Comparison of Serological and Physiological Classification of the Genus *Saccharomyces*

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SUMMARY

The physiological and morphological properties of 28 species of the genus *Saccharomyces* were subjected to numerical analysis. Ten groups were distinguished, which agreed well with serological groupings, thus: (1) *S. bailii*, *S. bisporus*, *S. mellis* and *S. rouxii*, of serological group A; (2) *S. carlsbergensis* and *S. cartilagenosus* (A); (3) *S. cerevisiae* (AB); (4) *S. oviformis*, *S. steineri* and *S. veronae* (AC) and *S. bayanus* and *S. pastorianus* (C); (5) *S. acidifaciens* and *S. elegans* (AD); (6) *S. fragilis* and *S. marxianus* (B); (7) *S. chevalieri* (BC); (8) *S. cerevisiae* var. *ellipsoideus*, *S. diastaticus*, *S. logos*, *S. uvarum*, *S. willianus* and a further group of strains of *S. carlsbergensis* (all of group C); (9) *S. fermentati*, *S. microellipsodes* and *S. rosei* (D); (10) *S. delbrueckii* (D).

Serological classification offers results sufficiently close to those obtained by morphological and physiological tests to provide a rapid and reliable method of identification of *Saccharomyces* species.

INTRODUCTION

A simple serological classification of the genus *Saccharomyces*, using four absorbed sera, has been described previously (Campbell, 1968). However, in some instances the serological classification does not coincide with the identification by the standard morphological and physiological tests of Lodder & Kreger-van Rij (1952). For example, there are two antigenically distinct subgroups of *S. carlsbergensis*, termed I and II by Campbell & Brudzynski (1966), and of *S. cerevisiae*, but in the latter case the antigenic variant closely corresponds to the morphological variant *ellipsoideus* (Campbell & Allan, 1964). Another discrepancy between generally accepted classification and the serological method is the large group of species, including *S. carlsbergensis* II, *S. cerevisiae* var. *ellipsoideus*, *S. logos*, *S. uvarum* and *S. willianus*, which are antigenically identical (Campbell, 1968).

Kockova-Kratochvilova & colleagues (1966*a*, *b*, 1967, 1968) have demonstrated by numerical taxonomy of many species of *Saccharomyces* that certain species recognized by Lodder & Kreger-van Rij (1952) do not on full analysis justify specific rank. *Saccharomyces pastorianus*, *S. bayanus* and *S. willianus* were sufficiently similar to be grouped as one species, *S. heterogenicus* was significantly different from that group, but *S. steineri* formed a transition between them (Kockova-Kratochvilova, Vojtkova-Lepsikova, Sandula & Pokorna, 1968).

In the present paper an analysis of 28 *Saccharomyces* species is presented in order to examine fully the compatibility of our serological identification scheme with the Lodder & Kreger-van Rij system, and with the condensed classification proposed by Kockova-Kratochvilova *et al.* (1968).

METHODS

Yeast cultures. Cultures of 28 *Saccharomyces* species were obtained from the National Collection of Yeast Cultures (NCYC), Nutfield; additional strains were isolated in this Department or obtained from other sources. The total of 136 cultures were identified on isolation or receipt by the system of Lodder & Kreger-van Rij (1952). Cultures were normally grown at 25° for 3 days; those for preparation of antisera and inoculation of identification tests were prepared on Sabouraud glucose agar (Oxoid).

Characters for numerical analysis. The 48 tests described below were performed on each strain.

Morphology (9 characters). Cells, grown in malt extract broth, were assessed as spherical (length up to $1\frac{1}{2} \times$ breadth), oval ($1\frac{1}{2}$ to 3×1) or long-oval (over 3×1); small (width up to 4 μ m.) or large (width over 4 μ m.). Production of pseudomycelium (slight, up to 5 cells long; extensive, over 5 cells) on Oxoid cornmeal agar was assessed without regard to morphology; for this and other tests on solid media nine cultures were conveniently spotted on one 10 cm. diameter plate. Colony morphology on malt extract agar was assessed after 3 days as smooth (circular outline, glossy surface) or rough (irregular outline, matt or rough surface).

