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Identification and Characterization of Osmotolerant Yeast Isolated from Soy Paste

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된장에서 분리된 내염성 효모의 동정 및 특성조사

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ABSTRACT: Osmotolerant yeast isolated from soy paste could grow on media with 2 M NaCl. This strain was identified as *Zygosaccharomyces rouxii* by biological characteristics, RFLP of ribosomal DNA and mating with compatible haploid strain. Growing rate of the *Z. rouxii* YDJ was slower than *Saccharomyces cerevisiae*. *Z. rouxii* YDJ accumulated trehalose, which is known as one of the osmolytic protectants, in cells cultured on media with salt. Enzyme activity of trehalose phosphate synthase related to trehalose biosynthesis of the YDJ was lower than those of *S. cerevisiae*. Trehalase activity related trehalose degradation was also lower in *Z. rouxii* YDJ than *S. cerevisiae*. However, as *Z. rouxii* accumulated trehalose by salt treatment, salt tolerancy of *Z. rouxii* was assumed to be related to trehalose in additon to glycerol.

KEYWORDS: *Zygosaccharomyces rouxii*, Trehalose, Salt tolerance

Soy paste is traditional fermented food which is very salty and preserved for a long period under ambient condition. *Zygosaccharomyces rouxii* was known as osmophillic yeast (Jermini, 1987) which is associated with spoilage of syrups or high sugar food and is important for the development of flavor of soy sauce or soy paste (Onishi, 1963). The organism has tolerance to high concentrations of NaCl in the growth medium and accumulates glycerol or other polyols in response to increasing concentrations of salt (Zyl, 1990). Osmotic stress induced the accumulation of trehalose in *Escherichia coli* (Giaever 1988, Styrvold 1991) and some cyanobacteria (Reed 1986). Trehalose was also observed to be accumulated in cells of *Saccharomyces cerevisiae* and *Tolulaspora delbrueckii* that have been osmotically shocked (Nakata 1995). Trehalose, is a non-reducing disaccharide of glucose, related to protection against environmental stresses such as desiccation, high osmolarity, frost, and heat. Trehalose also preserves various unstable products such as enzymes, foods, pharmaceuticals, and cosmetics.

In this paper we isolated a microorganism enduring on high salty soy paste and investigated its morphological characteristics, physiological traits, and DNA hybridization to identify the strain. We also estimated the carbohydrate production in cells to investigate the fundamental phenomenon related to salt tolerance.

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Materials and Methods

Strains and culture condition

Two yeast strains were used in this experiment and one is YDJ isolated from soy paste and stocked as KACC (Korean Agricultural Culture Collection) 201271 in NIAST and the other is *S. cerevisiae* KACC 201270 which is a commercially available yeast for bread making as a salt sensitive strain. *Z. rouxii* 14679 and 14680 were purchased from ATCC for mating type identification of *Z. rouxii* YDJ (Wickerham, 1960). Cultures were grown at 30°C on a rotary shaker (180 rpm) in YPD or YM (Difco). Ascospore formation was conducted on sporulation media (1% potassium acetate, 0.1% yeast extract, 0.05% glucose, and 1.5% agar) for *S. cerevisiae* (Ausubel, 1995) and yeast maintenance agar (3 g yeast extract, 3 g malt extract, 5 g peptone, 10 g glucose and 20 g agar per liter) containing 2% NaCl for *Z. rouxii* (Wickerham, 1960).

Identification of yeast strain

The following characteristics were tested: morphology of vegetative cells and modes of their reproduction, metabolic fingerprint, cellular fatty acid composition and analysis

of quinone by HPLC.

