Studies on Osmophilic Yeasts

Part I. Salt-tolerance and Sugar-tolerance of Osmophilic Soy-yeasts

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The characteristic of salt-tolerance of the osmophilic soy yeasts was different from their sugar-tolerance in the points of viability of the individual cells in a high concentration of salts or sugars of the same osmotic pressure, and of the limiting osmotic pressure for their growth. It became much more apparent that growth of the osmophilic yeasts in the medium of a high concentration of sodium chloride involved a process of physiological adaptation. The order of the toxicity of the salts for the growth of *Zygosaccharomyces major* was as follows: $K^+ < Na^+ < Mg^{++} < Ca^{++} < Li^+$ $Cl^- < SO_4^{--} \approx NO_3^-$

In the previous report⁽¹⁾, it was found that in the viable counts of the yeasts grown in soy sauce mash, which differed significantly from other brewage mashes in their high contents of sodium chloride (NaCl 18%) and nitrogenous compounds (as total N, 1.0~1.5%), a remarkable fall was observed in a single step transplantation when ordinary NaCl-free media were used for plating count, while there was not any significant fall to be observed when media containing sodium chloride in the same concentrations as soy sauce mashes were used, and also a fall in viability was similarly observed when, in a reverse direction, these yeasts grown in the plain medium (NaCl 0%) were directly transferred to the saline medium (NaCl 18%). Since the osmophilic property of soy yeasts against sodium chloride could be increased by culturing in media containing sodium chloride, the author has regarded this, to a certain extent, as an adaptive nature, though this property had hitherto been simply believed to be genetically constant and transmissible.

From the above facts, it seemed that fundamental studies on the influences of sodium chloride on growth and metabolism

of the osmophilic yeasts should be carried on further, and elucidation of these studies might be of great interest in the manufacture of soy sauce and miso paste, and furthermore in the preservation of salted food.

In this paper, the characteristics of salt-tolerance and sugar-tolerance of osmophilic soy yeasts are shown.

EXPERIMENTAL

(1) **Reversibility of Salt-tolerance of Osmophilic Soy Yeasts.** The stability of NaCl-tolerance which was acquired by growing in a medium containing sodium chloride was examined. Three strains of soy yeasts, *Zygosaccharomyces major*, N 5 and N 8 were cultured successively in NaCl-free and NaCl 18% koji extract media on the schedule shown in Table I. The salt-tolerance of the yeast cells grown in each cultural environment was measured by plating them on NaCl-free and NaCl 18% koji extract agar. As shown in Table I, salt-tolerance which was acquired by the yeast cells grown in a NaCl 18% medium, was easily lost if these yeasts were once cultured in a NaCl-free medium. Accordingly, even in case of the osmophilic yeasts, salt-tolerance was found to be much dependable on their cultural conditions, i.e. the presence and absence of NaCl.

(2) **Growth of Soy Yeasts on Various Media of Different Concentrations of Sodium Chloride.** As shown in Table II, when the yeasts cultured in

(1) H. Onishi, *J. Agr. Chem. Soc. Japan*, **28**, 546 (1954).

(Table III)

The growth curve in the NaCl 18% medium, manifested by *Z. major* which was precultured in the media of different NaCl concentrations as 0, 5, 10 and 18% is shown in Fig. 1.

The composition of the liquid medium employed

FIG. 1. The Effects of the Precultural Conditions on the Growth of *Z. major* in NaCl 18% Medium.

○: precultured in NaCl-free medium
 ×: precultured in NaCl 5% medium
 △: precultured in NaCl 10% medium
 □: precultured in NaCl 18% medium
 solid line: plating on NaCl-free medium
 broken line: plating on NaCl 18% medium

was as follows: glucose 5%; KH_2PO_4 0.1%; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.05%; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.01%; NaCl 0.01%; Bacto vitamin-free casamino acid 0.03% as total nitrogen; Bacto yeast extract 0.2%; thereto NaCl was added to the required amount.

The growth of yeast was measured by a plating count using NaCl-free and NaCl 18% media.

As shown in Fig. 1, when yeasts precultured in the medium containing more than 5% NaCl were inoculated in the NaCl 18% medium, the fall in viability at the lag phase of growth was not observed, but when precultured in the NaCl-free medium, the fall in viability at the beginning of the growth curve was remarkable.

