Effect of Sodium Chloride on Lipid Composition of *Saccharomyces rouxii*

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*Saccharomyces rouxii* is a salt-tolerant yeast, industrially important for miso- and shoyu-making. The change of intracellular lipid composition was examined in shaken cultures of *S. rouxii* ATCC 42981 grown in medium containing various concentrations of NaCl. Increases in sterol-esters and free fatty acids and a decrease in triacylglycerol were observed on adding 1 M or 2 M NaCl to the medium. With an increase in NaCl concentration to 3 M, sterol-esters decreased, triacylglycerol increased, and free fatty acids increased further. An increase in oleic acid and the decrease in linoleic acid were found with an increase in NaCl concentration. Effects of NaCl similar to these were also recognized in other strains of *S. rouxii*. The shape of *S. rouxii* cells changed from spherical to ellipsoidal with an increase in NaCl concentration. The size of cells was observed microscopically to be smaller in 2 M and 3 M NaCl-media than in 0 M and 1 M NaCl-media.

*MATERIALS AND METHODS*

**Yeasts.** *Saccharomyces rouxii* ATCC 42981 and IAM 4028 were provided by the National Food Research Institute, Ministry of Agriculture, Forestry, and Fisheries, Japan. *S. rouxii* S84, G10-1 and S2-B were provided by the Food Research Institute, Niigata Prefecture, Japan. *S. cerevisiae* 0-ll-4 was provided by Sankyo Co., Ltd., Japan.

**Medium.** The composition of YM medium employed was 3 g of yeast extract (from Nissui Chemical, Ltd., Japan), 5 g of polypeptone (from Daigo Eiyo Chemical, Ltd., Japan), 10 g of glucose in 1 liter of water, pH 4.8. The pH value of the medium containing NaCl was adjusted to 4.8 after the addition of NaCl. For strain S2-B it was adjusted to 4.6.

**Culture.** One loop of cells from a streak culture of *S. rouxii* was inoculated into 5 ml of YM medium without NaCl and incubated statically at 30°C for 48 hr. The pre-incubated culture was poured into 95 ml of YM medium containing NaCl (0, 1, 2 and 3 M) and incubated at 30°C by reciprocal shaking at 95 rpm. After 40 hr of incubation (Fig. 1), cells were harvested by centrifugation and washed 3 times with water. The growth of *S. rouxii* was estimated by measuring the number of cells with a Thoma haemacytometer. *S. cerevisiae* was cultured similarly in YM medium containing NaCl (0, 0.3, 0.6, and 1.0 M).

**Extraction of total lipids.** The absorbance at 600 nm of each yeast cell suspension was adjusted to 20. Ten ml of the suspension was transferred into a centrifuge tube (50 ml volume) with a glass stopper. The yeast cells were collected by centrifugation and resuspended in 4 ml of 0.1 M Tris-HCl (pH 7.5) containing 0.8 M KCl, 0.1 M Na2SO4 and 0.9 mg of Zymolyase 60,000 (from Kirin Brewery Co., Ltd., Japan). The mixtures were incubated at 30°C for 45 min. Total lipids were extracted from the Zymolyase digests by the method of Bligh and Dyer.

**Analysis of lipids.** Total lipids were dissolved in 200 μl
of chloroform–methanol (2:1, v/v). One μl of lipid solution was spotted on a Chromarod of Iatroscan TH-10 (from Iatron Laboratories, Inc., Japan) and developed with n-hexane–ether–formic acid (90:10:1, v/v) for analysis of neutral lipids. After the development, the amount of lipid in each chromatogram was determined by an Iatroscan TH-10 (TLC/FID analyzer). The analysis of phospholipids was carried out as follows: Fifty μl of lipid solution was spotted on a silica gel plate (from E. Merck A. G., 5 x 20 cm) and developed with n-hexane–ether–formic acid (90:10:1, v/v). The phospholipid fraction was scraped off of the plate and put into a screw cap test tube with a teflon liner. Phospholipids were eluted from silica gel scraped in 2 ml of chloroform–methanol (2:1, v/v). Two more washings were also collected. After drying, phospholipids were dissolved in 50 μl of chloroform–methanol (2:1, v/v). One μl of phospholipid solution was spotted on a chromarod, developed with chloroform–methanol–water (50:60:4.5, v/v) and analyzed by Iatroscan TH-10. The identification of the lipids was carried out by comparison with authentic compounds. After the correction of each area on the Iatroscan chart by the relative detector (FID) sensitivity, the area of each lipid was compared with the others. The relative detector sensitivity of sterol-esters, fatty acids, triacylglycerol, sterols, or phospholipids to stearyl alcohol is 1.2, 1.0, 1.3, 1.2, or 3.0, respectively. The determination of fatty acid composition was carried out as follows: Forty μl of lipid solution was put into a screw cap test tube. After removing traces of the solvent in vacuo, 4.5 ml of 2% (v/v) H₂SO₄-ab. methanol was added. The further procedures were carried out as described in our previous publication.⁴