Electron microscopic observation

Asci produced in sporulation medium were collected by centrifugation and solidified by addition of 5% agar. Samples were cut with 1×1×3 mm and prefixed in 1% glutaraldehyde at 4°C overnight, and washed 2~3 times with distilled water. The sample was post fixed with 1% osmic acid for 120 min. at 4°C and washed 2~3 times with distilled water. Staining was conducted with 1% uranyl acetate at 4°C overnight. Asci were dehydrated with increasing ethanol series. Embedding was sequentially conducted in the mixture of propylene oxide and Epon mixture (1 : 1 mixture for 2 hours and 2 : 1 mixture for 2 hours), Eppon overnight, the mixture of Eppon and 1.5% DMP-30 at 40°C for 24 hours, and then followed by heat treatment at 60°C for 48 hours. Thin sections of the sample was made with ultramicrotome (Sorvall M6000). The thin sectioned sample was stained with 5% uranyl acetate for 20 min. and lead citrate for 10 min before examination under transmission electron microscope (Hitachi H-800).

Determination of trehalose and trehalose related enzymes

Cells were harvested, washed once distilled water and suspended in 70% ethanol. This suspension was heated at 100°C for 5 min, cooled in a water bath, centrifuged at 10,000×g for 5 min. Trehalose, glucose and glucosamine in the resultant supernatant was measured by high performance liquid chromatography (HPLC) under the following conditions: column, high performance carbohydrate column (4.6×250 mm, Waters); mobile phase, 75% acetonitrile; flow rate, 1.4 ml/min.; detector, refractive index detector (Waters 410).

Trehalose phosphate synthase was assayed as described by Vandercammen *et al.* (1989).

Trehalase was assayed by incubating enzyme aliquots in MES(K^+) buffer (pH 6.3, 50 mM) containing 10 mM trehalose at 37°C for 30 min. The assay was stopped by boiling and the glucose generated was determined with the glucose oxidase -peroxidase method using a test kit (Boehringer Mannheim, Germany).

Protein extracts for determination of enzyme activities were prepared by extraction of yeast cells with glass beads in a buffer containing 50 mM tricine pH 7.0. Protein content was measured by BCA method (Sigma).

RFLP of rDNA

Total DNA of yeast was extracted using glass beads by the method of Ausubel (1995) seperated on the 1.0% agarose gel, and followed by deproteination and denaturation (ECL protocol, Amersham). DNA was transferred onto nylon memebrane and probe DNA was prepared by the ECL method with rDNA of *Schizophllum commune* to compare rDNA pattern of yeast. Hybridization and detection were conducted by the method of ECL protocol.

Results and Discussion

Isolation of osmotolerant yeast

The microorganisms isolated from soy paste showed exclusively yeast colonies on YPD plate, which appeared to be one species.

The morphology of colonies showed white mat type and morphology of vegetative cells was ovoid to ellipsoid shape. It was reproduced by multilateral budding. It was difficult to distinguish the *Z. rouxii* from *S. cerevisiae under* 400x magnification. Physiological characteristics of the strain showed that diazonium blue B reaction, nitrate reduction and urea hydrolysis were all negative.

In chemical taxonomic characteristics, main component of quinone was Q-6 and cellular fatty acid composition was 18 : 2 CIS 9, 12/18 : 0a, 18 : 1 CIS 9 (w9) and 16 : 1 CIS 9 (w7).

The strain assimilated maltose and glucose as a carbon source but not sucrose and lactose by Biolog metabolic fingerprint (Table 1). The results were similar to *Z. rouxii*, which ferments glucose and maltose but not galactose, saccharose and lactose (Onishi 1963).

Salt tolerance was tested with a plate growth assay. The strain YDJ grew well on MY plate containing 2.0 M NaCl, whereas *S. cerevisiae* KACC 201270 grew slowly on plate with 0.5 M NaCl and retarded its growth by increasing salt concentration in media and hardly grew on the plate with over 1.5 M NaCl (Fig. 1). *Z. rouxii* accumulates the

Table 1. Physiological characteristics of the microorganism

Item	Characteristics
DBB test	Negative
Urea hydrohysis	Negative
Nitrate reduction	Negative
Quinone system	Q-6
Cellular fatty acids	18 : 2 Cis 9 12/18 : Oa, 18 : 1 Cis 9 (W9) 16 : 1 Cis (W7)
Growth	
D-trehalose	V
D-galactose	V
Maltose	+
D-glucose	+
Sucrose	-
Lactose	-

V, Variable; +, Assimiation; -, No.

