeability barrier under adverse conditions by shielding the charged structural membrane phospholipids and regulating the lipid-protein interactions (Merymann, H. T., 1972). Calcium is also one of the typical cations that make complexes with yeast wall phosphomannans.

At growth temperatures, the target sites for ethanol-induced death are probably located in the plasma membrane (Sa-Correia, I. et al., 1986; Thomas et al., 1978). Ethanol may interact with membranes by insertion into the hydrophobic interior, increasing the polarity of this region, weakening the hydrophobic barrier to the free exchange of polar molecules, and weakening the hydrophobic interactions and affecting the positioning of proteins within the membranes (Ingram L. O., 1984).

The association of Ca^{2+} with the surface of yeasts is well documented, and this association has been used to protect the plasma membrane from the action of polyene antibiotics and butanol (Lee, T. C. et al, 1968; Lewis M. J., 1967; Stachiewicz, E. et al., 1963). It was shown that the presence of Ca^{2+} prevents the release of nucleotidic material by cells of *S. carlsbergensis* suspended in glucose solutions containing butanol and other membrane-damaging agents that increased cytoplasmic leakage (Lee, T. C. et al, 1968; Lewis M. J., 1967). Salgueiro et al. (1988) have reported that it is for concentrations of ethanol above the maximum for growth that the stimulation of the leakage of amino acids and other intracellular components in significant, suggesting a critical increase in membrane permeability near the ethanol concentration threshold. The dose response curves to ethanol support our hypothesis. The calcium ion could increase plasma membrane stability (Jones, R. P. et al., 1984) either by decreasing the ethanol-induced passive proton influx or stabilizing the ATPase activity inhibited by ethanol.

Ionic deficiencies do occur in some natural sources of carbohydrates that are used as alcoholic fermentation feedstock. On the other hand, high salt concentrations in substrates such as molasses are detrimental to growth and ethanol production, and the addition of EDTA has been found to improve their fermentation (Rotimi et al., 1985).

The optimization of Ca^{2+} (or any other ion) concentration in complex industrial fermentation media can be difficult. In fact, industrial media contain a range of chelating, sequestering, and adsorbing materials (amino acids, proteins, organic acids, polyphenols,

polyphosphates, and insoluble and colloidal materials) which act to reduce the effective available ionic concentration (Jones, R. P. et al., 1984).

Molasses, a common substrate used in alcoholic fermentations provides a high concentration of total calcium (0.4 to 0.6 % wt/wt), and the maximal concentration of ethanol produced did not surpass 9 to 10% (v/v). It remains to be seen whether the concentration of free Ca^{2+} in molasses would be sub-optimal if higher concentrations of ethanol could be produced. However, because of the presence of inhibitory compounds in molasses, ethanol concentrations higher than 13% can hardly be fermented. On the other hand, it is possible that the positive effect described in fermentations with immobilized yeasts entrapped in calcium alginate and carragenate gels could partially be attributable to the protection exerted by calcium toward the toxic effects of ethanol (Nabais, R. C. et al., 1988).

Conclusion:

Based on the results, both the calcium and potassium protective effects are expected to become more significant in continuous fermentations (cells fermenting continuously in the presence of high concentrations of ethanol) or in batch fermentations involving the production of high concentrations of ethanol (for example, during the secondary fermentation in the sparkling wine industry). As the presence of salt improves the cell viability in presence of exogenously added ethanol, we propose the addition of these salts to industrial ethanol fermentations to increase the yield, thereby increasing economic feasibility.

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