From these facts, it became much more clear that

the growth of osmophilic yeasts in the medium of a high concentration of sodium chloride involves a process of physiological adaptation, this not merely being a recovery of yeast cells from the osmotic effects produced by sodium chloride.

(3) **Limiting Osmotic Pressure for the Growth of Various Yeasts.** Limit concentrations of sodium chloride and sugars for the growth of ordinary yeasts such as *Saccharomyces saké* and *Sacch. ellipsoideus* were found to be 10% in NaCl, 50% in glucose and 60% in saccharose, and for osmophilic yeasts, such as *Zygosaccharomyces major* and *Zygosacch. soya* were 20~22% in NaCl, 80% in glucose and 80% in saccharose, respectively.

Osmotic pressure of the medium was calculated from the formula of Lewis²⁾.

$$\Pi = 12.06\Delta - 0.021\Delta^2$$

where

Π : osmotic pressure (atm.)

Δ : degree of freezing point depression ($^{\circ}\text{C}$)

Values for freezing point depression of NaCl solution were taken from the Table of Landolt-Börnstein³⁾. Published data on these measurements by Frazer and Myrick⁴⁾, Church⁵⁾ and International Critical Tables vol. 4 were also referred.

Limiting osmotic pressure for their growth was, therefore, calculated as 75 atm. in NaCl, 140 atm. in glucose and 90 atm. in saccharose for *Sacch. sake* and *Sacch. ellipsoideus*, and 150~165 atm. in NaCl, 220~260 atm. in glucose and 180~200 atm. in saccharose for *Zygosacch. major* and *Zygosacch. soya*.

It seemed quite natural that the soy yeasts, as a group of the so-called osmophilic yeasts show as above, much higher tolerance towards a high osmotic pressure than the ordinary yeasts; it was also noticeable that in general, the osmotic pressure which limits growth of the yeasts is much lower in NaCl than in sugar.

(4) **The Growth of Soy Yeasts on the Media of High Concentrations of Sugar.** Sugar concentration which corresponds to the osmotic pressure of a 18% NaCl solution (135 atm.) is 50% in glucose, 75% in saccharose and 50% in galactose respectively. Though a fall in viability was, as above-stated, very remarkable when soy yeasts cultured in NaCl-free

2) G.N. Lewis, *J. Am. Chem. Soc.* **30**, 668 (1908).

3) Landolt-Börnstein, *Physikalisch-Chemische Tabellen* **2**, 1452 (1923).

4) J.C.W. Frazer and R.T. Myrick, *J. Am. Chem. Soc.* **38**, 1907 (1916).

5) M.B. Church, *Science*, **74**, 492 (1931).

TABLE IV
CHANGES IN VIABILITY OF YEAST CELLS TRANSFERRED FROM THE PLAIN MEDIUM
TO MEDIA OF HIGH SUGAR CONCENTRATIONS

Strains	glucose 50%		saccharose 75%		galactose 50%	
	koji-agar	koji-agar	koji-agar	koji-agar	koji-agar	koji-agar
<i>Z. major</i>	532	524	468	460	1512	1496
<i>Z. soya</i>	292	302	304	328	832	800
A 31	486	464	1232	1304	1600	1632
A 34	132	144	176	182	456	460
N 5	352	332	660	688	1000	896
N 8	364	366	560	564	828	828
N 15	115	122	772	832	400	444
N 21	544	536	532	568	—	—
N 24	342	308	1624	1560	1568	1656
N 28	158	175	328	372	206	202

medium were transplanted into the NaCl 18% medium, it was examined whether a similar change in viability would really occur when in place of NaCl-medium, a sugar medium, whose osmotic pressure was adjusted

to be equal to that of a 18% NaCl solution was used. Unlike NaCl, a fall in viability for the sugars was not observed as shown in Table IV. In this test, it cannot occur that sugar concentration of the medium may decrease significantly according to the growth of yeasts, because tested yeasts cannot ferment galactose and saccharose, but can only assimilate them very weakly⁶⁾.