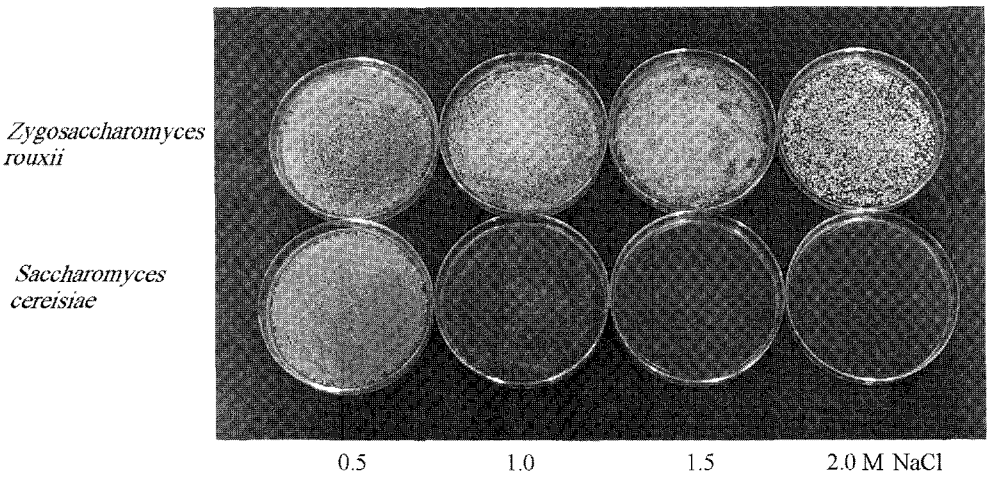


Fig. 1. Effect of Salt concentration on the yeast growth.

polyhydric alcohols (polyols) like glycerol and arabitol intracellularly when the water activity (a_w) is reduced. These solutes enable the organism to survive and grow under water stress. *Z. rouxii* isolated from soy paste is a typical yeast that plays an important role in the ripening of soy mashes and has tolerance to high concentrations of NaCl in growth medium (Brown 1976).

Ascopore formation

Both of *S. cerevisiae* and *Z. rouxii* collected in nature were wild types. We tried to form ascospores to distinguish the cultures by reproductive organs. *S. cerevisiae* could form ascus with four ascospores, while *Z. rouxii* could not form ascus on sporulation medium (Fig. 2). As *Z. rouxii* is haploid genetically, it could form asci containing ascospores after mating with compatible haploid strain. *Z. rouxii* YDJ was mated with *Z. rouxii* ATCC 14679 and Z. rouxii ATCC 14680 which are different mating type (Wickerham *et al.*, 1960). The mating of *Z. rouxii* YDJ with *Z. rouxii* ATCC 14680 produced asci with ascospore after 5 days same as the cross between ATCC 14680 and ATCC 14679 which produced conjugated asci with ascospores on yeast maintenance agar with 2% NaCl (Fig. 3). YDJ was suggested a same mating type as ATCC 14679. *S. cerevisiae* formed lots of ascospores from two to five days after inoculation on sporulation media. However *Z. rouxii* produced a few conjugated ascospores on yeast maintence agar with 2% NaCl after one week.

RFLP

Yeast total DNAs isolated by glass bead method were digested with restriction enzymes of *Eco*R 1 or *Pst* 1. The