RESULTS

Effects of NaCl on growth of S. rouxii and S. cerevisiae

Figure 1A shows the course of growth of S. rouxii. Increases in the concentration of NaCl resulted in retarded growth of S. rouxii, especially during the early incubation period. During prolonged incubation this effect was minimized. During the first 15 hr of incubation the growth in 0 m and 1 m NaCl-media was almost the same, though the growth in 2 m and 3 m NaCl-media was low. On incubating the cultures for 24 hr, no significant difference was observed in growth in 0 m, 1 m, and 2 m NaCl-media. Prolonged incubation over 40 hr allowed the growth in 3 m NaCl-medium to attain almost the same level as in 0 m, 1 m, and 2 m NaCl-media. During these investigations therefore, the cells were harvested after 40 hr of incubation. As a result the cells grown in 3 m NaCl-medium were in early stationary phase.

Fig. 1. Effects of NaCl on Growth of Saccharomyces rouxii ATCC 42981 and Saccharomyces cerevisiae 0-11-4.

Both yeasts were pre-incubated in 5 ml of YM medium (pH 4.8) at 30°C for 48 hr statically and then incubated in 100 ml of YM medium by shaking at 95 rpm reciprocally. Yeast cells obtained were washed 3 times with water. The growth of S. rouxii and S. cerevisiae were determined by measuring the number of cells with a Thoma haemacytometer and by measuring the absorbance of cell suspensions at 600 nm, respectively.

A shows the courses of growth of S. rouxii ATCC 42981 in the various concentrations of NaCl. ○—○, 0 m NaCl; △—△, 1 m NaCl; ▲—▲, 2 m NaCl; ▲—▲, 3 m NaCl.

B shows the ratios of growth of S. rouxii ATCC 42981 and S. cerevisiae 0-11-4. ○—○, S. rouxii; ●—●, S. cerevisiae.
Effect of NaCl on Lipid Composition of *S. rouxii*

2417

**Effect of NaCl on Lipid Composition of *S. rouxii***

The cells of *S. rouxii* grown in the medium containing NaCl (0 ~ 3 M) were observed under a microscope (Fig. 2). The average ratio of the length of the minor axis to that of the major axis was calculated from the photographs. The ratios were 0.90, 0.67, 0.65, and 0.50 in 0 M, 1 M, 2 M, and 3 M, respectively. These data indicate that the shape of *S. rouxii* cells changed from spherical to ellipsoidal with an increase in NaCl concentration in the medium.

**Effect of NaCl on shape of *S. rouxii* cells**

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**Lipid composition of *S. rouxii* ATCC 42981 cells**

Figure 3 shows the typical patterns of neutral lipids (A) and phospholipids (B) from *S. rouxii* cells (1 M NaCl) in the analysis with the Iatroscan TH-10.

Figure 4 shows the effect of NaCl on the content of each lipid component per mg of lyophilized yeast cells (*S. rouxii*). Sterol-esters...
Y. Watanabe and M. Takakuwa

Fig. 3. Typical Patterns of Neutral Lipids (A) and Phospholipids (B) from Saccharomyces rouxii ATCC 42981 Cells (1 m NaCl) by Iatroscan TH-10.

O, origin; F, front; PL, phospholipids; S, sterols; FFA, free fatty acids; TG, triacylglycerol; SE, sterol-esters; LPL, lysophospholipids; PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; UN, unknown components.

(SE) levels increased markedly in the cells grown in 1 m and 2 m NaCl-media as compared with cells grown in 0 m NaCl-medium. Growth in 3 m NaCl-medium, however, resulted in a fall in these levels to almost the same as cells grown in 0 m NaCl-medium. A marked decrease was observed in the triacylglycerol (TG) content of the cells when NaCl concentration in the medium was increased from 0 m to 2 m. A further increase in NaCl concentration to 3 m resulted in an increase in TG content. Intracellular levels of free fatty acids (FFA) and sterols (S) increased with an increase in NaCl concentration. An increase in NaCl concentration from 0 m to 2 m had no apparent effect on phospholipids level, although a further increase to 3 m resulted in a slight lowering of phospholipids level in the cells of S. rouxii.