As shown in Fig. 2, when the yeasts cultured in the plain medium were inoculated directly into the basal plain, 18% NaCl, 50% glucose or 50% galactose medium (the basal medium was the same as that shown in Fig. 1), not only in the basal plain medium but also in the glucose 50% and galactose 50% medium, the fall in viability at the lag phase of the growth curve was not found, but in 18% NaCl medium, a remarkable fall in viability was observed at the initial stage of growth.

Now, it is apparent that characteristics of salt-tolerance and sugar-tolerance of the osmophilic soy yeasts toward the medium of the same osmotic pressure, are quite different from each other, so some limiting factors other than the osmotic pressure itself are presumable to have influence on the salt-tolerance of the osmophilic yeasts.

(5) **Growth of Soy Yeasts in the Medium of a High Concentration of Various Salts.** The growth of *Zygosaccharomyces major* in the liquid media of various concentrations of sodium sulfate, sodium nitrate, potassium chloride, lithium chloride, calcium chloride and magnesium sulfate was observed. The basal medium was the same as that shown in Figs. 1 and 2. The growth curve is shown

FIG. 2. Growth Curve of *Z. major* in the high Sugar Concentration Medium.

- : inoculated into the basal plain medium
- ×: inoculated into the glucose 50% medium
- △: inoculated into the galactose 50% medium
- : inoculated into the NaCl 18% medium
- : plain koji agar
- - -: glucose 50% koji agar
- · ·: NaCl 18% koji agar

6) H. Onishi, This bulletin (in press).

FIG. 3. Growth Curve of *Z. major* in the Media of Various Concentrations of Several Salts.

FIG. 4. Relations between the Growth Constant and the Concentrations of Several Salts for the Growth of *Z. major*.

in Fig. 3.

Relations between the growth constant, e.g. total growth, exponential growth rate and time lag, and the concentrations of salts in the medium are shown in Fig. 4. For *Zygosaccharomyces major*, the order of

toxicity of ions per molar concentration of the tested salts was as follows:

The growth of *Zygosacch. major* was inhibited by relatively low concentrations of LiCl such as 0.5 mol

TABLE V
FALL IN VIABILITY OF YEAST CELLS TRANSFERRED FROM THE PLAIN
MEDIUM TO THE KCl 23% MEDIUM

Strains	KCl 23% koji agar		Strains	KCl 23% koji agar	
	koji agar	KCl 23% koji agar		koji agar	KCl 23% koji agar
<i>Z. major</i>	1072	139	N 8	153	9
<i>Z. soya</i>	172	62	N 15	744	96
A 31	358	200	N 21	524	100
A 34	312	145	N 24	960	99
N 5	780	113	N 28	287	119

per l, while on the contrary, in the case of KCl the growth was very good even in such a high concentration as 4 mols per l. Mg^{++} was found less toxic than Ca^{++} .

A fall in viability was also observed when yeasts cultured in a plain medium were transferred directly to the KCl 23% medium which was of the same molar concentration as 18% NaCl.

SUMMARY

(1) The salt-tolerance of soy yeasts acquired by growth in the saline medium with NaCl was easily lost if these yeasts were afterwards once cultured in the ordinary medium without NaCl. Accordingly, in the case of osmophilic soy yeasts salt-tolerance was much dependable upon their cultural environments with or without NaCl. Extreme salt-tolerance could be acquired even when the yeasts were cultured in the medium of NaCl of a relatively low concentration as 5%. Thus it becomes quite apparent that the growth of osmophilic yeasts in the medium of a high concentration of NaCl involves the process of physiological adaptation.

(2) A fall in viability was not observed, contrary to the case of NaCl, when the yeasts cultured in the plain medium were transferred directly to the medium consisting of a high concentration of sugars corresponding to the osmotic pressure of 18% NaCl (135 atm.).

The limiting osmotic pressure for their growth was higher in sugars (220~260 atm.) than in NaCl (150~165 atm.).

In these respects, the characteristic of the salt-tolerance of osmophilic soy yeasts differs from that of their sugar-tolerance and it is clear that some other limiting factors besides the osmotic pressure itself, will have effect on their salt-tolerance.

(3) The viability of the yeast cells when transferred from the plain medium to the various salt media generally fell. Judging from the relations between the growth constant and various concentrations of salts in the medium, the order of toxicity of the salts for the growth of *Zygosaccharomyces major* was estimated as follows:

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