3B2v7.51c GML4.3.1 YFMIC : 574 Prod.Type:COM pp.1–8(col.fig.:3) ED:MariammaOmmen PAGN: gopal SCAN: Shobha

ARTICLE IN PRESS

FOOD MICROBIOLOGY

Food Microbiology ▮ (▮▮▮▮) ▮▮▮–▮▮▮

www.elsevier.nl/locate/jnlabr/yfmic

Identification of yeasts isolated from wine-related environments and capable of producing 4-ethylphenol

L. Dias[a], S. Dias[a], T. Sancho[a], H. Stender[b], A. Querol[c], M. Malfeito-Ferreira[a,*], V. Loureiro[a]

[a] *Laboratório de Microbiologia, Departamento de Botânica e Engenharia Biológica, Instituto Superior de Agronomia, 1349-017 Lisbon, Portugal*
[b] *AdvanDx Inc., Concord, MA, USA*
[c] *Departamento de Biotecnologia, Instituto de Agroquímica y Tecnologia de Alimentos, P.O.Box 73, 46100 Burjassot, Valencia, Spain*

Received 4 June 2002; accepted 4 November 2002

Abstract

The ability to produce 4-ethylphenol from the substrate *p*-coumaric acid in synthetic media was evaluated for several yeast species associated with wine production. Molar conversion rates as high as 90% were found by only *Dekkera bruxellensis*, *D. anomala* and by some unidentified strains isolated from wine-related environments. Other unidentified strains produced traces of 4-ethylphenol. All unidentified strains showed the same cultural characteristics as *D. bruxellensis* when grown on DBDM (*Dekkera*/*Brettanomyces* differential medium) agar. The determination of long-chain fatty acid compositions and the utilization of peptide nucleic acid (PNA) probes specific for *D. bruxellensis* showed that the unidentified strains did not belong to this species. Further identification, by restriction pattern generated from PCR-amplification of the 5.8S rRNA gene and the two internal transcribed spacers (ITS), assigned the unidentified strains to *Candida cantarelli*, *C. wickerhamii*, *Debaryomyces hansenii*, *Kluyveromyces lactis* and *Pichia guilliermondii*. However, only some strains of *P. guilliermondii* were capable of converting *p*-coumaric acid into 4-ethylphenol with efficiencies close to those observed in *D. bruxellensis* and *D. anomala*.

1. Introduction

The volatile phenols are a group of compounds with a strong influence on wine flavour. The main molecules are 4-ethylphenol and 4-ethylguaiacol, in red wines, and 4-vinylphenol and 4-vinylguaiacol, in white wines (Chatonnet et al., 1992). In particular, high amounts of 4-ethylphenol in red wines result an unpleasant taint described as "phenolic odour" (Chatonnet et al., 1992; Rodrigues et al., 2001). The relation between high concentrations of 4-ethylphenol in wines and the activity of yeasts of the genus *Dekkera*, or its anamorph *Brettanomyces*, has been largely debated and was recently established (Chatonnet et al., 1995; Chatonnet et al., 1997). The fermenting yeast *Saccharomyces cerevisiae*, other yeast species or lactic bacteria are regarded as weak and non-ethylphenol producers under wine conditions (Chatonnet et al., 1992; Chatonnet et al., 1995; Chatonnet et al., 1997).

The isolation of *Dekkera*/*Brettanomyces* sp. from wine-related environments is not easy due to their slow growth rates and low relative occurrence (Fugelsang, 1997; Kunkee and Bisson, 1993). To overcome such difficulties several selective and differential media have been developed (see references cited in Rodrigues et al., 2001) and recently one medium (named DBDM, after *Dekkera*/*Brettanomyces* differential medium) was reported as being able to recover *Dekkera* sp. from wines where these yeasts were present in proportions lower than 1% of the total microbial flora (Rodrigues et al., 2001). Assuming that these yeasts were the single 4-ethylphenol producers isolated from wines, as stated by Chatonnet et al. (1992, 1995, 1997), the isolates obtained with this medium were assigned to *Dekkera*/*Brettanomyces* sp. according to their colonial characteristics and after the assessment of 4-ethylphenol production (Rodrigues et al., 2001). Therefore, these probable *Dekkera* sp. strains lacked identity confirmation.

