BARNETT, J. A., INGRAM, M. & SWAIN, T. (1956). *J. gen. Microbiol.* **15**, 529-555

The Use of β -Glucosides in Classifying Yeasts

BY J. A. BARNETT, M. INGRAM AND T. SWAIN

*Low Temperature Station for Research in Biochemistry and Biophysics,
University of Cambridge, and Department of Scientific
and Industrial Research*

SUMMARY: The β -glucosidase activity of 119 strains of yeast was studied with aesculin, arbutin, salicin or cellobiose as substrates. A medium which was sufficient for good growth was in many cases not sufficient to allow the development of β -glucosidase activity. A yeast capable of splitting one β -glucoside did not necessarily hydrolyse another. It is suggested that yeasts may contain different β -glucosidases. There is evidence that, grown under suitable conditions, most yeasts are capable of hydrolysing aesculin.

The ability to hydrolyse various β -glucosides has been used in classifying yeasts. Four β -glucosides have been used: aesculin, arbutin, salicin and cellobiose; all are β -D-glucopyranosides (see Ingram, 1955). The three phenolic β -glucosides on hydrolysis yield phenols, the presence of which may be shown by a suitable colour reaction such as that with ferric salts. The criterion used to determine the ability to hydrolyse cellobiose has been growth with this sugar as the sole source of carbon, it being assumed that the disaccharide is split before utilization for growth.

Stelling-Dekker (1931) was the first to use a β -glucoside for classifying yeasts, when she tested strains of *Pichia* and *Hansenula* against aesculin by streaking the yeast on yeast-water agar containing 0.5 % aesculin and a drop of iron ammonium citrate solution. Diddens & Lodder (1942) used arbutin plates instead of aesculin for classifying asporogenous yeasts, because arbutin was cheaper and gave the same results (Dr J. Lodder, private communication). Their medium contained 5 g. arbutin, 1 l. yeast water, 1 drop FeCl_3 solution, 20 g. agar, and was autoclaved; the results were observed after 2, 4 or 6 days. Except for adding a drop of FeCl_3 solution to each plate, the same method was followed by Lodder & Kreger-van Rij (1952) in classifying all types of yeast; they suggested (1954) that aesculin might be used as an alternative to arbutin. More recently, cellobiose and salicin have been used for identifying yeasts (Wickerham, 1951; Wiles, 1953); in both cases, growth was the criterion for utilization. Salicin was used by Gordon (1905) for differentiating streptococci and Henry & Auld (1905) showed that, like arbutin, it was hydrolysed by baker's yeast. Table 1 gives a chronological summary of some contributions to this subject.

Hitherto, there has been no attempt to compare the ability of a large number of yeasts to split several β -glucosides, although Lodder & Kreger-van Rij (1954) indicated that a yeast which splits arbutin also splits aesculin, and vice versa. Previous workers did not discuss their techniques at all critically.

Table 1. *Chronological summary of some studies concerned with β -glucoside splitting by micro-organisms*

Date	Author	Contribution
1893	Bourquelot	Extract from <i>Aspergillus niger</i> split amygdalin, coniferin and salicin
1903	Kobert	Aesculin, arbutin and salicin split by invertebrate enzymes
1905	Gordon	Differentiation of streptococci, using acid production with 1 % coniferin or salicin
1905	Henry & Auld	Baker's yeast capable of splitting amygdalin, arbutin and salicin
1906	Gonnermann	Certain intestinal bacteria split arbutin: tested with ammoniacal silver nitrate
1907	Beijerinck	Aesculin (0.1 %) + 1 drop ferric citrate in whey agar for studying milk-souring organisms
1909 (a & b)	Harrison & Van der Leek	Aesculin bile salt medium for isolating <i>Escherichia coli</i> and <i>Salmonella typhi</i> . Reaction only in glucose-free medium. Results in second paper suggest phosphate interferes
1917	Bau	<i>Saccharomyces ludwigii</i> and Saaz yeast both active against amygdalin
1917	Gosio	For diagnosing dysentery bacilli: arbutin added 1/200 to ordinary agar medium; hydroquinone thought to blacken by oxidation in air
1920	Van Steenberghe	Aesculin (0.1 %) + ferric citrate (0.1 %) in yeast water agar for classifying milk organisms
1924	Weintraub	Arbutin, salicin and β -methyl glucoside for <i>E. coli</i> ; acid and gas production
1929	Weatherall & Dible	Distinguished between splitting aesculin and growing in presence of bile
1929 & 1930	Weidenhagen	Top and bottom yeasts split both β -glucoside links of amygdalin and cellobiose
1931	Stelling-Dekker*	Aesculin (0.5 %) + 1 drop ferric citrate in yeast water agar: used for <i>Pichia</i> and <i>Hansenula</i> spp.
1932	Neuberg & Hofmann	Study of yeasts β -glucosidase: splitting of amygdalin, arbutin, cellobiose and salicin by extract from <i>Saccharomyces fragilis</i>
1935	Castellani & Douglas	Arbutin (1 %) agar (no added iron) for bacteria: explanation of reaction not known
1937	Castellani*	Arbutin agar (?no added iron) for pathogenic yeasts
1942	Diddens & Lodder*	Arbutin (0.5 %) agar + FeCl ₃ solution (1 drop/l.) for anascosporogenous yeasts
1948	Wickernam & Burton*	Cellobiose and salicin for yeast growth tests in liquid culture
1952	Lodder & Kreger-van Rij*	Arbutin (0.5 %) agar + FeCl ₃ solution (1 drop/plate) for most yeasts
1953	Wiles*	Aesculin, cellobiose, salicin for yeast growth tests in liquid culture
1954	Lodder & Kreger-van Rij*	Arbutin and aesculin tests are alternative

Authors marked with asterisk (*) were concerned with yeast taxonomy.

It seemed worth while, therefore, to develop methods for studying yeast β -glucosidases under standardized and relatively well-understood conditions. With rapid techniques for detecting β -glucosidase activity, the authors have attempted to answer the following questions:

- (1) Will a yeast which hydrolyses one β -glucoside hydrolyse others?
- (2) Are differences between the rates of hydrolysis of various β -glucosides of value in classifying yeasts?
- (3) Will tests with one β -glucoside alone give maximum information about β -glucosidase activity for purposes of classification, and if so, which test is the most sensitive and convenient, and how quickly can a consistent result be obtained?

METHODS

Media

- (1) Malt yeast glucose peptone agar (MYGPA) (Wickerham, 1951), pH 6.0.
- (2) Honey yeast glucose potato agar: (HYGSA) (cf. Lochhead & Farrell, 1931; Ingram, 1950; Scarr, 1951): honey (Cambridgeshire) 300 g.; glucose 200 g.; Difco Bacto potato dextrose agar 40 g.; fresh yeast water (Lodder & Kreger-van Rij, 1952), 1 l.; autoclaved at 115° for 15 min.; final pH 5.5.
- (3) Honey yeast glucose peptone agar (HYGPA). As for HYGSA, but 5 g. Difco Bacto peptone and 5 g. Difco Bacto agar in place of potato dextrose agar; final pH 5.5.
- (4) The carbon-free chemically defined medium of Barnett & Ingram (1955), was used (i) without additions (A 2); (ii) with 1 % (w/v) glucose and 2 % (w/v) agar (A 2G agar): vitamins and glucose were Seitz-filtered and added aseptically.
- (5) A 2 + 0.05 % (w/v) aesculin, Seitz-filtered.
- (6) A 2 + 0.5 % (w/v) arbutin, Seitz-filtered.
- (7) A 2 + 0.5 % (w/v) salicin, Seitz-filtered.
- (8) A 2 + 0.1 % (w/v) cellobiose, Seitz-filtered.
- (9) Arbutin agar (Lodder & Kreger-van Rij, 1952).
- (10) Aesculin agar, prepared as described (a) by Stelling-Dekker (1931) and (b) by Van Steenberge (1920).
- (11) Breakdowns of MYGPA: made up as for MYGPA, but omitting various constituents in turn: 'MYGA', 'MYPA', 'MGPA', 'YGPA', 'YGA' and 'YA' (see Table 6 for pH values).
- (12) Difco Bacto potato dextrose agar: (SDA), pH 5.8.
- (13) Difco Bacto wort agar with addition of 0.5 % (w/v) Bacto agar (WA) pH 5.2.
- (14) Hartley's meat digest agar (Mackie & McCartney, 1949; NA); pH 7.5.
- (15) Malt extract agar (Blakeslee, 1915; MEA): pH 5.9.
- (16) Malt yeast glucose potato agar (MYGSA): Difco Bacto malt extract broth 15 g.; glucose 10 g.; Difco Bacto potato dextrose agar 40 g.; Difco Bacto agar 5 g.; fresh yeast water 1 l. Autoclaved at 115° for 15 min.; final pH 5.7.

Glucosides. Aesculin and arbutin from L. Light and Co. Ltd., salicin and cellobiose from T. Kerfoot and Co. Ltd. The names of these glucosides are abbreviated to AE, ARB, SAL and CEL in the tables.

Compounds for analysis were of Analar quality or of the highest quality available.

Organisms. Most of the strains of yeast used in these experiments were selected for their interest with respect to food microbiology, therefore not all genera are represented. The names and sources of the strains are given in Table 2. Throughout this paper, the names used are those adopted by Lodder & Kreger-van Rij (1952); except for two strains with names which these authors do not consider and alternative names given in Table 2. Stock cultures were maintained at $+1^{\circ}$ on slopes of A2G agar, MYGPA or HYGSA. These stocks were subcultured every 6 months (see Kirsop, 1954).

