Study of Osmophilic Yeasts producing Invertase

By M. PAMELA SCARR AND D. ROSE

Tate and Lyle Ltd., Research Laboratories, Keston, Bromley, Kent, England

(Received 31 March 1966)

SUMMARY

Samples of sugar cane (*Saccharum officinarum*), cane juice, raw sugar and refinery samples were screened for osmophilic yeasts able to ferment sucrose. One of the isolates showed properties bordering on the original definition of an obligate osmophil. Therefore, diagnostic tests were adapted to fit ecological considerations. These modifications in technique were adopted for all isolates when it was found that more consistent results could thus be obtained. As a result of these tests relevant organisms were identified as *Torulopsis apicola*, *T. globosa*, *T. lactis-condensi*, *T. bacillaris*, *Candida guilliermondii*, *Saccharomyces florentinus* and a new species, *T. kestoni*.

INTRODUCTION

It has been known for some years (Scarr, 1951) that *Saccharomyces rouxii* and *S. mellis*, growing in impure concentrated sucrose solutions, will ferment any invert sugar present to form organic acids when the subsequent decrease in pH value allows a slow chemical inversion to follow. There was no detectable invertase production by normal diagnostic tests. *Torulopsis* species, growing more slowly, were also noted, but, under the conditions of test, did not appear to be biochemically active. Recently, however, an incident was reported of fermentation in a saturated sucrose solution containing no invert sugar and from it only *torulopsis* species were isolated. The following account describes the isolation and identification of osmophilic yeasts from this and other sources.

METHODS

Definition of concentration. Osmophilic yeasts grow in a highly selective medium. At very high concentrations of dissolved solids, the exact concentration is important and unless otherwise stated is defined here as ° Brix. This is the weight of dissolved solids as % of weight of solution determined at 20°.

Bacteria will grow, albeit slowly, in solutions up to 40° Brix and yeasts and moulds up to 75–79° Brix. For convenience, osmophilic organisms are defined here as those which can grow at concentrations over 65° Brix, which in pure sucrose solutions are conditions near to saturation (saturated sucrose solution at 20° = 66·7 °Brix).

Isolation, screening and storage. Plate cultures were made on osmophilic agar (Scarr, 1959) and colonies picked off into filter-sterilized 20 % (w/v) sucrose yeast-water solution containing a trace of bromocresol green, in McCartney bottles with a Durham tube. These were incubated for a maximum of 3 weeks at 27°. The contents of bottles showing acid and gas formation were then transferred to 65° Brix sucrose solutions containing 0·5 % Difco Bacto Peptone, 0·1 % $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0·2 % KH_2PO_4 (sterilized at 5 lb./in.² for 10 min.) and incubated at 27° for a further 3

weeks. Cultures still showing a positive result were then streaked on osmophilic agar and stored on slopes of this medium.

Isolates from cane, cane juice, air (entrapped on a membrane filter), raw sugar and intermediate refinery products were screened in this manner.

Diagnostic tests. Preliminary tests were made by the standard methods of Lodder & Kreger van-Rij (1952) but using liquid auxanogram tests and growth on vitamin-free medium according to Wickerham (1951). The fermentation tests were incubated for 3 weeks at 27°.

Subsequently modifications were made in the fermentation and carbon assimilation techniques by using 10% (w/v) of the appropriate sugar or carbon source, except for ethanol, inulin, succinic, lactic and citric acids which were tested at normal strength and salicin at 1% and in saturated solution. Assimilation of soluble starch was tested by the plate auxanogram.

Liquid assimilation media were prepared with Difco Yeast Nitrogen Base and carbon source at final strength, sterilized by filtration and dispensed aseptically as 5 ml. volumes into empty plugged sterile tubes. In the nitrogen assimilation test the Difco Yeast Carbon Base was re-inforced with extra glucose where necessary.

Inocula were taken from 72-hr slopes on osmophilic agar (incubated at 30°), washed and centrifuged in sterile Ringer's solution; one drop of washed suspension was used per tube. We noticed no loss of viability after this treatment.

RESULTS

One hundred isolates were examined. After screening tests and the elimination of obvious duplicates, the number of cultures was ten. For comparative purposes, a culture of *Saccharomyces rouxii*, Boutroux (code Sa 24) isolated from a Mauritius raw sugar, was included in the examination. The ten species isolated fell into three genera which are considered separately.

Cultures T7A, T8, T9, T10, T11 and T12

Data for this series of isolates are summarized in Tables 1 and 2.

