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RESEARCH ARTICLE

Comparative Study of Salt Tolerance in *Saccharomyces cerevisiae* and *Pichia pastoris* Yeast Strains

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ABSTRACT

The salt tolerance of both yeast strains have been compared in glucose media. Fermentation, cell numbers, cells viability, dry weight and glycerol determination was performed. The growth of cells was clearly reduced when the stress agent added at 5 hours fermentation. It was observed that the growth of cells was reduced with increased of concentration of stress agent from (0.5M to 1.5M). Pichia pastoris showed a better adaptation to high concentration of NaCl and sorbitol than Saccharomyces cerevisiae. Salt stress was accompanied by an increase in the intracellular level of glycerol. Saccharomyces cervisaie showed high glycerol production than Pichia pastoris strain. The cells viability remained at high levels under high salt stress even for more than 190 hours of fermentation.

***KEYWORDS**: Yeast, Pichia pastoris, Saccharomyces cerevisiae, osmotic stress, adaptation, fermentation.*

INTRODUCTION

Yeasts are an attractive group of lower eukaryotic microorganisms, some of which are used in several industrial processes for production of a variety of biochemical compounds [1-4]. Yeast likes others living organisms are subject to changing environmental conditions, to which they must adapt in order to survive [5-6]. Under extreme environmental circumstances, yeast undergo a rapid change in their cellular machinery, modulating metabolic pathways and changing gene expression [7-8]. These response mechanisms aim to protect cells against detrimental effects and to repair any molecular damage [9-10]. Microorganisms such as the yeast, *Saccharomyces cerevisiae,* develop systems to counteract the effect of osmotic stress such as salt stress (NaCl) [11]. Salt-induced stress results in two different phenomena: ion toxicity and osmotic stress [12]. Defense responses to salt stress are based on osmotic adjustments by osmolyte synthesis and cation transport systems for sodium exclusion [13]. Exposing yeast cells in a hyper osmotic environment leads to a rapid cell dehydration and arrest cell growth [14]. Under these conditions, cellular reprogramming or “adaptation" represents major defenses including accumulation of compatible solutes to balance the intracellular osmotic pressure with the external environment [15]. Under stress conditions yeast cells enhance intracellular accumulation of osmolytes, and polyols in particular glycerol and compatible ions such as, amino acids and fatty acids in cell membranes [16]. Other products synthesized by yeast during stress conditions are trehalose and glycogen that may collectively represent 25% of the dry cell mass depending on the environmental conditions [17]. The disaccharide trehalose accumulates has been shown to protect cells against high temperature by stabilizing proteins and maintaining membrane integrity [18). Elevated osmotic pressure of the medium also activates the plasma membrane H+-ATPase [19] and the Na+/H+ antiporter which use the proton electrochemical gradient on the plasma membrane as a driving force to eject excessive intracellular Na+ [20]. In some yeasts adaptation to higher salinity is accompanied by an increased viscosity of the membranes due to an increased level of unsaturated fatty acids or an increased length of fatty acyl residues [21].The goal of this study was to compare the relative tolerance to osmotic stress of two yeast stains during fermentation processing.

MATERIALS AND METHODS

Microorganisms

Yeast strains of *Saccharomyces cerevisiae* FY1679 and *Pichia pastoris* GS115 were from (Biotechnology center - EIPICO-Egypt).

Culture maintenance

Strains were routinely maintained on YPD (Yeast Peptone Dextrose) agar plates containing (w/v): 1% yeast extract, 2% peptone, 2% dextrose, 2% agar.