Plate techniques

Arbutin. Every strain was tested with the arbutin plate technique of Lodder & Kreger-van Rij (1952). Fresh yeast extract was prepared as these authors prescribed and 0.5 % (w/v) arbutin and 2 % (w/v) agar added. The medium was autoclaved at 120° for 15 min. The melted medium was poured into Petri dishes each of which contained one drop of sterile 1 % (w/v) FeCl_3 solution, and was thoroughly mixed (Lodder & Kreger-van Rij do not say what strength FeCl_3 solution they used).

Aesculin. The method of Van Steenberghe (1920) was used (see Table 1). A strip of agar was cut out across the centre of the plates with a sterile scalpel, giving two areas between which the aglycone could not diffuse (Kluyver & Custers, 1940). One area was inoculated and the other served as a non-inoculated control.

Attempts to use the medium of Stelling-Dekker (1931) were unsuccessful, perhaps because of uncertainty about the amount of iron to include. Dr G. C. Ingram (personal communication), with bacteria, found the concentration of iron in aesculin plates to be critical.

Growth experiments. The growth of certain strains was measured with cellobiose as the only source of carbon (Table 2). Shaken cultures in T-tubes were used (Barnett & Ingram, 1955); the inocula were cultivated in Roux bottles on A2G agar (except for NCYC 245 which had to be grown on MYGPA).

Preparation and use of the suspensions of organisms. For each experiment, a fresh slope was inoculated from the stock culture. After incubation for 3 days the organisms were washed off the slope with medium A2 and the entire crop used to inoculate a Roux bottle containing about 100 ml. of A2G agar, MYGPA, HYGSA or HYGPA. After 3 days of incubation at 25° , the cultures were harvested in 10 ml. of medium A2 and transferred to 1 oz (25 ml.) screw-capped bottles by means of sterile pipettes.

Estimation of splitting activity

In the routine experiments 2 ml. of suspension of organism was added aseptically to 2 ml. of medium A2, containing the required glucoside, in a T-tube (Barnett & Ingram, 1955). One T-tube was set up for each glucoside for each yeast strain; in addition, for each group of experiments there were control tubes without added yeast. The tubes were shaken (c. 2 cyc./sec. 5 cm.

Table 2 (cont.)

Classification of Lodder & Krieger-van Rij (1952)	Other name given previously	Quantitative tests with suspension of organisms grown on												Arbutin plate reactions for species given by Lodder & Krieger- van Rij (1952)		
		A2G agar					Complex medium					Cello- biase	Aesculin plates		Arbutin plates	
		No. of expts.	Aesculin	Arbutin	Salicin	Cello- biase	No. of expts.	Aesculin	Arbutin	Salicin						
<i>S. cerevisiae</i>		Strain														
		NCYC 77	1	-	-	-	1	+	+	+	0	0	-	-	-	-
		NCYC 231	1	-	(2)	-	2	+	+	+	(1)	0	+	-	-	-
		NCYC 353	2	-	-	-	1	+	+	+	0	0	-	-	-	-
		NCYC 369	1	-	+	-	1	+	+	+	+	0	-	-	-	-
		Guikerman 201	2	-	-	-	2	+	+	+	+	0	-	-	-	-
		Mossel	1	+	-	-	2	+	+	+	+	0	-	-	-	-
<i>S. cerevisiae</i> var. <i>ellipsoideus</i>	<i>Z. priorianus</i>	NCYC 176	2	+	-	-	2	+	+	+	+	0	-	-	-	-
		NCYC 365	1	+	(2)	-	1	+	+	+	+	0	-	-	-	-
<i>S. ellipsoideus</i>	<i>S. ellipsoideus</i>	NCYC 93	1	+	-	-	1	+	+	+	+	0	-	-	-	-
		NCYC 94	1	+	-	-	1	+	+	+	+	0	-	-	-	-
<i>S. fermentati</i>	<i>Z. fermentati</i>	CBS 818	1	-	-	0	1	+	+	+	+	0	-	-	-	-
<i>Z. mongolicus</i>	<i>Z. mongolicus</i>	CBS 705	1	+	+	0	1	+	+	+	+	0	-	-	-	-
<i>S. delbrueckii</i> var. <i>mongolicus</i>																
<i>S. fermentati</i>	<i>Torulaspora fermentati</i> /	NCYC 161	2	-	-	-	1	+	+	+	+	0	-	-	-	-
	<i>Z. eupagycus</i>	CBS 748	2	-	-	-(1)	1	+	+	+	+	0	-	-	-	-
<i>S. florentinus</i>	<i>Z. florentinus</i>	CBS 746	2	-	(3)	-	2	+	+	+	+	(1)	0	-	-	-
		NCYC 172	2	+	+	0*	1	+	+	+	+	0	-	-	-	-
<i>S. fragilis</i>		CBS 397	1	+	+	0*	1	+	+	+	+	0	-	-	-	-
		PHAFF 351	1	+	+	0*	1	+	+	+	+	0	-	-	-	-
<i>S. lactis</i>	<i>Z. casei</i>	CBS 739	1	+	+	-(1)*	1	+	+	+	+	0	-	-	-	-
	<i>Z. lactis</i>	CBS 683	2	+	+	+	1	+	+	+	+	0	-	-	-	-
	<i>Z. versicolor</i>	CBS 743	1	+	+	+	1	+	+	+	+	0	-	-	-	-
		ATCC 2628	1	+	+	+	1	+	+	+	+	0	-	-	-	-
<i>S. marrianus</i>		NCYC 111	1	+	+	+	1	+	+	+	+	0	-	-	-	-
	<i>S. macedoniensis</i>	NCYC 243	1	+	+	+	1	+	+	+	+	0	-	-	-	-
	<i>Z. ashbyi</i>	CBS 745	1	+	+	0*	1	+	+	+	+	0	-	-	-	-
	<i>Z. marrianus</i>	CBS 712	1	+	+	+	2	+	+	+	+	0	-	-	-	-
<i>S. mellis</i>	<i>Z. mellis</i>	CBS 736	1	+	+	+	1	+	+	+	+	0	-	-	-	-
		DBDR D 1 ^H	1	+	+	+	1	+	+	+	+	0	-	-	-	-
	<i>Z. nectarophilus</i>	DBDR D 1 ^H	1	+	+	+	1	+	+	+	+	0	-	-	-	-
		CBS 740H ²	1	+	+	+	1	+	+	+	+	0	-	-	-	-
		ATCC 8382 ^H	1	+	+	+	2	+	+	+	+	0	-	-	-	-
		DBDR 155Y ^{H2}	1	+	+	+	1	+	+	+	+	0	-	-	-	-
		DBDR S3 B2 ^H	1	+	+	+	2	+	+	+	+	0	-	-	-	-
<i>Z. perspicillatus</i>	<i>Z. perspicillatus</i>	ATCC 2620	1	+	+	+	1	+	+	+	+	0	-	-	-	-
<i>Z. raumunatis</i>		DBDR Z7 ^{B2}	1	+	+	+	1	+	+	+	+	0	-	-	-	-
<i>Z. richteri</i>	<i>Z. richteri</i>	CBS 742	1	+	+	+	1	+	+	+	+	0	-	-	-	-
		DBDR M1	1	+	+	+	1	+	+	+	+	0	-	-	-	-
<i>S. pastori</i>	<i>Z. pastori</i>	CBS 704	3	+	+	+	2	+	+	+	+	0	-	-	-	-
		NCYC 175	2	+	+	+	1	+	+	+	+	0	-	-	-	-
	<i>Z. pini</i>	CBS 744	2	+	+	+	2	+	+	+	+	0	-	-	-	-
<i>S. rosei</i>		Guikerman 401	1	+	+	+	1	+	+	+	+	0	-	-	-	-
	<i>Z. globiformis</i>	CBS 728	1	+	+	+	1	+	+	+	+	0	-	-	-	-

- or VW

Table 2 (cont.)