*Corresponding author. Tel.: +351-21-3653100; fax: +351-21-3635031.
E-mail address: mmalfeito@isa.utl.pt (M. Malfeito-Ferreira).

0740-0020/02/$ - see front matter © 2002 Elsevier Science Ltd. All rights reserved.
doi:10.1016/S0740-0020(02)00152-1

Classical identification techniques are not suitable for routine work in industrial microbiological control so other diagnostic techniques have been under great development during the last years (Loureiro and Querol, 1999). One of the possible strategies at the industrial level to identify foodborne yeasts relies on the use of long-chain fatty acid compositions followed by nucleic acid typing by means of species-specific selected ribosomal RNA (rRNA) sequences (Sancho et al., 2000). Recently, Esteve-Zarzoso et al. (1999) proposed a rapid and easy method for routine yeast identification based on the restriction analysis of the 5.8S rRNA gene and the internal transcribed spacers (ITS1 and 2). They presented an initial database to identify more than 132 yeast species belonging to 25 different genera, the majority isolated from food and some additional species representing related genera. However, molecular techniques are not yet easy enough to be performed in large scale routine identification of *Dekkera* sp. (Ibeas et al., 1996; Mitrakul et al., 1999). The search for highly specific easily applicable molecular techniques led to the development of peptide nucleic acid (PNA) probes (Egholm et al., 1993), which recently have been applied to a range of novel rapid microbiology applications (Stender et al., 2002). In particular, Stender et al. (2001) described a fluorescence in situ hybridization (FISH) assay using PNA probes targeting 26S rRNA of *D. bruxellensis* which avoids the nucleic acid extraction step. Thus, the objective of this work was to identify the strains able to grow in DBDM medium and assess their ability to produce 4-ethylphenol in synthetic media containing *p*-coumaric acid.

2. Material and methods

2.1. Yeast strains and growth in DBDM medium

The yeast strains tested were either obtained from culture collections or isolated in the ISA laboratory (Table 1). The strains were maintained in GYP medium (20 g l^{-1} glucose) (Merck, Darmstadt, Germany), 5 g l^{-1} yeast extract (Difco Laboratories, Detroit, USA), 10 g l^{-1} peptone (Difco) and 20 g l^{-1} agar, pH 6.0) in which 5 g l^{-1} of calcium carbonate (Merck) was added, at 4°C. Growth characteristics in DBDM medium were evaluated on the basis of medium acidification, colony morphology and detection of phenolic taint by smelling, after incubation at 25°C for up to 14 days (Rodrigues et al., 2001).

2.2. Production of volatile phenols

A loopful of fresh culture (24–48 h) was suspendend in Ringer solution and used to inoculate the YNB medium (Difco) (6.7 g l^{-1}) supplemented with glucose (20 g l^{-1}) and *p*-coumaric acid (100 mg l^{-1}) (Sigma Chemical Co., St. Louis, USA), adjusted to pH 5.4 and filter sterilized. Volatile phenols were measured according to a protocol described by Rodrigues et al. (2001). Briefly, the volatile phenols were extracted by ether-hexan from a 50 ml sample with pH adjusted to 8 with NaOH. The volatile phenols were separated by collecting the organic phase of the mixture. The quantitation was achieved by gas chromatography using a DB-Wax capillary column (J&W Scientific, Folsom, California, USA).

2.3. Determination of cellular fatty acid methyl ester compositions (FAME)

Pure cultures were grown on GYP slants for 24 h at 25°C. Biomass was suspended in 1 ml of Ringer solution (Oxoid, Hampshire, England) and inoculated in triplicate slants of 24 mm diameter tubes with cotton plugs. Incubation was carried out at 25°C for 48 h. Fatty acid extraction, derivatization and quantitation followed the procedures described by Sancho et al. (2000). Statistical analysis of data was performed by principal component and cluster analysis (PCA) using the software SPAD.N version 2.5 PC (Centre International de Statistique et d'Informatique Appliquées, Saint-Mandé, France). In the PCA the fatty acid compositions of identified strains obtained from culture collections were used as active variables while the profiles of yeast isolates were taken as illustrative variables which do not affect the statistical discrimination of the active variables (Anonymous, 1993).

