

Responses of Different Strains of *Saccharomyces cerevisiae* to Osmotic Stress

(Tindak Balas Pelbagai Strain *Saccharomyces cerevisiae* Terhadap Tekanan Osmotik)

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ABSTRAK

*Semasa fermentasi, sel berada dalam keadaan pelbagai tekanan. Salah satu tekanan yang penting adalah persekitaran osmotik yang tinggi yang mesti diatasi untuk meneruskan pertumbuhan. Untuk memahami bagaimana sel beradaptasi terhadap kesan ini, maklumat di peringkat genom, proteom dan metabolom adalah amat penting. Adalah dilaporkan, yis sel menghasilkan gliserol untuk mengelakkan kekurangan air dalam sel yang boleh membawa kepada mengecutan sel dan seterusnya kematian. Oleh itu kajian kesan fisiologi telah dijalankan dalam kelalang goncang menggunakan 3 jenis strain *Saccharomyces cerevisiae* yang berbeza iaitu s288c, IFO2347 dan FY834 yang telah ditumbuhkan dalam medium yis dekstrosa kentang (YPD) dengan penambahan natrium klorida dan sorbitol pada kepekatan 1M untuk menghasilkan keadaan osmotik. Kedua-dua agen ini ditambah kepada medium selepas 5 jam fermentasi iaitu sewaktu sel berada di fasa eksponen dan sumber karbon yang masih wujud. Keputusan membuktikan penambahan kedua-dua agen natrium klorida dan sorbitol dapat menghasilkan keadaan osmotik sewaktu pertumbuhan dengan pengumpulan gliserol dan trehalos bila dibandingkan dengan kawalan. Bagi ketiga-tiga strain ini, penghasilan gliserol (g gliserol/g sel berat kering) didapati tertinggi pada IFO2347, diikuti s288c dan FY834.*

*Kata kunci: *Saccharomyces cerevisiae*; tekanan osmotik; gliserol; trehalos*

ABSTRACT

*During fermentation cells are subjected to various kinds of stress. One of the stresses concerned is high osmotic environment, which cells need to encounter in order to continue growing. To understand how cells adapt to this stress condition, information from genome, proteome and metabolome levels are crucial. In yeast cells, it was report that they produce glycerol to avoid depletion of water in the cell that could lead to cell shrinkage and eventually death. Thus, investigation of physiological responses were executed by shake flask method using three different *Saccharomyces cerevisiae* strains namely s288c, IFO2347 and FY834 which were grown in yeast potato dextrose (YPD) medium under the treatment of sodium chloride (NaCl) and sorbitol at 1M concentration to create the osmotic condition. These agents were added into the medium after 5 hours of fermentation when the cells reached exponential phase and carbon source is still available. The results proved that addition of both NaCl and sorbitol created the osmotic condition during growth resulted in higher accumulation of glycerol and trehalose when compared to the control in all strains. Among these strains, production of glycerol (g glycerol/g cell dry weight) was found highest in IFO2347, followed by s288c and FY834.*

*Keywords: *Saccharomyces cerevisiae*; osmotic stress; glyserol; trehalose*

INTRODUCTION

Yeast (*Saccharomyces cerevisiae*) is widely used worldwide in many industries and it is one of the most extensive studied microorganism. Amongst the most interesting behaviors observed in yeast especially those strains use in brewing industries are their capability of surviving at high osmotic pressure. In the early stage of fermentation, cells need to encounter high osmotic condition due to high medium concentration. During fermentation, cells are subjected to various kinds of condition changes such as changing in

pH, nutrients availability and also producing product that could affect the osmotic condition. Cell experiencing these changes could opt to another metabolic pathway resulting in producing unwanted products, thus economically undesirable.