Growth conditions

Cells were routinely grown in 2L Erlenmeyer flasks in 100-ml well defined medium containing (g/liter): Glucose (30 g), (NH4)2SO4 (6g) , MgSO4(7H2O) (2.4g), H3PO4 (3g), KCl(2.4g), NaCl(0.12g), CaCl(0.12 g), ZnSO4(7H2O) (0.12 g), MnSO4(6H2O) (0.024 g), FeSO4(7H2O) (0.01g), CuSO4(5H2O) (0.006 g). Vitamin mixtures were also added from the stock (per liter) consisting of biotin (20mg), panthothenic acid (200 mg), folic acid (2 mg), thiamine hydrochloride (400 mg), riboflavin (200 mg), nicotinic acid (400 mg), pyridoxine hydrochloride (400 mg), *myo*-inositol (1000 mg) and *p*-amino benzoic acid (200 mg**).**

Preparation of inoculums

Yeast cells were grown on 500-ml conical flasks containing 100 ml of YPD medium. Cultures were held at 30°C on a rotary shaker to grow to early stationary phase.

Stress agents

Two types of stress agents (NaCl and sorbitol) were used in the experiments. Stock solution of both stress agents was prepared at different concentration.

Experiments

Cells were grown in 500 ml shake flasks containing 100 ml medium. The flasks were inoculated using 1% (v/v) seed culture and then put in the rotary shaker at 200 rpm, 30°C. To determine the time of stress addition, only NaCl was used as stress agent and was tested on FY1679 strain. The stress agent was added to the shake flaks at 5, 10, and 15 hours using 3 different concentrations (0.5, 1.0 and 1.5M) NaCl.

Fermentation

Fermentation using well defined medium was carried out in 20L fermenter (Applikon, Biotechnology -The Netherlands). The fermenter was calibrated for pH, dissolved oxygen, ethanol, CO2 in exit gas and temperature prior to its use and was operated using the following conditions: pH5.5, temperature 30°C, aeration 1l/ min and agitation 600 rpm. To inoculate the fermenter, 2 shake flasks, each containing 500 ml well defined medium were centrifuged (10,000 rpm, 15 minutes) and the cell pellets collected were transferred to the fermenter. Using these 2 flasks, the starting optical density value in the fermenter was around 0.5.

Cell number

The cells numbers were determined using Colony Forming Unit (CFU) on YPD agar plates. The plates were prepared by adding 10-15 ml of YPD-agar medium into each petri dish while the agar was still warm. Then, it kept to solidified and dried up before being stored at room temperature. Spreading technique was employed to spread 0.05 ml sample on each agar plate. Samples were serially diluted before used to produce colonies between 30-300 per plates. Colonies were counted after 2 days of incubation at 30°C.

Yeast cell viability

Cell viability was determined by vital staining using methylene blue stain 1 ml of yeast cells diluted in 9 ml of deionized water and then 1ml of this solution mixed with 1ml of 10% methylene blue solution. After 10 min 0.01 ml added to a haemocytometer. Continuously yeast total cell number and yeast living cell number took place by optical microscope (Helmut ***Hund*** Gmbh, Germany).

Optical density

Cell growth was monitored turbidimetrically by measuring the optical density at 600 nm using spectrophotometer (SmartSpec 3000-Biorad-Laboratories).

Dry weight

For dry-weight determination, 2 ml of sample medium were filtered using 0.45 um membrane filter (Millipore-France) under vacuum pump. The filtrate was dried in oven at 100°C for 24 hours to constant weight.

Glycerol determination

Samples (1 ml) were withdrawn from mid-exponential cultures supplemented with stress agents. Cells were collected by centrifugation for 5 minutes at 5,000 rpm and washed twice with a solution containing 1.5 M sorbitol

and 20 mM MgC1. Cells were resuspended in 1 ml 0.5 M Tris/HCl, pH 7.6, and heated at 95 "C for 12 min. After centrifugation, the supernatant was used to measure the internal glycerol content. For determination of extracellular glycerol, samples (1 ml) were withdrawn from the culture, cells were removed by centrifugation and the clarified supernatant was used for the assay. Glycerol content was determined by enzymatic analyzed using assay kit (Boehringer Mannheim, Germany).

