Development and use of a new medium to detect yeasts of the genera *Dekkera/Brettanomyces*

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Aims: The objectives of this work were to develop a selective and/or differential medium able to efficiently recover *Dekkera/Brettanomyces* sp. from wine-related environments and to determine the relationship between these yeasts and the 4-ethylphenol content in a wide range of wines.

Methods and Results: The selectivity of the developed medium was provided by the addition of ethanol, as single carbon source, and cycloheximide. The inclusion of bromocresol green evidenced acid-producing strains. The inclusion of *p*-coumaric acid, substrate for the production of 4-ethylphenol, enabled the differentiation by smell of *Dekkera/Brettanomyces* sp. from all other yeast species growing in the medium. The medium was used either by plating after membrane filtration or by the Most Probable Number (MPN) technique. In 29 white and 88 red randomly collected wines, these yeasts were found only in red wines at levels up to 2500 MPN ml^{-1}, but constituted less than 1% of the total microbial flora. In red wines, 84% showed detectable amounts of 4-ethylphenol up to 4430 $\mu g\ l^{-1}$ while 28% of the white wines showed detectable levels up to 403 $\mu g\ l^{-1}$.

Conclusions: The use of the medium proposed in this work evidenced the presence of low relative populations of *Dekkera/Brettanomyces* sp. even in wines contaminated by fast-growing yeasts and moulds.

Significance and Impact of the Study: Further ecological studies on *Dekkera/Brettanomyces* sp. should take into account the use of highly specific culture media in order to establish their true occurrence in nature.

INTRODUCTION

The yeasts of the genus *Brettanomyces*, or its teleomorph *Dekkera*, were first described by Claussen in 1904, in beer production (Gilliland 1961). The first reference to this genus in wines was made by Custers in 1940 who reidentified one strain of *Mycotorula intermedia* isolated by Krumbholz and Tauschanoff in 1933 from French grape must (Van der Walt and van der Kerken 1958). During the 1950s several authors reported the isolation of the genus *Brettanomyces* in French and Italian grape musts and wines (Van der Walt and van der Kerken 1958). Since then, several publications have mentioned the isolation of *Dekkera/Brettanomyces* sp. in many wine-producing countries such as France (Froudière and Larue 1990; Chatonnet *et al*. 1992), Italy (Gaia 1987; Ciolfi *et al*. 1988; Ciolfi 1991; Ciani and Ferraro 1997), Spain (Urbina and Beltran 1984; Longo *et al*. 1991; Ibeas *et al*. 1996; Alguacil *et al*. 1998), Australia (Heresztyn 1986), New Zealand (Wright and Parle 1974) and the USA (Kunkee and Bisson 1993; Fugelsang 1997; Mitrakul *et al*. 1999). However, in many other ecological surveys these yeasts are not referred to (see review by Deak and Beuchat 1996) which may be explained by the difficulties of their recovery from materials heavily contaminated with other

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yeasts or moulds which, by growing faster, prevented the detection of *Dekkera/Brettanomyces* sp. in plating media. To overcome such difficulties several selective culture media have been developed by manipulating the type and concentration of selected antimicrobial agents and carbon sources to suppress the growth of other yeast species and bacteria (Van der Walt and van der Kerken 1960; Wright and Parle 1974; Froudière and Larue 1990; Chatonnet *et al.* 1992; Fugelsang 1997; Alguacil *et al.* 1998; Mitrakul *et al.* 1999). In addition, bacteriological media have been described as being effective in the differentiation of *Dekkera* from other yeast genera (Davenport 1980). Fung and Liang (1990) also presented results demonstrating the ability of the dye basic fuchsin to identify *Brettanomyces anomalus*. However, the above-mentioned antimicrobial agents and carbon sources have not been systematically evaluated regarding the selective recovery of *Dekkera/Brettanomyces* sp.

The metabolic products responsible for wine spoilage by *Dekkera/Brettanomyces* sp. are mainly volatile phenols, acetic acid and tetrahydropyridines, being associated with large economic losses (Kunkee and Bisson 1993; Fugelsang 1997). The production of the volatile phenols 4-ethylphenol and 4-ethylguaiacol is due to the sequential activity of two enzymes which decarboxylate hydroxycinnamic acids into hydroxystyrenes which are then reduced to ethyl derivatives (*p*-coumaric acid is the substrate of 4-ethylphenol and ferulic acid is the substrate of 4-ethylguaiacol) (Steinke and Paulson 1964; Heresztyn 1986). The recent elucidation of the main role of *Dekkera/Brettanomyces* sp. in the development of wine 'phenolic taint', due to the presence of significant amounts of 4-ethylphenol and 4-ethylguaiacol (Chatonnet *et al.* 1992, 1995, 1997), renewed interest in their study. Before these works the origin of volatile phenols in wines was thought to be related to bacterial activity (Baumes *et al.* 1986; Cavin *et al.* 1993). In fact, lactic bacteria only produce traces of ethylphenols under wine conditions (Chatonnet *et al.* 1997) and the fermenting yeast *Saccharomyces cerevisiae* is unable to produce these compounds (Chatonnet *et al.* 1992). Therefore, in the present study, it was assumed that *Dekkera/Brettanomyces* sp. are the only producers of phenolic off-flavour in wines. This work was aimed at the development of a selective and/or differential culture medium able to efficiently recover *Dekkera/Brettanomyces* sp. in order to determine its relation with the 4-ethylphenol content in a wide range of wines.

MATERIALS AND METHODS

Yeast strains and wine samples

The strains of *Dekkera/Brettanomyces* sp. tested were either obtained from culture collections or isolated from wines in our laboratory (Table 1). The strains were maintained in GYP medium (20 g l^{-1} glucose, 5 g l^{-1} yeast extract, 10 g l^{-1} peptone and 20 g l^{-1} agar, pH 6·0) plus 5 g l^{-1} calcium carbonate at 4°C. The wine samples were randomly collected at Portuguese wine certification institutions and wine producers. The wines were accompanied by the respective chemical analyses. The vintage year ranged from 1992 to 1997 and the analysis was performed within 1 month of receipt.