Classification of Lodder & Kreger-van Rij (1952)	Other name given previously	Strain	Quantitative tests with suspension of organisms grown on										Aesculin Arbutin plates	Arbutin plates reactions for species given by Lodder & Kreger- van Rij (1952)
			A2G agar					Complex medium						
			No. of expts.	Aesculin	Arbutin	Salicin	Cello- biose	No. of expts.	Aesculin	Arbutin	Salicin	Cello- biose		
<i>S. rouzii</i>		NCYC 381	1	+	—	—	—	1	+	+	—	0	—	—
	<i>Z. cavaeae</i> var. <i>beauverie</i>	CBS 710	1	—	—	—	0	1	+	+	—	0	—	—
	<i>Z. gracilis</i>	CBS 731	2	+	—	—	— (1)	1	+	+	—	0	—	—
	<i>Z. gracilis</i> var. <i>italicus</i>	CBS 732	1	—	—	—	0	1	+	+	—	0	—	—
		ATCC 2623	2	+	—	—	0†	2	+	+	—	0	—	—
	<i>Z. major</i>	CBS 687	2	+	—	—	0	1	+	+	—	0	—	—
	<i>Z. major</i> var. <i>threntensis</i>	CBS 689	1	—	—	—	—	2	+	+	—	0	—	—
	<i>Z. major</i> st. <i>salsus</i>	CBS 688	1	—	—	—	—	2	+	+	—	0	—	—
	<i>Z. nadsonii</i>	CBS 686	1	+	—	—	—	1	+	+	—	0	—	—
		NCYC 174	1	—	—	—	—	1	+	+	—	0	—	—
	<i>Z. nussbaumeri</i>	CBS 738H	1	—	—	—	—	1	+	+	—	0	—	—
		DBDR J7	1	—	—	—	—	1	+	+	—	0	—	—
<i>S. rouzii</i> var. <i>polymorphus</i>	<i>Z. rugosus</i>	CBS 741	1	+	0	—	0	1	+	+	—	0	—	—
		ATCC 4898	1	— (2)	—	—	—	1	+	+	—	0	—	—
		DBDR Y 139	2	+	—	—	—	1	+	+	—	0	—	—
	<i>Z. variabilis</i>	CBS 730	1	+	—	—	—	1	+	+	—	0	—	—
		ATCC 2619	1	+	—	0	—	2	+	+	—	?	—	—
		DBDR Z1	1	+	—	—	—	1	+	+	—	0	—	—
	<i>Z. vini</i>	ATCC 10687	1	—	—	—	—	1	+	+	—	0	—	—
		CBS 1085	1	—	—	—	0	1	+	+	—	0	—	—
	<i>Z. dairenensis</i>	CBS 706	1	+	—	—	0	1	+	+	—	0	—	—
	<i>Z. amoeboides</i>	CBS 711	1	+	—	0	0	1	+	+	—	0	—	—
		DBDR Z3	1	+	—	—	—	1	+	+	—	0	—	—
	<i>Z. barkeri</i>	CBS 678	1	+	—	—	—	1	+	+	—	0	—	—
<i>Schizosaccharomyces octosporus</i>		NCYC 170	1	+	—	—	—	1	+	+	—	?	—	—
		ATCC 2606	1	+	—	—	—	1	+	+	—	0	—	—
		DBDR Z4	1	—	—	—	0	1	+	+	—	0	—	—
	<i>Z. citrus</i>	CBS 733	1	—	—	—	—	1	+	+	—	0	—	—
	<i>Z. felsineus</i>	CBS 734	1	+	—	—	0	1	+	+	—	0	—	—
		ATCC 2624	1	+	—	—	—	2	+	+	—	0	—	—
	<i>Z. polymorphus</i>	CBS 727	1	+	—	—	—	1	+	+	—	0	—	—
		CBS 713	1	+	—	—	—	1	+	+	—	0	—	—
	SCARR		1	+	—	—	—	1	+	+	—	0	—	—
		LTS 3	2	+	+	+	—	2	+	+	—	0	+	+
	<i>Torula homii</i>	NCYC 163	1	—	—	—	—	1	+	+	—	0	—	—
		NCYC 444	1	—	—	0	—	1	+	+	—	0	—	—
<i>Zygosaccharomyces nanivaeensis</i>	ATCC 11003	1	—	—	0	—	1	+	+	—	0	—	—	
<i>Schizosaccharomyces octosporus</i>	<i>Torulopsis homii</i>	ATCC 11388	1	—	—	0	—	1	+	+	—	0	—	—
	<i>Z. nishitaki</i>		1	—	—	—	—	1	+	+	—	0	—	—

NOTES. Media. The complex medium in each case was MYGPA, except where H or H2 is given by the strain number, indicating HYGSA or HYGPA respectively. Symbols. The symbols +, ++, +, —, for aesculin, arbutin and salicin results, are explained in Table 10. 0 = no test; V = variable; W = weak; VW = Very weak. No. of experiments. Where the number of tests is not the same for a particular glucose as that given in the appropriate column, the correct figure is indicated in brackets. Cellobiose growth response: * positive; † negative.

Strains. ATCC: American Type Culture Collection. CBS: Centraalbureau voor Schimmelmcultures (Delft, Holland). Strain numbers given by Mrs N. J. W. Kreger-van Rij. Ciukerman: from Dr I. Ciukerman, Agricultural Research Institute, Rehovot, Israel; but identified by CBS. DBDR: Division of Bacteriology and Dairy Research, Department of Agriculture, Ottawa, Canada. LTS: Low Temperature Research Station, Cambridge (England). Nos. 1 to 3 isolated at LTS, named by CBS. LTS 5 isolated by Lowings (1956). Mossel: from Dr D. A. A. Mossel, Central Institute for Nutrition Research (Utrecht, Holland). NCYC: National Collection of Yeast Cultures (England). Phaff: from Dr H. J. Phaff, University of California. Scarr: from Dr M. P. Scarr, Messers Tate and Lyle Ltd. (England).

G. Microb. xv

NOTES. *Media*. The complex medium in each case was MYGPA, except where H or H2 is given by the strain number, indicating HYGSA or HYGPA respectively. *Symbols*. The symbols +, +, +, —, —, —, for aesculin, arbutin and salicin results, are explained in Table 10. 0 = no test; V = variable; W = weak; VW = Very weak. *No. of experiments*. Where the number of tests is not the same for a particular glucoside as that given in the appropriate column, the correct figure is indicated in brackets. *Collobiose* growth response. * positive; † negative.

Strains. ATCC: American Type Culture Collection. CBS: Centraalbureau voor Schimmelcultures (Delft, Holland). Strain numbers given by Mrs N. J. W. Kreger-van Rij. Clukerman: from Dr I. Clukerman, Agricultural Research Institute, Rehovot, Israel; but identified by CBS. DBDR: Division of Bacteriology and Dairy Research, Department of Agriculture, Ottawa, Canada. LTS: Low Temperature Research Station, Cambridge (England). Nos. 1 to 3 isolated at LTS, named by LTS. LTS 5 isolated by Lowings (1956). Mossel: from Dr D. A. A. Mossel, Central Institute for Nutrition Research (Utrecht, Holland). NCYC: National Collection of Yeast Cultures (England). Phaff: from Dr H. J. Phaff, University of California. Scarr: from Dr M. P. Scarr, Messrs Tate and Lyle Ltd. (England).

travel) at 25° for 20 hr. The contents of the T-tubes were then centrifuged at *c.* 250 *g* for 5 min., and the supernatant treated as follows:

Aesculin. The supernatant fluid from each tube was poured separately into a test tube containing 5 ml. ethyl acetate. The contents of each tube were shaken thoroughly and allowed to stand for *c.* 30 min.; 3 ml. of ethyl acetate layer was added to 5 ml. of 1.5 M-Na₂CO₃ in a separating funnel and shaken. The colour of the lower (aqueous) layer was measured in $\frac{5}{8}$ in. diam. tubes at 450 m μ . (using the control as a blank) by means of a diffraction-grating spectrophotometer (Unicam SP 350; the instrument did not give a satisfactory response at wavelengths much below 450 m μ .), and at 400 and 450 m μ . in a Hilger 'Uvispec' spectrophotometer. This technique was described by Barnett & Swain (1956). The 4 ml. solution in which the yeast was suspended contained 1 mg. aesculin, capable of liberating approximately 500 μ g. aesculetin. Table 3 shows that, for purposes of classification, the results may be assessed in three arbitrary groups. A 5 % degree of splitting (25 μ g. aesculetin) is about the lowest degree which gives comparable replications. Though it is practicable to detect as little as 5 μ g. aesculetin, errors of measurement at this concentration are high. As can be seen from Fig. 1 *a*, Beer's law is obeyed approximately up to *c.* 25 % splitting. Higher concentrations of aesculetin could have been determined after dilution but this was not done in the routine determinations.

Table 3. *Quantitative assessment of β -glucoside splitting*

Glucoside	Wave-length (m μ .)	Assessment of degree of splitting	Amount of splitting (%)	Total amount of aglycone liberated (μ g.)	Colorimeter readings
Aesculin	450	—	< 5	< 25	< 0.1
		+	5–25	25–125	0.1–0.3
		++	> 25	> 125	> 0.3
Arbutin	470	—	< 0.5	< 20	< 0.24
		+	0.5–25	20–1000	0.24–0.8
		++	> 25	> 1000	> 0.8
Salicin	640	—	< 0.5	< 20	< 0.05
		+	0.5–25	20–1000	0.05–0.67
		++	> 25	> 1000	> 0.67

Measurements for arbutin above 1 % are approximate.

Arbutin (a) To 2 ml. of the supernatant fluid (or control) were added 2 ml. 0.5 % (w/v) phloroglucinol solution and 2 ml. N-NaOH. After 30 min. the solution was well shaken and the colour measured at 470 m μ . in $\frac{5}{8}$ in. diam. tubes with the diffraction spectrophotometer, using the control as a blank (Porteous & Williams, 1949). The aglycone (hydroquinone) is probably oxidized by oxygen in the solution to *p*-quinone, which reacts with the keto-form of phloroglucinol to give the coloured compound. This was shown to be likely, because no colour change occurred when the reaction was carried out under nitrogen in a Thunberg tube. At concentrations of hydroquinone > 10 μ g./ml. the method was unreliable (Fig. 1 *b*). This was probably because there was a deficiency of oxygen at higher concentrations of hydroquinone, and the

excess hydroquinone reacted with the coloured compound. Although it would have been possible to measure larger quantities of hydroquinone by dilution, this was not done as a matter of routine. With this technique it is practicable to detect in each tube quantities of hydroquinone $< 4 \mu\text{g.}$ (*c.* 0.1 % split).

(*b*) About 0.5 ml. of the original supernatant fluid was added to an equal volume of modified Tollen's reagent (0.5 %, w/v, AgNO_3 in 0.1 N-NaOH). The colour change was observed visually.

Fig. 1. Calibration curves for (*a*) aesculin splitting; (*b*) arbutin splitting; (*c*) salicin splitting.

Salicin. To 2 ml. of the supernatant fluid was added 2 ml. 0.04 % (w/v) 4-amino-antipyrin in 0.1 N-ammonia solution, followed after shaking by 2 ml. 0.2 % (w/v) $\text{K}_3\text{Fe}(\text{CN})_6$. The colour was measured spectrophotometrically at 515 and 640 m μ . against the control in $\frac{5}{8}$ in. diam. tubes. The use of 4-amino-antipyrin for estimating saligenin (see Martin, 1949) is described by Baruah & Swain (to be published).

