The Use of a Colloidal Silica Preparation in Media Used for Yeast Identification

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SUMMARY

Nutritional tests on yeasts which utilize *myo*-inositol as the sole carbon source or which have red pigment have been carried out on defined media solidified with silica gel (Ludox HS-40) in multicompartment polystyrene dishes. Yeasts of the above types isolated from soft fruits were tested together with 189 named strains representing 28 genera and 110 species and varieties. The results obtained were compared with those for growth on media solidified with several commercially prepared agars. Methods are described for purifying commercial samples of Ludox HS-40 and for removing contaminating sugars from some di- and trisaccharides used in the tests.

INTRODUCTION

In surveys of yeasts obtained from soft fruits, carried out between 1968 and 1975, over 80% of the isolates utilized *myo*-inositol as the sole carbon source or formed rugose colonies. Yeast species showing either of these two characteristics can be identified by their ability to utilize nine sugars as sole carbon sources, and nitrate as the sole nitrogen source, and by their ability to form starch, ballistospores or grow filamentously. This paper describes a simplified method for accurately testing the carbon and nitrate utilizing properties of these species on media gelled with Ludox HS-40, a commercial colloidal silica solution.

METHODS

Estimation of D-glucose and D-fructose contamination in commercially supplied sugars. D-glucose contamination in solutions of raffinose, melezitose, sucrose and maltose was estimated by the hexokinase/glucose-6-phosphate dehydrogenase method of Slein (1963). The D-fructose present in melezitose, sucrose and raffinose was subsequently estimated using phosphoglucose isomerase (Klotzsch & Bergmeyer, 1963).

Freshly made solutions of commercially supplied maltose contained up to 1% (w/w) D-glucose, and melezitose contained up to 0.7% D-glucose and 0.3% (w/w) D-fructose. In sucrose, the total contamination with D-glucose plus D-fructose was less than 0.02%, and raffinose contained less than 0.03% D-fructose. No increase in the level of contamination was observed in aqueous solutions of maltose or melezitose (pH 5.6) shaken for 26 days at 20 °C. In similar sucrose solutions, however, 0.06% D-glucose and 0.06% D-fructose were present after 7 days shaking at 20 °C, increasing to 0.2% of each sugar after 26 days incubation. Similarly, between 0.2 and 0.3% D-fructose was present in raffinose solutions shaken for 26 days at 20 °C.

Fig. 1. Apparatus for distributing media into Repli dishes. A Delta Micro-metering pump fitted with a specially constructed 15-tube module set to deliver 13 portions of media simultaneously. To the right of the pump is a standard Delta 10-tube module.

Removal of D-glucose and D-fructose from the sugar solutions. The D-glucose and D-fructose present in commercial supplies of melezitose, sucrose, maltose and raffinose were removed by incubating 200 mM solutions of these sugars with washed cells of *Kloeckera apiculata*, a yeast which is unable to utilize the four sugars concerned. Cells from cultures of *Kl. apiculata* (NCYC328) grown on Difco Bacto-YM agar for 3 days at 20 °C were washed twice with sterile distilled water and centrifuged at 4000 g for 10 min; the supernatant was discarded. The washed cells were added to the sugar solutions and the cell concentration was adjusted to about 0.8 mg dry wt yeast ml⁻¹. The suspensions (250 ml) were then incubated in 2 l flasks on a rotary shaker (150 rev. min⁻¹) for 18 h at 25 °C. The yeast cells were removed by centrifuging at 4000 g for 10 min followed by filtration through a 0.22 µm pore size membrane (Millipore) and the purified sugar solutions were used immediately in the required media. The absence of D-glucose and D-fructose was checked by inoculating media containing the purified sugars with cultures of *Kl. apiculata* (NCYC328) and *Torulopsis pustula* (CBS6528), neither of which can utilize the four oligosaccharides.

