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A Survey of Inhibitory Compounds for the Separation of Yeasts and Bacteria in Apple Juices and Ciders

BY F. W. BEECH AND J. G. CARR

Research Station, Long Ashton, Bristol

SUMMARY: Selected species of yeasts, lactic acid bacteria and acetic acid bacteria were tested against twenty-six antibiotics and twenty other organic compounds during a search for methods for separating chosen members of the mixed microflora of apple juices. Some of these compounds were found to be specifically inhibitory against either yeasts or lactic acid bacteria, but few inhibited completely such acetic acid bacteria as were tested.

The isolation of yeasts and lactic acid bacteria from apple juices and ciders calls for suitable differential media since the raw materials contain moulds and acetic acid bacteria whose growth must be restricted, otherwise they overgrow the primary isolation plates. In fresh and actively fermenting juices it is also difficult to make an accurate count of the lactic acid bacteria because of the large number of yeasts present, whereas in fully fermented ciders the counting problem is reversed since these lactic acid bacteria then form the major part of the microflora.

In preliminary work it was found that the following measures proved partially successful: (a) the use of apple juice media at pH 4.8 to discourage non-aciduric organisms; (b) anaerobic incubation to encourage the growth of lactic acid bacteria, followed by a further period of aerobic incubation when a yeast count was also required; (c) incorporation of 50 p.p.m. Acti-dione in media when isolating bacteria or 50 p.p.m. aureomycin when yeasts were being examined; (d) use of 100 p.p.m. diphenyl to suppress mould growth.

Certain yeast species resisted Acti-dione, and bacterial colonies occasionally grew in the presence of aureomycin. A search was therefore made for more selective inhibitory compounds which, incorporated in a medium, would permit only the growth of (i) yeasts, (ii) lactic and acetic acid bacteria, (iii) lactic acid bacteria or (iv) individual yeast genera or species of lactic acid bacteria. A simple screening method was used so that a large number of compounds could be tested against a wide range of yeasts and aciduric bacteria chosen from type culture collections. Each compound was tested in a standard malt wort at a number of concentrations to determine at which of these inhibition occurred; the true minimum inhibitory concentration was not determined.

METHODS

Organisms. The organisms used in these tests are given in Table 1. The yeasts were obtained from the Centraalbureau voor Schimmelcultures, Delft, representing at least one species of seventeen genera of non-pathogenic yeasts. With

the exception of *Candida pulcherrima*, all were type strains as given by Lodder & van Rij (1952); the strain of *C. pulcherrima* was that used by van der Walt (1952) in his tests for pigment production.

Both hetero- and homo-fermentative lactic acid bacteria were chosen from the genera *Leuconostoc*, *Streptococcus* and *Lactobacillus* (*Bergey's Manual*, 1948). In addition, two unidentified Gram-positive heterofermentative bacteria were included (both isolated from cider), one a coccus and the other a rod. Apart from these last two organisms and *Acetobacter mobile*, all the bacteria were obtained from the National Collection of Industrial Bacteria, Teddington, Middlesex.