Using the colorimeter at 515 m μ ., readings above about 0.65 were insensitive (Fig. 1 *c*); and, therefore, measurements were carried out also at 640 m μ . In this case, 0.5 % of splitting was taken as the lower limit (see Table 3). Initially 10 mg. salicin were present, capable of liberating about 4 mg. saligenin.

As for aesculetin and hydroquinone, it is practicable to detect as little as 1 $\mu\text{g./ml.}$ saligenin, though 5 $\mu\text{g./ml.}$ is the lower limit used. The concentration

of reagents was chosen to detect readily these lower limits. Thus when more than 25 % salicin was split, dilution was necessary for quantitative analysis; since 25 % is above the limit for a positive result, this was not done.

Cellobiose. In preliminary experiments the control tubes, containing yeast alone, as blanks, showed little variation, and were not used subsequently. The supernatant fluids from both tubes were filtered through Whatman no. 50 paper. To 0.5 ml. of filtrate in a $\frac{5}{8}$ in. test tube, 0.5 ml. 1 % (w/v) 2,3,5-triphenyl-tetrazolium chloride and 0.5 ml. 2 N-NaOH were added. The tubes were placed in boiling water for 30 ± 2 sec., removed, 5 ml. 10 % (v/v) glacial acetic acid in *iso*-propanol quickly added, and the mixture cooled to about 20°. The difference in colour between the control and the tube containing sugar was

Fig. 2

Fig. 2. Distribution of 69 cellobiose splitting measurements, for strains grown on A2G agar only

Fig. 3

Fig. 3. Development of formazan with time of heating:

	+ Cellobiose	Control
<i>P. membranaefaciens</i> (NCYC 54) (cellobiose-negative)	○	●
<i>C. utilis</i> (NCYC 359) (cellobiose-positive)	△	▲

measured at 540 mμ. and at 640 mμ. in $\frac{3}{4}$ in. matched tubes. Since the values for the controls were fairly constant when the yeast was grown on A2G agar, subsequent experiments with this medium were carried out without the use of controls.

The estimation of sugars with tetrazolium was described by Fairbridge, Willis & Booth (1951). The value of λ max. ≈ 485 mμ. for formazan agrees with their data, but the best response with blanks as above was obtained by reading at 540 mμ. When the readings were greater than 0.65 at this wavelength the depth of colour was also measured at 640 mμ. (see saligenin determination).

There appeared to be introduced into the harvesting medium with the yeast cells substances which reduced tetrazolium, although at slower rates than did cellobiose (see Fig. 3). Consequently, it was necessary to compare formazan formation in media which had contained yeasts + cellobiose with that in media which had contained only yeast. Heating for 30 ± 2 sec. in boiling water gave

the maximum differences between the experimental and control values (Fig. 3). When the yeasts were grown on MYGPA (more especially HYGSA) there was much more of the extraneous reducing substances. Although this method was satisfactory with MYGPA as growth medium, it was not so for yeasts grown on HYGSA. The amount of cellobiose introduced (0.5 mg./ml.) was so chosen that if it was not utilized, it could be detected readily by the above procedure. It was residual cellobiose, which was measured. Considering the relative quantities of yeast and cellobiose involved in these experiments, it has been assumed, *a priori*, that when the yeast splits cellobiose, the resulting glucose is all used. Since the experimental error in this determination is higher than that for the other β -glucosides, no attempt was made to study low activities. Instead, an arbitrary value of 75 % utilization was chosen as the dividing line between +ve and -ve organisms. The distribution of observations given in Fig. 2 shows that most of the yeasts fell either above or below this level.

Estimation of aglycones. Solutions of the aglycones aesculetin, hydroquinone and saligenin were made up in medium A 2 at concentrations equivalent to those of the corresponding β -glucosides. Mixtures of each of the glucosides with its corresponding aglycone were made in different proportions to give a series of solutions corresponding with known percentages of hydrolysis of the glucoside (glucose did not interfere with any of the measurements). Aglycone estimations were carried out with solutions containing medium A 2 + glucoside as a blank; and the spectrophotometer readings plotted against the percentage split (Fig. 1). Fig. 1 and Tables 6 and 8 give an idea of the size of errors involved.

Measurement of concentration of organisms in suspensions. After estimating the aglycones the yeast from each test tube was resuspended in 50 ml. water and the optical density measured in a $\frac{3}{4}$ in. diam. colorimeter tube at 580 m μ . When there were relatively few organisms present, only 10 ml. water was added. For 6 strains these colorimeter readings were compared with haemocytometer counts and dry-weight determinations; this comparison is shown in Table 4. The wavelength chosen was found by trial to be that at which a straight-line relationship was obtained between colorimeter readings and optical density, using serial dilutions over a tenfold range of cell concentration as obtained in these experiments (Table 4).

Determination of iron in yeast-water was carried out by the method described by Johnson (1955) with 1 : 10-phenanthroline.

Extraction of β -glucosidase from baker's yeast. Baker's yeast (100 g. wet weight, 'Ark' brand) was treated with three successive portions of ice-cold acetone (500 ml.); the resulting powder was dried at room temperature. The acetone powder was extracted with medium A 2 (5 ml./g.) for 20 min. at room temperature and the residue removed by centrifuging. For the determination of activity 2 ml. of the supernatant solution was incubated overnight with the glucoside solution as used for the yeasts, and the liberated aglycone determined as described previously. The extract showed no loss of activity against aesculin when dialysed overnight at +1° against distilled water. When a sample was heated to 100° for 5 min. no measurable activity remained.

Table 4. *Approximate quantities of yeast used in experiments*

Suspensions were prepared as described in the methods section.

Yeast strains	Organisms grown on					
	A2G agar			MYGPA medium		
	Organisms (no./ml.)	Dry wt. organism (mg.)	Colorimeter reading at 580 m μ .	Organisms (no./ml.)	Dry wt. organism (mg.)	Colorimeter reading at 580 m μ .
<i>Candida utilis</i>	NCYC 359	.	.	2.39×10^9	103	0.66
<i>Rhodotorula rubra</i> var. <i>longa</i>	CBS 326	1.67×10^9	67	3.37×10^9	116	0.80
<i>Pichia membranaefaciens</i>	NCYC 54	0.39×10^9	23	1.63×10^9	67	0.65
<i>Saccharomyces acidifaciens</i>	CBS 749	1.43×10^9	56	1.18×10^9	84	0.30
<i>Saccharomyces cerevisiae</i>	NCYC 77	0.65×10^9	25	1.35×10^9	59	0.46
<i>Saccharomyces rouzii</i>	CBS 710	1.37×10^9	35	2.75×10^9	101	0.37

RESULTS

The activity shown by each of the 119 strains studied in splitting aesculin, arbutin, salicin and cellobiose is summarized in Table 2. Besides the quantitative estimates, this includes results obtained by using the arbutin plate technique of Lodder & Kreger-van Rij (1952) and some with the aesculin plate method of Van Steenberge (1920).

The plate tests

The arbutin plate test described by Lodder & Kreger-van Rij (1952) gave repeatable results which, as can be seen (Table 2), were usually in agreement with those obtained by these authors; some of the exceptions are discussed later in the light of the quantitative tests. In contrast, aesculin plates prepared by Stelling-Dekker's (1931) method gave variable results, which may have been related to the amount of iron included; this was critical, as has been pointed out. Van Steenberge (1920) included 0.1 % ferric citrate in his medium, and this technique gave repeatable results (Table 2) though different from those obtained with arbutin. Castellani & Douglas (1935) and Castellani (1937) used an arbutin plate technique without added iron; and the present experiments confirm that the addition of iron is not essential. By contrast with aesculin, it seems that for arbutin plates, the concentration of iron is not critical. Indeed, whereas Diddens & Lodder (1942) advocated one drop of FeCl_3 solution/litre, Lodder & Kreger-van Rij (1952) recommend one drop/plate (concentration of FeCl_3 not given in either case). The concentration of iron in the yeast water used was 4.9 mg./l. as determined by the *o*-phenanthroline method. This concentration seems about comparable with that used by Diddens & Lodder (1942), calculated as 2 mg./l. on the assumption that their ferric chloride solution was 5 % (w/v) and that one drop was *c.* 0.1 ml.

Glucoside concentrations. The concentration of 0.25 % (w/v) used for salicin and arbutin was chosen since it was of the order of that used in usual β -glucoside tests (e.g. Lodder & Kreger-van Rij, 1952; 0.5 % for arbutin). The concentration of aesculin (0.025 %) was limited by its solubility (given as *c.* 0.15 % in water at 10°; Heilbron & Bunbury, 1946), though Stelling-Dekker (1931) recommended 0.5 %. It is likely that it would have been satisfactory to use each of these glucosides at, say, 0.01 %. As discussed previously, the concentration of cellobiose was minimal.

