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Changes in the Membrane Properties of *Zygosaccharomyces rouxii* in Response to Osmotic Stress

– Review –

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Abstract

***Zygosaccharomyces rouxii* is a salt-tolerant yeast which plays an important role during the ripening stage of soy sauce fermentation. *Z. rouxii* used in the experiment could grow in YPD (1% yeast extract, 2% peptone and 2% glucose, pH5.0) medium with 18% (w/v) NaCl, whereas *Saccharomyces cerevisiae* could only grow in YPD medium with less than 8% NaCl. In the presence of 15% NaCl, *Z. rouxii* accumulates a large amount of glycerol as a compatible solute within the cells in the exponential phase. It is a characteristic of salt-tolerant yeasts. From the chemical analyses on membrane lipid fluidity, the membrane structure of the cells grown in 15% NaCl was suggested to become more rigid and its fluidity was decreased to keep glycerol within the cells in response to surrounding medium with high concentrations of salt.**

Key words : salt-tolerant yeast, *Zygosaccharomyces rouxii*, membrane fluidity

INTRODUCTION

We often see mold growing on bread under household conditions and recognize that microbial deterioration of food proceeds more rapidly in humid atmospheres than under very dry conditions. It is well known that food can be preserved from microbial spoilage by adjusting physicochemical conditions to retard or prevent the growth of microbes. Ways to decrease available water in food are the addition of sugar or salt, freezing or drying. Moisture level in the solution or solid substrate is described as water activity(a_w) ; this is a concept that was introduced by Scott[1]. Thus, minimal water activity is important from the view point of food presevation. Microorganisms can grow in foods with a low level of water activity such as those containing a large amount of sugar or salt. Minimal water activity required for the growth of microorganisms[2] is shown in Table 1. In general, bacteria require high water activity for the growth, whereas fungi can thrive in a rather dry environment.

On the other hand, the growth condition of yeasts is generally somewhere between these two groups. It has been observed that the concentrations of one or more intracellular components increase as osmotolerant microbes respond to low water activity in the growth medium[3]. These components are thought to be acting as osmoregulators and to have a secondary function to protect enzyme activities. These components have been called compatible solutes by Brown[4].

Algae, bacteria, yeasts and fungi accumulate different compatible solutes in response to surrounding medium with low water activity[5].

Since ancient times yeasts have been used to brew alcoholic beverages and bake breads. *Saccharomyces cerevisiae*, in particular is a common popular yeast in the fields of fermentation and molecular

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genetics. While, *Zygosaccharomyces rouxii* is a salt-tolerant yeast which plays an important role during the ripening stage of soy sauce fermentation. A non-salt-tolerant yeast like *S. cerevisiae* can only grow in medium above 0.90 a_w. However, *Z. rouxii* can grow at a much lower level of water activity. Depending on how the water activity of the medium is adjusted, the growth-limiting water activity for *Z. rouxii* changes accordingly. The minimal water activity is 0.85 a_w when adjusted with salts, whereas it changes to 0.60 a_w when adjusted with sugars[3]. This dependency of growth-limiting water activity on salt and nonelectrolyte is not presently understood.

The characteristic of salt-tolerance in yeasts has long been recognized, but the mechanism of salt-tolerance has not been fully elucidated.

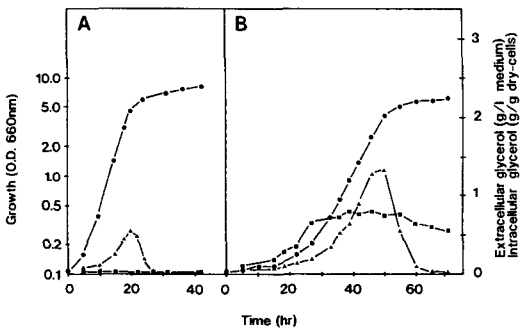

Fig. 1. Changes in the accumulation of intracellular and extracellular glycerol by *Z. rouxii* grown in YPD medium without (A) or with 15% NaCl(B).
Symbol : (●) growth, (▲) extracellular glycerol and (■) intracellular glycerol.