Medium development

Inoculum preparation and culture media.

A loopful of fresh culture (24–48 h) was suspended in Ringer solution and used to inoculate the several media described below by streaking on plates. Growth, change in colour of medium and colony colour were recorded after different periods of incubation at 25°C. The periods of incubation and concentration of some of the compounds mentioned below are referred to in the Results. The media used were as follows.

i Mannitol Salt Agar (Difco, Detroit, USA), Kanamycin Aesculin Azide Agar Base (Oxoid, UK) and KF *Streptococcus* Agar (Difco).

ii YM with basic fuchsin was prepared by adding 0·1 g l^{-1} of this dye to YM agar (5 g l^{-1} peptone, 10 g l^{-1} glucose, 3 g l^{-1} yeast extract, 3 g l^{-1} malt extract, 20 g l^{-1} agar).

iii Several media were prepared by modification of the carbon source in the mineral medium described by Van Uden 1967). To the basal medium were added glucose, sucrose, maltose and ethanol, alone or in combination. The pH was adjusted to 4·5. To these media were added cycloheximide and sorbic acid in different concentrations.

iv GYP with different carbon sources (glucose, ethanol and galactose, alone or in combination) at pH 5·4.

v YNB (Difco) (6·7 g l^{-1}) supplemented with different carbon sources (glucose, ethanol, glycerol and several organic acids), adjusted to pH 5·4 and filter sterilized; agar (2 g l^{-1}) was added after sterilization in an autoclave and cooling to about 50°C; cycloheximide was added at 10 mg l^{-1}.

vi The medium WLN (Difco) with the addition of different concentrations of cycloheximide and sorbic acid.

vii To the medium GYP were added several antibiotics at different concentrations: 5-fluorocytosine (Sigma), fluconazole (Pfizer, Sandwich, UK), nystatin (Sigma), econazole (Sigma), ketoconazole (Jansen, Beerse, Belgium) (dissolved in 0·1 N HCl), myconazole (Sigma) (dissolved in dimethylsulphoxide) and cycloheximide (Sigma).

The media described above as (iii)–(vii) were supplemented with 100 mg l^{-1} *p*-coumaric acid (Sigma) and 22 mg l^{-1} bromocresol green (Panreac, Barcelona, Spain).

Table 1 Yeast strains

Species	ISA no.	Origin
Brettanomyces nanus	1985	MUCL 31149^T
Dekkera anomala	1521	UOFS-wy505
16 *B. anomalus*	1652	CBS 77^T, IGC-5153, stout beer
D. anomala	1653	IGC-5160, spoiled beer
D. anomala	1654	IGC-5161, bees wine culture
D. bruxellensis	1146	UCD-605
D. bruxellensis	1327, 1328	Sparkling wine*
D. bruxellensis	1329, 1331	Cane molasses†
B. bruxellensis	1649	CBS 72^T, IGC-4179, lambic beer
D. bruxellensis	1650	IGC-4801, tea fungus
D. bruxellensis	1655	IGC-5162
D. custersiana	1981	MUCL 27704^T
B. naardenensis	1721	CBS 6043, IGC-5163, tonic water
B. naardenensis	1722	IGC-5164
B. naardenensis	1723	CBS 6042^T, IGC-5165, lemonade
Dekkera sp.	1147	UCD-615
Dekkera sp.	1600, 1601	Sparkling wine*
Dekkera sp.	1699, 1700, 1701, 1702, 1703, 1704, 1791, 1792	Red wine*
Dekkera sp.	1717	White wine*
Dekkera sp.	1793	Cider‡
Dekkera sp.	1794	Wine‡
Candida halophila	1982	MUCL 29967^T
C. mannitofaciens	1984	MUCL 30038, CBS 5981
C. tropicalis	1359	Beer
C. versatilis	1983	MUCL 30048, CBS 1752
C. vini	1007	IGC-2597^T
Debaryomyces hansenii	1507	IGC-2968^T
D. polymorphus	1587	IGC-2606
Kloeckera apiculata	1189	CECT-1120
Kluyveromyces marxianus	1538	IGC-3886
Lodderomyces elongisporus	1421	Wine filler*
Pichia anomala	1478	IGC-4121^T
P. membranifaciens	1005	IGC-3796
P. membranifaciens	1449	IGC-4875B, tea fungus
Saccharomyces cerevisiae	1631	IGC-4543^T
S. exiguus	1183	CECT-1206
S. unisporus	1097	IGC-2500, kefyr grains
Schizosaccharomyces pombe	1190	CECT-1375
Torulaspora delbrueckii	1037	ACA-4§
Yarrowia lipolytica	1718	IGC-4159^T
Zygosaccharomyces bailli	1307	Sparkling wine production line*
Z. fermentati	1215	DBVPG-6475
Z. florentinus	1724	IGC-4169^T
Z. microellipsoides	1725	IGC-2534^T
Unidentified strains¶		
10, 15, 18, 19		Grapes
24, 30, 31,43, E		Grape juice
48, 57, 66, 68, 80, A, B, D, I, J, L		Red wine
70		Grape pump outlet
73		Destemming roll
75		Press roll
81		Press
C, H		Wine lees
G		Vineyard insect (*Drosophila* sp.)4

ISA, Instituto Superior de Agronomia, Lisboa, Portugal; IGC, Instituto Gulbenkian de Ciências, Oeiras, Portugal; UCD, University of California, Davis, CA, USA; DBVPG, Dipartamento di Biologia Vegetale, Università di Perugia, Italy; UOFS, University of the Orange Free State, Bloemfontein, Republic of South Africa; MUCL, Micothéque de la Université Catholique de Louvain, Belgium; CECT, Coleccion Española de Cultivos Tipo, Valencia, Spain.

* Strain isolated by ISA.

† Strain supplied by Union Nationale des Groupements de Distillateurs d'Alcool, Paris, France.

‡ Strain supplied by Escola Superior de Biotecnologia, Porto, Portugal.