Effects of differences in growth media. It seemed desirable to use a defined medium in order to minimize variation in the experiments (Barnett & Ingram, 1955). Some strains (such as *Kloeckera apiculata* NCYC 245), did not grow well on A2G agar and had to be grown on MYGPA; it was noticed that they all hydrolysed aesculin and tended to be more active than comparable strains grown on A2G agar. A similar difference in activity was also found when strains able to grow on A2G agar were cultivated on MYGPA (Table 5). Of strains grown on A2G agar about 65 % were aesculin-negative; but when they were grown on MYGPA only one of 71 was in this category. Table 5 shows that whenever there was a difference between strains grown alternately on A2G

agar and MYGPA, organisms from the complex medium were the more active. This striking relation between aesculinase and salicinase activity, and the kind of medium on which the organisms had grown, led to an investigation of the β -glucoside splitting capabilities of a yeast grown on 14 different media; the CBS 240 strain of *Pichia membranaefaciens* was used. This is a good example of an organism negative to aesculin when grown on A2G agar, but positive when

Table 5. Summary of differences between patterns of response to β -glucosides for yeasts grown alternately on A2G agar and on MYGPA media

Each figure indicates number of strains				A2G agar							
				AE	ARB	SAL					
MYGPA	AE	ARB	SAL	AE	ARB	SAL					Σ
	+	+	+	3	0	1	3	4	3	14	
	+	+	-	0	0	0	1	0	1	2	
	+	-	+	0	0	2	8	1	14	25	
	+	-	-	0	0	0	4	0	11	15	
	-	+	-	0	0	0	0	0	0	0	
	-	-	-	0	0	0	0	0	0	0	
			Σ 8	0	3	16	5	29	56		

In this Δ greater splitting follows change from A2G agar to MYGPA medium

In this Δ decreased splitting follows changing from A2G agar to MYGPA medium

AE = aesculin;
ARB = arbutin;
SAL = salicin.

grown on MYGPA, although there was about the same amount of growth on both media. The results (Table 6) from organisms grown on media 9 to 14 (breakdowns of MYGPA) indicate that aesculinase activity was developed regardless of whether the N-source was peptone, yeast or malt extract, provided growth was sufficient. The assessment of aesculin-negativity for *P. membranaefaciens* (CBS 240), when grown for example on MYPA, may have been because there was little growth. On the other hand, this observation does not apply to β -glucosidase activity with some other substrates as can be seen from the response to salicin when this yeast was grown on meat digest agar (NA), where growth is very small. Glucose is obviously important in supplying energy for growth, but certain amino acids or peptides may be essential for synthesizing specific β -glucosidases; some strains of yeasts appear to do this only very slowly with ammonia as the sole source of nitrogen. Peptone seemed to provide a factor essential for aesculinase activity by two strains of *Saccharomyces mellis* (CBS 740; DBDR 155 Y) which gave negative responses to aesculin when grown on HYGSA but which were positive on HYGPA. This situation may be comparable with that found by Jermyn (1955), who showed that the production of β -glucosidase by one strain of *Stachybotrys atra* required

Table 6. *β-glucoside splitting activity of Pichia membranaefaciens* (CBS 240) *grown on 14 different media*
Media no. 9 to 14 are breakdowns of MYGPA.

Medium for growth of organism	pH	First experiment										Second experiment									
		Colorimeter reading*										Colorimeter reading*									
		Assessment of results					Concentration of organisms†					Concentration of organisms†					Concentration of organisms†				
		AE	ARB	SAL	AE	ARB	AE	ARB	AE	ARB	AE	ARB	AE	ARB	AE	ARB	AE	ARB	AE	ARB	SAL
1 A2G agar	5.0	—	—	—	0	0	0.01	0.03	0.01	0.03	0.01	0.03	0.01	0.03	0.01	0.03	0.01	0.03	0.01	0.03	0.01
2 HYGSA	5.6	+	?	—	0.15	?	0.07	0.04	0.07	0.04	0.07	0.04	0.07	0.04	0.07	0.04	0.07	0.04	0.07	0.04	0.07
3 SDA	5.8	+	—	—	0.14	0	0.02	0.01	0.02	0.01	0.02	0.01	0.02	0.01	0.02	0.01	0.02	0.01	0.02	0.01	0.02
4 WA	5.2	+	+	—	0.14	0.09	0.06	0.03	0.06	0.03	0.06	0.03	0.06	0.03	0.06	0.03	0.06	0.03	0.06	0.03	0.06
5 MYGSA	5.7	++	+	—	0.46	0.11	0.11	0.04	0.11	0.04	0.11	0.04	0.11	0.04	0.11	0.04	0.11	0.04	0.11	0.04	0.11
6 NA	7.5	—	—	+	0	0.03	0.20	0.05	0.20	0.05	0.20	0.05	0.20	0.05	0.20	0.05	0.20	0.05	0.20	0.05	0.20
7 MEA	5.9	+	—	—	0.08	0.03	0.02	0.03	0.02	0.03	0.02	0.03	0.02	0.03	0.02	0.03	0.02	0.03	0.02	0.03	0.02
8 MYGPA	6.0	++	—	—	0.45	0.01	0.06	0.03	0.06	0.03	0.06	0.03	0.06	0.03	0.06	0.03	0.06	0.03	0.06	0.03	0.06
9 YA	6.7	—	—	—	0.02	0	0.04	0.03	0.04	0.03	0.04	0.03	0.04	0.03	0.04	0.03	0.04	0.03	0.04	0.03	0.04
10 YGA	6.7	++	—	—	0.45	0.05	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03
11 YGPA	6.8	++	—	—	0.46	0.05	0.06	0.02	0.06	0.02	0.06	0.02	0.06	0.02	0.06	0.02	0.06	0.02	0.06	0.02	0.06
12 MGPA	5.8	++	—	—	0.46	0	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03
13 MYPA	6.3	—	—	—	0.05	0.01	0.05	0.03	0.05	0.03	0.05	0.03	0.05	0.03	0.05	0.03	0.05	0.03	0.05	0.03	0.05
14 MYGA	6.0	++	—	—	0.46	0.06	0.07	0.03	0.07	0.03	0.07	0.03	0.07	0.03	0.07	0.03	0.07	0.03	0.07	0.03	0.07

* The relations between these colorimeter readings and amount of splitting are indicated in Fig. 1.
† The concentration of organisms here is measured in terms of colorimeter readings comparable with those in Table 4.
AE = aesculin; ARB = arbutin; SAL = salicin.

the presence of a factor in yeast extract. This ' β -glucosidase factor' was not a growth factor and has not yet been characterized.

The effect of washing. In the routine experiments, as already described, the yeasts were washed from the agar by 10 ml. of medium A 2. This suspension was then used in 2 ml. volumes, each of which was added to 2 ml. of medium A 2 containing, for example, 0.5 % (w/v) aesculin. Table 7 indicates that there was little difference in the response of the organism to aesculin, even when the suspension was washed 3 times. The fluid from the first wash (the initial 10 ml. medium A 2 with which the cells had been harvested) was used in the same way as the suspension, diluting it 1/1 with 0.05 % (w/v) aesculin in medium A 2. The results (Table 7) show that there was some aesculinase activity in this fluid, particularly when the yeast was grown on MYGPA. This might have been due to autolysis which liberated intracellular enzyme, since colonies grown on MYGPA developed faster than those cultivated on A 2G agar. MYGP broth alone did not hydrolyse aesculin.

Table 7. *Effect of washing on aesculin splitting by five yeasts grown on two different media*

Each figure is for a different suspension of organisms differently treated as indicated, and represents the quantity of aesculetin liberated (in $\mu\text{g.}$), calculated from colorimeter readings at 450 m μ . as in Fig. 1.

Organism		Organism grown on					
		A 2G agar			MYGPA		
		Un-washed cells	Washed cells	Liquor from first wash	Un-washed cells	Washed cells	Liquor from first wash
<i>Pichia membranaefaciens</i>	CBS 240	13	13	13	250	238	58
	NCYC 54	13	10	13	225	275	70
<i>Saccharomyces cerevisiae</i>	NCYC 176	90	25	10	225	250	163
<i>S. marzianus</i>	CBS 712	200	250	20	250	250	90
<i>S. pastori</i>	CBS 704	13	38	10	185	238	25

Amount of hydrolysis

With every strain studied it has been possible to detect some hydrolysis of aesculin: those which were negative when grown on A 2G agar were all positive when grown on MYGPA. Only 2 strains (*Saccharomyces mellis* DBDR Z7 and *Schizosaccharomyces octosporus*) are given in Table 2 as aesculin-negative when grown on complex media. The colorimeter readings for these observations were 0.08 and 0.04, respectively, corresponding with the liberation of about 20 and 10 $\mu\text{g.}$ of aesculetin (Fig. 1 a). At this level, errors can arise which are of the same order as the values observed; the colour of an alkaline solution containing about 3 $\mu\text{g./ml.}$ aesculetin could be distinguished from that of the control by eye.

In these experiments strains were found which, on both complex and defined media, split almost any proportion of the aesculin present (0.25 mg./ml.), from 0 to nearly 100 %; and there is a tendency for the final degree of splitting to be characteristic for the strain. The same appears true for salicin and arbutin.

In the hope of discovering why some yeasts give a lower degree of splitting than others, replicate experiments were run for 5 strains with different characteristics, aesculetin estimations being made at intervals up to 72 hr. The results (Fig. 4) show that in most cases after 20 hr. of shaking (the time at which analyses were made in the routine experiments), most of the hydrolysis had occurred. Ten A2G agar-grown strains, aesculin-negative at 20 hr., were shaken with aesculin for 120 hr. and of these two passed the positive level of hydrolysis, liberating more than 25 μ g. aesculetin (*Pichia fermentans* NCYC 246, 120 μ g.; *Saccharomyces cerevisiae* NCYC 353, 50 μ g.); the 8 other strains showed no significant change.

Fig. 4. The course of aesculin splitting by 5 strains over 3 days. Organisms grown on A2G agar. *C. tropicalis*, NCYC 4, ●; *D. kloekeri*, LTS 1, △; *K. africana*, NCYC 26, ○; *S. cerevisiae*, NCYC 353, ×; *S. cerevisiae* var. *ellipsoideus*, NCYC 365, ▲.

It is possible that the differences in the maxima of the curves in Fig. 4 are due to inhibitory activity by aesculetin. If this be so, then aesculetin seriously inhibited hydrolysis at about 3 μ g./ml. (cf. Fig. 1 a) for NCYC 353 and 12 μ g./ml. for NCYC 4. If the aesculetin simply inactivated the enzyme, these levels may be directly related to the quantity of enzyme which has been synthesized in the organisms before they were introduced into the aesculin medium.