Deionization of the Ludox HS-40 colloidal silica solution. An aqueous solution of Ludox HS-40 (75%, v/v; Du Pont Co. (U.K.), London EC1) was passed at 4 ml min⁻¹ through the cation exchanger Dowex 50W-X8 (hydrogen form; Dow Chemical Co., Michigan, U.S.A.) and then through the anion exchanger Amberlite IR-45 (hydroxyl form; Rohm & Haas Co., Pennsylvania, U.S.A.). The resins were prewashed (Hirs, Moore & Stein, 1952; Steven & Tristram, 1962) and packed into 500 × 30 mm columns. Resin beds containing 200 ml Dowex 50W-X8(H) and 300 ml Amberlite IR-45(OH) were used to deionize 1 l of 75% (v/v) Ludox HS-40 solution.

Preparation and dispensing of the silica gel media. The media for testing the utilization of the carbon sources and of KNO₃ were those used by Wickerham (1951) except that the amino acids were omitted (Barnett & Ingram, 1955) and 50 mM-carbon sources were added

(Buhagiar & Barnett, 1971). In the medium containing KNO_3 as the sole nitrogen source, the concentration of KH_2PO_4 was increased to 5 g l^{-1} .

To make 1 l medium, 10 ml of filter-sterilized vitamin solution was mixed aseptically with 250 ml of a filter-sterilized 200 mM solution of the required carbon source (raffinose, melezitose, sucrose, maltose, lactose, erythritol, *myo*-inositol, L-rhamnose, melibiose or D-glucose) and with 200 ml of a solution containing the appropriate mineral salts sterilized by autoclaving for 15 min at 121°C . Deionized Ludox HS-40 solution (533 ml), sterilized by autoclaving for 15 min at 121°C , was then added and the volume was made up to 1 l with sterile distilled water. This silica gel medium was adjusted to pH 5.6 with sterile 1 M-HCl or 1 M-NaOH.

A Delta Micro-metering pump (Watson-Marlow, Falmouth, Cornwall) fitted with a specially constructed 15-tube module (Fig. 1) was used to deliver 3 ml medium into individual compartments of polystyrene Repli dishes (Sterilin, Richmond, Surrey). The various test media were pumped simultaneously from the flasks through a series of silicone rubber tubes (3 mm internal diam.), the ends of which were attached to stainless-steel nozzles inserted through a stainless-steel template that fitted on top of a Repli dish (Fig. 1). The colloidal silica medium in the unvented Repli dishes gelled after 24 h at room temperature (about 20°C). The prepared Repli dishes were inoculated within 3 days.

The chemicals used were obtained from BDH (AnalaR grade), and the sugars and vitamins were from Sigma.

Yeast inoculation of the silica gel media. The individual compartments of the Repli dishes containing the colloidal silica media were inoculated with an aqueous yeast suspension ($30 \mu\text{l}$; containing about $0.025 \text{ mg dry wt yeast ml}^{-1}$) obtained from a 2 to 3-day-old culture growing at 20°C on Difco Bacto-YM agar. The inoculated dishes were incubated lid uppermost at 20°C for 7 days.

RESULTS

Preliminary treatment of Ludox HS-40 silica solution

Earlier tests showed that untreated Ludox HS-40 contained inhibitors since many of the yeasts grew poorly or not at all on medium containing over 6.5% (v/v) Ludox HS-40 [2.6% (w/v) silica content]. However, with 6.5% Ludox HS-40, medium containing the salt concentration used by Wickerham (1951) gelled only after lengthy periods. This delay could be reduced to about 21 days by increasing the NaCl and KH_2PO_4 content of the basal medium 10-fold. With a lower concentration of silica, the medium failed to gel. In contrast, no inhibition of yeast growth was observed on medium prepared with up to 40% deionized Ludox HS-40 [16% (w/v) silica content], at which concentration the carbon and nitrate utilization media (see Methods) gelled after 24 h. Similar results were obtained with five random samples of Ludox HS-40 supplied by Du Pont. After treatment with cation-exchange resin, the pH of the five samples (75% aqueous solutions) was lowered from about 10 to between 1.6 and 2.8. Subsequent treatment with anion-exchange resin raised the pH of these solutions to between 3.4 and 5.8. After autoclaving (15 min at 121°C) the pH of four of these solutions ranged from 7 to 7.5; the pH of the remaining solution was 5.6. No gelling occurred with any of these solutions although the solution at pH 5.6 became progressively more viscous after storage for 3 months at room temperature. None of the solutions adjusted to pH 7 before autoclaving showed any tendency to become glutinous on subsequent storage.

