Studies on Osmophilic Yeasts

Part III. Classification of Osmophilic Soy and Miso Yeasts

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Most of the salt-tolerant yeasts isolated from soy-sauce and miso-paste belonged to *Saccharomyces rouxii* Boutroux. Some of them produced luxuriant pellicles on the medium containing sodium chloride, though pellicles were not formed on the medium without salts. These strains were separated from the species, in a variety, *Saccharomyces rouxii* Boutroux var. *halomembranis* nov. var. A few strains were identified as *Saccharomyces acidifaciens* (Nickerson) Lodder et Kreger-van Rij. Still further, two new species, *Torulopsis halophilus* nov. sp. and *Torulopsis nodaensis* nov. sp. were also described. These two species showed high salt-tolerance, especially the former was facultative halophile. Both species were able to assimilate nitrate but not to form pellicles, and differed from each other in the fermentation and assimilation of sugars.

INTRODUCTION

As for the osmophilic yeasts, many new species and varieties have been described¹⁾²⁾. Most of them belong to *Zygosaccharomyces* and some others to *Debaryomyces*, *Pichia*, *Torulopsis* and *Saccharomyces* etc. It seems that they generally exist in haplophase in an environment of a high concentration of sugars or salts. In the monograph on the taxonomy of yeasts by Lodder and Kreger-van Rij³⁾, subgenera *Zygosaccharomyces* is no longer recognized, on the basis of the fact that no fundamental difference exists between the types of sexual reproduction shown by the species of *Saccharomyces* and *Zygosaccharomyces*. In this classification, almost all of the *Zygosaccharomyces* known as the osmophilic yeasts, were included into only two species and one variety, *Saccharomyces rouxii* Boutroux, *Saccharomyces rouxii* Boutroux var. *polymorphus* Lodder et Kreger-van Rij and *Saccharomyces*

mellis (Fabian et Quinet) Lodder et Kreger-van Rij. For instance, *Zygosaccharomyces major*⁴⁾, *Z. soya*⁵⁾, *Z. salsus*⁴⁾ and *Z. japonicus*⁶⁾ known as soy yeasts, were all included in one species, *Saccharomyces rouxii*.

This paper is concerned with a taxonomic study on the yeast strains which play an important role in soy-brewing, isolated from soy-mashes by the author and the collected strains labelled as *Z. major*, *Z. soya*, *Z. salsus*, *Z. japonicus*, *Z. miso*⁷⁾ and *Sacch. miso*⁷⁾.

METHODS

The methods employed and the classification system referred here were essentially the same as those outlined in the monograph by Lodder and Kreger-van Rij³⁾, though certain modifications and additions were made in connection with the taxonomic test carried out by the Dutch workers.

The outlines of the examination were as follows:

1) A.T. Henrici, *Bact. Rev.* 5, 97 (1941).

2) E.M. Mrak and H.J. Phaff, *Ann. Rev. Microbiol.* 2, 1 (1948).

3) J. Lodder and N.J.W. Kreger-van Rij, *The Yeasts, a taxonomic study* (1952).

4) T. Takahashi and M. Yukawa, *J. Coll. Agr. Tokyo Imp. Univ.* 5, 227 (1915).

5) K. Saito, *Centr. Bakt. Parasitenk. Abt. II.* 17, 20, 101, 152 (1907).

6) K. Saito, *Botan. Magazine Tokyo*, 23, 96 (1909).

7) M. Mogi, *J. Agr. Chem. Soc. Japan*, 12, 367 (1936), 14, 951 (1938); 15, 921, 1023, 1221 (1939); 16, 7 (1940); 18, 543, 733, 940 (1942).