The indication of cell responses toward osmotic stress can be physiological observed in terms of cell shrinkage in size followed by dehydration resulted in decreasing cell volume and eventually cell loss its viability (Morris et al. 1986). To avoid this condition, it has been cited that most fungi cell will synthesize polyol including glycerol,

trehalose, arabitol and sorbitol (Onishi 1963). In *Saccharomyces cerevisiae* glycerol is said to play an important role in overcoming the stress, for example to balance the osmotic pressure across the cell membrane (Blomberg & Adler 1992) and adjusting external water pressure (Nevoigt & Stahl 1997). The role of glycerol in exponentially grown *S. cerevisiae* cell under salt-stress (NaCl) has been also reported by many authors (Reed et al. 1987, Bellinger & Larher 1987, Meikle et al 1991) using ¹³C nuclear magnetic resonance (NMR) spectroscopy proving its importance for osmotolerance by maintaining the osmotic properties of cell. Glycerol was also reportedly involved in yeast tolerance toward ethanol stress (Sharma et al. 1996).

Some authors suggested trehalose which act as membrane protectant (Hounsa et al. 1998) also contributes to the survival of cells when exposed to various stress conditions including osmotic stress. Trehalose has been reported to involve in stabilizing cellular protein during heat stress (de Virgillio et al. 1994, Lewis et al. 1995), dehydration (Gadd et al. 1987, Hottiger et al. 1987, d'Amore et al. 1991), freezing (de Virgillio et al. 1994) and when exposed to certain toxic chemical (Attfield 1994). Apart from these conditions, there is also an indication of resistance to osmotic stress during growth also which might be related to accumulation of intracellular trehalose (MacKenzie et al. 1988, Sharma 1997). However, there was argument that the amount of trehalose accumulated during salt stress does not have major impact on the osmotic properties of the cells (Olz et al. 1993), thus the increased amount of glycerol is far most important (Blomberg 2000).

In the process of understanding this stress respond, knowledge and information at all levels (genome, proteome and metabolome) is needed. Many studies have been reported in determining and understanding the gene expressed (Ivorra et al. 1999) and protein (Norbeck & Blomberg 1996) produced during osmotic stress. Few genes were already reported responsible for the production of glycerol, namely GPD1 and GPD2 (Larsoon et al. 1993, Albertyn et al. 1994). These two genes were responsible for controlling the activity of the key enzyme, glycerol-3-phosphate dehydrogenase but only GPD1 was believed to be induced by stress (Errikson et al. 1995).

In this introductory research, three strains of *S. cerevisiae* representing laboratory (s288c and FY834) and industrial strain (IFO2347) were used to study the effect of different stress agent (NaCl and sorbitol) towards their metabolome.

MATERIALS AND METHODS

MICROORGANISMS

Laboratory strain of *Saccharomyces cerevisiae* S288c and FY834 (genotype: *MATα hisΔ200 ura3-52 leu2Δ1 lys2Δ202*) were kindly given by Prof. F. Winston (Dept. of

Genetics, Harvard Medical School, USA) (Winston et al. 1995) while industrial strain IFO 2347 was kindly given by Prof. S. Harashima (Department of Biotechnology, Osaka University, Japan). All strains were stored at -80 °C in 15% glycerol. From these frozen stocks, yeast cells were grown on Yeast Potato Dextrose (YPD) agar prior to its use to obtain a single colony. The typical yeasts form white-cream of colonies upon growth on the agar.