RESULTS AND DISCUSSION

Effect of addition of NaCl at different time

To study the effect of time addition of stress agent NaCl on the growth of *Saccharomyces cerevisiae* FY1679 and *Pichia pastoris* GS115 cells. Three different concentration (0.5, 1.0 and 1.5M) of NaCl were added to shake flask fermentation medium at 5, 10, and 15 hours. The growth of FY1679 cells was clearly reduced when the stress agent added at 5 hours fermentation (Fig. 1A). After this period, differences between stressed and unstressed cells became apparent, especially around 25 hours. The effect of addition of NaCl at 5 hours was less effect on *Pichia pastoris* GS115 (Fig. 1D). No effect was observed when the stress agent was added after 15 hours fermentation in both strains (Fig. 1C&F). Previous studies by Luisa et al*.,* [22]; Blomberg [6]; Sathyanarayana and Sawan [23] and Gummadi et al., [24] have reported that yeast cell can tolerate high concentration of NaCl (up to 2.0M). However, Kogej et al., [20] reported that 3% NaCl represents a concentration that is toxic for *S. cerevisiae*. Black yeast *Hortaea werneckii* can grow, albeit extremely slowly, in a nearly saturated salt solution (5.2M NaCl) [25 -27]**.**

It was also observed that the growth of cells was reduced with increased of concentration of stress agent from (0.5 to 1.5M) comparing to growth of control (Fig. 1A&D). The reduction of growth might be due to the disproportional deviation of carbon and energy into production of the cellular sub-products [28-29). These results are agree with Fernanda et al., [30] whose reported that, *Schizosaccharomyces* pombe and *Pichia* membranaefaciens showed a marked decrease in growth the when level of NaCl increased from 0·5 to 1M and from 1 to 3M, respectively. Based on these observations, in all stress experiments addition was made at 5 hours fermentation and only 1.5M stress agent concentration was used.

Effect on different strains

The results in Figure 2 indicate that the growth of yeast species differ in their relative sensitivities to both stress agents (NaCl and sorbitol) and also showed that the growth of these strains was decrease under stress condition comparing to unstressed. The effect of reduction of growth was less in GS115 compared to FY1679 strain (Fig. 2A, B) respectively. Also these results showed highest concentration of NaCl (1.5M) showed more effective in reduction of growth of both stains than 1.5M sorbitol (Fig. 2 A&B). These results might be due to different in the uptake of two stress agent or might be due to high accumulation of Na+ in *Saccharomyces cervisiae* compared to *Pichia postris*. High accumulation level of Na+ might cause metabolic poisoning to yeast cells [31]. This results are agree with Lahava et al., [32] whose reported that *Pichia guilliermondii* able to grown in NaCl concentrations ranging up to 3.5M. Previous studies showed that yeast species *Saccharomyces cerevisiae and Pichia guillierondii* were differ in their relative sensitivities to Sorbitol and NaCl [33].

Glycerol production under salt stress

S. cervisaie FY1679 strain (Fig. 3A) showed high glycerol production than *Pichia pastoris* GS115 (Fig. 3B) strain. Cells started to accumulate glycerol after 5 hours of addition of salt and reached steady-state levels in 30 hours. This results indicating that cells were highly produced glycerol under salt stress and glycerol was mean metabolic products under stress condition using NaCl and sorbitol. Studies carried out on over 20 different yeasts and filamentous fungi show that glycerol seems to be the main osmolyte accumulated upon salt stress [34-35].

Higher permeability to glycerol would also explain the higher extracellular levels of glycerol detected at high salinities in a previous study [36]. The accumulation of chemically inert osmolytes, such as glycerol, trehalose, and other organic compatible solutes, plays a central role in osmoadaptation of most of yeast and fungi [37-39].

Effect of salt stress on cell viability

The viability of yeast cell was monitored in the presence of stress agents. The results in figure 4 showed that the viability of cells was remained high under high osmotic stress of 1.5M NaCl and sorbitol compared to control cells (Fig. 4A&B,). These results indicated that positive effect of both NaCl and sorbitol on viability of cells and might be due to synthesis of trehalose and glycerol [40].