Physiology (35 characters). Suspensions of cells from Sabouraud agar were prepared in 0.1 M-phosphate buffer, pH 6, washed by centrifugation and incubated at 25° in the same buffer for 2 h. to deplete intracellular reserves of nutrients. After two further washings the suspensions were adjusted to approximately 10^7 cells/ml. and the test media inoculated with 0.02 ml. of suspension. All tests were performed in duplicate or triplicate on separate occasions. Fermentation and assimilation of galactose, sucrose, maltose, raffinose, lactose, melibiose and maltotriose (Lodder & Kreger-van Rij, 1952; Gilliland, 1956) were tested by the method of Campbell & Brudzynski (1966). Maltotriose, added at 3 % to the basal medium, was prepared by hydrolysis of pullulan (Ueda, Fujita, Komatsu & Nakashima, 1963) by pullulanase (Bender & Wallenfels, 1961). Assimilation alone of sugars was scored + - . Fermentation, which included assimilation, was scored + +, except in the case of maltotriose when only fermentation was scored as +. No distinction was made between partial and complete fermentation of raffinose. Assimilation of ethanol, glycerol or L-arabinose, each at 2 %, as sole carbon source, and of 0.25 % glycine, L-histidine, L-lysine, L-methionine or L-tryptophan as sole nitrogen source (Wickerham, 1952) were tested on the appropriately supplemented Difco yeast nitrogen or carbon medium, solidified with 1 % Oxoid agar no. 1. In addition to growth on tryptophan, cultures were examined for production of a brown halo which was observed to surround the colonies of certain species on tryptophan agar. Requirements for vitamins (Schultz & Atkin, 1947) were assessed in terms of 'bios factors' 1, 2, 3 and 6 (inositol, pantothenate, biotin and pyridoxine + thiamine) on agar based on the formula for Difco yeast vitamin medium but prepared from salts and amino acids supplied by B.D.H. Chemicals Ltd (Poole, Dorset) and vitamins by Koch-Light Ltd (Colnbrook, Buckinghamshire).

As additional identification tests, the following were examined: growth in malt extract broth containing 8, 12 or 16 % (v/v) ethanol (added aseptically to sterile broth) and growth under conditions of high osmotic pressure in a medium of 20 g. glucose, 5 g. NaCl and 1 g. Oxoid mycological peptone per 100 ml. ('Osmophilic medium')

(Kockova-Kratochvilova *et al.* 1966a) and at 30 and 37° over 2 days on malt extract agar. Lipolysis was detected on Oxoid tributyrin agar and acid production on the chalk agar described by Lodder & Kreger-van Rij (1952). Strains were also tested for growth in the presence of 20 p.p.m. crystal violet (Kato, 1967).

Flocculation and fining (4 characters). These properties were assessed as described by Campbell, Robson & Hough (1968) and each scored —, + — or ++.

Table 1. *Antigenic structure of various Saccharomyces species*

Species and NCYC* numbers	Antigenic structure
<i>Saccharomyces bailii</i> 385, 580	A
<i>S. bisporus</i> 171, 697	A
<i>S. cartilaginosus</i> 76	A
<i>S. mellis</i> 418	A
<i>S. oviformis</i> 374, 482	AC
<i>S. steineri</i> 406, 701	AC
<i>S. fructuum</i> 410, 609, 620	C
<i>S. heterogenicus</i> 415, 484	C
<i>S. italicus</i> 108	C
<i>S. microellipsodes</i> 698	D
<i>S. rosei</i> 566, 585	D

* National Collection of Yeast Cultures, Nutfield, Surrey.