§ Strain supplied by Universidad de Sevilla, Sevilla, Spain.

¶ Strains supplied by Universidade de Évora, Évora, Portugal.

Recovery of Dekkera/Brettanomyces.

Fresh cultures of the strain *Dekkera* sp. ISA 1791 were used to inoculate 50 ml YNB supplemented with ethanol (10% v/v) and glucose (0·2% p/v) at pH 3·5. At mid-exponential phase (O.D. 0·5), the culture was inoculated into filter-sterilized wines to give an initial inoculum of 2×10^3 viable cells ml^{-1}. One white wine (12·4% v/v ethanol, pH 3·30) and one red wine (12·4% v/v ethanol, pH 3·60) were inoculated after reducing the free sulphur dioxide to trace levels with hydrogen peroxide. These wines (in triplicate) were incubated in Erlenmeyer flasks at 25°C with orbital agitation. Wine samples (0·1 ml) were inoculated onto GYP and *Dekkera/Brettanomyces* Differential Medium (DBDM) (6·7 g l^{-1} YNB, ethanol (6% v/v), 10 mg l^{-1} cycloheximide, 100 mg l^{-1} *p*-coumaric acid, 22 mg l^{-1} bromocresol green and 20 g l^{-1} agar). The pH was adjusted to 5·4 in DBDM. In this medium, all components were sterilized by membrane filtration, except agar which was sterilized in an autoclave. Colonies were counted after 5 d (GYP) and 12 d (DBDM) incubation at

25°C. Means of three replicates of yeast counts were transformed to $\log_{10}$ and subjected to statistical analysis
8 (Student's *t*-test, $P < 0{\cdot}05$).

Microbial counts

Total microbial counts were obtained by membrane filtration (0·45-μm pore size) of 20 ml wine and plating on GYP medium for 5 d at 25°C. Yeasts of the genera *Dekkera*/*Brettanomyces* were detected using the medium DBDM, after membrane filtration of 20 ml wine and incubation at 25°C for up to 14 d. These yeasts were differentiated by medium acidification, colony morphology and detection of phenolic taint by smelling. The recovery of stressed cells was tested by preincubation of membranes after wine filtration in tryptone soy broth (30 g l^{-1}) with yeast extract (20 g l^{-1}) and agar (7 g l^{-1}) for 24 h at 25°C.

The contamination by moulds and the frequency of samples heavily contaminated by yeasts led to the utilization of the Most Probable Number (MPN) technique according to the procedures described by Harrigan (1998), using broths of the above-mentioned media under the same incubation conditions. Briefly, 1-ml portions of each decimal dilution of wine samples were inoculated into three tubes of GYP or DBDM with daily vortex agitation. Positive growth in GYP tubes was indicated by visual observation of medium turbidity. In DBDM tubes positive results were indicated by medium turbidity and by detection of a phenolic smell. The MPN was obtained by using the tables and rules provided by Harrigan (1998). The presence of *Dekkera*/*Brettanomyces* sp. was confirmed after strain purification and determination of the 4-ethylphenol produced in GYP broth with 100 mg l^{-1} *p*-coumaric acid (see below) and by analysis of long-chain fatty acid compositions according to Malfeito-Ferreira *et al.* (1997).

Determination of 4-ethylphenol in culture media and wines

The production of 4-ethylphenol was measured according to a protocol adapted from Bertrand (1981). The volatile phenol was extracted from samples (10 ml GYP broth or 50 ml wine) by ether-hexane at a previously adjusted pH of 8. The quantification was achieved by adding 10 mg l^{-1} 3,4-dimethylphenol (Fluka, Buchs, Switzerland) as an internal standard followed by gas chromatography (model 8130; Fisons Instruments, Rodano, Italy) with a DB-Wax column (30 m, 0·53 mm ID, 0·25-μm film thickness; J & W
9 Scientific, Folsom, USA). The programme conditions were as follows: initial temperature 50°C, increase in temperature 5°C min^{-1}, second temperature 215°C, second increase in temperature 20°C min^{-1}, final temperature 250°C for 15 min. The injector and detector temperatures were 260°C. Hydrogen was used as the carrier gas at 80 kPa pressure (2·0 ml min^{-1}). The sample volume injected was 1 μl.

Evolution of 4-ethylphenol content in wines

After determination of 4-ethylphenol and microbial counts, the remaining wine of the samples was kept in 0·375-l bottles with rubber caps, at room temperature, and the 4-ethylphenol determined after 57 d. Separately, a selection of four wines was transferred to 25-ml screw-capped Macartney flasks and incubated at 25°C for 35 d. Each flask was used for a single determination of 4-ethylphenol content. One flask of each wine, after membrane filtration (0·22-μm pore size), was analysed at the end of the experiment to check the stability of 4-ethylphenol concentrations in sterile samples.

RESULTS

Development of culture medium

Bacteriological and basic fuchsin media. The bacteriological media did not support the growth of any *Dekkera* strain tested and the basic fuchsin medium did not enable their differentiation from other yeast species within 2 weeks of incubation (results not shown).

Selection of carbon sources and antimicrobial agents. The results of yeast growth in mineral and GYP media with different carbon sources are shown in Table 2. The mineral media did not appear to be selective with any carbon source tested. However, in GYP media, ethanol alone inhibited the growth and change in medium colour for a wider range of non-*Dekkera* species. Higher concentrations of ethanol (10% v/v) inhibited the growth or acid production of some strains of *Dekkera* (Table 2) and increased the time for appearance of colonies.