Although a low rate of enzyme synthesis seemed a possible cause of the low activity on a minimal medium, other explanations are possible. For instance, it is conceivable that a specific inhibitor was synthesized when the yeast was

cultivated in these conditions, but not on a richer medium, as for pyrophosphatase in *Proteus vulgaris* (Swartz, Kaplan & Frech, 1956).

The concentration of organisms used in the routine experiments was not strictly controlled, though it was measured turbidimetrically at the end of each experiment (see Tables 4 and 8). The number of organisms involved in each experimental tube was, for those grown on A2G agar, *c.* 2 to 6×10^9 ; and for MYGPA experiments, *c.* 4 to 14×10^9 (Table 4). The measurements given for replicated experiments in Table 8 indicate that variations in numbers were not usually of importance in determining the results. It is possible to try to put some of the data into terms of molecules split/organism, as advocated by Pirie (1955). Thus, for the data in Table 8, roughly 6×10^9 organisms of CBS 240 strain of *Pichia membranaefaciens*, grown on MYGPA, split *c.* 1 mg. aesculin in 20 hr.; i.e. about 350×10^{11} molecules/organism.

The activities shown in Fig. 4 are expressed in Table 9 in terms of molecules/org./sec. during the phase of greatest activity: the errors (chiefly from the estimate of numbers of cells) should be less than a factor of 5. Probably those strains classed as negative split less than 1000 aesculin molecules/org./sec.; those given as + *c.* 1000 to 10,000; and as ++ > 10,000.

Assessment of the measurement

The symbols ++, + and -, used in Table 2 for indicating results of the quantitative tests for the hydrolysis of the phenolic β -glucosides, are explained in Table 3. It is frequent in microbiology to use + and - only, but this gives a minimum of information about a quantitative process. With the system used in Table 2, however, it is possible to be fairly certain of the reality of a distinction between ++ and -. In the opinion of the present writers, three such categories are the least to be expected from biochemical tests used in classification. After 20 hr. have elapsed, variations in the time at which the experiment is stopped and the analysis carried out probably have little effect on the assessment (Fig. 4). It can be seen that the activity of a strongly aesculin-positive strain (e.g. NCYC 26, *Kloeckera africana*) may be detected after shaking for 1 hr., but a yeast such as NCYC 365 (*Saccharomyces cerevisiae* var. *ellipsoideus*), which gives a curve suggestive of adaptation, would be assessed as negative (-) after 8 hr. + at 20 hr., and ++ after 2 days. Table 3 shows that the values chosen for the difference between negative and positive are comparable for each glucoside in terms of the amount of aglycone liberated. On the other hand, the dividing line between + and ++ was drawn at 25 % splitting in each case.

Cellobiose

Results of measuring growth responses to cellobiose as the sole source of carbon are given in Table 2; and they are identical with those of other workers in the four cases where a growth technique has been used (Table 10). The results obtained by the tetrazolium method agree reasonably well, although with the CBS 683 strain of *Saccharomyces lactis* the tetrazolium measurement is probably aberrant. Auxanographic growth responses to cellobiose for three

Table 8. *Results of some replicated experiments*

Organism	Medium for growth of organism	Aesculin			Arbutin			Salicin			
		Splitting		Concentration of organisms†	Splitting		Concentration of organisms†	Splitting		Concentration of organisms†	
		Assessment	Colorimeter reading*		Assessment	Colorimeter reading*		Assessment	Colorimeter reading*		
			450 mμ.		470 mμ.		515 mμ.	640 mμ.			
<i>Pichia membranaefaciens</i>	CBS 240 A 2G agar	—	0	0.45	—	0	0.42	—	?	0	0.46
		—	0	0.42	—	0	0.42	—	0.02	0.01	0.44
		—	0	0.42	—	0	0.37	—	0.01	0.03	.
		—	0.03	0.45	—	0	0.44	—	0.03	0	.
		—	0	0.58	—	0	0.45	—	0.03	?	0.58
<i>Saccharomyces bisporus</i>	CBS 702 A 2G agar	+	0.43	0.49	—	0.05?	0.42	—	0.05	0	0.46
		+	0.39	0.46	—	0.03	0.43	—	?	0.02	0.43
		+	0.37	0.45	—	0.02	0.50	—	0.05	0.02	0.49
		+	0.45	0.52	—	0.01	0.52	—	0.06	0.03	.
		—	0.09	0.29	—	0	0.27	—	0.01	?	0.27
<i>S. cerevisiae</i>	NCYC 176 A 2G agar	—	0.07	0.20	—	0	0.22	—	0.01	0.01	0.22
		+	0.39	0.52	—	0.03	0.56	—	0.14	0.05	0.44
		+	0.37	0.62	—	0	0.50	+	0.19	0.05	.
		—	0.07	0.46	—	0	0.46	—	0.01	?	0.45
		+	0.22	0.20	—	0	0.19	—	0.02	0.02	0.22
<i>S. pastori</i>	CBS 704 A 2G agar	+	0.38	0.64	—	0.14	0.66	+	0.51	0.14	0.66
		+	0.39	0.58	—	0.20	0.54	+	0.85	0.23	.
		—	0.06	0.29	—	0.02	0.30	—	?	0.01	0.29
		—	0	0.45	—	0.02	0.40	—	0.03	0.01	0.42
		+	0.22	0.66	—	0.05	0.58	—	0.05	0.01	0.60
	MYGPA	+	0.13	0.60	—	0.02	0.59	—	0.07	0.03	.

* Relations between colorimeter readings and amount of splitting are indicated in Fig. 1.

† The concentration of organisms here is measured in terms of colorimeter readings comparable with those in Table 4.

Table 9. Rates of aesculin-splitting by five yeasts

See Fig. 4. The organisms were grown on A2G agar.

Organism	Extent of phase of greatest activity (hr.)	Total number of org.* $\times 10^6$	Amount aesculin split (mg.)	μ g. aesculin split/org./hr. $\times 10^{-9}$	Molecules aesculin split/cell/sec. in active phase $\times 10^3$	Assessment (after 20 hr.)
<i>Candida tropicalis</i>	0-4	6	0-075	3	1-5	+
<i>Kloeckera africana</i>	0-4	2	0-55	70	33	++
<i>Saccharomyces cerevisiae</i>	0-4	2-6	0-02	1-9	0-9	-
<i>S. cerevisiae</i> var. <i>ellipsoideus</i>	8-48	4	0-55	3-4	1-8	+
<i>Debaryomyces hloekeri</i>	0-8	2	0-825	20	13-8	++

* These quantities were contained in the usual 4 ml. of medium in a T-tube.

Table 10. Comparison of results with those of other workers

Organism	Quantitative results (from Table 2)						Results of other workers					
	AE			SAL			Results			References		
	ARB	CEL	AE	ARB	SAL	CEL	ARB	SAL	CEL	ARB	SAL	CEL
<i>Candida guilliermondii</i>	A	+	+	+	+	+	+	+	+	6	6	6
<i>C. krusei</i>	+	+	+	+	+	+	+	+	+	6	4, 6	4, 6
<i>C. mycoderma</i>	A	+	+	+	+	+	+	+	+	6	6	6
<i>C. tropicalis</i>	+	+	+	+	+	+	+	+	+	2	4	4
<i>Hansenula anomala</i>	M	+	+	+	+	+	+	+	+	1, 3, 6	1, 4, 5, 6	4, 5, 6
<i>H. saturnus</i>	A	+	+	+	+	+	+	+	+	1, 3	2	5
<i>H. suavolens</i>	A	+	+	+	+	+	+	+	+	1, 3	2	5
<i>H. subpelliculosa</i>	A	+	+	+	+	+	+	+	+	1	2	5
<i>Kloeckera apiculata</i>	+	+	+	+	+	+	+	+	+	6	6	6
<i>Pichia fermentans</i>	M	+	+	+	+	+	+	+	+	1	2	1
<i>P. membranefaciens</i>	+	+	+	+	+	+	+	+	+	1, 3, 6	2	6
<i>P. polymorpha</i>	+	+	+	+	+	+	+	+	+	3	2	6
<i>Saccharomyces carlsbergensis</i>	+	+	+	+	+	+	+	+	+	6	6	6
<i>S. cerevisiae</i>	+	+	+	+	+	+	+	+	+	6	6	6

±, some strains +, others -. ±, some strains -, others weak. M: organisms grown on MYGPA; A: organisms grown on A2G agar.
 1, Bedford (1942); 2, Lodder & Kreger-van Rij (1952); 3, Stelling-Dekker (1931); 4, Teunissen (1954); 5, Wickerham (1951); 6, Wiles (1953).

species were given by Barnett & Ingram (1955) as: *Saccharomyces bailii* (CBS 680) -ve; *S. marxianus* (CBS 712) +ve; a strain of *S. rouxii* -ve. These results agree with the observations described in the present paper.

DISCUSSION

Although the arbutin plate test gives fairly consistent results and may therefore have some use as a crude instrument of sorting, it does not seem to give much useful information about the arbutin-splitting capacities of a yeast. For one thing, it is probably rather insensitive. Some species of *Kloeckera*, for instance, are shown as arbutin-negative, though they can be demonstrated to be quite active splitters of arbutin (Table 2). Further, Lodder & Kreger-van Rij (1952) described all *Kloeckera* species as arbutin-negative, and the National Collection of Yeast Cultures place their strains NCYC 37 of *K. antillarum* and NCYC 328 of *K. apiculata* in the same category. Careful observation of plates with these two strains showed that the brown iron-hydroquinone complex was formed more slowly than its rate of diffusion across the plate and thus no distinct brown area was formed round the colony. When a diagonal strip of agar was cut from the plate with a sterile scalpel and the surface then inoculated on one side only (see Kluyver & Custers, 1940), it soon became obvious that the inoculated side was appreciably darker than the control; this was confirmed by Dr E. O. Morris (personal communication).