When *Z. rouxii* is grown in medium with low water activity, it accumulates glycerol in cells as the primary osmoregulatory solute[2,6,7]. *Z. rouxii* used in the experiment[8] could grow in YPD medium with about 18% (ca. 3.1M) NaCl, whereas *S. cerevisiae* could only grow in YPD medium with less than 8% NaCl.

The changes in the accumulation of intracellular and extracellular glycerol are shown in Fig. 1[7]. *Z. rouxii* accumulated a large amount of intracellular glycerol at exponential growth phase when it was grown in YPD medium with 15% (ca. 2.6M) NaCl, but the same cells did not accumulate intracellular glycerol when it was grown in YPD medium. The intracellular amount of glycerol in *Z. rouxii* was shown to be proportional to the concentration of NaCl in the growth medium[9]. The accumulation of intracellular glycerol is a characteristic of *Z. rouxii* and is considered to be a prerequisite for tolerance toward high concentration of salts in the growth medium[10]. When grown in YPD medium with 15% NaCl, *Z. rouxii* at the end of exponential growth phase produced about 2.5-fold more extracellular glycerol compared to the amount of extracellular glycerol when it was grown in YPD medium. A number of hypotheses has been proposed to explain the mechanism of intracellular glycerol accumulation when *Z. rouxii* is grown in medium with low water activity. The proposed mechanisms include glycerol and the change of membrane properties to retain glycerol in cells.

Table 1. Minimal water activity required for the growth of microorganisms

Bacteria		Yeasts		Fungi	
Bac. mycoides	0.99	*Torulopsis utilis*	0.94	*Rhizopus*	0.92~0.94
Pseudomonas	0.97	Beer yeast	0.94	*Botrytis*	0.93
Achromobacter	0.96	*Candida utilis*	0.94	*Mucor*	0.92~0.93
E. coli	0.935~0.96	*Schizosaccharomyces*	0.93	*Oospora lactis*	0.895
Bac. subtilis	0.95	Baker yeast	0.905	*Asp. niger*	0.88~0.89
Cl. botulinum	0.95	*Mycoderma*	0.90	*Penicillium*	0.80~0.83
Aerobactor aerogenes	0.945	*Sacch. cerevisiae*	0.895	*Asp. flavus*	0.80
Salmonella newport	0.945	*Rhodotorula*	0.89	*Asp. candidus*	0.75
Sc. faecalis	0.94	*Endomyces*	0.885	*Asp. chevalieri*	
Sarcina	0.915~0.930	*Willia anomala*	0.88	*Asp. repens*	
Mc. roseus	0.905	*Zygo. rouxii*	0.60~0.61	*Asp. ruber*	0.65
Staphy. aureus	0.86			*Asp. amstelodami*	
Halophilic bacteria	0.75			*Xeromyces bisporus*	

GLYCEROL METABOLISM

The pathways for glycerol metabolism are shown in Fig. 2[17]. Fructose-1,6-diphosphate is changed to dihydroxyacetone phosphate and glyceraldehyde-3-phosphate by fructose diphosphate aldolase (EC 4.1.2.13).

A part of glyceraldehyde-3-phosphate is converted to dihydroxyacetone phosphate by triosephosphate isomerase (EC 5.3.1.1) and the rest is dissimilated into the glycolytic pathway.

Dihydroxyacetone phosphate is converted to glycerol-3-phosphate by cytoplasmic NAD-dependent glycerol-3-phosphate dehydrogenase (EC 1.1.1.8) and subsequently it is converted to glycerol by phosphatase[11,12]. Glycerol production via dihydroxyacetone has not yet been reported in any yeasts. The specific activities of fructose diphosphate aldolase, triosephosphate isomerase and glycerol-3-phosphate dehydrogenase were examined in extract from *Z. rouxii* grown in YPD medium with 15% NaCl. These three enzymes are closely related to glycerol production. Both activities of triosephosphate isomerase and glycerol-3-phosphate dehydrogenase in the extract increased about 2 times and the activity of fructose diphosphate aldolase decreased to 80% when *Z. rouxii* was grown in YPD medium with 15% NaCl. These results suggest that the enzyme activities are not greately influenced to increase the amount of glycerol by the presence of 15% NaCl.

