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Salt-Induced Changes in Lipid Composition and Ethanol Tolerance in *Saccharomyces cerevisiae*

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ABSTRACT

The effect of salt stress on lipid composition and its relationship with ethanol tolerance in *Saccharomyces cerevisiae* was studied. Amounts of phospholipids as well as that of sterols decreased, whereas that of protein and glycolipids increased with increasing salt concentration. Relative proportion of amino phospholipids (phosphatidylethanolamine and phosphatidylserine) decreased, whereas that of phosphatidylcholine showed a reverse trend. Cells grown under increasing salt concentration were more resistant to ethanol-induced leakage of UV-absorbing substances, an index of ethanol endurance. Results showed an overlap between osmotolerance and ethanol tolerance in this strain.

Index Entries: Salt stress; *S. cerevisiae*; phospholipids; ethanol endurance; osmotolerance.

INTRODUCTION

It is well known that the plasma membrane is the first organelle to establish contact with alcohol and thereby be subject to an environmental stress. Studies on ethanol tolerance by microorganisms have suggested that cell membrane composition is an important aspect of end-product tolerance (*1–4*). It has been demonstrated that incorporation of unsaturated fatty acids and/or sterol(s) as well as proteolipids into cellular membrane

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helps to alleviate ethanol inhibition. Van Uden (*5*) has shown that ethanol tolerance of microorganisms is considerably diminished as the temperature rises, and a suggestion has been made that sites that determine maximum temperature are located in the cell membranes. The probable sites where ethanol interacts are the membranes lipids (*1*). There is ample evidence that microbes as well as higher organisms preadapted to one type of stress acquire a state of tolerance. This state of acquired tolerance to one type of stress can also be obtained during conditioning to another type of stress (*6*). During such types of studies, osmotolerance has been found to confer thermotolerance to *Saccharomyces cerevisiae* (*7*). Retrospectively, there seems to be an overlap between osmotolerance and thermotolerance.

Salt stress is well known to alter lipid composition of bacteria and halotolerant yeast species (*8–10*). In the past, the ability of microorganisms to grow under osmotic stress has received much attention from general physiological aspects (*6,11*). Present studies were carried out to examine changes in the lipid composition of *S. cerevisiae* NCYC 366, a brewing yeast, under various salt concentrations. A possible correlation between ethanol endurance of those cells under salt stress conditions has been explored.

METHODS

Yeast Strain and Growth Conditions

S. cerevisiae NCYC 366, generously sent by A. H. Rose, was maintained at 4°C on YPD slants (1% w/v yeast extract, 2% w/v peptone, 2% w/v dextrose, 2% agar, pH 5.5). The yeast was grown in YPD broth at 30°C with or without 0.5*M* NaCl. Since this yeast strain failed to grow in YPD supplemented with 1.0 or 1.5*M* NaCl, therefore, it was first grown for 16 h (midlong phase) in YPD supplemented with 0.5*M* NaCl. These NaCl-preconditioned cells were then grown in presence of the higher NaCl concentrations. The cells were harvested by centrifugation at 2000*g* for 5 min, and washed two times with distilled water followed by washings with phosphate buffered saline (PBS; 20 m*M* phosphate containing 600 m*M* KCl, pH 6.0).

Ethanol Tolerance

Cells grown in various salt concentrations were suspended in PBS containing ethanol (16% v/v) in conical flasks fitted with rubber septa. The flasks were incubated at 30°C at 175 rpm, and fixed aliquots withdrawn with a hypodermic syringe. The leakiness of the cells during incubation was measured by measuring the absorbance at 260 nm of supernatant, obtained after centrifuging the incubation mixture at 2000*g* for 15 min at 4°C.

Viability Measurement

Viability of the cells was determined by methylene blue staining method of Lees et al. (*12*) and also by colony counting after spreading appropriate dilutions on YPD-agar, thereafter keeping the plates at 30°C for 72 h. All the plates were run in duplicate.

Lipid Extraction and Analysis

Lipids from the washed cells were extracted following the procedure of Bligh and Dyer (*13*). The total lipid content was measured gravimetrically, and the phosphorus content determined according to Ames (*14*). Values for phosphorus contents were multiplied by 25 (assuming 775 being average molecular weight of a phospholipid molecule divided by 31) to obtain total amount of phospholipids. Total and esterified sterols were estimated by the method of Sperry and Webb (*15*), using ergosterol (Sigma Chemical Co., St. Louis, MO) as the standard. For estimating esterified sterols, free sterols were precipitated by an ethanolic solution or digitonin (1%). Glycolipids were quanitified following the procedure of Dubois et al. (*16*). Individual phospholipids were separated by two-dimensional thin-layer chromatography (TLC) on silicagel G-60 F254 plates (E. Merck) using chloroform/methanol/ammonia (65:35:5, v/v), in the first dimension, and chloroform/methanol/acetone/acetic acid/water (10:2:4:2:1, v/v), in the second dimension, as solvent systems. The various phospholipid spots visualized after staining the plate with iodine vapor were identified by comparing their RF values with standard phospholipids (Sigma Chemical Co.), and also by specific staining as described by Mangold (*17*). The iodine-stained (phosphorus +ve) spots were cut out and eluted with chloroform/methanol (1:1 v/v) several times. The solvents were removed, and phosphorus contents of the various eluates determined as described earlier. The recovery of phospholipids from silica gel plates was never $< 95\%$.