Table 2 also shows representative results of growth with 250 mg l^{-1} sorbic acid and 10 mg l^{-1} cycloheximide in mineral medium with glucose and sucrose. The use of higher concentrations of sorbic acid (500 mg l^{-1}) and cycloheximide (50 mg l^{-1}) did not improve the selectivity of the media and increased the incubation period by 2–3 d (representative results shown in Table 2 only for mineral medium with maltose as single carbon source). In addition, the use of sorbic acid together with cycloheximide did not improve the selectivity provided by cycloheximide alone and so the preservative was not used for further experiments (results not shown). The selectivity was not improved either by high concentrations of cycloheximide (1000 mg l^{-1}) (results not shown). Table 3 shows the results of growth

Table 2 Growth of yeast strains in mineral media and GYP with different carbon sources

Species	ISA strain	MGV	MSV	MMV	MEV	G2E6	E06	E6	E10
Dekkera anomala	1331	++*	++	++	++	++	++	++	++
D. anomala	1521, 1652	++	++	−†	++	++	++	++	−
D. anomala	1653, 1654	nd	nd	nd	++	++	++	++	++
D. bruxellensis	1146	++	++	++	++	++	++	++	++
D. bruxellensis	1649	++	++	−	++	++	++	++	++
D. bruxellensis	1327, 1328, 1650	nd	nd	nd	++	++	++	++	++
D. bruxellensis	1329	nd	nd	nd	++	++	++	++	+‡
D. bruxellensis	1655	nd	nd	nd	−	++	++	++	++
D. naardenensis	1721	++	++	−	++	++	+	−	−
D. naardenensis	1722, 1723	++	++	−	++	+	+	+	+
Candida tropicalis	1359	++	++	−	++	++	++	++	++
C. vini	1007	−	−	−	−	−	−	−	−
Debaryomyces hansenii	1507	++	++	−	−	++	+	−	−
D. polymorphus	1587	++	++	−	++	+	+	+	+
Kloeckera apiculata	1189	++	++	−	++	++	++	++	++
Kluyveromyces marxianus	1538	++	++	−	++	++	+	+	+
Lodderomyces elongisporus	1421	++	++	−	++	++	+	+	+
Pichia anomala	1478	−	−	−	++	−	−	−	−
P. membranifaciens	1005	−	−	−	−	−	−	−	−
P. membranifaciens	1449	−	−	−	++	−	−	−	−
Saccharomyces cerevisiae	1631	−	−	−	−	−	−	−	−
S. exiguus	1183	−	−	−	−	−	−	−	−
S. unisporus	1097	++	++	−	++	+	+	+	+
Schizosaccharomyces pombe	1190	++	++	−	++	++	−	−	−
Torulaspora delbrueckii	1037	−	−	−	−	−	−	−	−
Yarrowia lipolytica	1718	++	++	−	++	++	+	+	+
Zygosaccharomyces bailli	1307	++	++	−	−	−	−	−	−
Z. fermentati	1215	++	++	−	++	+	+	+	+
Z. florentinus	1724	++	++	−	++	+	+	+	+
Z. microellipsoides	1725	++	++	−	−	−	−	−	−

* Growth and change in medium colour.

† Absence of growth.

‡ Growth without change in medium colour.

Mineral media: MGV, 2% (p/v) glucose, 10 mg l^{-1} cycloheximide, 250 mg l^{-1} sorbic acid; MSV, 2% (p/v) sucrose, 10 mg l^{-1} cycloheximide, 250 mg l^{-1} sorbic acid; MMV, 0·5% (p/v) maltose, 50 mg l^{-1} cycloheximide, 500 mg l^{-1} sorbic acid; MEV, 0·6% (v/v) ethanol, 10 mg l^{-1} cycloheximide (readings after up to 12 d incubation). GYP media: G2E6, 2% (p/v) glucose, 6% (v/v) ethanol; E06, 0·6% (v/v) ethanol; E6, 6% (v/v) ethanol; E10, 10% (v/v) ethanol (readings after up to 6 d incubation). All media contained 100 mg l^{-1} *p*-coumaric acid.

ISA, Instituto Superior de Agronomia, Lisboa, Portugal; nd, not determined.

in the presence of representative concentrations of different antibiotics. Cycloheximide (10 mg l^{-1}), by permitting the growth of *Dekkera* strains and inhibiting the growth or acid production of a higher number of other species, gave the best differentiating ability.

Selection of selective and differential media. Growth on mineral media required longer incubation periods than growth on GYP media (see legend to Table 2) and mineral media were not more selective. In addition, the best selectivity was achieved by ethanol as sole carbon source and by the antibiotic cycloheximide. Therefore, further tests were directed to improving the results obtained with GYP-based media. Table 4 shows the results demonstrating the better performance of YNB plus 6% (v/v) ethanol (DBDM), particularly due to the inhibition of growth of *Kloeckera apiculata*. In this medium the most important wine contaminant yeasts were inhibited, such as *Candida vini, K. apiculata, Pichia* sp., *S. cerevisiae, Schizosaccharomyces pombe, Torulaspora delbrueckii* and *Zygosaccharomyces bailli.* The other species growing in this medium (*C. tropicalis, Debaryomyces hansenii, D. polymorphus, Kluyveromyces marxianus, Lodderomyces elongisporus, S. unisporus, Yarrowia lypolytica, Z. fermentati, Z. florentinus* and *Z. microellipsoides*), besides being rare in the wine industry, were distinguished by the absence of a phenolic smell and

Table 3 Yeast growth in GYP medium with addition of different antibiotics

Species	ISA strain	T15	F15	N10	E10	K10	M10	C10
Dekkera anomala	1521	++*	++	++	−†	−	−	++
D. anomala	1652	++	−	++	−	−	−	++
D. bruxellensis	1146	−	++	−	++	++	−	++
D. bruxellensis	1327	++	++	−	++	++	−	++
D. bruxellensis	1328	++	++	−	++	−	++	++
D. bruxellensis	1649, 1650	++	++	−	−	−	++	++
D. bruxellensis	1655	++	−	−	−	−	−	++
D. naardenensis	1723	++	++	++	−	++	−	++
Candida tropicalis	1359	+‡	+	+	+	+	+	+
C. vini	1007	+	+	+	+	−	−	−
Kloeckera apiculata	1189	+	+	+	+	+	+	++
Pichia anomala	1478	+	+	+	+	+	+	−
P. membranifaciens	1005	+	+	+	+	+	−	−
P. membranifaciens	1449	+	+	+	+	+	+	−

* Growth and change in medium colour.