Table 1. *List of test organisms used*

Yeasts	Bacteria
Family ENDOMYCETACEAE	Lactic acid bacteria
1. <i>Schizosaccharomyces pombe</i>	Genus <i>Leuconostoc</i>
2. <i>Saccharomyces cerevisiae</i>	24. <i>L. mesenteroides</i>
3. <i>S. cerevisiae</i> var. <i>ellipsoideus</i>	25. <i>L. dextranicum</i>
4. <i>S. pastorianus</i>	26. <i>L. citrovorum</i>
5. <i>S. rouxii</i>	27. C.1*
6. <i>S. carlsbergensis</i>	Genus <i>Streptococcus</i>
7. <i>S. fragilis</i>	28. <i>S. cremoris</i>
8. <i>Pichia membranaefaciens</i>	Genus <i>Lactobacillus</i>
9. <i>Hansenula anomola</i>	Heterofermenters
10. <i>Debaryomyces hansenii</i>	29. <i>L. brevis</i> var. <i>rudensis</i>
11. <i>Saccharomycoides ludwigii</i>	30. <i>L. brevis</i>
12. <i>Hanseniaspora valbyensis</i>	31. <i>L. fermenti</i>
13. <i>Lipomyces starkeyi</i>	32. <i>L. pastorianus</i>
Family SPOROBOLOMYCETACEAE	33. <i>L. buchneri</i>
14. <i>Bullera alba</i>	34. <i>L. hilgardii</i>
Family CRYPTOCOCCACEAE	35. V822†
15. <i>Cryptococcus laurentii</i>	Homofermenters
16. <i>Torulopsis colliculosa</i>	36. <i>L. casei</i>
17. <i>Brettanomyces bruxellensis</i>	37. <i>L. plantarum</i>
18. <i>Candida utilis</i>	38. <i>L. leichmanni</i>
19. <i>C. mycoderma</i>	<i>Acetobacter</i> spp.
20. <i>C. pulcherrima</i>	39. <i>A. keutzingianum</i>
21. <i>Kloeckera apiculata</i>	40. <i>A. pasteurianum</i>
22. <i>Trigonopsis variabilis</i>	41. <i>A. aceti</i>
23. <i>Rhodotorula glutinis</i>	42. <i>A. orleanense</i>
	43. <i>A. mobile</i>

* Heterofermentative coccus isolated from cider.

† Heterofermentative rod isolated from cider.

Medium. The organisms were maintained and tested on a wort medium (pH 5.4) prepared from the same batch of malt by the method of Lodder & van Rij (1952), but sterilized by intermittent steaming instead of autoclaving. Two liquid worts were prepared, one at sp.gr. 1.060 for subculturing yeasts and the other at sp.gr. 1.040 for bacteria. The solid medium for the actual tests was prepared by adding 2% agar to wort of sp.gr. 1.040.

Compounds tested. The compounds used in these tests are listed in Table 2, which also, where necessary, gives the sources from which they were obtained.

Table 2. *Compounds tested for possible inhibitory action against the yeasts and bacteria*

Antibiotics	
Compound	Address of supplier
Compound A 18	Glaxo Laboratories Ltd., Sefton Park, Stoke Poges, Buckinghamshire
„ A 228	
„ A 432	
„ A 583	
Acti-dione	The Upjohn Co., Kalamazoo, Michigan, U.S.A.
Actinomycin	Merck and Co. Ltd., Rahway, New Jersey, U.S.A.
Aspergillie acid	Northern Regional Research Laboratory, Peoria 5, Illinois, U.S.A.
Aureomycin	Lederle Laboratories Division, Aberdare, Glam., S. Wales
Bacitracin	National Institute for Medical Research, Mill Hill, London, N.W. 7
Chloramphenicol	Parke, Davis and Co. Ltd., Hounslow, Middlesex
Clavatin	Biochemical Laboratories, Imperial College, London, S.W. 7
Frequentin	I.C.I. Ltd., Butterwick Laboratories, Welwyn, Hertfordshire
Gliotoxin	Northern Regional Research Laboratory, Peoria 5, Illinois, U.S.A.
Kojic acid	Biochemical Laboratories, Imperial College, London, S.W. 7
Licheniformin	National Institute for Medical Research, Mill Hill, London, N.W. 7
Neomycin	The Upjohn Co., Kalamazoo, Michigan, U.S.A.
Nisin	Applin and Barrett Ltd., Yeovil, Somerset
Polymyxin B	Burroughs Wellcome and Co., Euston Road, London, N.W. 1
Penicillin G	Antibiotics Research Station, Clevedon, Somerset
Streptomycin	
Streptothricin	Merck and Co. Inc., Rahway, New Jersey, U.S.A.
Subtilin	National Institute for Medical Research, Mill Hill, London, N.W. 7
Terramycin	I.C.I. Ltd., Butterwick Laboratories, Welwyn, Hertfordshire
Thiolutin	Northern Regional Research Laboratory, Peoria 5, Illinois, U.S.A.
Tomatine	Eastern Regional Research Laboratory, Philadelphia 18, Pa., U.S.A.
Tyrothricin	Sharp & Dohme Ltd., Hoddesdon, Hertfordshire