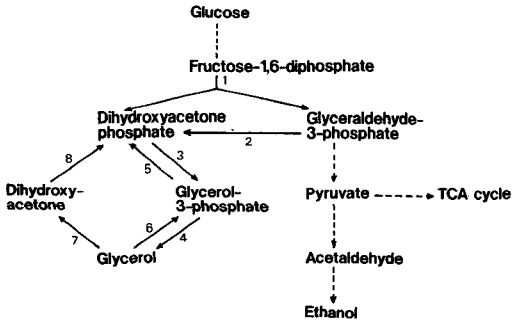

Fig. 2. Scheme for glycerol metabolism in *Z. rouxii*.
(1) Fructose diphosphate aldorase(EC 4.1.2.13) ; (2) triosephosphate isomerase(EC 5.3.1.1) ; (3) NAD-dependent glycerol-3-phosphate dehydrogenase(EC 1.1.1.8) ; (4) phosphatase ; (5) mitochondrial glycerol-3-phosphate dehydrogenase(EC 1.1.99.5) ; (6) glycerol kinase (EC 2.7.1.30) ; (7) glycerol dehydrogenase (EC 1.1.1.6) ; and (8) dihydroxyacetone kinase

Glycerol dissimilation can occur via either glycerol-3-phosphate or dihydroxyacetone. The metabolism of glycerol in *S. cerevisiae*[13] occurs via glycerol-3-phosphate and involves glycerol kinase (EC 2.7.1.30) and mitochondrial glycerol-3-phosphate dehydrogenase (EC 1.1.99.5). An alternative dissimilatory pathway via dihydroxyacetone involving glycerol dehydrogenase (EC 1.1.1.6) and dihydroxyacetone kinase occurs in *Schizosaccharomyces pombe*[14,15]. Both glycerol dehydrogenase and dihydroxyacetone kinase activities have been reported in *S. cerevisiae*[16], but the significance of these enzymes in glycerol dissimilation was not established. The route via dihydroxyacetone in *Z. rouxii* has been suggested by van Zyl *et al.*[17] to be significant in osmoregulation ; the activities of glycerol dehydrogenase (EC 1.1.1.6) and dihydroxyacetone kinase in cell extract were found to be increased 9- and 4-folds, respectively, during osmotic stress as compared to non-stressed conditions. From their earlier paper[18], the initial response of *Z. rouxii* to osmotic stress is suggested to be glycerol retention inside cells and glycerol accumulation from growth medium by means of an active sodium-driven transport system.

Subsequently, the pathway that utilizes excess accumulated glycerol is induced. The activities of glycerol dehydrogenase and dihydroxyacetone kinase in this pathway are then regulated to adjust the amount of glycerol during the growth at low water activity. This similar mechanism of accumulating intracellular glycerol has been reported for *Debaryomyces hansenii*[19].

In high concentrations of NaCl, both *Z. rouxii* and *S. cerevisiae* produced a large amount of glycerol when it was grown in YPD medium with 6% NaCl and this amount was much more than that produced by *Z. rouxii* when it was grown in YPD medium with 15% NaCl(unpublished results). However, the intracellular amount of glycerol in *Z. rouxii* was much more than that in *S. crevisiae* ; a large part of glycerol produced by *S. cerevisiae* was leaked into the surrounding medium. *Z. rouxii* thus seems to have a special mechanism to retain glycerol in cells. The mechanism of glycerol accumulation is thought to depend not on the increase in glycerol production, but rather

on the change of plasma membrane function in retaining intracellular glycerol.

PLASMA MEMBRANE PROPERTIES

The plasma membrane controls the entry of nutrients and the exit of waste products, and thus generate differences in their concentrations between the interior and exterior of the cell.

Singer and Nicolson[20] had offered the fluid mosaic model of the structure of cell membranes in 1972. This model has been widely accepted by many researchers. Biological membranes are generally thought to be composed of lipid bilayer and proteins which are dispersed in this lipid bilayer.

Membrane fluidity influences various important functions such as permeability and transport of some low molecular weight substances. The membrane lipids play an important role in controlling membrane fluidity.

Several factors are involved in the maintenance of the proper fluidity : the type of fatty acyl chain, the amount of sterols and, to a lesser extent, the nature of the polar head-groups of phospholipids.