† Absence of growth.

‡ Growth without change in medium colour.

T15, 15 mg l^{-1} fluorocytosine; F15, 15 mg l^{-1} fluconasole; N10, 10 mg l^{-1} nystatin; E10, 10 mg l^{-1} econazole; K10, 10 mg l^{-1} ketoconazole; M10, 10 mg l^{-1} miconazole; C10, 10 mg l^{-1} cycloheximide (readings up to 13 d incubation). All media contained 100 mg l^{-1} *p*-coumaric acid.
ISA, Instituto Superior de Agronomia, Lisboa, Portugal.

by the shorter incubation period (less than half of the minimum 8 d required for the appearance of *Dekkera/Brettanomyces* colonies). Therefore, the medium selected was DBDM. In this medium *Dekkera/Brettanomyces* colonies were yellow cream pinpoint, changing frequently to green with increasing incubation time. In addition, the medium colour changed from blue to yellow due to acid production and the production of 4-ethylphenol was detected by its phenolic smell. Other smell precursors (ferulic acid for medicinal odour and lysine plus ethanol for mousy taint) were less well perceived than the phenolic smell given by the production of 4-ethylphenol from *p*-coumaric acid. The supplementation of culture media with thiamine and biotin did not improve cell recovery and the use of organic acids (YNB plus 5 g l^{-1} of each of acetic, malic, lactic, succinic, formic or citric acids) (results not shown) and glycerol (YNB plus 0·24% v/v glycerol) (Table 4) resulted in lower selective and differential abilities than those of DBDM. The medium WLN, commonly used in wineries for microbial counts, was tested as a reference and was also found to be less selective than DBDM (Table 4).

Recovery of Dekkera/Brettanomyces *sp. in DBDM.* The comparison of plate counts of *Dekkera* sp. ISA 1791, previously inoculated in white and red wines, in the general purpose GYP medium and in DBDM demonstrated that enumeration results were not statistically different in both media (results not shown).

Testing of wild strains

A group of unidentified strains isolated from natural environments and cellar equipment was tested in DBDM medium. The results, shown in Table 5, demonstrate the differentiating ability of this medium. For most strains, either positive or negative results were clear-cut. However, in four (strains 15, 19, 43 and 73) difficulty in the perception of the phenolic taint led to the assessment of the production of 4-ethylphenol by chromatography. Another strain (strain G in Table 5) produced a phenolic-like smell but its rapid growth eliminated the possibility of it being *Dekkera*, which was confirmed by the absence of 4-ethylphenol production. The presumptive identification as *Dekkera/Brettanomyces* was confirmed by microscopic observation (e.g. search for ogival or missile-shaped cells with multilateral budding).

Contents of 4-ethylphenol and *Dekkera/Brettanomyces* sp.

The 4-ethylphenol content was evaluated in 29 white and 88 red randomly collected wines. In red wines, 84% showed detectable amounts of 4-ethylphenol up to 4430 μg l^{-1} while 28% of the white wines showed detectable levels up to 403 μg l^{-1}. Chatonnet *et al.* (1992) defined the limit preference threshold as the minimum concentration above which a compound depreciates the wine aroma. The reported values were: (i) 426 p.p.b. of a mixture of 4-ethylphenol plus

Table 4 Yeast growth in WLN, YNB with addition of 0·24% (v/v) glycerol and *Dekkera/Brettanomyces* differential medium (DBDM) (readings after 8–10 d incubation)

Species	ISA no.	WLN	YNB glycerol	DBDM
Dekkera anomala	1331, 1521, 1652, 1653, 1654	++*	++	++
D. bruxellensis	1146, 1327, 1328, 1329, 1649, 1650, 1655	++	++	++
Dekkera sp.	1147, 1600, 1601, 1699, 1700, 1701, 1702, 1703, 1704, 1717, 1791, 1792, 1793, 1794	nd	++	++
D. naardenensis	1721, 1722, 1723	++	++	++
Brettanomyces custersianus	1981	nd	+†	++
B. nanus	1985	nd	+	−‡
Candida halophila	1982	nd	+	−
C. manitofaciens	1984	nd	+	−
C. tropicalis	1359	+	++	++
C. versatilis	1983	nd	+	−
C. vini	1007	−	−	−
Debaryomyces hansenii	1507	+	++	++
D. polymorphus	1587	+	+	++
Kloeckera apiculata	1189	+	−	−
Kluyveromyces marxianus	1538	+	++	++
Lodderomyces elongisporus	1421	+	++	++
Pichia anomala	1478	+	++	−
P. membranifaciens	1005	−	++	−
P. membranifaciens	1449	+	++	−
Saccharomyces cerevisiae	1631	−	−	−
S. exiguus	1183	−	−	−
S. unisporus	1097	+	++	++
Schizosaccharomyces pombe	1190	++	−	−
Torulaspora delbrueckii	1037	−	−	−
Yarrowia lipolytica	1718	nd	++	++
Zygosaccharomyces bailli	1307	++	++	−
Z. fermentati	1215	+	++	++
Z. florentinus	1724	nd	++	++
Z. microellipsoides	1725	nd	−	++

* Growth and change in medium colour.

† Growth without change in medium colour.

‡ Absence of growth.

All media contained 100 mg l^{-1} *p*-coumaric acid and 10 mg l^{-1} cycloheximide.ISA, Instituto Superior de Agronomia, Lisboa, Portugal; nd, not determined.

4-ethylguaiacol and (ii) 620 p.p.b. 4-ethylphenol. Based on these thresholds, our results showed that: (i) 48% of the red wines from 88 samples analysed had levels higher than 426 μg l^{-1} 4-ethylphenol plus 4-ethylguaiacol and (ii) 43% had amounts of 4-ethylphenol higher than 620 μg l^{-1}. The concentration of 4-ethylguaiacol is smaller than that of 4-ethylphenol and the typical phenolic taint is due to the presence of both molecules in a proportion close to 8 : 1 (Chatonnet *et al.* 1992). In our study, 4-ethylguaiacol was absent from many samples and so the proportion close to 8 : 1 (4-ethylphenol : 4-ethylguaiacol) observed by Chatonnet *et al.* (1992), in lower concentrations of both compounds, was not observed with our data.