In Culture T7A (isolated from a refinery sample) 10% sugar broths only gave a fermentation pattern corresponding to *Torulopsis apicola* Hajsig (1958). From this aspect and its very poor growth on wort agar, it resembles the 'obligate osmophil' of Kroemer & Krumboltz (1931); but many osmophilic yeasts can be gradually accustomed to more standard conditions, so we do not consider this a difference of taxonomic significance. Assimilation of L-sorbose, xylose and adonitol were also positive at 10% concentration only.

Culture T8 (isolated from raw, unrefined syrup) was a typical isolate of *Torulopsis globosa* (Olson & Hammer); but here again, fermentation tests gave more consistent results at the higher concentration.

The biochemical reactions of Culture T9 (isolated from the air) at first indicated a connexion with *Torulopsis dattila*; but this was discounted as our isolate was a very much smaller organism than the authentic strain from the Centraal Bureau voor Schimmelcultures, Delft, Holland (CBS) and differed in growing without added vitamins, and growing strongly with ethanol as carbon source. It is smaller than *T. bacillaris* and utilizes more carbon compounds in the full auxanogram. Any con-

nexion with *T. apicola* can also be eliminated, since Culture T9 ferments glucose and sucrose more quickly, with the additional fermentation of raffinose; and again, it assimilates more compounds. We confirm the results of Dr W. Slooff (private communication), that the assimilation pattern resembles that of *Candida guilliermondii*, although more significant differences in fermentation become apparent when tested at 10 % concentrations. Culture T9 may be related to *C. guilliermondii*, but it must be ascribed to *Torulopsis* as it is asporogenous, forms no pseudomycelium on potato or corn-meal agar slide-cultures, and does not split arbutin. It is also a point of difference that we found the assimilation tests on salicin and cellobiose were only

Table 1. *Morphological characteristics of osmophilic yeasts*

Code	Growth in wort broth	Growth on wort agar	Pseudo-mycelium	Ascospores
T7	(1.1-1.5) × (2-2.5) μ* sediment only	(1.1-1.5) × (1.5-2.5) μ growth poor, browns	Negative	Not formed
T8	(4-6.5) × (4.5-6.5) μ sediment only	(4.5-7.5) × (5-7.5) μ growth poor, cream	Negative	Not formed
T9	(1.5-2.5) × (2-3.5) μ sediment only	(0.75-1.5) × (1.5-3) μ browns, growth poor	Negative	Not formed
T10	(1.5-3) × (2-5) μ sediment only	(1.5-3) × (2.5-5) μ dark brown, growth poor	Negative	Not formed
T11	(3.5-5) × (2.5-5.5) μ sediment, thin ring and faint islets	(2.7-4.5) × (3.5-6.5) μ white, creamy, margin entire	Negative	Not formed
T12	(2.5-5) × (3.5-5) μ sediment only	(2.5-4) × (2.5-5) μ pale brown, growth poor	Negative	Not formed
P4A	(1.5-4) × (2-5) μ sediment, ring and islets	(1-4) × (1.5-4) μ smooth, glistening, creamy-white	Blastodendrion type	Not detected
P4B	(1.5-4) × (2-5) μ sediment, ring and islets	(1-4) × (1.5-4) μ smooth, glistening, creamy-white	Blastodendrion type	Not detected
Sa24	(2-4) × (3-7.5) μ sediment only	(3-6) × (3.5-7.5) μ white—dirty-white, viscous	Negative	In newly isolated cultures
Sa26	(3-5.5) × (5.5-9) μ sediment, thin ring and islets	(2.5-6) × (4.5-8.5) μ creamy-white, margin crenated	Long, slender cells, sparse	Not detected

* Cell sizes of 8-day cultures, macroscopic appearance after 1 month at ambient temperature.

positive with inocula prepared by Wickerham's (1951) starvation method. With washed organisms as inocula (a necessity here, particularly with some of the 'obligate osmophils') the results were negative; β-glucosidase production is probably therefore very slight. With this combination of characteristics, we consider this to be a new species which we have named *T. kestoni*. A culture has been deposited at the Centraal Bureau voor Schimmelcultures. The Latin diagnosis is included at the end of this account.