CULTURE MEDIUM

All strains (s288c, FY834 and IFO2347) were capable of growing on YPD medium and in all experiments this medium was used. The composition of YPD is listed as follows (per liter) : Yeast extract (Difco) 20 g, Peptone (Difco), 20 g and glucose, 20 g. When preparing agar medium, 20 g of agar was added into 1 liter solution. For experiment using jar fermenter, well defined medium was used. The composition of YPD and well-defined medium are listed as follows (per liter). YPD : Yeast extract (Difco) 20g, Peptone (Difco), 20 g and glucose, 20 g. When preparing agar medium, 20 g of agar was added into 1 liter solution. Well-defined medium : Glucose (30 g); nitrogen source was (NH₄)₂SO₄ (6g) and was autoclaved together with H₃PO₄ (3g), KCl(2.4g) and NaCl(0.12g); minerals consisting of MgSO₄(7H₂O) (2.4g), FeSO₄(7H₂O) (0.01g), ZnSO₄(7H₂O) (0.12 g), MnSO₄(6H₂O) (0.024 g), CuSO₄(5H₂O) (0.006 g) and CaCl(0.12 g). Glucose was autoclave separately with nitrogen source and minerals to avoid browning reaction. 3 mL of vitamin mixtures were also added from the stock (per liter) consisting of biotin (20 mg), 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); Amino acid stock (81 ml) as per 100 ml consisting of tryptophan (0.2 g), Histidine (1.0 g), Leucine (1.0 g) and Lycine (1.0 g). All vitamins and Uracil were autoclave by filter (0.2 μm) to avoid denaturation.

SEED CULTURE

Single colony was obtained after growing the yeast on YPD agar at 30 °C for 2 days. A single colony was used to inoculate 3 ml YPD medium in a test tube. The inoculum was incubated at 30 °C using a water bath shaker rotating at 120 rpm for 20 hours. For jar fermenter seed culture was prepared using well-defined medium.

STRESS AGENTS

Two types of stress agents (NaCl and sorbitol) were used in the experiments. Stock solution of both stress agents were prepared at 1M concentration.

EXPERIMENTS

All shake flask experiments were carried out in 500 ml

Sakaguchi flask containing 150 ml medium. The flasks were inoculated using 1% (v/v) seed culture and then put in the waterbath shaker at 120 rpm, 30 °C. To determine the time of stress addition, only NaCl was used as stress agent and was tested on strain s288c. The stress agent were added to the Sakaguchi flasks at 5 and 12 hours using 3 different concentrations (0.5, 0.7 and 1.0M).

FERMENTATION

Fermentation using well defined medium was carried out in 5L jar fermenter (Mituwa, Co. Ltd., Japan-model KMJ-5A) with 3L working volume. 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: pH6, temperature 30 °C, aeration 1l/min and agitation 300 rpm. To inoculate the fermenter, 3 Sakaguchi flasks, each containing 100 ml well defined medium were centrifuged (10,000 rpm, 15 minutes) and the cell pellets collected were transferred to the fermenter. Using these 3 flasks, the starting optical density value in the fermenter was around 0.6.

ASSAYS

Cell number. The determination of Colony Forming Unit (CFU) was carried out using YPD agar plates. The plates were prepared by adding 10-15 ml agar solution into each petri dish while the agar was still warm. Then, it was 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 to produce between 30- 300 colonies per plates. Colonies were counted after 2 days of incubation at 30 °C.

Optical Density. The absorbance of yeast suspension was measured using UV-VIS spectrophotometer (Shimadzu) at 660 nm.

Dry weight. 2 ml of sample medium were filtered using 0.45 µm membrane filter (Toyo Roshi Kaisha, Ltd., Japan) under vacuum pump. The filtrate was dried in oven at 100 °C for 24 hours together with silica gel to obtain a constant weight.

Glycerol measurements. Total glycerol : Samples (1.0 ml) of culture broth were heat treated at 75 °C for 15 minutes. Cell debris was removed by centrifugation for 5 minutes at 15,000 rpm. Supernatant was kept frozen (-20 °C) until analyzed. Extra-cellular : Samples (1.0ml) of culture broth were centrifuged (15,000 rpm) for 5 minutes and the supernatant was heat treated for 75 °C for 15 min, then was kept frozen (-20 °C) until analyzed. Samples were enzymatically analyzed using enzyme combination kits (Biochemica Test Combination, Boehringer Mannheim GmbH, Mannheim, Germany) for glycerol content. The intra-cellular glycerol concentration was calculated by

subtracting the total glycerol concentration with the extra-cellular concentration.