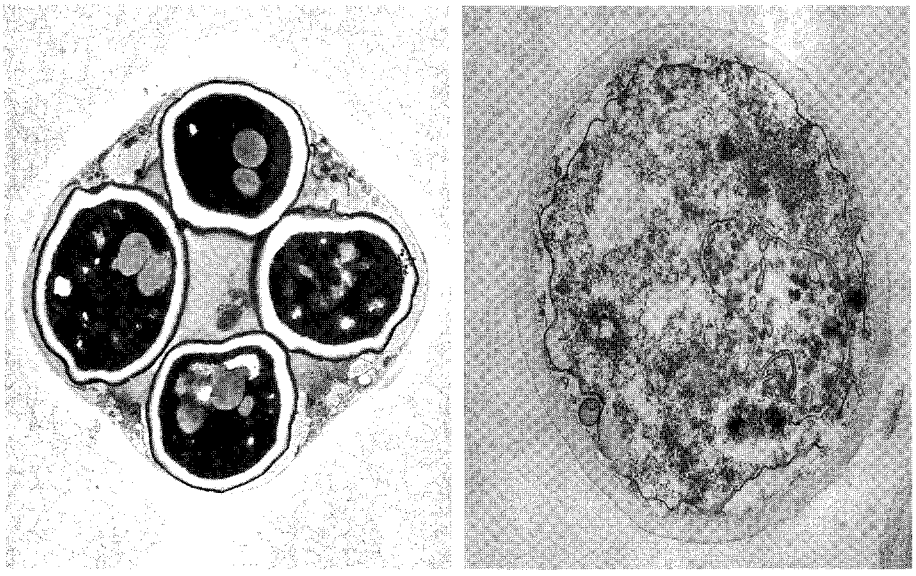
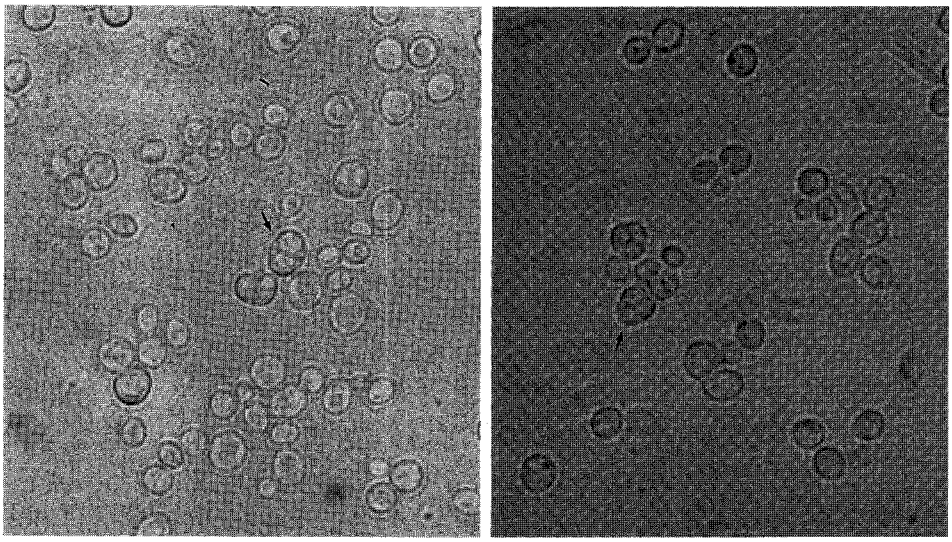


Fig. 2. Observation of yeasts incubated on sporulation media under transmission electron microscope.



Zygosaccharomyces rouxii 14679 X 14680 | *Zygosaccharomyces rouxii* 14680 X YDJ

Fig. 3. Ascospore formation of *Zygosaccharomyces rouxii* YDJ after mating with compatible haploid strain. Arrow indicates conjugated asci with ascospores (×400).

Table 2. Effects of salt on the amount of carbohydrates in yeast cells

Yeast strain	NaCl (M)	Carbohydrates (mg/ml)		
		Trehalose	Glycerol	Glucosamine
Saccharomyces cerevisiae	0	1.22	0.00	0.00
	0.5	9.51	0.20	0.00
	1.0	1.33	0.00	0.00
Zygosaccharomyces rouxii	0	0.87	0.06	0.26
	0.5	0.31	0.19	1.58
	1.0	1.13	0.07	0.35

digested DNA with restriction enzyme was seperated on agarose gel and blotted onto nylon membrane and hybridized with the probe derived from 5S and 18S rDNA fragment of *Schizophyllum commune*. Two bands were hybridized in DNA digested with *Eco*R 1 but one band was obtained in DNA digested with *Pst* 1. *Z. rouxii* ATCC 14679, ATCC14680, and YDJ showed polymorphism with 3 Kb and 500 bp fragments in *Eco*R 1 digested DNA, while *S. cerevisiae* showed polymorphism at 2 kb and 700