Culture T10 (isolated from unrefined liquor) can be directly typed as *Torulopsis lactis-condensi* (Hammer). Culture T11 (also isolated from unrefined liquor) shows

Table 2. *Biochemical characteristics of Torulopsis spp.*

	Cultures											
	T7		T8		T9		T10		T11		T12	
	10 %	2 %	10 %	2 %	10 %	2 %	10 %	2 %	10 %	2 %	10 %	2 %
Fermentation												
Glucose	+	—	+	V*	+	+	+	+	+	+	+	+
Sucrose	+	—	+	VW	+	VW	+	+	+	+	+	+
Raffinose	—	—	+	VW	+	—	+	+	+	—	+	+
Maltose	—	—	—	—	—	—	—	—	—	—	—	—
Galactose	—	—	—	—	—	—	—	—	—	—	—	—
Lactose	—	—	—	—	—	—	—	—	—	—	—	—
Melibiose	—	—	—	—	—	—	—	—	—	—	—	—
Assimilation of carbon compounds												
	10 %	1 %	10 %	1 %	10 %	1 %	10 %	1 %	10 %†	10 %	1 %	
Glucose	+	+	+	+	+	+	+	+	+	+	+	
Galactose	W	—	—	—	+	—	—	—	+	W	—	
Sucrose	+	+	+	+	+	+	+	+	+	+	+	
Maltose	—	—	+	+	+	—	—	—	+	W	—	
Lactose	—	—	—	—	—	—	—	—	—	—	—	
L-sorbose	+	—	+	+	+	+	—	—	+	+	—	
Cellobiose	—	—	—	—	+	—	—	—	—	+	—	
Trehalose	—	—	+	+	—	—	—	—	nt	+	+	
Melibiose	—	—	—	—	+	—	—	—	+	+	—	
Raffinose	+	+	+	+	+	+	+	+	+	+	+	
Melizitose	—	—	—	—	W	W	—	—	nt	—	—	
Inulin	.	—	.	W	.	—	.	—	+	.	+	
Starch	.	—	.	—	.	—	.	—	—	.	—	
Xylose	+	—	—	—	+	—	—	—	nt	—	—	
L-arabinose	—	—	—	—	—	—	—	—	—	—	—	
D-arabinose	—	—	—	—	—	—	—	—	—	—	—	
D-ribose	+	W	—	—	+	+	—	—	nt	—	—	
L-rhamnose	—	—	—	—	—	—	—	—	—	—	—	
Ethanol	.	+	.	+	.	+	.	—	—	.	W	
Glycerol	+	+	+	+	+	+	—	—	+	+	—	
Erythritol	—	—	—	—	W	—	—	—	—	—	—	
Adonitol	+	—	+	+	+	—	—	—	nt	—	—	
Dulcitol	—	—	—	—	—	—	—	—	nt	—	—	
D-mannitol	+	+	+	+	+	+	—	—	+	+	+	
D-sorbitol	+	+	+	+	+	+	—	—	+	+	W	
α -methyl glucoside	—	—	+	+	+	+	—	—	+	+	+	
Salicin	—	—	—	—	W	—	—	—	—	—	—	
Lactic acid	.	—	.	—	.	W	.	—	—	.	VW	
Succinic acid	.	—	.	—	.	+	.	—	nt	.	+	
Citric acid	.	—	.	—	.	+	.	—	+	.	W	
Inositol	—	—	—	—	—	—	—	—	—	—	—	
Arbutin	—	—	—	—	—	—	—	—	—	—	—	
KNO ₃	—	—	+	—	—	—	+	—	+	—	—	
Growth in vitamin-free medium	W	—	+	—	+	—	—	—	+	—	+	
Growth at 37°	—	—	—	—	+	‡	—	—	—	—	—	

* V = variable; W = weak.

† In T11, 10 % solutions only were tested, except where the lower concentrations were used as in ethanol, starch, etc. nt = not tested.

‡ Growth at 37° positive on osmophilic agar only.