TABLE I MORPHOLOGICAL, CULTURAL AND PHYSIOLOGICAL CHARACTERISTICS OF THE TESTED YEASTS

Species allocation	<i>Saccharomyces rouxii</i> Boutroux	<i>Saccharomyces rouxii</i> Boutroux var. <i>halomem-branis</i> nov. var.	<i>Saccharomyces acidifaciens</i> (Nicker-son) Lodder et Kregger-van Rij	<i>Saccharomyces acidifaciens</i> (Nick-erson) Lodder et Kregger-van Rij var. <i>halomem-branis</i> nov. var.	<i>Torulopsis halophilus</i> nov. sp.	<i>Torulopsis no laensis</i> nov. sp.
Vegetative cells (after 6 days' cultivation in koji extract, at 30°)	Cells are round or single or in pairs. The cells usually measure (5~8) $\times$ (5~10) μ .	Same as <i>Saccharomyces rouxii</i>	Cells are round or oval, single or in pairs. The cells usually measure (4~6) $\times$ (7~10) μ .	Same as <i>Saccharomyces acidifaciens</i>	Cells are oval or short ellipsoidal, single or in pairs. The cells usually measure (2~4) $\times$ (3~5) μ .	Cells are oval, single or in pairs. Cells usually measure (2~4) $\times$ (3~5) μ .
Growth on koji agar, after 2 weeks, at 30°	The streak culture is light cream colored, dull glistening, raised and almost smooth.	The streak culture is light cream colored, dull, raised and wrinkled.	The streak culture is cream-colored, dull glistening, raised and smooth.	The streak culture is cream-colored, dull, raised and slightly wrinkled.	The streak culture is white grey, glistening, flat and smooth.	The streak culture is greyish brown, almost flat, dull shining and almost smooth.
Slide culture on potato agar	No pseudomycelium	No pseudomycelium	No pseudomycelium	No pseudomycelium	No pseudomycelium	No pseudomycelium
Ascospore formation	Isogamous or heterogamous conjugation may precede ascus formation. 1~4 spores are found per ascus. Spores are round.	Isogamous or heterogamous conjugation may precede ascus formation. 1~4 spores are found per ascus. Spores are round.	Isogamous or heterogamous conjugation may precede ascus formation. 1~4 spores are found per ascus. Spores are round.	Isogamous or heterogamous conjugation may precede ascus formation. 1~4 spores are found per ascus. Spores are round.	Not found on three sporulation media observed over a three months-period.	Not found on three sporulation media observed over a three months-period.
Growth in koji extract	NaCl-free Ring—no film	NaCl-free Ring—no film	NaCl-free Ring—no pellicle	NaCl-free Ring—no pellicle	NaCl-free Ring—no pellicle	NaCl-free Ring—no pellicle
Fermentation of sugars	Good growth Ring—no film	Good growth and a thick wrinkled film is formed.	Good growth. Ring—no pellicle	Good growth and a thick wrinkled pellicle is formed.	Good growth. Ring—no pellicle	Good growth. Ring—no pellicle
Assimilation of sugars	Glucose and maltose Galactose, saccharose and lactose	Glucose and maltose Galactose, saccharose and lactose	Glucose Galactose, maltose, saccharose and lactose	Glucose Galactose, maltose, saccharose and lactose	Glucose Galactose, maltose, saccharose and lactose	Glucose and galactose Maltose, saccharose and lactose
Assimilation of sugars	Glucose, galactose and maltose. Saccharose (variable)	Glucose, galactose and maltose. Saccharose (variable)	Glucose and Galactose.	Glucose and Galactose	Glucose, galactose, saccharose and maltose	Glucose, galactose and saccharose
Assimilation of sugars	Lactose	Lactose	Maltose, saccharose and lactose	Maltose, saccharose and lactose	Lactose	Maltose and lactose
Assimilation of nitrate	Negative	Negative	Negative	Negative	Positive	Positive
Growth in ethanol	No growth	No growth	No growth	No growth	Growth	Good growth
Splitting of arbutin	Negative	Negative	Negative	Negative	Negative	Negative
Outstanding characteristics						Besides biotin, thiamin is essentially required.

(1) Characteristics of vegetative reproduction.

The formation of pseudomycelium was observed in a slide culture with potato agar.

(2) Shape and size of the cells.

(3) Ascospore formation.

Three media, Gorodokowa agar, V-8 agar⁸⁾ and diluted soy-sauce (four times) were used. The final observation was made after three months' cultivation.

(4) Macromorphological characteristics of the cultures on koji agar.

(5) Pellicle formation.

It was observed in koji extract with and without sodium chloride, salt-tolerance of the tested yeasts being examined at the same time.

(6) Fermentation of sugars.

The fermentation of 2% sugar solution in yeast extract was tested in a Durham tube and an Einhorn tube. The sugar were: glucose, galactose, maltose, saccharose and lactose. The final reading was made after 10 days.

(7) Assimilation of sugars.

Bacto yeast-nitrogen base was employed as the basal liquid medium.

(8) Assimilation of nitrate was tested with a Bacto yeast-carbon base.

(9) Ethanol as a sole source of carbon.

(10) Splitting of arbutin.

EXPERIMENTAL AND RESULTS

First of all, the salt-tolerance of the tested yeasts was examined and it was confirmed that the strains classified below, were all able to grow in a 18% NaCl medium.

The 87 tested strains were classified as follows:

(1) *Saccharomyces rouxii* Boutroux

(2) *Saccharomyces rouxii* Boutroux var. *halomembranis* nov. var.

(3) *Saccharomyces acidifaciens* (Nickerson) Lodder et Kreger-van Rij

(4) *Saccharomyces acidifaciens* (Nickerson) Lodder et Kreger-van Rij var. *halomembranis* nov. var.