Protein Estimation

The total protein was estimated according to Bradford (*18*), using bovine serum albumin (Sigma Chemical Co.) as the standard.

RESULTS AND DISCUSSION

The cells grown in the presence of different salt concentrations were analyzed for lipid and protein contents. As shown in Table 1, the amounts of phospholipids as well as that of sterols decreased with an increase in halotolerant character of yeast. These observations are in agreement with earlier reports (*9*). Since sterols play an important role in modulating

Table 1
Total Phospholipid, Glycolipid, Sterol, and Protein Contents of *S. cerevisiae* Grown in YPD with or without NaCl

	YPD	YPD + 0.5*M* NaCl	YPD + 1.0*M* NaCl	YPD + 1.5*M* NaCl
Phospholipid	27.5 ± 1.2	21.6 ± 1.8	16.2 ± 2.1	10.5 ± 1.4
Glycolipid	26.0 ± 2.1	28.5 ± 1.9	30.0 ± 1.6	31.6 ± 2.8
Total sterols	16.5 ± 0.8	13.8 ± 0.9	11.9 ± 0.1	6.2 ± 0.1
Free sterols	9.0 ± 0.2	7.6 ± 0.4	5.8 ± 0.2	2.8 ± 0.1
Protein	15.0 ± 0.4	18.3 ± 1.1	21.2 ± 1.2	22.0 ± 1.8
Phospholipid/ protein ratio	1.80	1.18	0.80	0.55
Free sterol/ phospholipid ratio (molar)	0.35	0.35	0.37	0.27
Glycolipid/ phospholipid ratio (molar)	0.94	1.31	1.85	3.01

Values are expressed as mg/g dry wt and are means of four determinations ± SD.

Table 2
Phospholipid Composition of Yeast Cells Grown in YPD with or without NaCl

Phospholipid	YPD	YPD + 0.5*M* NaCl	YPD + 1.0*M* NaCl	YPD + 1.5*M* NaCl
PC	44.4 ± 2.0	48.8 ± 1.8	53.8 ± 4.0	57.4 ± 1.2
PE	25.0 ± 1.5	18.5 ± 0.6	16.5 ± 2.0	15.6 ± 1.0
PS	9.1 ± 1.0	8.5 ± 1.3	6.7 ± 0.2	4.5 ± 0.2
PI	20.9 ± 1.3	24.3 ± 2.9	22.5 ± 2.4	22.2 ± 2.0
PC/PE ratio	1.77	2.64	3.26	3.67

Values are expressed as means of four determinations ± SD. Trace amounts (< 5%) of cardiolipins and lysophospholipids were also detected. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PE, phosphatidylinositol.

membrane fluidity (*19*) and consequently membrane stability, the effect of halotolerance on the free-sterols-to-phospholipid ratio was also examined in *S. cerevisiae*. This ratio was reduced from 0.35 to 0.27 when cells were grown in the presence of 1.5*M* NaCl. This is consistent with earlier findings (*9*).

The protein content of yeast cells grown under conditions of salt stress increased with the increase in salt concentration. A similar result had been observed earlier (*10*) with the salt-tolerant yeast *Zygosaccharomyces rouxii*. As proteins of salt-stressed cells are enriched with acidic amino acids and hydrated at high saline conditions, this may be an adaptive mechanism, where many new proteins, called stress proteins, are generated (*5,6*).

Increased salinity of growth medium affected not only the total phospholipid content, but also the relative amounts of individual phospholipids (Table 2). The amounts of aminophospholipids, viz. phosphatidyl-ethanolamine (PE) and phosphatidylserine (PS), decreased, but that of phosphatylcholine (PC) increased with an increase in salt stress. However, no significant change was registered in the amounts of another major lipid, phosphatidylinostol (PI), under identical conditions. These observations are different from those reported earlier for the salt-tolerant yeast *Debaryo-*