In one case studied, the arbutin plate test may have been actually misleading in the opposite way, showing a strong positive result for a 'negative' organism. From Table 2 it can be seen that *Pichia polymorpha* (NCYC 56) was arbutin-negative by the phloroglucinol test when grown on A2G agar or MYGPA. Dr Morris (personal communication) described three arbutin plates for this strain as having a deep brown zone around each colony which was of a black metallic colour. Analysis by the phloroglucinol method of a portion of the agar in the dark zone revealed that no more hydroquinone was liberated than in the case of an 'arbutin-negative' strain such as *Saccharomyces cerevisiae* (NCYC 231). Presumably, therefore, either this strain of *P. polymorpha* was capable of making a coloured complex directly with arbutin; or it split arbutin and made the hydroquinone unavailable by coupling it (or the iron complex) with, for example, an amino acid (cf. pulcherrimin: Walt, 1952).

Further, as for instance Table 5 shows, the nutrition of the growing yeast is critical in determining the result of a β -glucoside test. In the arbutin-plate test, the organisms were grown on yeast water agar, which, as has been seen at least with aesculin, does not appear to be a good medium for developing β -glucosidase activity (Table 6). This point is well illustrated by the activity of the strains of *Saccharomyces cerevisiae*, which were arbutin-plate negative (Lodder & Kreger-van Rij, 1952), but quite active as β -glucoside splitters when grown on MYGPA. Henry & Auld (1905) described baker's yeast (*S. cerevisiae*) as capable of splitting arbutin and salicin (see Table 1). Commercial samples of baker's yeast ('Ark Brand'), substantially free from

bacterial contaminants, were found in the present experiments to be active aesculin-splitters when tested directly without further cultivation; Brunet & Kent (1955) used baker's yeast as a source of β -glucosidase. Baker's yeast is cultivated for commercial purposes on a rich medium (White, 1954); assuming it to be *S. cerevisiae*, this alone would account for its β -glucosidase activity in contrast with Lodder & Kreger-van Rij's results on arbutin plates: the latter authors were using yeast cultivated on less rich media.

It is evident from the foregoing results that any observations on β -glucoside splitting are much affected by the techniques used. With this reservation, some general relationships do emerge from the quantitative estimate of splitting activity:

(i) When grown on a 'maximal' medium, probably all these yeasts can split aesculin to a measurable degree. Table 5 shows that the largest group (39 %) comprises those which split aesculin and salicin but not arbutin. This percentage might, however, be very different for different groups of yeast. And, of course, changing their nutritional status would give a different response pattern. For instance, a suspension which was negative for aesculin when grown on A2G agar, was also negative for salicin or cellobiose, though in five out of the thirty-eight cases the suspension split arbutin. Yeasts grown on MYGPA never split arbutin only.

(ii) In agreement with the findings of Stelling-Dekker (1931) it may be said that *Hansenula* strains are very strong splitters and *Pichia* strains weak. The active splitting by the strains of *Rhodotorula* studied agrees with the data of Lodder & Kreger-van Rij, but the results with members of the genus *Kloeckera* do not.

(iii) *Candida* and *Saccharomyces* strains varied a great deal in their splitting capacities. The weak *Saccharomyces* included many osmophilic strains, formerly called *Zygosaccharomyces*. *Saccharomyces mellis* and *S. baillii* are very weakly active groups; and also *S. rouxii* (in particular the former *Zygosaccharomyces nussbaumeri*). In the species *S. pastori*, the strains which had been named *Z. pastori* were very weak splitters, whilst the one representative of *Z. pini* was very strong. This difference cuts across their classification as the same species, thus agreeing with the observations of Wickerham (1952), Kudriavzev (1954) and Barnett & Ingram (1955). *S. lactis* and *S. florentinus* were the two most active β -glucoside splitters amongst Lodder & Kreger-van Rij's species of *Saccharomyces*.

If it be held, as hitherto (cf. Lodder & Kreger-van Rij, 1954), that tests with different β -glucosides are completely interchangeable, then the genus *Kloeckera* already provides an interesting anomaly: Lodder & Kreger-van Rij (1952) said it was arbutin-negative; Wiles (1953) described *K. apiculata* as aesculin-negative but salicin- and cellobiose-positive (Table 10) and Ingram (1955) stated that the apiculate yeasts utilize cellobiose. The work described in the present paper does indicate that *Kloeckera* species are active β -glucoside splitters. So much so that the demonstration by Lowings (1956) that a strain of *K. apiculata* was capable of collapsing the structure of picked strawberries led the present writers to investigate possible cellulase activity in Lowing's

strain (LTS 5); the results, however, were negative. The only indication from previous workers that a strain might be able to split one β -glucoside and not another was that quoted above for *K. apiculata* (Wiles, 1953). The results given in Table 2 suggest that yeasts do vary in this way but that it would have been wrong to draw this conclusion from Wiles's results. As Table 2 shows, *K. apiculata*, including Wiles's T70 strain, is a strong aesculin-splitter. Perhaps his negative results were because the aesculin growth test was insufficiently sensitive to detect the splitting.

For a full understanding of the β -glucoside splitting capacities of yeasts it is necessary to know much more about their enzymic basis. Veibel (1950) suggested that β -glucosidase acts irrespective of the nature of the aglycone, though the rate of reaction may vary a great deal. But it appears difficult to explain the results described in the present paper on the basis that all yeasts produce only one β -glucosidase with different quantitative potentialities for each substrate, because the relative potentialities are so different for different yeasts. Thus, for instance, though *Candida tropicalis* (NCYC 4) was a strong salicin-splitter, it was not possible to detect any arbutinase activity at all with this strain. In contrast, several strains of *Saccharomyces cerevisiae* did not split salicin so strongly but gave positive results with arbutin (see MYGPA results in Table 2). Also, the results discussed previously for *Pichia membranaefaciens* grown on different breakdowns of MYGPA suggest that more than one enzyme is present. It cannot be certain from such data that yeasts do produce more than one β -glucosidase, because the cell surface is selective and differences might arise in the transport of the substrates into the cell. It should, however, be easy to avoid this uncertainty by experiments with enzyme preparations. A cell-free extract of baker's yeast, the active part of which was thermolabile and non-dialysable, was found capable of splitting aesculin (see Brunet & Kent, 1955); and an appreciable degree of extracellular β -glucosidase activity was present in the suspensions (see Table 7). In view of this, the data just quoted have an added significance. At one time it was thought that there was only one α -glucosidase in yeasts (see Ingram, 1955). The study of yeast β -glucosidase seems likely to reveal that it, too, includes a number of distinct enzymes (cf. Jermyn, 1952).

It should be clear from this discussion that the data given in this paper are unlikely to throw much light on the classification of yeasts, except iconoclastically. For example, before deciding that *Torulopsis holmii* does not split salicin (see Table 2) it is necessary to think not only in terms of the yeast's nutrition (the growth medium), but also quantitatively; how many organisms split how much glucoside during what period? The quantitative interpretation of differences held previously to be qualitative is always likely to confuse distinctions used in classification (Mayr, 1942).

It is possible to answer, at least partially, the questions asked in the introduction. Within the limits imposed by the sensitivity of the methods for detecting aglycones it appears that any one yeast able to split one β -glucoside will not necessarily split all the others. Thus when grown on A2G agar *Pichia polymorpha* NCYC 56 split all the β -glucosides tested except arbutin, whereas

Saccharomyces delbrueckii var. *mongolicus* CBS 705 split aesculin only, and *S. cerevisiae* NCYC 353 split arbutin only. As already pointed out, the ability to split the β -glucosides is dependent markedly on the kind of medium on which the organism is grown. Even when grown on the complex medium, however, there are still many yeasts which do not split arbutin, salicin or cellobiose, though all are perhaps capable of splitting aesculin. It may be suggested with some confidence therefore that yeasts vary in the β -glucosidases they contain and that some yeasts are not capable of synthesizing certain enzymes, notably arbutinase, even under very favourable conditions of growth.

The factors which influence the differences in the rate of splitting have not been examined in sufficient detail to decide whether these rates are of value for classification. It is known (e.g. Veibel, 1950) that, besides the influence of pH and temperature (not studied here), the activity of β -glucosidases is dependent on the concentration of glucoside, and is usually decreased in the presence of glucose, aglycone and of certain inhibitors such as heavy metal ions. It was found in the present work (cf. Fig. 4) that splitting was arrested at a degree, sometimes far short of completion, characteristic for the different strains. Because the only major variable was the increase in aglycone concentration, probably this was responsible. Thus it might be possible to classify yeasts by measuring their ability to split glucosides in the presence of different amounts of aglycone, but such a technique would require rigid control of the size of the inoculum and other conditions and may seem impracticable.

It would seem desirable that for purposes of classification yeasts should be grown on the richest possible medium so that their full potentialities can be developed. For example, taxonomists have abandoned most of the nitrogen-assimilation tests (contrast Lodder, 1934, with Lodder & Kreger-van Rij, 1952) following Wickerham's (1946) demonstration that many yeasts, previously said to be unable to grow on $(\text{NH}_3)_2\text{SO}_4$, asparagine or urea as N-sources, could in fact do so when they had an adequate supply of vitamins. It is difficult, however, to decide what constitutes a maximal medium. For example, *Pichia membranaefaciens* shows salicinase but no aesculinase or arbutinase activity when grown on nutrient agar (Table 6). On all other media this yeast showed varying activities against aesculin and arbutin but not against salicin. It seems likely therefore that even on an optimum medium not all yeasts will hydrolyse arbutin, salicin or cellobiose. It would be worth while to utilize all these three compounds for classification purposes, but it is obviously important to carry out quantitative estimation of the degree of splitting. Further work, however, on the rates of hydrolysis and the effect of inhibitors (including aglycones), will be necessary before a satisfactory rationale can be fixed for using these tests for purposes of classification.