The change of plasma membrane properties under osmotic stress was examined to elucidate the mechanism of glycerol accumulation in *Z. rouxii*[8]. To isolate plasma membranes, yeast cells were converted to protoplasts by cell wall lytic enzyme, Zymolyase, as previously reported[21]. Then protoplasts were lysed osmotically and crude plasma membranes were collected by centrifugation. The contents of total lipid, phospholipid, protein and ergosterol in plasma membranes were examined. Total lipid and phospholipid in plasma membranes isolated from cells grown in YPD medium with 15% NaCl decreased to 83% and 56%, respectively, as compared to those of control cells. But protein and ergosterol increased 1.4- and 2.9-folds, repectively. The ratio of phospholipid to protein and the ratio of ergosterol to phospholipid had often been used as the index of membrane fluidity in the past. The ratio of phospholipid to protein in plasma membranes decreased from 0.05 to 0.02, but the ratio of ergosterol to phospholipid increased 5-fold from 1.34 to 6.83 when *Z. rouxii* was grown in YPD medium with 15% NaCl. These results suggested that the membrane fluidity was decreased with the presence of 15% NaCl in the growth medium.

Fatty acid composition of plasma membranes of *Z. rouxii* is shown in Fig. 3[8].

Generally, *S. cerevisiae* has a large amount of unsaturated fatty acids such as palmitoleic acid ($C_{16:1}$) and oleic acid ($C_{18:1}$), and a small amount of saturated fatty acids such as lauric acid (C_{12}), Myristic acid (C_{14}), palmitic acid (C_{16}) and stearic acid (C_{18})[22]. In addition to these fatty acids, *Z. rouxii* has a large amount of linoleic acid ($C_{18:2}$). The percentage of linoleic acid decreased from 46.3% to 28.7% and that of oleic acid increased from 32.6% to 46.0% in plasma membranes when *Z. rouxii* was grown in YPD medium with 15% NaCl[18]. Desaturation of fatty acids from oleic acid to linoleic acid was inhibited by the presence of 15% NaCl in the growth medium.

Structural changes of the acyl chain such as unsaturation, length and branching are thought to affect membrane fluidity. In these experiments, the degree of unsaturation of fatty acids in plasma membranes was calculated by the equation of Kates and Hagen[23].

It was found that the index of the degree of unsaturation decreased from 1.37 to 1.18 when yeast cells were grown in the presence of 15% NaCl. These results suggested that the membrane fluidity decreased under osmotic stress.

Sterol also plays an important role in determining the structural organization of biological membranes.

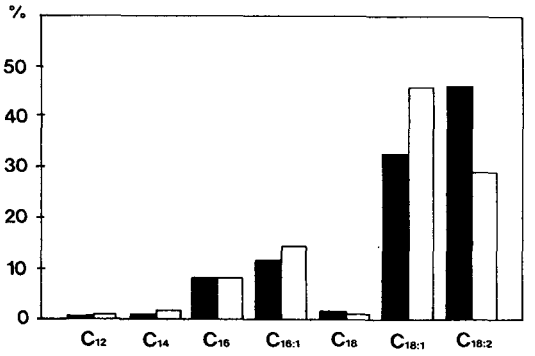

Fig. 3. Fatty acid composition of plasma membranes of *Z. rouxii* grown in YPD medium with or without 15% NaCl.
Open and closed bars show percentages of fatty acids extracted from the cells grown in the medium with and without 15% NaCl, respectively.

The structural organization of membranes in turn influences such functions as the permeability of solutes and the activities of membrane-bound enzymes[24,25]. The major sterol of yeast has been reported to be ergosterol[26]. There are two types of ergosterol : free type that is easily extracted by conventional lipid extraction procedure, and bound type that is released after saponification of isolated membranes. Free type of ergosterol was found to increase 2.9-fold from 5.5 to 15.7mg/g dry weight of plasma membranes when *Z. rouxii* was grown in YPD medium with 15% NaCl[8] . This increase in the amount of free ergosterol in plasma membranes might cause a decrease in the membrane fluidity. Bound type of ergosterol in plasma membranes had not been determined in this study, whereas the content in whole cells was 0.6 and 0.2 mg/g dry weight of the cells grown in without and with 15% NaCl, respectively.