Numerical analyses. Properties, other than serological, were recorded on 80-column punched cards and the percentage similarity between strains calculated on an I.C.L. 4130 computer. Both positive and negative matches were assessed as similarity, using the formula for S_s of Sneath (1962). Similarity matrices and thence dendrograms were prepared as described by Sneath (1962), plotting results at 5 % intervals. Relationships between groups of strains were drawn at the highest mutual similarity level, as at lowest or mean similarity level the differences between groups were unjustifiably enhanced. Normally the percentage similarity between strains in a cluster fell within a 5 % range.

Since the large number of strains involved in the project could not be handled conveniently in a single run of the computer, sets of up to 20 strains of similar serological properties were processed together, and compared in turn with a similar number of strains of other serological groups. Thus all strains of species of serological group A (*Saccharomyces bailii*, *S. bisporus*, *S. carlsbergensis* I, *S. cartilaginosus*, *S. mellis* and *S. rouxii*) (Campbell, 1968; see also Table 1) were compared in successive analyses with groups of strains of serological groups AB, AC, AD, B, BC, C and D, which were compared in turn with all other groups. A small number of strains were chosen of each species to confirm in a final survey the relationships indicated by the preliminary findings.

RESULTS

Serological properties of species. All available strains of 28 *Saccharomyces* species were tested by absorbed antisera A, B, C and D (Campbell, 1968). Agglutination patterns of species not classified in the earlier communication are listed in Table 1. In addition, sera were prepared against at least one strain of each species not tested previously. On analysis by a full programme of absorption tests with strains of various antigenic groups, no antigenic factors additional to those already listed in Table 1 were

Table 2. *Properties of strains*

Saccharomyces species	No. of strains	Assimilation of										Growth at 37°	Hydrolysis of tributyrin agar	Clearing of chalk agar	Growth on C.V.A.	Malto-triose fermentation	Flocculence	Fining
		Glycine	Histi-dine	Lysine	Methio-nine	Tripto-phan	Arabi-nose	Ethanol	Glycerol	Bios number	Alcohol tolerance (%)	Osmotic tolerance						
<i>S. acidifaciens</i>	6	-	x	+	+	x	-	+	+	0	8 to 12	x	x	-	-	-	-	++
<i>S. bayanus</i>	1	-	-	-	+	+	-	-	+	236	8	-	-	-	-	+	-	-
<i>S. baltii</i>	3	x	x	x	-	x	-	-	-	x	8 to 12	-	+	-	-	-	x	+
<i>S. bisporus</i>	3	x	+	x	+	+	-	-	-	0	8 to 12	-	+	-	-	-	-	++
<i>S. carlsbergensis</i> I	3	-	-	-	+	+	-	-	-	0	8 to 12	-	-	-	+	x	-	++
<i>S. carlsbergensis</i> II	12	-	x	-	x	x	-	x	x	x	8 to 12	-	-	-	x	x	x	x
<i>S. carlinaginosus</i>	1	-	-	-	+	+	-	-	-	2	8 to 12	-	-	-	+	-	-	++
<i>S. cerevisiae</i>	12	-	-	-	x	x	-	-	-	x	8 to 12	-	-	-	-	x	x	++
<i>S. cerevisiae</i> var. <i>ellipsoideus</i>	12	-	x	-	x	x	-	x	x	x	12 to 16	-	-	-	+	x	x	x
<i>S. chevalieri</i>	5	-	-	-	+	+	-	-	-	x	8	-	-	-	+	-	-	-
<i>S. delbrueckii</i>	3	+	+	-	+	x	-	-	-	x	8	-	+	+	+	-	-	++
<i>S. diastaticus</i>	4	-	-	-	+	x	-	+	x	3	12 to 16	-	-	-	x	+	x	+
<i>S. elegans</i>	1	-	+	-	+	+	-	+	+	36	8	-	-	-	-	-	-	++
<i>S. fermentati</i>	3	-	+	+	+	+	-	x	x	x	12	+	+	-	-	-	-	+
<i>S. fragilis</i>	5	x	+	+	+	+	+	+	-	236	8 to 12	+	+	+	-	-	-	+
<i>S. fructuum</i>	3	x	-	+	+	+	-	-	-	3	12 to 16	+	+	+	+	-	-	+
<i>S. heterogenicus</i>	3	-	-	-	+	x	-	+	+	0	≤ 8	-	-	-	+	-	-	x
<i>S. italicus</i>	1	-	-	-	+	+	-	+	+	3	8	+	-	-	-	-	-	-
<i>S. logos</i>	3	-	-	-	x	-	-	-	-	236	8 to 12	-	-	-	+	x	x	+
<i>S. marxianus</i>	2	x	+	+	+	+	+	+	-	236	8	+	+	+	-	-	-	+
<i>S. mellis</i>	1	+	+	+	-	+	-	-	-	2	< 8	+	-	-	-	-	-	++
<i>S. microellipsoides</i>	1	-	+	+	+	+	-	-	-	0	8	-	-	+	-	-	-	+
<i>S. oviformis</i>	12	-	-	-	+	x	-	x	+	x	12 to 16	x	-	-	x	-	-	-
<i>S. pastorianus</i>	3	-	-	-	x	+	-	-	+	236	8	-	-	-	-	x	-	+
<i>S. rosei</i>	3	-	+	+	+	+	-	+	+	x	12 to 16	+	+	-	-	-	-	++
<i>S. rouxii</i>	6	-	-	+	+	+	-	-	+	0	8	+	x	-	+	-	x	++
<i>S. sttheri</i>	8	-	-	+	+	x	-	-	+	x	8 to 12	+	-	-	x	-	-	++
<i>S. uvarum</i>	5	-	-	+	+	+	-	x	x	236	8 to 12	-	x	-	+	-	-	+
<i>S. veronae</i>	3	-	x	+	+	+	-	-	+	236	8	+	-	-	-	-	-	x
<i>S. williamsii</i>	8	-	x	-	+	+	-	+	+	0	8 to 12	+	-	-	-	-	-	++