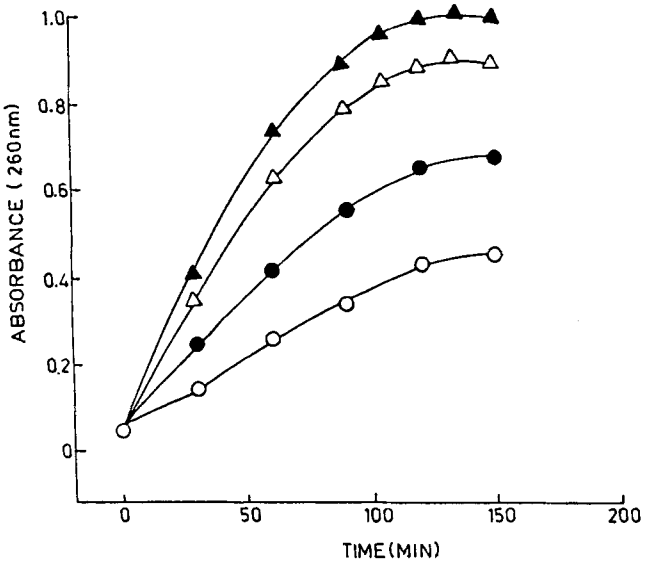

Fig. 1. Ethanol-induced leakage of 260 nm of absorbing substances from *S. cerevisiae* grown under various concentrations of NaCl and suspended in PBS containing 16% ethanol (v/v). Control (▲), 0.5*M* (△), 1.0*M* (●), 1.5*M* (○).

myces hansenii (*9*). In this yeast species (unlike the present case), amounts of PS increased and that of PI decreased by increasing salt concentration of growth medium.

The amount of glycolipid, another major constituent, enhanced when the yeast cells were grown in the presence of different salt concentrations. This could be a compensatory mechanism evolved by such cells, where contents of phospholipids and sterols are decreased. A similar trend was documented by Russell (*8*). The glycolipids can play a significant role in stabilizing membrane structure owing to their high phase transition temperature and ability to form nonbilayer structures (*20,21*).

It is well established that when microorganisms are cultivated under high osmolarity conditions, compatible solutes accumulate (*22*). Under high osmolarity, it is possible that plasma membrane might become rigid. However, as indicated by a decrease in phospholipid-to-protein and sterol-to-phospholipid ratios as well as an increase in PC-to-PE ratio, membrane fluidity should have been increased under salt stress, which in turn might be compensated for by an increase in glycolipid-to-phospholipid ratio, providing an optimal level of membrane fluidity. In the past, the role of lipids and proteins has been established to enhance alcohol endurability of microorganisms (*2–4*). Resistance to ethanol-induced leakage of UV-absorbing substances has been adopted as a means of rapid screening of ethanol-tolerant yeast strains (*23*). In an attempt to determine whether changes in the membrane composition of *S. cerevisiae*, under high-salinity conditions, have a correlation with ethanol tolerance of the organism, ethanol endurability of such cells was investigated (Fig. 1). Evidently, cells adapted to high-saline conditions were capable of checking ethanol-

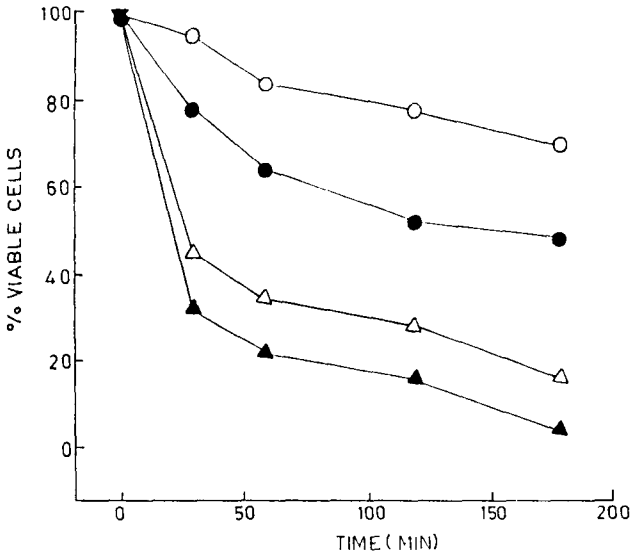

Fig. 2. Viability (methylene blue method) of *S. cerevisiae* cells grown at different concentrations of NaCl and then incubated in PBS with 16% ethanol (v/v) added. Control (▲), 0.5*M* (△), 1.0*M* (●), 1.5*M* (○).

induced leakage to a greater extent. To confirm these results, viability of such cells was also studied (Fig. 2). It was evident that adaptation of yeast cells to high osmotic conditions renders a major part of the population ethanol-tolerant. These findings thus confirm a previous proposition of acquired tolerance (*6,20*). Cells with increased fluidity can tolerate higher ethanol concentrations (*3*). Increased fluidity as a result of salt stress further corroborates the previous findings.

Therefore, such osmotolerant yeast strains might have a potential application in fermentation industries, where yeast cells are exposed to high osmotic stress and high ethanol concentrations (*24*). Present findings suggest an overlap between osmotolerance and ethanol-endurance in *S. cerevisiae*.

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