Other compounds

β -Phenylethyl alcohol; Benzoic acid; Methyl-*p*-hydroxybenzoate; Ethyl-*p*-hydroxybenzoate; Propyl-*p*-hydroxybenzoate
 Dehydroacetic acid; Diphenyl; Ferbam (Ferric dimethyl dithiocarbamate)
 Pandurol, J. M. Collet & Co. Ltd., Bristol Road, Gloucester
 Pentachlorophenol; *p*-Nitrophenol; 2:4-Dinitrophenol; *o*-Phenylphenol; 1:3-Dichloro-2-naphthol
 8-Hydroxyquinoline; Salicylanilide; Sorbic acid; Mercaptoacetic acid; Thiourea.

Method of testing for possible inhibitors

Preparation of inocula. Actively growing cultures (5 ml.) were centrifuged and the organisms resuspended in 0.5 ml. sterile saline.

Preparation of test plates. Stock solutions of the inhibitory compounds were freshly prepared, water-soluble compounds were dissolved in sterile water without further sterilization; others were dissolved in absolute ethanol, acetone or pyridine. For antibiotics, 1.66 ml. of solutions of 5000, 500 and 100 p.p.m. dilutions were added to 15 ml. lots of molten (50°) wort agar in 1 oz.

screw-capped bottles to give final concentrations of 500, 50 and 10 p.p.m. respectively. The contents of the bottles were mixed, poured into sterile Petri dishes, allowed to set and dried at 25° for 2 hr. before inoculation. Similarly, 0.63, 0.46, 0.31 and 0.15 ml. of 50,000 p.p.m. solutions of the other inhibitory compounds were used to give final concentrations of 2000, 1500, 1000 and 500 p.p.m. Occasionally it was necessary to depart from these methods when using less soluble compounds or where intermediate concentrations in the agar were required. Control plates containing the greatest concentration of solvent used in each test were also prepared.

Inoculation of plates. Three complete sets of plates were prepared for each compound, each set being inoculated with one group of the test organisms, i.e. yeasts, acetic acid bacteria or lactic acid bacteria. Individual plates were placed upon a card marked with a numbered grid which acted as a guide for the inoculation pattern. This ensured that every organism was spotted in its correct position on the appropriate set of plates. A 2 mm. wire loop was used for all inoculations.

Records. All plates were incubated at 25° aerobically for yeasts and acetic acid bacteria, but for lactic acid bacteria the plates were kept under partial vacuum in a vessel containing pyrogallol and sodium carbonate (Millis, 1951). The sizes of the colonies were estimated visually after 6 and 12 days of incubation; only the later observations are recorded in this paper. When the lowest concentration used for any compound entirely inhibited one or more groups of organisms, tests were repeated at still lower concentrations.

RESULTS

Since it is not possible to present in detail here the effect of each compound on every organism over the whole range of concentrations tested, a copy of the complete results will be deposited with the Librarian, General Library, British Museum (Natural History), London S.W. 7, for reference purposes.

The compounds are classified under three headings according to their action on the different organisms; all concentrations are given in p.p.m., unless stated otherwise. When combinations of compounds are given it is probable that the effective concentrations of the constituents would be lower in practice since the values quoted relate to their effect when used singly.

Compounds suitable for selective media

These compounds differentially inhibited one or more groups of the organisms shown in Table 1.

Inhibition of bacteria. All the bacteria tested were inhibited by the following compounds at the stated concentrations while leaving the yeasts unaffected: aureomycin, 500; chloramphenicol, 500; pentachlorophenol, 10; actinomycin, 0.5 plus aureomycin, 50; penicillin G, 500 plus aureomycin, 50; terramycin, 500 plus aureomycin, 50.