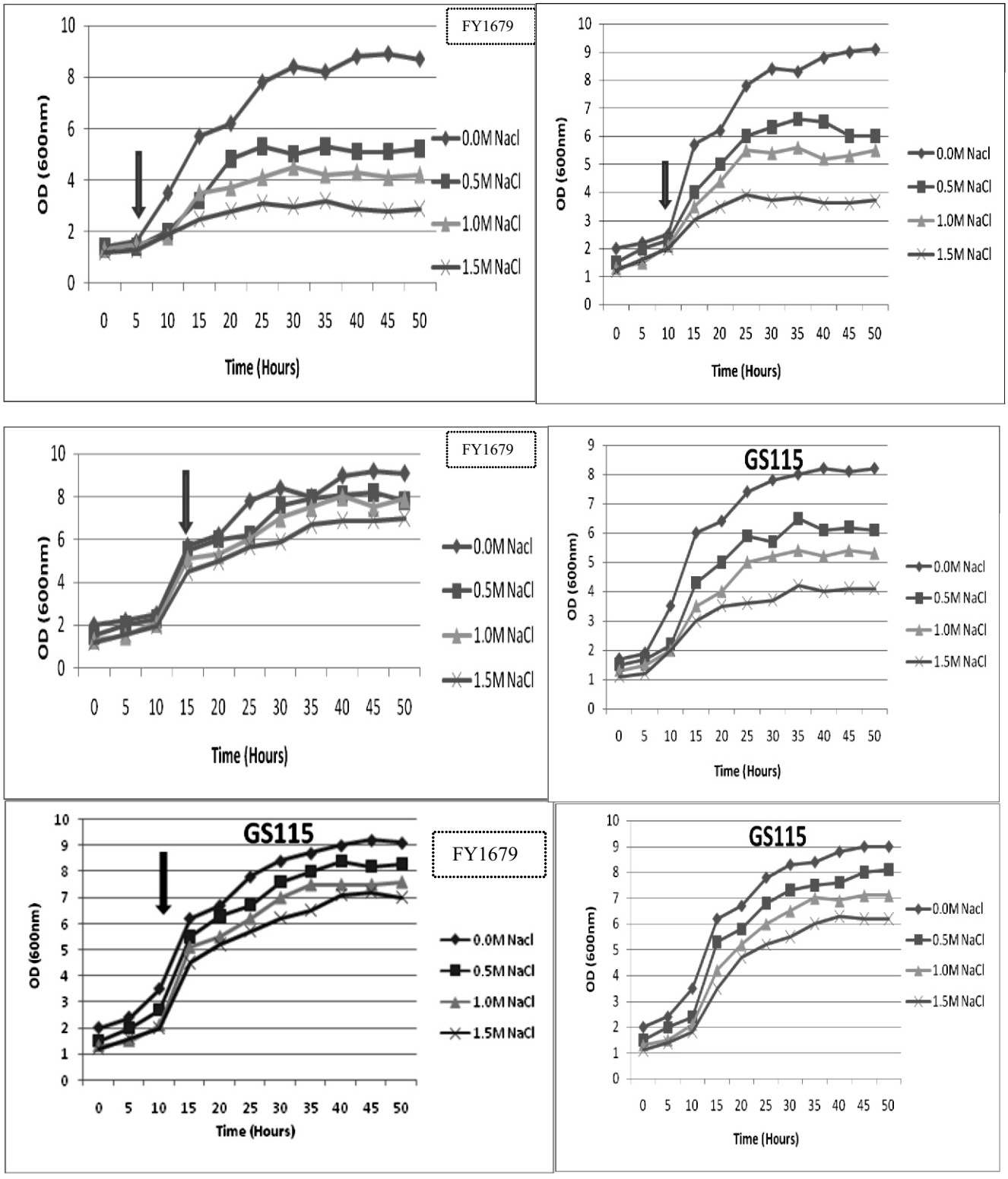