Triptophan: + = growth; ++ = brown halo round colony; - = no growth.
 Flocculence, fining: - = absent; +, ++ = increasing fining or flocculence.

Tributyrin and chalk agars: + = zone of clearing round colony; - = no effect.
 Other tests: + = growth; - = no growth; x = various reactions between strains, or between tests with same strain.

Fig. 1

Fig. 2

Fig. 1 Dendrogram of typical NCYC strains reacting with serum A, and selected group C strains (*Saccharomyces carlsbergensis* 1047, *S. ellipsoideus* 506 and *S. willianus* 106) for comparison.

Fig. 2. Dendrogram of typical NCYC strains reacting with serum B (*Saccharomyces marxianus* and *S. fragilis*) and serum C (other species).

found. Sera to *Saccharomyces oviformis* and *S. steineri* agglutinated cultures of both groups A and C; absorption by a culture of one group left agglutinating activity to all cultures of the other group. All species agglutinated by serum A, other than *S. rouxii*, were also agglutinated by the serum 1 described previously (Campbell, 1968). No reactions were noted between the newly examined strains and sera 2, 3, 4 and 5.

Numerical analyses. The identification tests of Lodder & Kreger-van Rij (1952) accounted for 20 morphological and physiological characters. Some tests, e.g. fermentation of glucose, were not applicable because of consistently positive results in the genus *Saccharomyces*; other tests, e.g. pellicle formation, were consistently negative. The remaining properties of each species are summarized in Table 2. Typical examples of

Fig 3. Dendrogram of typical NCYC strains reacting with serum D, and selected group A strains (*Saccharomyces mellis* 418 and *S. rouxii* 381) for comparison.

dendrograms are presented in Fig. 1 to 3, which show only NCYC strains, although additional strains of most species, from the NCYC and elsewhere, were examined. The total number of strains tested of each species is shown in Table 2.