Table 1 (a). Nitrate-positive strains tested

Yeast	Strain*	Yeast	Strain*
<i>Aeossosporon salmonicolor</i>	CBS5937	<i>Rhodospiridium capitatum</i>	FRI55
<i>Aureobasidium pullulans</i>	ATCC11942	<i>Rhodotorula glutinis</i>	NCYC59
<i>A. prunorum</i>	FRI32	<i>Rh. lactosa</i>	CBS5826
<i>Brettanomyces anomalus</i>	NCYC449	<i>Sporidiobolus johnsonii</i>	CBS522, 50
<i>Cryptococcus albidus</i> var. <i>albidus</i>	LTS19, FRI58	<i>Sporobolomyces holsaticus</i>	NCYC420
<i>Cr. albidus</i> var. <i>aerius</i>	CBS155	<i>Sp. odoros</i>	CBS483
<i>Cr. albidus</i> var. <i>diffuens</i>	NCYC460	<i>Sp. roseus</i>	NCYC423
<i>Cr. infirmo-miniatus</i>	FRI50	<i>Torulopsis bacarum</i>	CBS6526, FRI41
<i>Cr. kuetzingii</i>	CBS1926	<i>T. domercqii</i>	IGC3067
<i>Cr. macerans</i>	FRI59, NCYC586	<i>T. fragaria</i>	CBS6253, FRI53
<i>Cr. terreus</i>	NCYC393	<i>T. magnoliae</i>	IGC3521, IGC3191
<i>Hansenula anomala</i>	CBS2577	<i>T. pustula</i>	CBS6527, CBS6528
<i>H. saturnus</i>	NCYC22	<i>Trichosporon pullulans</i>	FRI25, FRI47,
<i>H. silvicola</i>	NCYC413		CBS5108, CBS2532,
<i>H. subpelliculosa</i>	NCYC16		CBS2534, CBS2535,
<i>H. wingei</i>	NCYC809, NCYC810		CBS2536, CBS2538,
<i>Leucosporidium scottii</i>	FRI13		CBS2539, CBS2540,
<i>Oosporidium margaritifera</i>	FRI24		CBS2541, CBS2542