Table 3. *Biochemical characteristics of osmophilic yeasts*

	Cultures							
	P4A		P4B		Sa24		Sa26	
	10 %	2 %	10 %	2 %	10 %	2 %	10 %	2 %
Fermentation								
Glucose	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	—	—	+	+
Raffinose	+	W	+	W	—	—	+	+
Maltose	—	—	W	—	+	—	+	+
Galactose	+	+	+	+	—	—	+	+
Lactose	—	—	—	—	—	—	—	—
Melibiose	—	—	—	—	—	—	+	+
Assimilation of carbon compounds								
	10 %	1 %	10 %	1 %	1 %	10 %	10 %	1 %
Glucose	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	W	—	+	+
Sucrose	+	+	+	+	—	—	+	+
Maltose	+	+	+	+	+	VW	+	+
Lactose	—	—	—	—	—	—	—	—
L-sorbose	+	+	+	+	—	—	—	—
Cellobiose	+	+	+	+	—	—	—	—
Trehalose	+	+	+	+	—	—	+	+
Melibiose	+	+	—	—	—	—	+	+
Raffinose	+	+	+	+	—	—	+	+
Melzitose	+	+	+	+	—	—	+	+
Inulin	.	+	.	+	.	—	.	—
Starch	.	—	.	—	.	—	.	—
Xylose	+	+	+	+	—	—	—	—
L-arabinose	+	+	+	+	—	—	+	W
D-arabinose	—	—	+	+	—	—	—	—
D-ribose	—	—	+	+	—	—	—	—
L-rhamnose	+	+	+	+	—	—	—	—
Ethanol	.	+	.	+	.	—	.	—
Glycerol	+	+	+	+	W	—	—	—
Erythritol	+	+	+	+	—	—	—	—
Adonitol	—	—	+	+	—	—	—	—
Dulcitol	+	+	+	+	—	—	—	—
D-mannitol	+	+	+	+	—	—	—	—
D-sorbitol	+	+	+	+	—	—	—	—
α -methyl glucoside	+	+	+	+	W	W	+	+
Salicin	+	+	+	+	—	—	—	—
Lactic acid	.	+	.	+	.	—	.	+
Citric acid	.	+	.	+	.	W	.	—
Succinic acid	.	+	.	+	.	—	.	—
Inositol	—	—	—	—	—	—	—	—
Arbutin		+		+		—		—
KNO ₃		—		—		—		—
Growth in vitamin-free medium		+		+		—		+
Growth at 37°		+		+		+		+

* Growth at 37° on osmophilic agar only.

typical morphological characteristics of *T. globosa* (Olson & Hammer) together with a strong reaction in the vitamin-free medium. It is unusual, however, in that it assimilates galactose and on the potato-agar slide cultures produces occasional organisms which show an abortive elongation similar to those in the authentic strain of *T. dattila*. It may represent an intermediate form with species of the genus *Candida*, but these differences are not considered sufficiently decisive to justify the creation of a new variety.

Culture T12 (from a South American cane juice sample) was typed as *Torulopsis bacillaris* (Kroemer & Krumboltz). The negative reaction with KNO_3 eliminated any relationship with *T. lactis-condensi*; the configuration of the organisms (singly and in pairs) in wort broth also eliminated the possibility of its being *T. stellata* which produces star-like clusters, or the variety *T. stellata* var. *cambresieri* which splits arbutin.

Cultures P4A and P4B

Culture P4A was isolated from a West Indian sugar cane and Culture P4B from a South American cane juice. These two isolates were obviously identical and were identified as *Candida guilliermondii* (Cast.) Langeron et Guerra (Tables 1, 3). Formation of pseudomycelium on potato agar was observed after 7 days at 27°, but sporulation was not seen on V8 and Gorodkova agar slants or carrot plugs even when mixed with a possible mating strain (Kreger van Rij, 1964). Culture P4B is similar to a strain described by Kreger van Rij which assimilated melibiose. Of interest is the very high osmotic tolerance of these strains, a further indication of the cosmopolitan distribution of this species.

Cultures Sa24 and Sa26

Culture Sa24 is a typical strain of *Saccharomyces rouxii*, but the fermentation of maltose was only definite in 10 % solutions. This observation is similar to that of Santa Maria (1964) with Spanish cane molasses.

Culture Sa26 was isolated from the air in a sugar factory and was identified as *Saccharomyces florentinus*, Castelli. Small amounts of mycelium only were seen on potato agar slide cultures after 7 days incubation. Sporulation was not observed on V8 or Gorodkova agar or carrot plugs after 12 weeks at 27°. To our knowledge, this is the first time that this organism has been found in an osmophilic habitat.

DISCUSSION

Barnett (1957) advocated the use of special biochemical tests for specific groups of yeasts, but the fat-splitting enzyme test used for the identification of *Candida lipolytica* shows that such an approach has been used for some time. The limitations of such tests are that often the media used are not sufficiently standard for biochemical use and merely form a medium for morphological observation, for example, Koji extract (Onishi, 1957) and honey agar (Lochead & Heron, 1929); malt extract can be included in media of this type. On the other hand, increased sugar concentrations can be used in more chemically defined media.