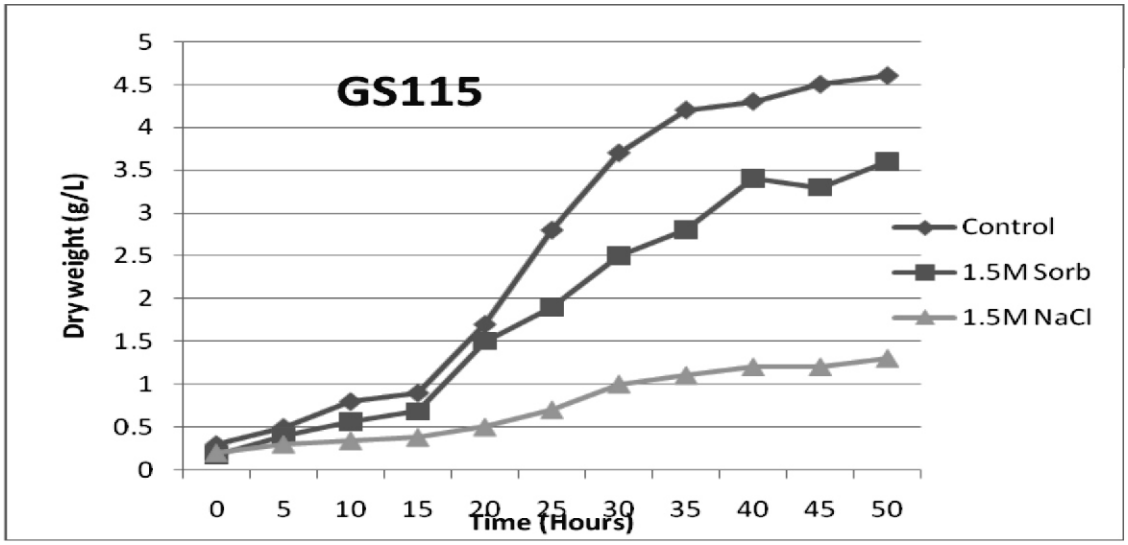