Table 1 (b). Nitrate-negative strains tested

Yeast	Strain*	Yeast	Strain*
<i>Aeossosporon dendrophilum</i>	CBS6074	<i>Rhodotorula minuta</i>	FRI38, FRI39
<i>Bullera alba</i>	NCYC425	<i>Rh. rubra</i>	CBS316, NCYC142
<i>Candida curvata</i>	FRI6	<i>Saccharomyces bailii</i> var. <i>bailii</i>	CBS749, ATCC2602
<i>C. diddensii</i>	FRI44, FRI45	<i>S. bailii</i> var. <i>osmophilus</i>	DBDR58
<i>C. humicola</i>	NCYC461	<i>S. bisporus</i> var. <i>bisporus</i>	NCYC171
<i>C. krusei</i>	NCYC338	<i>S. cerevisiae</i>	FRI1, CBS2823,
<i>C. lambica</i>	CBS603, CBS2066		NCYC77, NCYC78,
<i>C. mesenterica</i>	FRI9, FRI10		NCYC81, NCYC89,
<i>C. parapsilosis</i>	CBS604		NCYC93, NCYC114,
<i>C. tropicalis</i>	FRI14, FRI15		NCYC176, NCYC231,
<i>C. valida</i>	NCYC335		NCYC365, NCYC1026,
<i>C. zeylanoides</i>	FRI11, FRI12		NCYC1141, NCYC1235,
<i>Cryptococcus ater</i>	CBS4685		NCYC1244, LTS22
<i>Cr. dimennae</i>	CBS5770	<i>S. eupaegyus</i>	CBS748
<i>Cr. flavus</i>	CBS331	<i>S. fermentati</i>	CBS818
<i>Cr. gastricus</i>	CBS1927	<i>S. florentinus</i>	CBS746, NCYC172
<i>Cr. hungaricus</i>	CBS4214	<i>S. kluyveri</i>	NCYC616
<i>Cr. lactativorus</i>	CBS5771	<i>S. rosei</i>	CBS728
<i>Cr. laurentii</i> var. <i>laurentii</i>	NCYC139, FRI48	<i>S. rouxii</i>	NCYC568, ATCC4898
<i>Cr. laurentii</i> var. <i>flavescens</i>	NCYC553	<i>S. uvarum</i>	NCYC74, NCYC324,
<i>Cr. laurentii</i> var. <i>magnus</i>	CBS140		NCYC354, NCYC463,
<i>Cr. luteolus</i>	NCYC591		NCYC534
<i>Cr. melibiosus</i>	CBS2102	<i>Saccharomycodes ludwigii</i>	NCYC521
<i>Cr. skinneri</i>	CBS5029	<i>Saccharomycopsis capsularis</i>	NCYC127
<i>Cr. uniguttulatus</i>	CBS1730	<i>Saccharomycopsis lipolytica</i>	FRI8, IGC3345,
<i>Debaryomyces hansenii</i>	CBS811, LTS1		IGC3346
<i>D. marama</i>	FRI46	<i>Schizosaccharomyces octosporus</i>	NCYC131
<i>Filobasidium capsuligenum</i>	CBS4736	<i>Schiz. pombe</i>	CBS2627, NCYC132
<i>Hanseniaspora valbyensis</i>	NCYC17		NCYC380
<i>Kloeckera africana</i>	NCYC58	<i>Sporobolomyces pararoseus</i>	CBS484
<i>Kl. apiculata</i>	NCYC245, NCYC328	<i>Torulopsis candida</i>	FRI17, FRI18,
<i>Kluyveromyces dobzhanskii</i>	CBS2104		NCYC389, NCYC576
<i>K. drosophilae</i>	CBS2896	<i>T. colliculosa</i>	NCYC141
<i>K. fragilis</i>	CBS397	<i>T. inconspicua</i>	FRI19
<i>K. lactis</i>	CBS683, CBS739	<i>T. multis-gemmis</i>	CBS6524, CBS6525
	ATCC2627	<i>T. xestobii</i>	CBS5975
<i>K. marxianus</i>	CBS712, CBS745	<i>Trichosporon aculeatum</i>	CBS5578
	NCYC243	<i>Tr. capitatum</i>	NCYC473
<i>Lipomyces starkeyi</i>	NCYC533	<i>Tr. cutaneum</i>	FRI23, FRI54
<i>Metschnikowia pulcherrima</i>	NCYC166		CBS2485, CBS2486,
<i>Nadsonia elongata</i>	NCYC379		CBS2488, CBS2489,
<i>Pichia burtonii</i>	NCYC439, NCYC 440		CBS2493, CBS2545,
<i>P. deliensis</i>	LTS24		CBS2820, CBS5785,
<i>P. fermentans</i>	CBS187		NCYC444, NCYC472
<i>P. kudriavzevii</i>	FRI57	<i>Tr. fermentans</i>	FRI37, CBS2529
<i>P. membranacea</i>	CBS5567, NCYC52	<i>Tr. inkin</i>	CBS5585
<i>P. pastoris</i>	CBS704, NCYC175	<i>Tr. penicillatum</i>	CBS5586
<i>P. pinus</i>	CBS5097, CBS744		
<i>P. rhodanensis</i>	FRI51, FRI52		
<i>P. trehalophila</i>	CBS5361		
<i>P. vint</i>	CBS4050		