In identifying these highly osmophilic yeasts either by the standard methods or by using an increased sugar concentration as here, consideration must be made of the nature of the original microhabitat; simply to record growth on an increased con-

centration of sugar in an agar medium would appear to be insufficient (Scarr & Rose, 1965). The practical assessment of the industrial importance of this group of osmophilic yeasts; either alone or with *Saccharomyces rouxii* and *S. mellis* would be in relation to the storage of raw sugar and other sucrose products for any length of time. This has yet to be determined.

Latin diagnosis

Torulopsis kestoni sp. nov.

In musto maltato cellulae subovoidae $(1.5-2.5) \times (2.0-3.5) \mu$, singulae, binae et glomerae. Post unem mensem, sedimentum.

In agara maltato cultura (post unem mensem 17°) fusca, tempore breve moritar. Pseudomycelium nullum.

Fermentatio ambis concentrationibus 2 et 10 % glucosi et sacchari. Fermentatio raffinosi concentratione sola 10 %. In media minerale cum 1 et 10 % glucoso, saccharoso, L-sorbose, raffinose, D-riboso, glycerolo, D-mannitolo, D-sorbitolo et α -methyl glucosido crescit. Cum 10 % sola galactoso, maltoso, cellobioso, xyloso, adonitolo et salicino (exiguum) crescit. Cum 1 % sola acido succinico, acido citrico et acido lactico (exiguum) crescit.

Arbutin non finditur. Formatio esterium nulla. Kalium nitricum non assimilantur. Non necessariae ad crescentium sunt vitaminae externae.

The authors wish to thank the Directors of Tate and Lyle Ltd., for permission to publish this paper and Miss W. Slooff of the Centraal-Bureau voor Schimmelcultures for her criticism and very valuable suggestions.

REFERENCES

- BARNETT, J. A. (1957). Some unsolved problems of yeast taxonomy. *Antonie van Leeuwenhoek* **23**, 1.
- HASIG, M. (1958). *Torulopsis apicola*, nov. spec. New isolates from Bees. *Antonie van Leeuwenhoek* **24**, 18.
- KREGER-VAN RIJ, N. J. W. (1964). *Taxonomic Study of the Yeast Genera Endomycopsis, Pichia and Debaromyces*. PhD. Thesis, University of Leiden, Netherlands.
- KROEMER, K. & KRUMBHOLTZ, G. (1931). Untersuchungen über osmophile Sprosspilze. I. Beiträge zur Kenntnis der Gärungsvorgänge und der Gärungserreger der Trockenbeerenauslesen. *Arch. Mikrobiol.* **2**, 352.
- LOCHEAD, A. G. & HERON, D. A. (1929). Microbiological studies of honey. *Bull. Dep. Agric. Dom. Can.* No. 116 *New Series*.
- LODDER, J. & KREGER-VAN RIJ, N. J. W. (1952). 'The Yeasts'. *A Taxonomic Study*. Amsterdam: North-Holland Publishing Co.
- ONISHI, H. (1957). Studies on osmophilic yeasts. Part III. Classification of osmophilic soy and miso yeasts. *Bull. agric. chem. Soc. Japan* **21**, 157.
- SANTA MARIA, J. (1964). Utilizacion de sacarosa y maltosa por levaduras. *Bohn. Inst. nac. Invest. agron. Madr.* **50**, 1.
- SCARR, M. P. (1952). Osmophilic yeasts in raw beet and cane sugars and intermediate sugar-refining products. *J. gen. Microbiol.* **5**, 704.
- SCARR, M. P. (1959). Selective media used in the microbiological examination of sugar products. *J. Sci. Food Agric.* **10**, 678.
- SCARR, M. P. & ROSE, D. (1965). Assimilation and fermentation patterns of osmophilic yeasts in sugar broths at two concentrations. *Nature, Lond.* **207**, p. 887.
- WICKERHAM, L. J. (1951). Taxonomy of yeasts. *Tech. Bull. U.S. Dep. Agric.* No. 1029.

EXPLANATION OF PLATE

Cultures mounted in glycerine jelly from a slope of osmophilic agar.

- (a) *Torulopsis kestoni* (T9). (b) *T. bacillaris* (T12). (c) *Saccharomyces rouxii* (Sa24).
(d) *Candida guilliermondii* (P4B).

M. P. SCARR AND D. ROSE

(Facing p. 16)