From our results it is clear that high levels of 4-ethylphenol may be attained a short time after the end of wine fermentation (two wines from the 1997 vintage with about 4-month bulk storage showed 1600 and 2340 μg l^{-1} 4-ethylphenol) and so this problem is not only characteristic of wines with long ageing periods. In addition, in a small group of red wines where it was possible to trace their origin, we have also detected 'phenolic' wines stored in concrete or stainless steel tanks without contact with wood (results not shown).

The enumeration of *Dekkera/Brettanomyces* sp. was carried out in all wine samples analysed for volatile phenol content. These yeasts were not recovered from white wines or from red wines without 4-ethylphenol. In the 74 red wine samples with detectable amounts of 4-ethylphenol, 57% were contaminated by *Dekkera/Brettanomyces* sp. This incidence increased to 69% in samples with levels of

Table 5 Probable identification of *Dekkera/Brettanomyces* sp. according to growth characteristics in *Dekkera/Brettanomyces* differential medium (DBDM) and 4-ethylphenol production of yeast strains isolated from wine and related environments (readings up to 10 d incubation)

Number	Growth in DBDM	DBDM acidification	Phenolic smell	4-ethylphenol production	Probable *Dekkera/Brettanomyces* sp.
10, 18, 24, 70, 75	+	+	+	+	+
15	+	+	a*	+	+
19, 43, 73	+	+	a	−	−
G	+†	+	a	−	−
30, 31, 48, 66, 68, 81, C, D, E	+	+	−	nd	−
80	+	−	−	nd	−
57, A, B, H, I, J, L	−	−	−	nd	−

* Doubtful identification of phenolic smell.

† In this strain growth was observed after 2 d incubation.

nd, Not determined.

All strains were observed microscopically, first to check for the presence of bacteria and afterwards to evaluate any particular morphology.

4-ethylphenol/4-ethylguaiacol higher than the limit preference threshold (426 μg l^{-1}). The introduction of a preincubation step using TSY broth was not efficient either in increasing yeast recovery or decreasing the incubation time (results not shown).

Contamination by moulds is a serious concern when counting slow-growing yeasts like *Dekkera* sp. and has been reported to be prevented by oligomycin (Eliskases-Lechner and Prillinger 1996). However, we found that some *Dekkera* sp. were inhibited by this antibiotic (results not shown). In order to overcome these difficulties and as yeast colonies were frequently too numerous to be enumerated, the count was obtained by using the MPN technique. The enumeration of the total contaminating flora and of *Dekkera/Brettanomyces* yeasts is shown in Table 6 for a representative group of wines. The total microbial flora attained levels higher than $2{\cdot}8 \times 10^6$ MPN ml^{-1} while *Dekkera/Brettanomyces* sp. were detected in counts up to $2{\cdot}5 \times 10^3$ MPN ml^{-1}. Total microbial counts were relatively high because samples were not bottled wines from the market but samples supplied by certification institutions.

Evolution of 4-ethylphenol in wines contaminated by *Dekkera/Brettanomyces* sp.

Table 6 shows that *Dekkera/Brettanomyces* sp. were detected in four samples which had rather low amounts of 4-ethylphenol. The 4-ethylphenol content of these wines was monitored for 35 d showing that, in three samples, the amount increased but kept below the threshold of 426 μg l^{-1} (Table 7). However, one sample showed much higher amounts than the initial value determined about 1 month before, which continued to increase thereafter (sample R 90/T97). Yeast growth is inhibited by ethanol and sulphur dioxide and so we checked their values in these wines (see Table 6). As expected, the sample showing a higher susceptibility to an increase in 4-ethylphenol (R 90/T97) contained lower amounts of molecular sulphur dioxide (0·43 mg l^{-1}) and ethanol (11·4% v/v). This sample also showed the highest counts of *Dekkera*; it was not determined whether this high level of contamination was due to heavy winery contamination or to the absence of growth inhibition or both. Because of these observations, 4-ethylphenol concentrations were determined in all the wine samples contaminated by these yeasts after storage for 57 d, after initial 4-ethylphenol evaluation, in 0·375-l bottles. The results showed that all wines suffered an increase in 4-ethylphenol content (see Table 6). These results are very significant because they show that 4-ethylphenol may increase in bottled wines during storage and distribution, if they are contaminated by *Dekkera/Brettanomyces* sp. It should be noticed, however, that oxygen stimulates yeast growth (Malfeito-Ferreira *et al.* 2000) and sample preparation for this experiment might have dissolved more oxygen than the usual amounts in wineries.

In agreement with the data in Table 7, other wines with a high alcohol content suffered small relative increases (samples R 01/T94, R 09/T96 and R 11/T96 in Table 6). However, wine R 07/T95, with the lowest ethanol content, suffered the higher relative increase. These results led us to investigate a possible correlation between 4-ethylphenol and the levels of ethanol and molecular sulphite in all wines analysed. As seen before, volatile phenols may increase during bottle storage and may not correspond to the concentration present at the time when the chemical analysis was performed. Therefore, the following correlations should be regarded as indicative. We found that the correlations with ethanol and sulphur dioxide were weak but very high levels of ethanol (higher than about 13% (v/v))

Table 6 Contents of 4-ethylphenol and microbial counts in wine samples and change of 4-ethylphenol during storage