Fig. 1. The effect of addition time and concentration of stress agent (NaCl) on *Saccharomyces cerevisiae* FY1679 strain at 5 hours (A), 10 hours (B) and 15 hours (C) and *Pichia pastoris* GS115 at 5 hours (D), 10 hours (E) and 15 hours (F). (point of addition showed by an arrow).

(A)



(B)

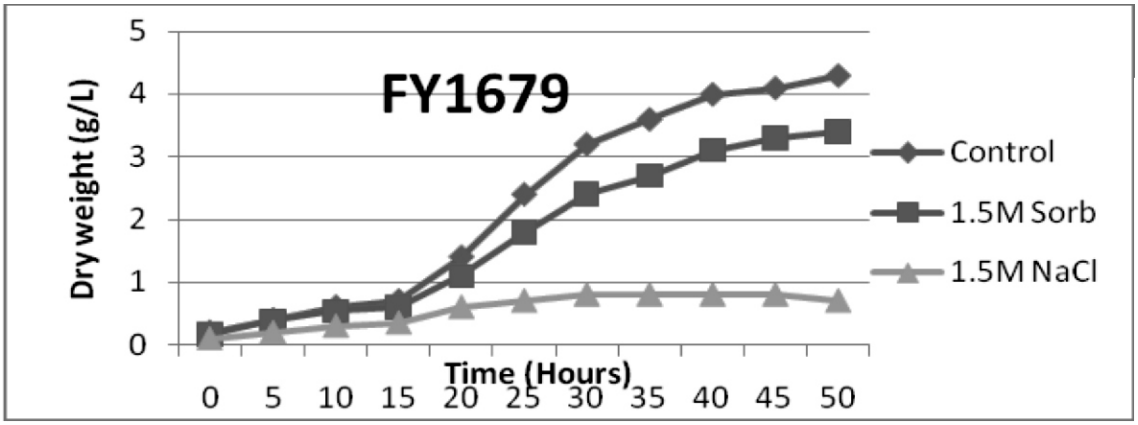
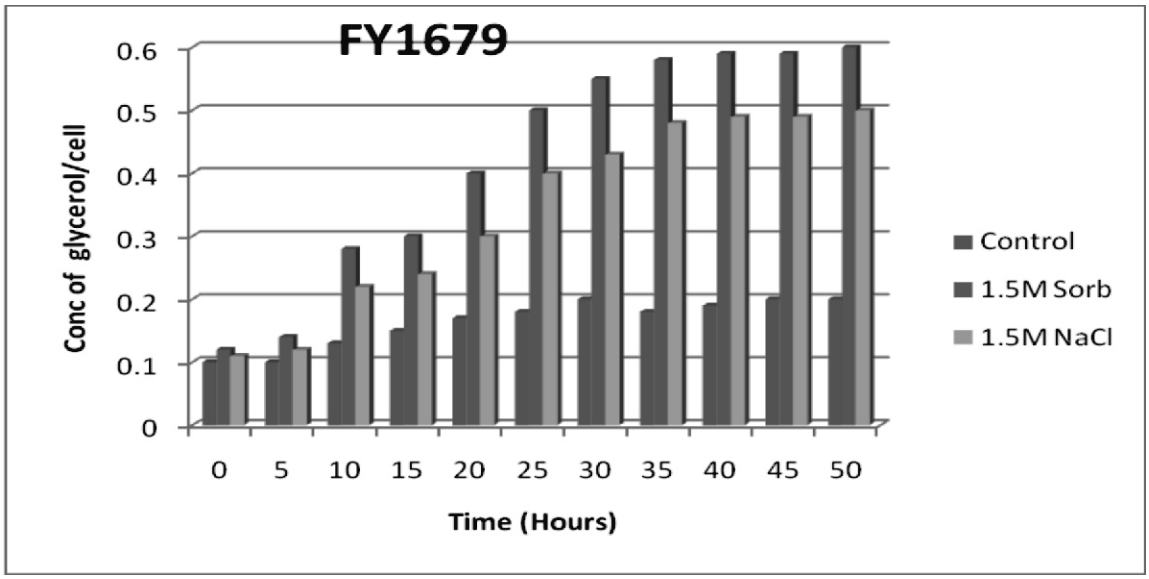
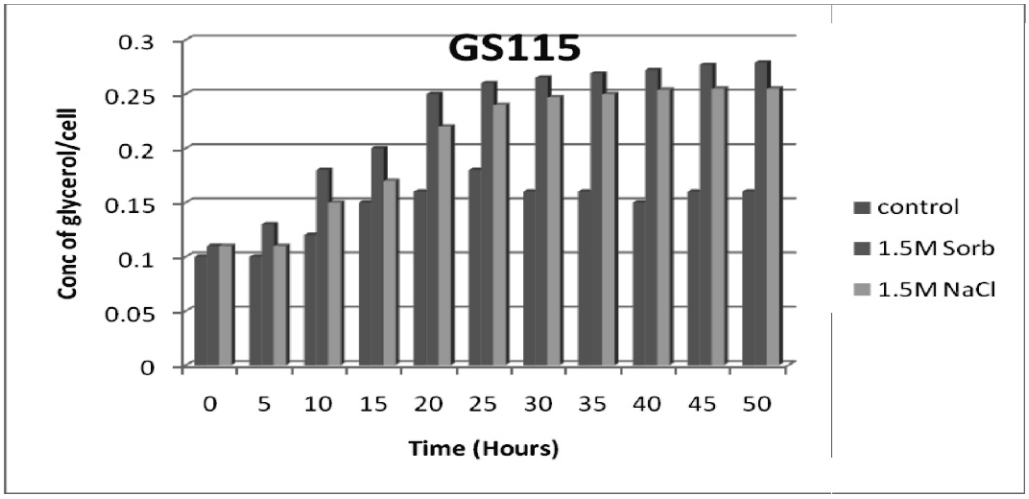