Figure 4 shows the effect of NaCl on the proportions of components of neutral lipids (A), polar-head groups in phospholipids (B), and cellular fatty acids (C), in S. rouxii cells. The proportions of TG, SE, and FFA changed markedly. In the assay of polar-head groups in phospholipids, it is noteworthy that the percentage of phosphatidylinositol (PI) increased 3.5-fold. The changes in percentages of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were surprisingly small. In the assay of cellular fatty acids, it was observed that oleic acid (18:1) increased but linoleic acid (18:2) and palmitoleic acid (16:1) decreased with an increase in NaCl concentration. The saturated fatty acids (16:0, 17:0, and 18:0) were present only in trace amounts and were almost unaffected. The unsaturation index, which indicates the extent of unsaturation of fatty acids (U.I. = Σ% of fatty acid × the number of double bonds), decreased with an increase in NaCl concentration. Such find-
Effect of NaCl on Lipid Composition of S. rouxii

Fig. 5. Effects of NaCl on Composition of Neutral Lipids, Polar-head Groups in Phospholipids, and Cellular Fatty Acids from Saccharomyces rouxii ATCC 42981 Cells.

(A): The composition of neutral lipids. △—△, sterol-esters; ○—○, triacylglycerol; ▲—▲, sterols; □—□, free fatty acids; ■—■, unknown components.

(B): The composition of polar-head groups in phospholipids. ○—○, phosphatidylcholine; ●—●, phosphatidylethanolamine; △—△, phosphatidylinositol; ▲—▲, phosphatidylserine; □—□, lysophospholipids. The percentage of unknown component was omitted.

(C): The composition of cellular fatty acids. ○—○, linoleic acid; ●—●, oleic acid; ▲—▲, palmitoleic acid; △—△, palmitic acid; ■—■, heptadecanoic acid; □—□, stearic acid; ○—○, unsaturation index (U.I.), Σ% of fatty acid × the number of double bonds.

Lipid composition of S. cerevisiae 0-11-4 cells

Figure 6 shows the effect of NaCl on the proportions of neutral lipids (A), polar-head groups in phospholipids (B), and cellular fatty acids (C) in S. cerevisiae cells. As shown in Fig. 1B, the growth of S. cerevisiae in 1.0 M NaCl was too low. Lower concentrations of NaCl were hence employed during the present studies to obtain sufficient amounts of cells for these assays. In S. cerevisiae cells TG and SE levels changed markedly contrary to those in S. rouxii cells. S and FFA levels were almost unaffected. In the assay of polar-head groups in phospholipids, PC increased slightly with an increase in NaCl concentration. The other components were in trace amounts and their changes were also small. The changes in cellular fatty acids in S.
Table I. Effects of NaCl on Composition of Neutral Lipids from Various Strains of Saccharomyces rouxii

Total lipids were extracted from Zymolyase digests of cell pellets obtained from 10 ml of cell suspension ($A_{600nm}=20$). Analysis of neutral lipids was carried out as described in Materials and Methods. Several unknown components were omitted from this table.

<table>
<thead>
<tr>
<th>Strains</th>
<th>NaCl (M)</th>
<th>Growth ($A_{600}$)</th>
<th>% of neutral lipids</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>SE</td>
</tr>
<tr>
<td>S84</td>
<td>0</td>
<td>13.8</td>
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<td>23.6</td>
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</tr>
<tr>
<td></td>
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<td>26.3</td>
</tr>
<tr>
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<td>5.5</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>17.1</td>
<td>26.2</td>
</tr>
</tbody>
</table>

Table II. Effects of NaCl on Composition of Cellular Fatty Acids from Various Strains of Saccharomyces rouxii

Analysis of fatty acids was carried out as described in Materials and Methods. Myristic acid, heptadecanoic acid, and linolenic acid were only traces and thus were omitted.

<table>
<thead>
<tr>
<th>Strains</th>
<th>NaCl (M)</th>
<th>% of fatty acids</th>
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<td></td>
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</tr>
<tr>
<td>IAM 4028</td>
<td>2</td>
<td>11.0</td>
</tr>
</tbody>
</table>

cerevisiae were insignificantly small. U.I. was almost unaffected in S. cerevisiae cells.