The work described in this paper was carried out as part of the programme of the Food Investigation Organization of the Department of Scientific and Industrial Research. The authors wish to thank Dr R. Davies for a much helpful criticism and Mrs B. M. Nightingale for technical assistance.

REFERENCES

- BARNETT, J. A. & INGRAM, M. (1955). Technique in the study of yeast assimilation reactions. *J. appl. Bact.* **18**, 131.
- BARNETT, J. A. & SWAIN, T. (1956). Splitting of aesculin by yeasts. *Nature, Lond.* **177**, 133.
- BAU, A. (1917). Über das Verhalten des Amygdalins gegen Gärungsorganismen, *Wschr. Brau.* **34**, 29.
- BEDFORD, C. L. (1942). A taxonomic study of the genus *Hansenula*. *Mycologia*, **34**, 628.
- BEIJERINCK, M. W. (1907). Melkzuurgisting in melk. *Versl. gewone Vergad. Akad. Amst.* **15**, 883.
- BLAKESLEE, A. F. (1915). Linder's roll tube method of separation cultures. *Phytopathology*, **5**, 68.
- BOURQUELOT, E. (1893). Les ferments solubles de l'«*Aspergillus niger*». *Bull. Soc. mycol. Fr.* **9**, 230.
- BRUNET, P. C. J. & KENT, P. W. (1955). Observations on the mechanism of a tanning reaction in *Periplaneta* and *Blatta*. *Proc. Roy. Soc. B*, **144**, 259.
- CASTELLANI, A. (1937). A short general account for medical men of the genus *Monilia*, Persoon, 1797. *J. trop. Med. (Hyg.)*, **40**, 293.
- CASTELLANI, A. & DOUGLAS, M. (1935). Reaction of organisms on arbutin agar. *J. trop. Med. (Hyg.)*, **38**, 197.
- DIDDENS, H. A. & LODDER, J. (1942). *Die Hefesammlung des Centraal-bureau voor Schimmelcultures* II. Teil. *Die anaskosporogenen Hefen*. Amsterdam: N.V. Noord-Hollandsche Uitgevers Maatschappij.
- FAIRBRIDGE, R. A., WILLIS, K. J. & BOOTH, R. G. (1951). The direct colorimetric estimation of reducing sugars and other reducing substances with tetrazolium salts. *Biochem. J.* **49**, 423.
- GONNERMANN, M. (1906). Über das Spaltungsvermögen von Leberhistozym und einiger Enzyme auf einige Glykoside und Alkaloide. *Pflüg. Arch. ges. Physiol.* **113**, 168.
- GORDON, M. H. (1905). A ready method of differentiating streptococci and some results already obtained by its application. *Lancet*, ii, 1400.
- GOSIO, B. (1917). Valore batterio-diagnostico dell' arbutina con speciale riguardo ai bacilli dissenterici. *Ann. Igiene (sper.)*, **27**, 213.
- HARRISON, F. C. & VAN DER LECK, J. (1909*a*). Aesculin bile salt media for the isolation of *B. coli* and *B. typhosus*. *Zbl. Bakt. (Abt. 1. Orig.)*, **51**, 607.
- HARRISON, F. C. & VAN DER LECK, J. (1909*b*). Aesculin bile salt media for water analysis. *Zbl. Bakt. (Abt. 2)*, **22**, 547.
- HEILBRON, I. & BUNBURY, H. M. (1946). *Dictionary of Organic Compounds*. London: Eyre and Spottiswoode.
- HENRY, T. A. & AULD, S. J. M. (1905). On the probable existence of emulsin in yeast. *Proc. Roy. Soc. B*, **76**, 568.
- INGRAM, M. (1950). Osmophilic yeasts from concentrated orange juice. *J. gen. Microbiol.* **4**, ix.
- INGRAM, M. (1955). *An Introduction to the Biology of Yeasts*. London: Pitman.
- JERMYN, M. A. (1952). Fungal cellulases. II. The complexity of enzymes from *Aspergillus oryzae* that split β -glucosidic linkages, and their partial separation. *Aust. J. Sci. Res. (Ser. B)*, **5**, 433.
- JERMYN, M. A. (1955). Fungal cellulases. IV. Production and purification of an extracellular β -glucosidase of *Stachybotrys atra*. *Aust. J. Biol. Sci.* **8**, 541.
- JOHNSON, W. C. (1955). *Organic Reagents for Metals*, vol. 1. Chadwell Heath: Hopkin and Williams.
- KIRSOP, B. (1954). The National Collection of Yeast Cultures. *J. Inst. Brew.* **60**, 210.

- KLUYVER, A. J. & CUSTERS, M. TH. J. (1940). The suitability of disaccharides as respiration and assimilation substrates for yeasts which do not ferment these sugars. *Leeuwenhoek ned. Tijdschr.* **6**, 121.
- KOBERT, R. (1908). Über einige Enzyme wirbelloser Thiere. *Pflüg. Arch. ges. Physiol.* **99**, 116.
- KUDRIAVZEV, V. I. (1954). *The Systematics of Yeasts*. Moscow: Academy of Sciences.
- LOCHHEAD, A. G. & FARRELL, L. (1931). The types of osmophilic yeasts found in normal honey and their relation to fermentation. *Canad. J. Res.* **5**, 665.
- LODDER, J. (1934). *Die anaskosporogenen Hefen*. Amsterdam: N.V. Noord-Hollandsche Uitgeversmaatschappij.
- LODDER, J. & KREGER-VAN RIJ, N. J. W. (1952). *The Yeasts. A Taxonomic Study*. Amsterdam: North Holland Publishing Co.
- LODDER, J. & KREGER-VAN RIJ, N. J. W. (1954). Classification and identification of yeasts. I. *Lab. Pract.* **3**, 483.
- LOWINGS, P. H. (1956). The fungal contamination of Kentish strawberry fruits in 1955. *Appl. Microbiol.* **4**, 84.
- MACKIE, T. J. & MCCARTNEY, J. E. (1949). *Handbook of Practical Bacteriology*, 8th ed. Edinburgh: Livingstone.
- MARTIN, R. W. (1949). Rapid colorimetric estimation of phenol. *Analyt. Chem.* **21**, 1419.
- MAYR, E. (1942). *Systematics and the Origin of Species*. New York: Columbia University Press.
- NEUBERG, C. & HOFMANN, E. (1932). Neue Beobachtungen über β -glucosidase. *Biochem. Z.* **256**, 450.
- PIRIE, N. W. (1955). Summing up of discussion on the principles of microbial classification. *J. gen. Microbiol.* **12**, 382.
- PORTEOUS, J. W. & WILLIAMS, R. T. (1949). Metabolism of benzene in rabbits. Studies in detoxication. 19. *Biochem. J.* **44**, 46.
- SCARR, M. P. (1951). Osmophilic yeasts in raw beet and can sugars and intermediate sugar-refining products. *J. gen. Microbiol.* **5**, 704.
- STELLING-DEKKER, N. M. (1931). *Die sporogenen Hefen*. Amsterdam: De Koninklijke Akademie van Wetenschappen.
- SWARTZ, M. N., KAPLAN, N. O. & FRECH, M. E. (1956). Significance of 'heat-activated' enzymes. *Science*, **123**, 50.
- TEUNISSON, D. J. (1954). Yeasts from freshly combined rough rice stored in a sealed bin. *Appl. Microbiol.* **2**, 215.
- VAN STEENBERGE, P. (1920). Les propriétés des microbes lactiques; leur classification. *Ann. Inst. Pasteur*, **34**, 803.
- VEIBEL, S. (1950). β -glucosidase. In SUMNER, J. B. & MYRBACK, K. *The Enzymes*. New York: Academic Press.
- WALT, J. P. VAN DER (1952). *On the Yeast Candida pulcherrima and its Pigment*. De Technische Hogeschool Te Delft, Thesis.
- WEATHERALL, C. & DIBLE, J. H. (1929). Aesculin fermentation and haemolysis by enterococci. *J. Path. Bact.* **32**, 413.
- WEIDENHAGEN, R. (1929). Zur Kenntnis der β -Glucosidase. I. Spaltung von Amygdalin. *Z. Ver. deutsch. Zuckerind.* **79**, 591.
- WEIDENHAGEN, R. (1930). Zur Kenntnis der β -Glucosidase. II. Spaltung von Cellobiose. *Z. Ver. deutsch. Zuckerind.* **80**, 11.
- WEINTRAUB, A. (1924). Über Glukosidsplaltung durch Bakterien der Coli-Gruppe. *Zbl. Bakt. (Abt. 1)*, **91**, 273.
- WHITE, J. (1954). *Yeast Technology*. London: Chapman and Hall.
- WICKERHAM, L. J. (1946). A critical evaluation of the nitrogen assimilation tests commonly used in the classification of yeasts. *J. Bact.* **52**, 293.

- WICKERHAM, L. J. (1951). Taxonomy of yeasts. *Tech. Bull. U.S. Dep. Agric.* no. 1029.
- WICKERHAM, L. J. (1952). Recent advances in the taxonomy of yeasts. *Annu. Rev. Microbiol.* **6**, 317.
- WICKERHAM, L. J. & BURTON, K. A. (1948). Carbon assimilation tests for the classification of yeasts. *J. Bact.* **56**, 363.
- WILES, A. E. (1953). Identification and significance of yeasts encountered in the brewery. *J. Inst. Brew.* **59**, 265.

(Received 17 May 1956)