The relationship between *Saccharomyces cerevisiae*, *S. carlsbergensis* I, two strains of *S. willianus* and the one strain available of *S. cartilaginosus*, all of which react with serum A, is illustrated in Fig. 1. The 90 to 97 % mutual similarity of all strains of *S. rouxii*, and the more distant relationship of other species of serological group A is also shown. However, the small-celled species *S. bailii*, *S. bisporus*, *S. mellis* and *S. rouxii* show a percentage similarity of 75 or less to the large-celled species *S. carlsbergensis* and *S. cartilaginosus* which similarly react only with serum A. The '*S. carlsbergensis* group' shows more affinity to *S. cerevisiae* and the two strains, 134 and 618, of *S. willianus* reacting with sera A and B, but these *S. willianus* strains in turn show a closer similarity to the group C strains of their own species, of which strain 106 appears as an example in Fig. 1. *Saccharomyces willianus*, irrespective of serological properties, is taxonomically closer to *S. cerevisiae* var. *ellipsoideus* and those strains of *S. carlsbergensis* which react with serum C, than to *S. cerevisiae*.

Fig. 2 illustrates the close relationship between the strains of groups AC and C, e.g. the 80 to 85 % similarity between *Saccharomyces oviformis* (AC) and *S. cerevisiae* var. *ellipsoideus* (C). *Saccharomyces fructuum*, *S. heterogenicus* and *S. italicus*, serologically of group C, occupy an intermediate position on the dendrogram between the 'S.

Fig. 4. Dendrogram of all species tested, from NCYC and other sources. Spread of proposed groups is denoted by shading.

carlsbergensis-*S. willianus* group' (C) and the '*S. oviformis* group' (AC), thus producing a large cluster of species with no significant subgrouping. Only *S. chevalieri* and the *S. fragilis*-*S. marxianus* group showed a significant difference from other species shown in Fig. 2, which agrees well with their different serological properties, BC and C respectively.

Fig. 3 shows all NCYC strains which reacted with the specific group D serum. *Saccharomyces mellis* and *S. rouxii* are included for comparison with organisms of serological group A; both groups are mainly composed of species which ferment few of the sugars tested, or only glucose, but on taking into account all 48 properties the

similarity between species of serological groups A and D is no more than 75 %. *Saccharomyces acidifaciens* and *S. elegans* react with both sera A and D, the serological properties reflecting a marked taxonomic difference.

Comparison of serological and physiological groupings. Strains were allocated to distinct groups according to all suitable physiological and morphological tests (Fig. 1 to 3). In Fig. 4, compiled from a final analysis of representative NCYC strains of each species, or taxonomic group as determined above, the observed groups are compared with our serological classification (Campbell, 1968; and Table 1 above). Distinct groups, corresponding to antigenic properties, include *Saccharomyces acidifaciens* and *S. elegans* (group AD), *S. fragilis* and *S. marxianus* (B), *S. chevalieri* (BC), and *S. fermentati*, *S. microellipsodes* and *S. rosei* (D). *Saccharomyces rouxii* forms a taxonomically and serologically distinct subgroup of a group of small-celled poorly fermentative species of serological group A. *Saccharomyces delbrueckii*, although also of group D, is only distantly related to *S. fermentati*.

DISCUSSION

Many antigenically identical species (Campbell, 1968) have been shown by Kockova-Kratochvilova *et al.* (1966*a*, 1968) to be very similar in general properties. The present report describes an investigation by numerical taxonomy of a full range of *Saccharomyces* species of which the antigenic structure has been determined by our system using four sera A, B, C and D.