(5) *Torulopsis halophilus* nov. sp.

(6) *Torulopsis nodaensis* nov. sp.

Their morphological, cultural and physiological characteristics are summarized in Table I.

DISCUSSION

Of the 87 tested strains, 78 strains belonged

to *Saccharomyces rouxii* Boutroux, and among them 24 strains produced thick wrinkled pellicles on the medium containing sodium chloride, though pellicle formation was absent in the liquid media without salts. This characteristic has previously been recognized by Takahashi and Yukawa⁴⁾ on *Z. salsus*, the film yeast of soy, and also by Mogi⁷⁾ on some strains of miso yeasts. Recently, Etchells and Bell⁹⁾ described *Zygosaccharomyces halomembranis* which was isolated from films on commercial cucumber brines and showed the same characteristic of pellicle formation as mentioned above. This species is presumed to be synonymy with *Sacch. rouxii* for it ferments glucose and maltose, and not galactose, saccharose and lactose. They are, of course, clearly distinguished from *Pichia* and *Hansenula* in the point of lacking early development of dry matte pellicles on ordinary media without salts. However, the property of luxuriant pellicle formation on the media containing NaCl, e.g. soy-sauce, is considered not only to be a very obvious characteristic for the taxonomy of yeasts but also a very important one pertaining to the industrial and applied standpoints, e.g. the preservation of soy-sauce. The author, therefore, is of opinion that these strains should be separated from the species, *Sacch. rouxii* Boutroux, in a variety, *Sacch. rouxii* Boutroux var. *halomembranis* nov. var.

It was noticeable that a half of the collected strains labelled as *Z. salsus* and *Z. japonicus*, could not form pellicles on the medium containing NaCl, though it was not apparent whether their ability of pellicle formation was lost or not during the stock culture for many years, yet, however they agreed with *Sacch. rouxii* in all other properties.

Pellicle formation on the media of NaCl-free and 6~9% NaCl by many species of *Saccharomyces* and *Zygosaccharomyces* received from the Nagao Institute in Tokyo, Japan, and by the strains placed in *Sacch. rouxii*

8) L.J. Wickerham, M.H. Flickinger and K.A. Burton, *J. Bact.* 52, 611 (1946).

9) J.L. Etchells and T.A. Bell, *Food Technol.* 4, 77 (1950).

TABLE II
SALT-TOLERANCE AND SUGAR-TOLERANCE OF VARIOUS STRAINS OF *SACCH. ROUXII*.

Strains	NaCl					Glucose		Saccharose	
	0%	10%	12.5%	15%	18%	80%	90%	80%	90%
<i>Zygosacch. amoeboideus</i>	+++	+++	++	++	—	+	—	+	+
<i>Z. citrus</i>	+++	+++	++	++	++	+	—	+	+
<i>Z. felsineus</i>	+++	+++	++	++	—	+	—	+	+
<i>Z. gracilis</i>	+++	+++	+++	++	—	+	—	+	+
<i>Z. gracilis</i> var. <i>italicus</i>	+++	+++	++	++	±	+	—	+	+
<i>Z. mellis</i>	+++	+++	+++	++	±	+	—	+	+
<i>Z. nussbaumeri</i>	+++	+++	—	—	—	+	—	+	+
<i>Z. polymorphus</i>	+++	+	+	—	—	+	—	+	+
<i>Z. rugosus</i>	+++	+++	++	++	—	+	—	+	+
<i>Z. variabilis</i>	+++	++	++	—	—	+	—	+	+
N 5*	+++	+++	+++	+++	++	+	—	+	+
N 8*	+++	+++	+++	+++	++	+	—	+	+
A 34*	+++	+++	+++	+++	++	+	—	+	+
<i>Torulopsis halophilus</i>	+++	+++	+++	+++	+++	+	—	+	+
<i>T. nodaensis</i>	+++	+++	+++	+++	+++	+	—	+	+

* These strains were isolated from soy-mashes by the author and then placed in *Sacch. rouxii*.

supplied from Centraalbureau voor Schimmelcultures, Delft, Holland was observed. The strains tested were as follows:

Sacch. anamensis, *S. awamori*, *S. bayanus*, *S. cartinoginosus*, *S. chevalieri*, *S. dairensis*, *S. exiguus*, *S. fragilis*, *S. intermedius*, *S. lindneri*, *S. logos*, *S. marxianus*, *S. monacensis*, *S. monogenicus*, *S. paradoxus*, *S. pasteurianus*, *S. unisporus*, *S. validus*, *S. willianus*, *Zygosacch. bisporus*, *Z. dairensis*, *Z. fermentati*, *Z. mellis acidii*, *Z. naniwaensis*, *Z. vini*, *Z. amoeboideus*, *Z. citrus*, *Z. felsineus*, *Z. gracilis*, *Z. gracilis* var. *italicus*, *Z. mellis*, *Z. nussbaumeri*, *Z. polymorphus*, *Z. rugosus*, *Z. variabilis*.