From the above chemical analysis on the lipid composition of plasma membranes, their structure was suggested to become more rigid in the presence of 15% NaCl as compared to that of control cells. In order to measure the fluidity of plasma membranes without the extraction of lipids, lipids in plasma membrane were labelled with a fluorescence probe, 1,6-diphenyl 1,3,5-hexatriene (DPH) and fluorescence polarization was measured[8]. The polarization value reflects the structural order of membrane lipids. In this study, the degree of fluorescence polarization was calculated by the equation of Litman and Barenholz[27]. High degree of fluorescence polarization represents high structural order (i.e. low membrane fluidity). Polarization value of DPH in plasma membranes prepared from the cells grown in medium without and with 15% NaCl was 0.250 and 0.308, respectively. This result suggested that the membrane fluidity of cells grown in the presence of 15% NaCl was decreased.

ROLE OF PLASMA MEMBRANE ATPASES IN OSMOTOLERANCE

Pysiological and biochemical studies have characterized the plasma membrane ATPase as a type of ion pump. The fungal plasma membrane contains a proton-translocating ATPase that is closely related, both structurally and functionally, to the (Na^+, K^+)-, (H^+,K^+)-, and (Ca^{2+})-ATPases of animal cells, the plasma-membrane (H^+)-ATPase of higher plants, and several bacterial cation-transporting ATPases[28]. The plasma membrane (H^+)-ATPase is an integral membrane protein of yeast cells. The enzyme hydrolyzes ATP and transports protons from the cytosol to the extracellular medium. It was proposed that the (H^+)-ATPase plays an important role in controlling several cellular functions such as nutrient uptake, intracellular pH and cell growth[29]. Plasma membranes of *Z. rouxii* have a typical (H^+)-ATPase as judged by testing with various ATPase inhibitors. The ATPase activity from *Z. rouxii* was higher in cells grown in medium with 2M NaCl than that of control cells grown without NaCl[30]. The (Na^+,K^+)-activated Mg^{2+}-dependent ATPase, which exhibits some properties similar to those of animal cells, has been reported to exist in *Z. rouxii*[31].

The ATPase activity of cells adapted to medium with 18% NaCl was significantly higher than that of cells unexposed to high salt. There may be close relationship between ATPase activity and osmoregulation. In order to keep the homeostasis of yeast cells in medium with high concentrations of salt, plasma membrane ATPases seem to play a very important role in adjusting intracellular concentration of cations. However, inorganic solutes are generally thought to play only a minor role in the osmoregulation of yeasts[32]. It is difficult to associate the role of plasma membrane ATPases with the accumulation of glycerol directly at present.

Z. rouxii, a salt-tolerant yeast, can grow in the medium with low water activity. This characteristic seems to depend on the ability to change the membrane functions in order to retain intracellular glycerol in response to osmotic stress.

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(1992년 7월 23일 접수)

삼투압 스트레스에 대응하는 *Zygosaccharomyces rouxii* 의 막성질의 변화

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요 약

*Zygosaccharomyces rouxii*는 간장의 숙성 기간동안 중요한 역활을 하는 내염성 효모이다. 이 실험에서 사용된 *Z. rouxii*는 18% NaCl함유 YPD (1% yeast extract, 2% peptone과 2% glucose, pH5.0)에서 잘 성장하는 반면 *Saccharomyces cerevisiae*는 8% NaCl보다 적은 YPD에서만 자란다. 15% NaCl존재하에서 *Z. rouxii*는 대수기에서 세포내에서 적합한 용질로서 많은 양의 글리세린을 축적하는데 이것은 내염성 효모의 특성이다. 세포막 지질 유동층의 화학 분석에 따르면 15% NaCl에서 자란 세포의 막구조는 더욱 단단하였고 그 유동층은 고농도의 식염의 배지에서는 세포내에 글리세린이 감소되었다.

바로 잡습니다.

21권 6호 (1992년 12월호) p.668, Screen 2. Input of cooking type code, food code and food amount. 바로 윗줄에 "완두콩밥 완두콩"을 삽입합니다.