Fig. 2. Growth response of GS115 (A), and FY1679 (B) strains to NaCl and sorbitol addition

(A)

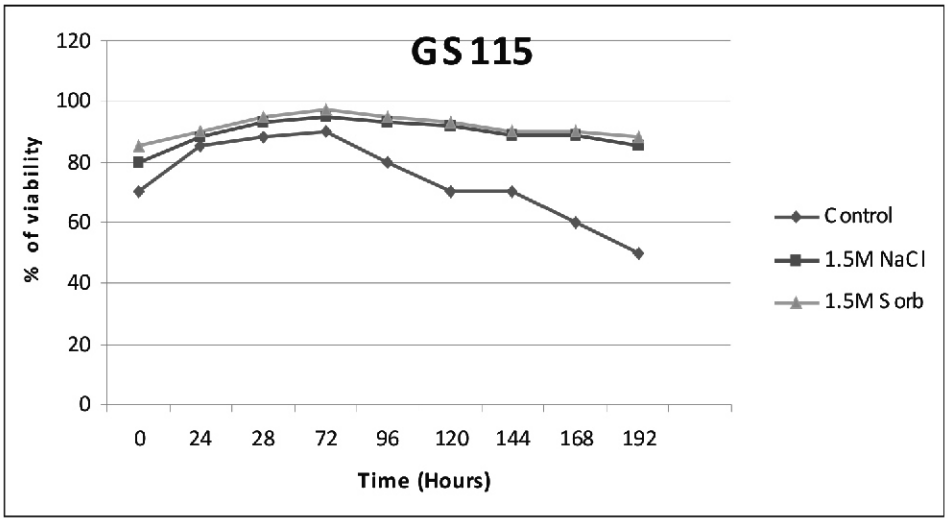




(B)

Fig. 3. Effect of hypoosmotic shock on the intracellular amounts of glycerol on FY1679 (A), GS115 (B) cells grow on 1.5M NaCl and 1.5M sorbitol.

(A)



(B)

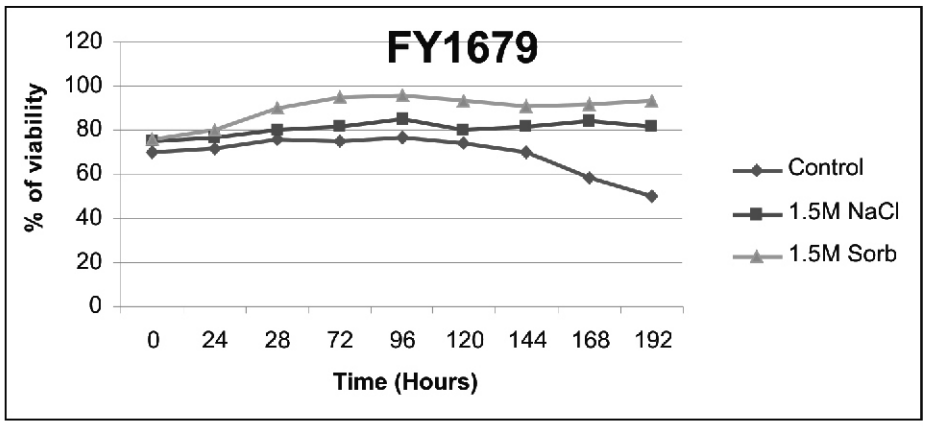


Fig. 4. Viability of GS115 (A), and FY1679 (B) after exposure for various times to 1.5M of NaCl and sorbitol.

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