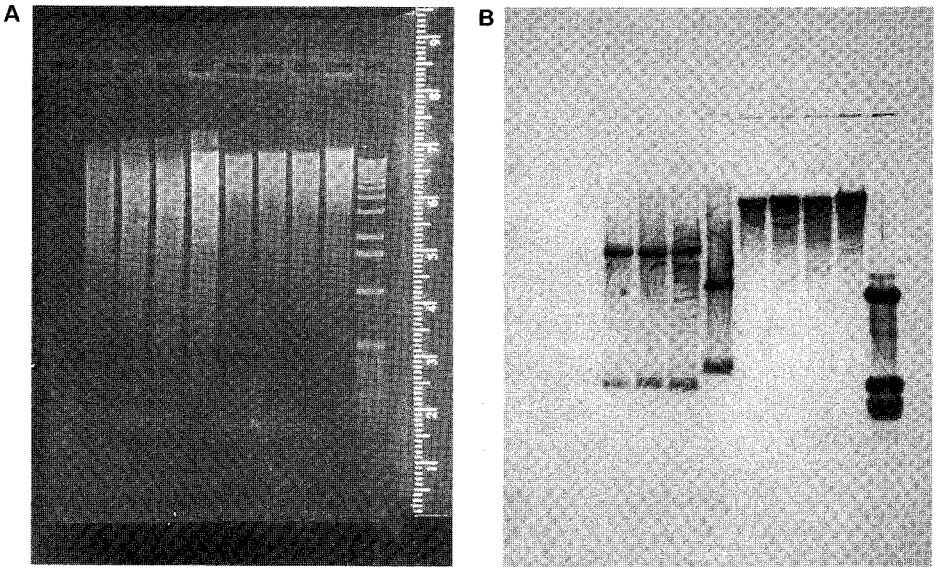


Fig. 4. Southern hybridization of yeast total DNA with 5S and 18S of *Schizophyllum commune* rDNA. Lane 1-4: *Eco*R1 digested DNA. Lane 5-8: Pst1 digested DNA. Lane 1, 5: *Zygosaccharomyces rouxii* 14679. Lane 2, 6: *Zygosaccharomyces rouxii* 14680. Lane 3, 7: *Zygosaccharomyces rouxii* YDJ. Lane 4, 8: *Saccharomyces cerevisiae*. Lane 9: 1 Kb Ladder.
A: Agarose gel, B. Hybridized membrane by ECL method.

bp fragment (Fig. 4).

The results of RFLP with rDNA probe suggest the new strain is *Z. rouxii.*

Trehalose accumulation

To investigate the trehalose change in cells depending on the culture age of *Z. rouxii* YDJ, ATCC 14680 strain, and *S. cerevisiae*, trehalose was assayed from cells grown from 8 hours to 93 hours in the medium of MY at 28°C. Trehalose content reached a maximum point at 31 hours in *Z. rouxii* YDJ and ATCC 14680, and then decreased, whereas *S. cerevisiae* increased rapidly at 23 hours and remained at high level from 31 hours to 93 hours (Fig. 5). Accumulation of trehalose was found to coincide generally with periods of stationary phase, starvation, or heat shock.