* FRI, Food Research Institute; CBS, Centraal Bureau voor Schimmelcultures; NCYC, National Collection of Yeast Cultures; ATCC, American Type Culture Collection; LTS, Department of Scientific and Industrial Research, Low Temperature Research Station; IGC, Instituto Gulbenkian de Ciencia; DBDR, Division of Bacteriology and Dairy Research Service.

1	2	3	4	5
6	7	8	9	10
11	12	13	12	11
10	9	8	7	6
5	4	3	2	1

Fig. 2. Growth of yeasts on media gelled with Ludox HS-40. (a) *Cryptococcus albidus* var. *albidus*, (b) *Cr. laurentii* var. *laurentii*, (c) *Cr. infirmo-miniatus* (rugose yeast) and (d) *Rhodotorula glutinis* (rugose yeast) growing in Repli dishes filled with basal utilization medium gelled with Ludox HS-40 and containing: (1) raffinose; (2) melezitose; (3) sucrose; (4) maltose; (5) lactose; (6) erythritol; (7) *myo*-inositol; (8) L-rhamnose; (9) melibiose; (10) D-glucose with KNO₃ as sole nitrogen source; (11) D-glucose; (12) D-glucose and no added nitrogen source; (13) no added carbon source. The Repli dishes were incubated at 20 °C for 7 days.

Purity of the treated melezitose, sucrose, maltose and raffinose

After incubation for 26 days at 20 °C with *Kl. apiculata* (NCYC328) or *T. pustula* (CBS6528), no growth occurred in freshly made liquid defined media (Wickerham, 1951) without amino acids and containing 50 mM purified maltose or melezitose solutions. However, the continuing production of constituent monosaccharides from the purified sucrose and raffinose was sufficient to support traces of growth with the same two species after 7 days incubation at 20 °C. With further incubation this growth progressively intensified.

Carbon and nitrate utilization tests

Tests carried out with 189 yeasts (Table 1) representing 28 genera showed that after incubation for 7 days at 20 °C growth on the carbon utilization medium containing D-glucose and on the KNO₃ utilization medium, both gelled with the deionized Ludox HS-40 solution, was generally comparable with that obtained on similar media gelled with 1.5% (w/v) of either Agarose (Sigma), Special Agar-Noble (Difco), Ionagar no. 2 (Oxoid), washed Ionagar no. 2 (Oxoid), washed New Zealand Agar (Davis), Bacto-Agar (Difco) or Purified Agar (Oxoid). There was limited growth on both the agar and the Ludox HS-40 media of *Trichosporon cutaneum* (NCYC444; CBS2486), *Saccharomycodes ludwigii* (NCYC521), *Kloeckera africana* (NCYC58), *Saccharomycopsis capsularis* (NCYC127), *Schizosaccharomyces pombe* (NCYC380), *Torulopsis xestobii* (CBS5975), and *Cr. albidus* var. *aerius* (CBS155). Growth with *Tr. cutaneum* (CBS2486; CBS2545; NCYC444) and with *Cr. laurentii* var. *laurentii* (NCYC139) was noticeably more abundant on the Ludox HS-40 medium. Of the yeasts isolated from fresh fruit surveys, 173 were grown on the Ludox HS-40 media with the appropriate carbon sources (see Methods). These included *Cr. laurentii* var. *flavescens*, *Cr. laurentii* var. *laurentii*, *Cr. albidus* var. *albidus*, *Cr. albidus* var. *diffluens*, *Cr. macerans*, *Cr. infirmo-miniatus*, *Rhodotorula glutinis* and *Sporobolomyces roseus*, together with named species and varieties of the genera *Cryptococcus*, *Rhodotorula*, *Rhodospiridium*, *Sporobolomyces* and *Sporidiobolus* (Table 1). Growth was clearly differentiated after 7 days at 20 °C (Fig. 2). Yeast growth on carbon utilization medium containing no sugar and gelled with silica, was absent or minimal after incubation for 7 days at 20 °C. In contrast, there was often limited growth with the same basal medium gelled with the seven types of agar tested. Of the various types, Oxoid Purified Agar and the laboratory washed agars showed least organic impurities capable of sustaining yeast growth. Similarly, with the nitrate-negative yeasts tested (Table 1), less growth was encountered on the silica/KNO₃ medium than with the same medium gelled with the seven agars tested. Incidental growth supported by the nitrogen introduced by the cell inoculum (Thorne, 1945) can be minimized by reducing the concentration of the inoculum. No advantage was noted with washed cell inocula.