Of the tested strains, only two, *Z. mellis acidii* and *Z. nussbaumeri*, were able to form prominent pellicles, only on the saline medium. Therefore, it is estimated that few strains with the exception of osmophilic yeasts show such property.

Concerning the fermentability of saccharose by *Sacch. rouxii*, it was shown by Terui and Sase¹⁰⁾ that the utilization of saccharose is detectable, only when saccharose adaptation

is permitted under the condition applied, that is to say, a negative result is obtained with the Lodder medium, but on fortification with 0.2% of L-aspartic acid and 1 µg per ml of the biotin, the result becomes positive. The author's observation on the fermentation of saccharose by the strains precultured on koji agar, was in good agreement with the description of Lodder and Kreger-van Rij though some strains showed extremely poor saccharose fermentation after 15~20 days' incubation.

The salt-tolerance of 10 strains of *Sacch. rouxii* received from Centraalbureau voor Schimmelcultures, Delft, Holland, was lower than that of soy and miso yeasts, in spite of their same level of sugar-tolerance. (Table II) It is suggested that the salt-tolerance and sugar-tolerance of *Sacch. rouxii* differ considerably, strain by strain, according to their origin.

Spore formation of the 78 strains that belonged to *Sacch. rouxii* and its variety was very difficult, being observable in only two strains. Lodder and Kreger-van Rij have also recognized the difficulty of this

¹⁰⁾ G. Terui and M. Sase, *Technol. Rep., Osaka Univ.*, 5, 229 (1955).

species and stated that it might be due to its strong tendency to live in haplophase and to easily lose its ability to form ascospores.

Four strains were identified as *Sacch. acidifaciens* (Nickerson) Lodder et Kreger-van Rij due to the facts that they fermented glucose only, their cell size being relatively large (6~9 μ), and the streak culture on koji agar being smooth. Two strains among them, which formed prominent pellicles only on the medium containing NaCl were separated from

FIG. 1. Fermentation Curve of *Sacch. rouxii* in Media of High Concentrations of NaCl and Sugars.

- : the plain medium
- ×: the 10% NaCl medium
- △: the 18% NaCl medium
- : the 50% glucose medium
- ▲: the 50% saccharose medium

FIG. 2. Fermentation Curve of *Torulopsis halophilus* in the Media of High Concentrations of NaCl and Sugars.

- : the plain medium
- ×: the 10% NaCl medium
- △: the 18% NaCl medium
- : the 50% glucose medium
- ▲: the 50% saccharose medium

the species in a variety, *Sacch. acidifaciens* var. *holomembranis* nov. var.

Two strains isolated from soy mashes in

Noda district by the author, belonged to genus *Torulopsis*, due to the characteristics that are shown in Table I. It is an interesting fact that though both are able to assimilate nitrate very well, neither is capable of forming pellicles on any liquid media with or without NaCl. Furthermore, they required biotin and thiamin essentially¹¹. They were different from each other in both the fermentability and assimilability of sugars. As shown in Figs. 1 and 2, one of them showed a better fermentation in the medium of high concentrations of NaCl or sugars than in the ordinary plain medium, unlike the fermentation curve shown by *Sacch. rouxii*, that is to say, it is facultative halophilic.

This halophilic yeast, in many taxonomic properties, resembles *Torulopsis etchellsii* Lodder et Kreger-van Rij which was isolated from cucumber pickles and named *Brettanomyces sphericus* by Etchells and Bell¹², and thereafter renamed as above by Lodder and Kreger-van Rij, but that is distinguished from *T. etchellsii* in respect of its fermentability and assimilability of sugars, and utilization of ethanol. It also differs from *Hansenula minuta*¹³ in its assimilability of sugars, splitting of arbutin and halophilic property, even when it is sporulated. Accordingly, the author regards it as a new species and has given it the name *Torulopsis halophilus* nov. sp.

The other strain has close resemblance to *Torulopsis halophilus* but differs in its fermentability of galactose and salt-tolerance (non-halophilic). And also, it differs from *Hansenula silvicola* in its assimilation of sugars, pseudomycel formation and utilization of ethanol besides sporulation. It, therefore, is named *Torulopsis nodaensis* nov. sp.

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11) H. Onishi, This Bulletin in press.

12) J.L. Etchells and T.A. Bell, *Farlowia*, 4, 87 (1950).

13) L.J. Wickerham, *Tech. Bull.* No. 1029, U.S. Dep. Agric. Washington, D.C., May (1951).

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