Trehalose change in *Z. rouxii* by salt concentration was compared with those in *S. cerevisiae*. Osmophillic yeast *Z. rouxii* could grow on media with 2 M NaCl but *S. cerevisiae* could only endure on medium with 1 M NaCl. Trehalose content in cells cultured in medium without salt was much higher in *S. cerevisiae* than *Z. rouxii*. Trehalose contents were increased in cells grown on media with 0.5 M NaCl but declined in 1 M NaCl in *S. cerevisiae* which is salt sensitive species, whereas *Z. rouxii*, osmophillic yeast, accumulated highly trehalose at 1 M NaCl than 0.5 M NaCl. The carbohydrates were estimated in cells grown in media with different salt concentration. *S. cerevisiae* increased the accumulation of trehalose and glycerol by the addition of 0.5 M NaCl. *Z. rouxii* increased the trehalose, glycerol and glucosamine in cells in case of salt supplementation. Of those, glycerol and glucosamine were accumulated at 0.5 M NaCl but trehalose was increased at 1 M NaCl (Table 2). Onishi (1963) reported that osmophillic yeast formed a large amounts of polyols like glycerol and arabitol in saline environment. Carbohydrate

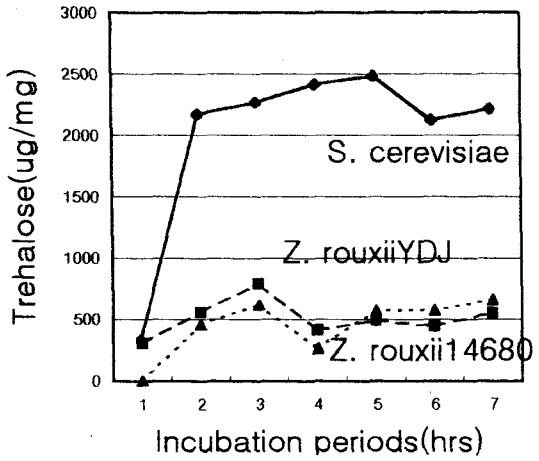


Fig. 5. The time course of trehalose contents in the cells of different yeast strain during cell culture.

Table 3. Enzyme activity related to trehalose metabolism in *Saccharomyces cerevisiae* and *Zygosaccharomyces rouxii* YDJ

Organism	Trehalose phosphate synthase (10^3 U/mg protein)	Trehalase (10^3 U/mg protein)
Saccharomyces cerevisiae	4.1	6.2
Zygosaccharomyces rouxii YDJ	1.0	2.1

data of *Z. rouxii* in saline environment exhibited that trehalose might play a role for osmosis in addition to polyols. Enzyme activity for trehalose metabolism was estimated in cells. Trehalose phosphate synthase and trehalase activity in *Z. rouxii* was lower than those of *S. cerevisiae* (Table 3). Trehalose content in *Z. rouxii* was lower than those of *S. cerevisiae* but trehalose was one of the osmoprotectants in *Z. rouxii.*

적 요

염농도가 높은 전통식품인 된장에서 호염성 효모를 분리하였다. 2M NaCl에서도 생육할 수 있는 이 균은 *Zygosaccharomyces rouxii*로 동정되었으며 생리적 특성, rDNA를 probe로 이용한 RFLP 및 화합성 단핵 동종 효모 균주와 교배 결과에 의하여 이루어졌다. *Z. rouxii* YDJ 균은 생장속도가 *S. cerevisiae* 보다 느려 대수기를 완료하는데 2~3일 소요되었다. *S. cerevisiae*에서는 배지에 염농도가 높아질수록 glycerol 보다 trehalose 함량이 크게 증가하였으나 *Z. rouxii*는 세포 내에 glucosamine, glycerol, trehalose 함량이 증가하였다. 따라서 *Z. rouxii*의 호염성은 glycerol과 같은 polyol이외에도 trehalose와 glucosamine의 효과가 크게 나타났다. Trehalose 생합성 효소인 trehalose phosphate synthase는 *S. cerevisiae* 보다 *Z. rouxii*에서 낮았으며 trehalose 분해효소인 trehalase도 *S. cerevisiae* 보다 *Z. rouxii*에서 낮았다. 그러나 *Z. rouxii*는 NaCl 처리시 trehalose가 증가하므로 내염성은 glycerol 이외에 trehalose에 의해서도 영향을 받는 것으로 생각된다.

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