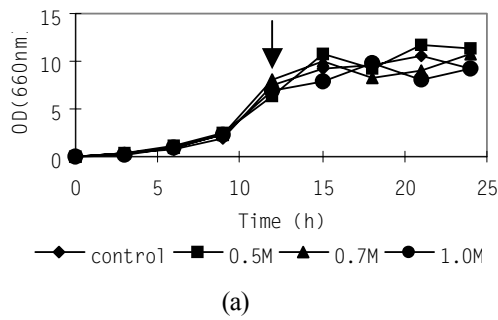
Glucose Glucose was measured using glucose analyzer YSI2700 (Yellow Spring Inc.,USA).

Ethanol and acetate. For ethanol and acetate determination, culture broth (1.0 ml each) were centrifuged (5 min, 15,000 rpm) and the supernatants were kept frozen (-20 °C) until analyzed. Determination of these metabolites was performed using gas chromatography G-3000 (Hitachi, Japan). Different columns, TC-1 (I.D. = 1.5 mm, L = 30 x 0.53 mm, GL Science, Inc.) and TC-FFAP (I.D. = 1.5 mm, L = 0.53 mm x 15m, GL Science Inc.) were used for measuring ethanol and acetate, respectively.

Trehalose concentration. Anthrone reagent was prepared according to Spiro (1966) using concentrated sulphuric acid (720 ml) which was added into 280 ml distilled water. While the solution mixture is still warm, 500 mg of anthrone and 10 g of thiourea were added and the solution was mixed until dissolved. The solution was cooled and stored at 4 °C and only used after 4 hours preparation. Cells for trehalose determination were chilled quickly in ice, collected by centrifugation at 4 °C, and washed 3 times with chilled distilled water. The final pellets were frozen and stored at -20 °C. The cells were thawed by suspending them in approximately three times their volumes using 500 mM trichloroacetic acid (TCA) and letting the suspension standing for 40-60 min at room temperature to extract the sugar. After centrifugation, a second extraction of the pellets was repeated. The combined supernatants from 2 extractions were assayed for anthrone-positive material according to method described by Lillie & Pringle (1980). Samples (200 µl) were pipetted into test tube to which 1 ml of cold anthrone reagent was added and shaken vigorously to ensure complete mixing, then capped properly before heating in boiling water for 15 minutes. The tubes were cooled immediately in a water bath. After 20 minutes the absorbance was measured at 620 nm. Water was used as a blank and standard of varying glucose concentrations should be included with each analysis. Since trehalose is a dimer of glucose, the concentrations are expressed as glucose equivalents.

RESULTS AND DISCUSSION

Point of stress addition. The experiments to determine the point of addition of stress agent (NaCl) on s288c strain were executed using addition at 5 and 12 hours shake flask fermentation. The results (Figure 1) showed that addition at 12 hours did not affect growth. This might be due to that fact that, at this hour, the cells almost completed their growth since glucose was completely depleted. Thus, addition of stress agent did not retard their metabolism any longer.



(b)

FIGURE 1. The effect of different time of stress agent (NaCl) addition and concentration on strain s288c for (a) 12 hours and (b) 5 hours (point of addition showed by an arrow)

Whereas for addition at 5 hours fermentation, the stress agent clearly reduced the growth as exhibited by decreasing absorbance of the cultured medium.

Effect of different concentrations. Different strength of osmotic stresses were imposed by using 3 different concentrations (0.5, 0.7 and 1.0M) of NaCl. Using these varying concentrations, cells were still maintaining their growth, in agreement with observation reported by Blomberg (2000) that most yeast cells can stand up to 1.7M NaCl. It is also observed that the higher the concentration resulted in the lowest growth values (based on absorbance of culture medium) as compared to control. These observations suggest that cells did experience the stress imposed upon them by reducing its growth. The reduction might be the result of the disproportional deviation of carbon and energy into production of the cellular sub-products (Blomberg & Adler 1992, Brown 1978). Figure 1 summarized the effect of different time of stress agent addition at different concentrations on the growth of strain s288c. Based on these observations, in all stress experiments addition was made at 5 hours fermentation and only 1M stress agent concentration was used.