Wines*	4-ethyl-phenol ($\mu g\ l^{-1}$)	*Dekkera* counts (cfu 20 ml^{-1})	*Dekkera* counts (MPN ml^{-1})	Total counts (MPN ml^{-1})	Ethanol (% v/v)	Molecular SO_2 (mg l^{-1})	4-ethyl-phenol ($\mu g\ l^{-1}$)†	Relative increase‡
R 01/T94	240	56	0·0	$2{\cdot}5 \times 10^4$	13·1	0·30	405	1·69
C 02/T95	2440	a§	250	$> 2{\cdot}8 \times 10^6$	12·4	0·38	5205	2·13
R 07/T95	440	TNTC	25	$1{\cdot}1 \times 10^5$	11·4	0·54	2799	6·36
A 05/T96	800	TNTC	250	2×10^4	12·0	0·54	2568	3·21
A 08/T96	590	TNTC	250	$> 2{\cdot}8 \times 10^6$	11·9	0·54	3274	5·55
R 02/T96	1070	TNTC	250	$1{\cdot}1 \times 10^5$	12·6	0·53	3109	2·91
R 09/T96	130	1	0·0	$> 2{\cdot}8 \times 10^6$	12·9	0·47	286	2·20
R 10/T96	420	TNTC	250	$> 2{\cdot}8 \times 10^6$	12·3	0·33	1516	3·61
R 11/T96	720	1	0·0	$> 2{\cdot}8 \times 10^6$	12·9	0·33	1719	2·39
R 14/T96	860	TNTC	250	$> 2{\cdot}8 \times 10^6$	12·1	0·68	2298	2·67
R 16/T96	90	TNTC	4·5	$2{\cdot}5 \times 10^4$	13·0	0·50	–¶	–
T 01/T96	260	1	0·0	$1{\cdot}1 \times 10^5$	12·8	0·45	796	3·06
R 02/T97	980	305	45	$4{\cdot}5 \times 10^4$	11·7	0·35	2365	2·41
R 17/T97	20	a	0·0	4·5	12·5	0·56	–	–
R 31/T97	60	13	2·5	$4{\cdot}5 \times 10^3$	12·6	0·46	–	–
R 33/T97	1600	TNTC	45	$> 2{\cdot}8 \times 10^6$	12·6	0·24	5074	3·17
R 89/T97	2340	TNTC	250	$> 2{\cdot}8 \times 10^6$	11·8	0·43	7506	3·21
R 90/T97	60	TNTC	2500	$> 2{\cdot}8 \times 10^6$	11·4	0·43	–	–

*Reference a/bc, where a is the sample code, b the type of wine (T, red) and c the vintage year.
†Determined after 57 d storage at room temperature in airtight bottles.
‡Proportion between final and initial 4-ethylphenol concentrations.
§Spreading moulds prevented the counting of typical *Dekkera* colonies present in the plates.
¶Data presented in Table 7.
TNTN, Too numerous to count; MPN, most probable number.

Table 7 Evolution of 4-ethylphenol in wine samples contaminated by *Dekkera/Brettanomyces* sp. but with a low initial concentration of 4-ethylphenol

	4-ethylphenol ($\mu g\ l^{-1}$)			
Time (d)	R 16/T96	R 17/T97	R 31/T97	R 90/T97
Initial *	90	20	60	60
0†	101	70	–‡	1958
22	–	84	102	4287
26	95	73	199	4504
31	125	76	127	4462
35	120	104	184	4366
Dekkera counts (MPN ml^{-1})*	4·5	0·0§	2·5	$2{\cdot}5 \times 10^3$

*Results from Table 6.
†Experiment began 1 month after initial determinations and wines were kept in 25-ml screw-capped Macartney flasks and incubated at 25°C.
‡Not determined.
§Colonies of *Dekkera/Brettanomyces* sp. were found on plates covered by moulds.

and sulphur dioxide (higher than 0·6 mg l^{-1} molecular sulphur dioxide) yielded low levels of 4-ethylphenol (Fig. 1). These results agree with the respective minimum inhibitory concentrations of *Dekkera/Brettanomyces* sp., as determined by Froudière and Larue (1990) and Chatonnet *et al.* (1993). In addition, we checked whether other analytical parameters could indicate any correlation with the levels of 4-ethylphenol. Concerning volatile acidity (Fig. 1) the result ($r^2 = 0{\cdot}24$) suggested the absence of a relationship between the production of acetic acid and of 4-ethylphenol, and so these two spoiling features of *Dekkera/Brettanomyces* sp. did not appear to be related. The correlation with the dry extract (Fig. 1) was suggested by our empirical observation that the phenolic taint seems to be more frequent in full-bodied red wines. However, this hypothesis did not find much support in the correlation obtained which, although positive, was low ($r^2 = 0{\cdot}42$). Other correlations with wine pH and total acidity were meaningless (results not shown).

DISCUSSION

With the exception of wines spoiled by pure cultures of *Dekkera/Brettanomyces* sp. (Ciani and Ferraro 1997; Gaia 1987), the use of a selective and/or differential medium is indispensable in the study of the occurrence of these yeasts in

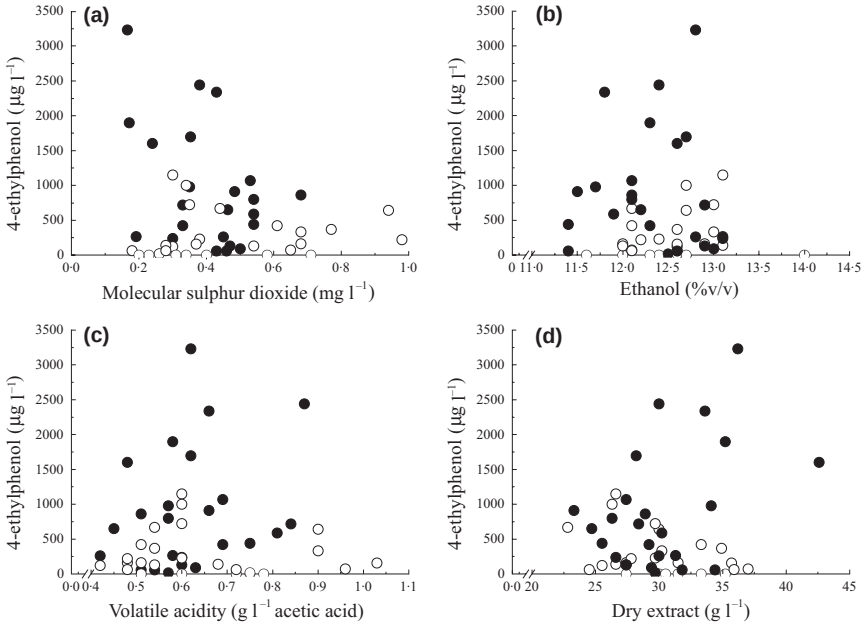

Fig. 1 Relationship between 4-ethylphenol and (a) molecular sulphur dioxide, (b) ethanol, (c) volatile acidity and (d) dry extract in red wines contaminated (●) or not (○) by *Dekkera/Brettanomyces* sp. Correlation coefficients (r^2) between 4-ethylphenol content and the referred analytical parameters for wines not contaminated by *Dekkera/Brettanomyces* sp. were –0·34 (molecular sulphur dioxide), 0·23 (ethanol), 0·24 (volatile acidity) and 0·42 (dry extract)