Lactobacillus brevis, *L. brevis* var. *rudensis* and *L. buchneri* were the most resistant species of lactobacilli and were inhibited only at the highest concentrations of aureomycin, chloramphenicol or pentachlorophenol. Actino-

mycin inhibited the lactobacilli at very low concentrations, but actinomycin and penicillin G had no effect on the acetic acid bacteria and inhibited both groups of organisms only when combined with 50 p.p.m. of aureomycin. The mixture tyrothricin, 500 plus aureomycin, 50 also inhibited the yeasts *Debaryomyces hansenii*, *Lipomyces starkeyi*, *Cryptococcus laurentii* and *Kloeckera apiculata*; this mixture is therefore only suitable if these are either absent from the sample or need not be isolated.

Inhibition of yeasts. All the yeasts were inhibited by the following compounds, which did not affect the bacteria: compound A. 228, 500 units/ml.; methyl-*p*-hydroxybenzoate, 1000; ethyl-*p*-hydroxybenzoate, 1000; dehydroacetic acid, 250; Ferbam, 50; Acti-dione, 125 plus gliotoxin, 50; 8-hydroxyquinoline, 250 plus Acti-dione, 10; thiolutin, 250 plus Acti-dione, 10; *p*-nitrophenol, 250.

In general, the film-forming yeasts were very resistant to most of the inhibitory compounds. *Saccharomyces cerevisiae* var. *ellipsoideus* was the most resistant of the fermenting yeasts. This property might prove useful as a supplementary test to differentiate it from its less resistant parent species, if other strains of the two yeasts reacted in a similar manner to the test organisms. *Lipomyces starkeyi*, *Kloeckera apiculata* and *Rhodotorula glutinis* were very sensitive to most of the compounds yet, surprisingly, resisted the action of Acti-dione alone.

Acti-dione and gliotoxin had a complementary inhibitory action. The suggested concentration of Acti-dione in this combination may be reduced to 50 p.p.m. when *Cryptococcus laurentii* is known to be absent from the sample.

Isolation of lactic acid bacteria. The specific isolation of lactic acid bacteria proved most difficult because many compounds which prevented the growth of acetic acid bacteria also inhibited the lactic acid bacteria at the same or lower concentrations. Only sorbic acid at 2000 p.p.m. inhibited the acetic acid bacteria without affecting the lactic acid bacteria. Sorbic acid did not inhibit all the yeasts, and in order to prevent yeast growth completely this compound had to be used in admixture with dehydroacetic acid, 250; or gliotoxin, 500; or Acti-dione, 50. The last mixture may still permit the growth of *Brettanomyces bruxellensis* and *Trigonopsis variabilis*, but as these are encountered only infrequently this may be of little consequence.

Compounds of limited value for selective media

Few raw materials would contain all the yeasts and bacteria used in these tests, so that the compounds in this section may be equally valuable for isolating members of a more limited microflora.

Resistant yeasts. Each of the inhibitory compounds listed below allowed the selective isolation of a restricted number of yeasts which are coded as in Table 1. Bacterial contaminants may, if necessary, be inhibited by the addition of one of the compounds which inhibited all the bacteria tested (see above paragraphs labelled 'Inhibition of bacteria').

In the tabulated records which follow, the data are given in the following

way: compound, concentration used in p.p.m., code number (from Table 1) of resistant organisms.

Compound A. 228, 500: 7, 8, 9, 18, 19, 22.	Clavatin, 500: 3, 22.
Acti-dione, 500: 7, 12, 13, 17, 21, 22, 23.	Frequentin, 125: 8, 9, 19.
Acti-dione, 50 + gliotoxin, 50: 15.	Frequentin, 250: 19.
Acti-dione, 50 + sorbic acid, 2000: 17, 22.	Gliotoxin, 500: 8, 9, 11, 19.
Aspergillid acid, 500 or 8-hydroxy-quinoline, 250: 9.	Phenol, 2000: 3, 7, 8, 9, 17, 18.
<i>n</i> -propyl- <i>p</i> -hydroxy-benzoate, 250: 3, 7, 9, 10, 15, 18.	Polymyxin B, 500: 1, 3, 5, 7, 8, 9, 22.
	Salicylanilide, 1500: 3, 4, 12, 17.
	Sorbic acid, 1000: 2, 3, 5, 6, 16, 17, 18, 22.
	Thiolutin, 250: 8, 19.