Different strains. The results of different stress agent on 3 different strains are depicted in Figure 2. Both stress agents were found to decrease growth and consumption rate of glucose (Figure 2a). After fermentations were stopped, glucose was still available and not completely utilized by all strains in all experiments. The accumulation of ethanol also decreased but the effect of stress agent cannot be pointed clearly in term of acetate and trehalose accumulation. Comparing amongst strains, industrial strain IFO2347 was the most superior in term of growth and ethanol production compared to s288c and FY834 strains.

Production of intracellular glycerol (g glycerol/g dry cell) were increased in all strains for stressed cell compared to control. This proved that glycerol is indeed metabolites produced when cells are exposed to osmotic condition. Yet again, accumulation of glycerol was found highest in IFO2347 strain, followed by s288c and FY834 as shown in Figure 2b.

Different stress agents. Cells exposed to physiological acceptable stress agent concentrations, will slow their growth because of the need to respond to the stress. This adaptation respond which correlated to the accumulation of glycerol is dependent on yeast strains, cell age, type (Blomberg 2000) and solutes concentrations and period of exposure. Since the effect of agent dependent on solute used, it is inappropriate to compare the effect of different agents at the same concentration. At 1M concentration, NaCl and sorbitol gave different strength of osmotic stress to cell due to different ionic dissociation of water chemical. NaCl is “stronger” than sorbitol as far as osmotic strength is concerned thus, can be seen in growth data observed. However, the results (Table 1) showed that under the influence of 1M sorbitol, higher accumulation of glycerol (g/g dry cell) as recorded in s288c and IFO2347 strains than the 1M NaCl was used. In FY834 strain, the pattern is totally the opposite as sorbitol effect was lower than the NaCl.

TABLE 1. Effect of sorbitol and NaCl on the production of intracellular glycerol (g glycerol/g cell) in different yeast strain grown in shake flask culture

Jar fermentation. Strain FY834 was used in jar fermentation using well defined medium and results confirmed that glycerol was indeed the metabolites produced when cell exposed to osmotic stress agent. As for growth, the dry weight in stress was lower compare to control, so did ethanol, acetate and trehalose. Intracellular glycerol in stress experiment was almost twice higher than that in control pointed in Figure 3. Although more solid confirmation needed, our preliminary *in vivo* NMR analysis indicated that there was a peak observed believed to be glycerol when cell treated with NaCl stress.