DISCUSSION

Tests carried out between 1968 and 1975 have shown that the majority of the yeast isolates obtained from fresh strawberries, raspberries, blackberries, blackcurrants, loganberries and gooseberries utilize *myo*-inositol as the sole source of carbon or form red-pigmented colonies. These yeasts are rarely fermentative, sporogenous or filamentous and are identified largely on the basis of the results of nutritional tests. Maximum separation of the species can be achieved by testing with only nine of the 37 carbon sources used by Wickerham (1951) provided that growth due to impurities in the media is reduced to acceptably low levels. When tested in this way, the identification of these species depends greatly upon the accuracy of the results of the nutritional tests and demands media free of traces of extraneous nitrogen and carbon sources. When solid media are used, the gelling agent must also be free of utilizable nitrogen or carbon. Carbon source impurities have been reported in commercially available maltose and melezitose (Buhagiar & Barnett, 1971) and D-galactose (Shifrine, Phaff & Demain, 1954), and van der Walt (1970) states that certain oligosaccharides such as sucrose and maltose may undergo slow hydrolysis after prolonged storage in basal medium. Shifrine *et al.* (1954) treated a suspension of D-galactose with washed cells of *Candida*

monosa (*C. krusei*) to remove the sugar contaminants and then added the purified D-galactose to the basal utilization medium which was subsequently gelled with washed agar. The present experiments have shown that media solidified with deionized Ludox HS-40 have marked advantages over media solidified with washed or unwashed commercial agars. With Ludox HS-40 media, utilization of a substrate could be clearly distinguished from growth resulting from contaminating constituents in the agars tested. Silica gel prepared from several starting materials, including Ludox, is subject to influences such as temperature, silica content, pH, nature of ions present and method of sterilization. It is necessary at times to vary these factors empirically to obtain satisfactory media. The deionized Ludox HS-40 tested has advantages over the silica solutions used by other workers (Ingelman & Laurell, 1947; Temple, 1949; Taylor, 1950; Kingsbury & Barghoorn, 1954) in that the gelling time is predictable, it does not gel on autoclaving, it is non-toxic to all the yeasts tested and can be stored for lengthy periods. All the yeasts tested grew on media containing concentrations of KH_2PO_4 and NaCl 10 times higher than those recommended by Wickerham (1951), and, if required, the gelling time can be reduced by increasing the concentration of these salts to at least those levels.

Since gelling with deionized Ludox HS-40 is independent of heat, the automatic dispensing of media in large batches is clearly facilitated. Unlike agar media, however, thinly spread silica gel medium is normally subject to shrinkage and fragmentation. With Repli dishes this problem was avoided by dispensing 3 ml amounts into each compartment. The blocks of media ($20 \times 20 \times 7$ mm) rarely showed any shrinkage, fragmentation or condensation in sealed Repli dishes stored for 6 months at 20 °C.

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