Resistant lactic acid bacteria. The compounds given below allowed the preferential isolation of certain of the lactic acid bacteria (code numbers as in Table 1). Certain of these compounds will also suppress yeasts and acetic acid bacteria present as contaminants. Where they are ineffective in this respect they may be combined with a compound from among those listed above in the paragraph *Isolation of lactic acid bacteria*.

Aureomycin, > 50 and < 500: 24, 29, 30, 33.	2:4-Dinitrophenol, 100: 29, 30, 31, 32, 33, 34, 35, 36, 37, 38.
Benzoic acid, > 2000: 33.	<i>o</i> -Phenylphenol, 250: 24, 26, 29 to 35, 37, 38.
Gliotoxin, 500: 33.	8-Hydroxyquinoline, 2000: 25, 29, 30, 36, 37.
<i>n</i> -Propyl- <i>p</i> -hydroxy-benzoate, 1000: 29, 30, 31, 32, 33, 35, 36, 38.	Salicylanilide, > 2000: 33, 36.
Chloramphenicol, 500: 27, 33, 36, 37.	Streptomycin, 500: 27, 29, 30, 31, 33, 37.
Clavatin, 500: 29, 30, 33.	Mercaptoacetic acid, 200: 31, 32, 34, 37.
Neomycin, > 500: 34, 36, 37.	Thiolutin, > 250: 29.
Pandurrol, 10: 29, 30, 31, 32, 33, 35, 38.	

But for the fact that 250 p.p.m. *o*-phenylphenol inhibited *Leuconostoc mesenteroides*, C. 1, *Streptococcus cremoris* and *Lactobacillus casei*, this compound could have been included among those listed above as useful for the isolation of lactic acid bacteria since it inhibited all the yeasts and acetic acid bacteria at this concentration.

The heterofermentative members of *Lactobacillus*, e.g. *L. buchneri*, were more resistant than the homofermentative species to the action of the test compounds. Similarly, the heterofermentative *Leuconostoc* spp. were less easily suppressed than the homofermentative *Streptococcus cremoris*, but the resistance of the cocci as a group was less than that of the rods.

Compounds useless for selective media

The remaining substances tested, namely, compounds A 18 and A 583, bacitracin, kojic acid, licheniformin, nisin, β -phenylethyl alcohol, streptothricin, subtilin, thiourea and tomatine, were unsuitable for preparing differential media since they were not sufficiently selective in their action even at the highest concentrations used in these tests.

Compounds suitable for suppressing moulds

A number of the compounds tested are normally used as fungicides, but of these only diphenyl was found suitable for incorporation in media for the suppression of mould contaminants. When used at 2000 p.p.m. the lactic acid and acetic acid bacteria were unaffected, and the only yeasts inhibited were *Debaryomyces hansenii*, *Bullera alba*, *Cryptococcus laurentii*, *Kloeckera apiculata* and *Rhodotorula glutinis*. In routine practice diphenyl was used at 100 p.p.m. and all the yeasts grew satisfactorily at this concentration while mould contaminants were suppressed.

Effect of solvents

Of the inhibitory compounds tested twenty-five were soluble in water, eighteen in ethanol, two in dilute pyridine water and one in acetone. The effects of these solvents on the test organisms were as follows.

Yeasts. The control plates contained 10% (v/v) ethanol, the highest concentration used in the actual tests. When the experiments were begun it was found that seven of the yeasts (*Debaryomyces hansenii*, *Hanseniaspora valbyensis*, *Lipomyces starkeyi*, *Bullera alba*, *Cryptococcus laurentii*, *Kloeckera apiculata* and *Rhodotorula glutinis*) sometimes failed to grow or the colonies were much decreased in size compared with those on the normal control plates. This was largely overcome by using inocula not more than 3 days old, preparing fresh media every 14 days and standardizing the period of plate-drying before inoculation. Under these conditions only the ethanol sensitive yeasts, *Bullera alba*, *Kloeckera apiculata* and *Rhodotorula glutinis* failed to develop fully. For these organisms no results are recorded in cases when it was found that a particular concentration of ethanol was reinforcing the inhibitory effect of a given compound.