The largest group of *Saccharomyces* species, reacting with sera A, A and B, A and C, or C alone, contains the large-celled species actively fermenting a wide range of sugars: glucose, sucrose, maltose, often galactose and raffinose, and occasionally maltotriose. These species are important in the fermentation industries, both as culture yeasts and as contaminants. Antigenically they form four distinct groups, as shown in Fig. 4, but numerical analyses (above, and Kockova-Kratochvilova *et al.* 1966*a*, 1968) show that they are very closely related; all show a similarity by our method of calculation of 80 to 85 % between antigenically different strains and 85 to 90 % between strains of the same antigenic group. The species of antigenic group C, *S. carlsbergensis*, *S. cerevisiae* var. *ellipsoideus*, *S. diastaticus*, *S. uvarum* and *S. willianus*, show a particularly close interrelationship, and instances were noted of strains more closely related to strains of other species than to other strains of their own species, e.g. between *S. cerevisiae* var. *ellipsoideus* and *S. diastaticus* (Fig. 2).

In Fig. 2 and 4, *Saccharomyces pastorianus*, *S. bayanus* and the species reacting with sera A and C are grouped separately from those reacting with serum C only. Although *S. pastorianus* is not agglutinated by serum A, it possesses a more complex antigenic structure than species of the *S. carlsbergensis*-*S. willianus* 'C-only' group. Campbell (1968) described five minor sera as an aid to species classification; one of these, serum 5, agglutinated *S. pastorianus*, but the 'C-only' group does not react with any of the sera 1 to 5. Thus the species of simplest antigenic structure are distinguishable by numerical taxonomy from those species reacting with sera A and C or C and 5.

Saccharomyces carlsbergensis I (of serological group A), *S. cerevisiae* (group AB) and groups AC, including C5, and C may be valid subdivisions, separable at about 85 % similarity, but the results of Kockova-Kratochvilova *et al.* (1966*a, b*, 1967, 1968) obtained from a similar but not identical series of tests, combined with the above

observations suggest that the serological groups overlap to form a single large group. It is nevertheless useful, for diagnostic and epidemiological purposes, to subdivide serologically. In our experience, two strains showing a similarity of over 85 % are, almost invariably, serologically identical; conversely, two isolates which are serologically identical are normally over 85 % similar in other respects. The two antigenic types of *S. willianus*, and the difference between *S. delbrueckii* and the other species of serological group D, provided the only exceptions, and we have found the rapid serological method a reliable preliminary identification system for *Saccharomyces* yeasts isolated from spoilt beers, wines and foods. Although our basic system (Campbell, 1968), using only four sera A, B, C and D, gives more limited information than the more comprehensive antigenic analyses of Tsuchiya *et al.* (1965), it is sufficient for identification within the genus *Saccharomyces*. Thus any yeast isolate which ferments glucose actively, i.e. overnight at 25°, and is therefore presumed to be of the genus *Saccharomyces*, is quickly allocated to the appropriate antigenic group by a slide agglutination test. In most cases we found it was sufficient simply to record the isolation of 'S. oviformis type' (group AC), 'S. bailii type' (group A), etc. Following the precedent of Beech, Davenport, Goswell & Burnett (1968), no distinction was attempted between *Saccharomyces* species and the corresponding species of *Candida* or *Torulopsis*, and sporulation was not considered in our numerical analyses.

For more accurate identification, a set of seven sera was necessary: A, B, C and D, and the sera 1, 2 and 5. Serum 2 distinguished *Saccharomyces rouxii* from other species reacting with serum A; also, all other group A species were agglutinated by serum 1. Serum 5 separated *S. pastorianus* from the various species which reacted only with serum C. Thus, by agglutination test and a morphological examination of cells, e.g. to distinguish *S. carlsbergensis* or *S. cartilaginosus* from the small-celled species reacting with serum A, the various serological subgroups of the genus *Saccharomyces* may be rapidly distinguished. The numerical analyses of Kockova-Kratochvilova *et al.* (1966*a, b*, 1967, 1968) and the above results suggest that serological methods reliably identify groups of *Saccharomyces* species to the limit of taxonomic validity.

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