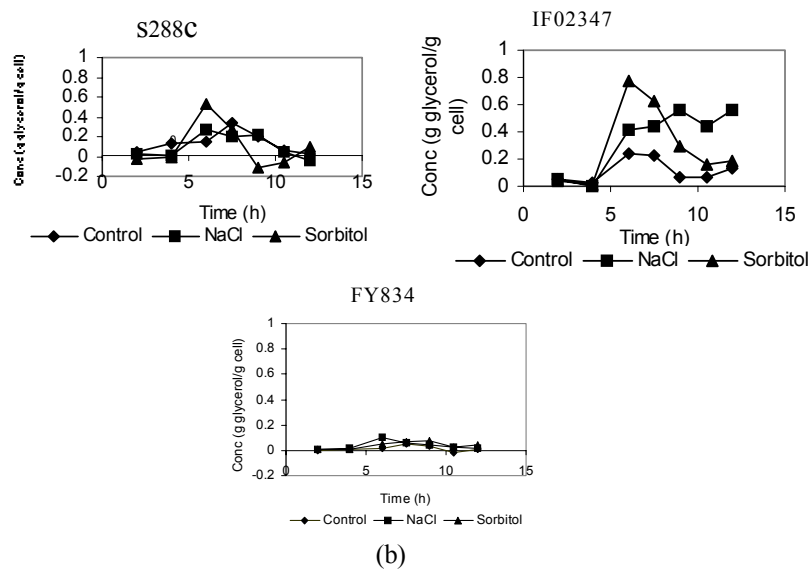
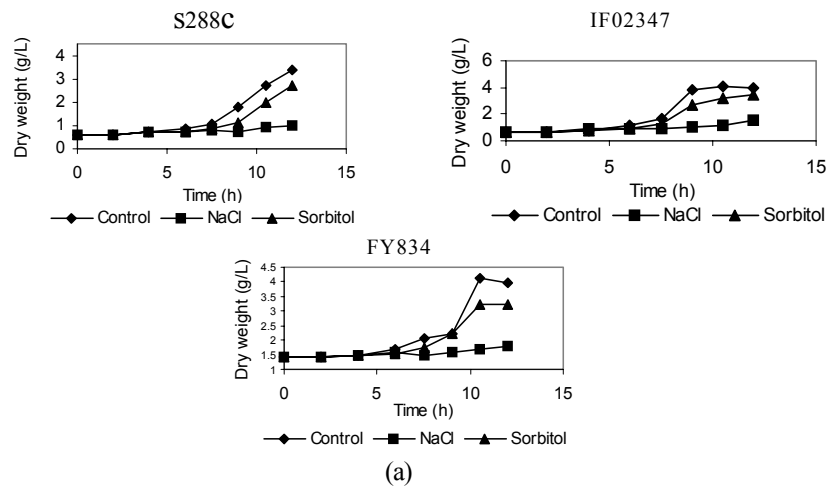


FIGURE 2. Different strains respond on NaCl and sorbitol addition (a) Growth and (b) Intracellular glycerol

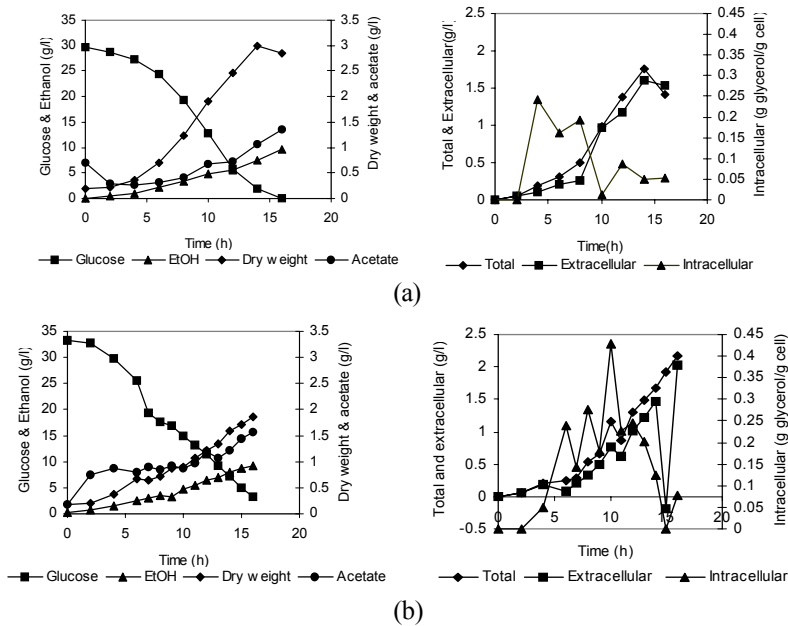


FIGURE 3. Jar fermentation results for control and stress (1M NaCl) experiment (a) Control and (b) Stress

CONCLUSIONS

From metabolic point of view, it can be concluded that both NaCl and sorbitol were suitable in creating osmotic condition as compare to control. Industrial strain IFO2347 was found to be the best strain which can stand high osmotic stress proving suitability usage in breweries industries, followed by s288c and FY834. Cells reacted toward these stress agents by accumulating intracellular glycerol to accommodate the changes as proved by our preliminary *in vivo* NMR. Further research to confirm these findings is still in progress.

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