wine environments. In this work several media referred to in the literature were evaluated. The use of bacteriological media (Davenport 1980) and basic fuchsin (Fung and Liang 1990) did not support the growth of all strains under study or did not enable a clear differentiation from other species, respectively. The use of sucrose and/or maltose (Van der Walt and van der Kerken 1960; Wright and Parle 1974; Chatonnet *et al*. 1992) did not prove to be an advantage over the use of glucose. However, ethanol at 6% (v/v) as single carbon source was more selective than glucose, preventing the growth of species frequently recovered from wine environments. Higher concentrations of ethanol (10% v/v), as used by Alguacil *et al*. (1998), acted as an antimicrobial agent and not as a carbon source for some *Dekkera/Brettanomyces* sp. The use of glycerol, suggested by Fugelsang (1997), was found to be less selective than the use of ethanol as single carbon source. The preservative sorbic acid, used by Chatonnet *et al*. (1992), was found to be less selective than the antibiotic cycloheximide. The addition of *p*-coumaric acid was directed to the production of a metabolite detected by smell. This was only found to be positive in *Dekkera/Brettanomyces* sp., demonstrating that 4-ethylphenol may be regarded as a chemical marker for these yeasts. The species *C. halophila*, *C. mannitofaciens* and *C. versatilis* were reported as producers of 4-ethylphenol (Suezawa 1995), but did not grow in our medium. In conclusion, the medium developed in this work was partially selective and completely differential for *Dekkera/Brettanomyces* sp., based on colony morphology, time for growth, change in colour of medium and production of phenolic flavour.

The trial with unidentified strains revealed that it is possible to have doubts when identifying the phenolic smell. Under these conditions an additional step of 4-ethylphenol production in synthetic medium should be carried out. Our efforts did not succeed in reducing the normal 6–7 d incubation for the detection of these yeasts (Fugelsang 1997), but the increase of 2–3 d when ethanol was used as single carbon source is compensated by the higher selectivity obtained, especially towards common prefermentation fast-growing contaminants (e.g. *C. vini*, *K. apiculata* and *Pichia* sp.) which must be inhibited to allow the growth of *Dekkera/Brettanomyces* sp. colonies. The long incubation period may even be considered as a convenient differentiating feature because fast-growing strains are not *Dekkera/Brettanomyces* sp. In addition, the screening of these yeasts is mainly relevant during wine maturation (e.g. storage in oak barriques) and so a short incubation time is not essential for monitoring the development of *Dekkera/Brettanomyces* sp. populations.

The high incidence of phenolic wines, comparable to those observed in Italy (Di Stefano 1985), France (Chatonnet *et al*. 1992) and Australia (Pollnitz *et al*. 2000), was not accompanied by equivalent recovery of *Dekkera* yeasts when using solid plate media. In fact, the use of the MPN technique was essential both to avoid mould growth and to demonstrate the presence of these yeasts among the total contaminating flora. The observed low proportion of *Dekkera/Brettanomyces* sp. (less than 1%) together with the utilization of inadequate culture media may explain the difficulty in their recovery even from wines with a high concentration of 4-ethylphenol. In

addition, the absence of these yeasts from 'phenolic' wines may be explained by cell death after production of volatile phenols (Fugelsang 1997; Malfeito-Ferreira *et al.* 2000). These facts justify, at least partially, the possible absence of correlation between levels of 4-ethylphenol and isolation of *Dekkera/Brettanomyces* sp. which has contributed to the uncertainty regarding the origin of 4-ethylphenol in wines. Our results with the MPN technique, where the most diluted DBDM-positive tubes were only contaminated by these yeasts, suggest that the remaining microbial flora, especially bacteria, play a minor role, if any, in the production of 4-ethylphenol. These observations, at winery level, are in accordance with the hypothesis presented by Chatonnet *et al.* (1995, 1997).

Dekkera/Brettanomyces sp. have been regarded as typical cellar contaminants (Froudière and Larue 1990; Mitrakul *et al.* 1999), being absent from vineyard environments. However, our results demonstrating the presence of probable *Dekkera* strains isolated from grapes, in agreement with
11 Alguacil *et al.* (1998), suggest that ecological studies using appropriate culture media should be further developed in order to establish their true occurrence in nature.

The overall results suggest that the absence of *Dekkera/Brettanomyces* sp. proliferation is the main aim in the adequate control of phenolic taint. Even in wines with low initial levels of 4-ethylphenol, the increase in its content was sharp when these yeasts were present in relatively high numbers. The total amount of 4-ethylphenol also depends on the amount of *p*-coumaric acid present in grapes, which is a function of the grape variety (Gunata *et al.* 1986) and the processing technology (Romeyer *et al.* 1985). However, these facts do not explain the variability in 4-ethylphenol levels in different bottles of the same wine (Chatonnet *et al.* 1993) or in wines of the same grape variety produced in the same vintage and winery (Di Stefano 1985). Thus, in our opinion, the regulation of the activity of these yeasts is the key to understanding the above-mentioned variability in levels of 4-ethylphenol in wines. Further experiments are being carried out to determine the factors affecting the production of volatile phenols by *Dekkera/Brettanomyces* sp.

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