Lipid composition of various strains of S. rouxii

Table I shows the effects of NaCl on the proportion of four neutral lipids from various strains of S. rouxii. The percentages of SE and FFA were observed to increase and that of TG was observed to decrease in all strains upon adding 2 M NaCl to the medium. Changes in the percentage of S varied among the strains used in these experiments. Table II shows the effects of NaCl on the relative percentage of cellular fatty acids from various strains of S. rouxii. On adding NaCl to the medium, the increase in 18:1 acid and the decreases in 18:2 acid and U.I. were recognized in all strains. However the change in the percentage of 16:1 acid varied among the strains used.

DISCUSSION

Among the salt-tolerant species of Torulopsis, Candida, Hansenula, Debaryomyces, and Saccharomyces we wish to discuss mainly the relationship between the lipid composition of salt-tolerant S. rouxii cells and
the NaCl concentration in the medium.

With respect to the composition of fatty acids of S. rouxii cells the increase in 18:1 acid and the decreases in 18:2 acid and U.I. were observed with an increase in NaCl concentration (Fig. 5C). Such observations may suggest that the cell membrane may reduce its fluidity to be more rigid, although the analysis was not carried out on the lipids of the cell membrane. Mogi et al.\(^6\) reported similar changes of fatty acid composition with S. rouxii cells cultured statically in 0 and 18% NaCl-media for a long period, although the extent of unsaturation and the percentage of 18:2 acid were low since their culture conditions were more anaerobic than that in the present experiments. Such phenomena could not be observed in S. cerevisiae, a salt-sensitive yeast (Fig. 6C). Moreover, it is noteworthy that the above findings are contrary to the effect of ethanol on fatty acid composition of S. cerevisiae cells, where the greater viability was retained in cells enriched in 18:2 residues as compared with 18:1 residues.\(^7\)

It has been postulated that sterols exist in bilayer membranes and may play some role in the regulation of fluidity\(^8\) and permeability\(^9\) of cell membranes. However, the location of SE in cell membrane and its functions are not yet known. At present, there are two hypotheses about the location of SE.\(^12\) Valic et al.\(^13\) reported that SE exists in the bilayer membrane, facing the ester-linkage of SE to the exterior. On the other hand, Hossack and Rose\(^14\) reported that SE could not be detected in cell membranes prepared carefully.

With respect to the latter, it is recently reported that SE exists as "droplets" or "pockets" which are attached very strongly to cell membranes.\(^15\) S. rouxii cells produced greater amounts of SE with the increased concentration of NaCl (Figs. 4 and 5A). S. cerevisiae 0-11-4 cells originally contained a larger amount of SE, which was reduced in proportion with an increase in NaCl concentration (Fig. 6A). These results suggest that the increased SE in S. rouxii cells may have a significant role which may be different from that in S. cerevisiae cells. Some information on the significance of SE in yeast cells can be made available by pursuing the relationship between SE in the salt-tolerant yeast cells (S. rouxii) and NaCl. We are now researching the molecular species of sterol in the increased SE and its localization in the cells.

Yeast cells are known to accumulate TG as storage product.\(^16\) It is noteworthy that S. rouxii cells accumulate larger amount of TG than S. cerevisiae cells in the absence of NaCl (Figs. 4, 5A and 6A). In S. rouxii cells, the TG content decreased with an increase in NaCl concentration (Fig. 4), suggesting that the synthesis of TG was depressed by the addition of NaCl to the medium. On the other hand, S. rouxii cells grown in the presence of NaCl contained larger amount of free fatty acids (Figs. 4 and 5A), suggesting that in S. rouxii cells the synthesis of fatty acids may not be depressed by the addition of NaCl. Additionally the content of phospholipids was almost constant from 0 M to 2 M NaCl but decreased slightly in 3 M NaCl (Fig. 4), indicating that the content of phospholipids may be little affected by the addition of NaCl. The depressive effect of NaCl was observed only in the synthesis of TG. Moreover, as shown in Fig. 1A, cells grown in 0 M, 1 M, and 2 M NaCl-media were in the late stationary phase and those in 3 M NaCl-medium were in the early stationary phase. Since it appears that the lipid composition is affected by differences in age of the culture,\(^6\) we are now researching the relationship between lipid composition and the culture age.

The shape of S. rouxii cells changed from spherical to ellipsoidal with an increase in NaCl concentration of the medium (Fig. 2). Since it is known that the shape of cells is defined by cell walls, such changes of cell shape may suggest that the participation of cell walls in the salt-tolerance of yeasts is not negligible.

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REFERENCES