The initial rate of growth of the other yeasts was slightly delayed by ethanol, but after 12 days incubation, the colonies were the same size as those on the normal control plates. Similar results were obtained with acetone and pyridine water.

Bacteria. The lactic acid bacteria were not affected by any of the four solvents, while the growth of the acetic acid bacteria was slightly retarded on the ethanol control plates during the early stages of incubation.

DISCUSSION

These results show that some of the compounds tested are suitable for the selective isolation of yeasts and lactic acid bacteria from mixed microfloras such as are found in apple juices and ciders. Similar differential isolation techniques have been used in other fermentation industries, although a smaller number of inhibitory compounds have been tested (Strandskov, Brescia & Bockelmann, 1953; Gray & Kazin, 1946). One of the main experimental uses of antibiotics in such instances has been the suppression of undesirable organisms in several fermentation products (Day, Serjak, Stratton & Stone, 1954; Strandskov & Bockelmann, 1953; Strandskov, Baker & Bockelmann,

1953). Much smaller amounts of inhibitory compounds were required to suppress such species under these conditions than were needed in pure culture methods, no doubt because of the lack of competition for nutrients in the latter. A wide range of compounds was tested by Fitzgerald & Jordan (1953) for the suppression of pure cultures of oral lactobacilli; the inhibitory levels quoted agree closely with those presented here. The compounds of the greatest interest in the present tests are summarized below.

Acti-dione. Whiffen (1948) found that this substance had no effect on bacteria but inhibited many, although not all, yeast genera. Similar results were obtained in the present tests and in the routine plating of apple juice samples on media containing Acti-dione. Acti-dione has been used for counting bacterial contaminants in brewery yeasts and beers (Phillips & Hanel, 1950; Green & Gray, 1951; Strandkov & Boeckelmann, 1951); it was found ideal for this purpose because brewery yeasts are especially sensitive to it.

Actinomycin was the most effective inhibitor for the lactic acid bacteria; according to Reilly, Schatz & Waksman (1945) it is also fungistatic.

Aureomycin was valuable for inhibiting bacterial growth, especially acetic acid bacteria; it had no effect on yeast growth at 500 p.p.m. (Hesseltine, Hauck, Hagen & Bohonos, 1952). Terramycin, which has a similar chemical constitution, was less effective.

Chloramphenicol, often used to inhibit Gram-negative bacteria, did not suppress the acetic acid bacteria differentially.

Dehydroacetic acid was a good yeast inhibitor at 250 p.p.m. without affecting bacterial growth. It is only fungicidal at higher concentrations (Eeckhaut, 1952; Mossel & de Bruin, 1950; Wolf, 1950).

Diphenyl has been used for several years at Long Ashton to prevent mould growth (Hertz & Levine, 1942). At 100 p.p.m. it does not affect yeast or bacterial growth, in contrast with many other fungicides which suppress these organisms at fungistatic concentrations.

Frequentin was found to be an effective yeast inhibitor; its use for this purpose has not been previously reported. It is also fungicidal (Curtis, Hemming & Smith, 1951).

Gliotoxin inhibited most of the test yeasts at 500 p.p.m. but was not as selective as frequentin since it was also anti-bacterial at this level.

8-Hydroxyquinoline was a most effective yeast inhibitor at concentrations which left the test bacteria unaffected. Previous reports have concerned its effect on fungi and bacteria (Albert, 1953).

Sorbic acid was the only compound that inhibited the acetic acid bacteria but not the lactobacilli (Emard & Vaughn, 1952). It also inhibited many of the test yeasts. Phillips & Mundt (1950) used this compound to inhibit film yeasts in pickle brines to allow the preferential growth of species of *Leuconostoc* and *Lactobacillus*.

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