2.4. PNA FISH hybridization

Colonies were smeared onto microscope slides (Erie Scientific, Portsmouth, New Hampshire, USA), heat fixed and analysed by FISH using PNA probes targeting 26S rRNA of *D. bruxellensis* (CGGTCTCCAGC-GATT) as described by Stender et al. (2001). Microscopic examinations were performed using a fluorescence microscope (Dialux 20, Leica Microsystems, Cambridge, United Kingdom) and *D. bruxellensis* was identified as bright fluorescent yeast cells.

2.5. PCR reaction and DNA digestions of the 5.8S-ITS region

Isolated colonies were identified by PCR amplification of the 5.8S-ITS region and subsequent restriction analysis according to Esteve-Zarzoso et al. (1999) using DyNAzyme™ II DNA Polymerase (Finnzymes OY, Espoo, Finland). PCR products and restriction fragments were separated on 1.4% or 3% agarose gels, respectively. CfoI, DdeI, HaeIII and HinfI (Roche Molecular Biochemicals, Mannheim, Germany) were used as restriction endonucleases to identify all yeasts

Table 1
Yeast strains studied

Species	ISA number	Origin[a]
Dekkera bruxellensis	1146	UCD-605
D. bruxellensis	1327, 1328	Sparkling wine[b]
D. bruxellensis	1329, 1331	Cane molasses[c]
Brettanomyces bruxellensis	1649	CBS 72^T, IGC-4179, Lambic beer
D. bruxellensis	1650	IGC-4801, Tea fungus
D. bruxellensis	1655	IGC-5162
D. anomala	1521	UOFS-wy505
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. custersiana	1981	MUCL 27704^T
B. nanus	1985	MUCL 31149^T
B. naardenensis	1721	CBS 6043, IGC-5163, tonic water
B. naardenensis	1722	IGC-5164
B. naardenensis	1723	CBS 6041, IGC-5165, lemonade
Dekkera sp.	1147	UCD-615
Dekkera sp.	1600, 1601	Sparkling wine[b]
Dekkera sp.	1699, 1700, 1701, 1702, 1703, 1704, 1791	Red wine from region C[b]
Dekkera sp.	1717	White wine[b]
Dekkera sp.	1793	Cider[d]
Dekkera sp.	1792, 1794	Wine[d]
Candida halophila	1982	MUCL 29967^T
C. mannitofaciens	1984	MUCL 30038, CBS 5981
C. versatilis	1983	MUCL 30048, CBS 1752
Saccharomyces cerevisiae	1631	IGC-4543^T
Zygosaccharomyces bailli	1307	Sparkling wine production line[b]
Unidentified strains	2001, 2002	Wines from winery 6, region A[e]
	2004	Wine from winery 3, region A[e]
	2010	Wine from winery 4, region A[e]
	2013, 2031, 2032, 2033	Wines from winery 7, region A[e]
	2014 to 2021	Wines from region B[b]
	2027 to 2030	Wines from region C[b]
	2003	Grapes from winery 1, region A[e]
	2005, 2022	Grapes from winery 5, region A[e]
	2006, 2023	Grapes from winery 6[e]
	2007, 2008, 2009, 2012	Insect (*Drosophila* sp.) from winery 1[e]
	2011	Insect (*Drosophila* sp.) from winery 3[e]
	2024	Grape juice from winery 5[e]
	2025	Grape juice pump outlet from winery 5[e]
	2026	Press roll from winery 5[e]

[a] ISA: Instituto Superior de Agronomia, Lisboa, Portugal; IGC: Instituto Gulbenkian de Ciências, Oeiras, Portugal; CBS: Centraalbureau voor Shimmelcultures, Delft, The Netherlands; UCD: University of California, Davis, USA; UOFS: University of the Orange Free State, Bloemfontein, Republic of South Africa; MUCL: Micothéque de la Université Catholique de Louvain, Belgium.
[b] Strains isolated by Instituto Superior de Agronomia (ISA), Lisboa, Portugal.
[c] Strain supplied by Union Nationale des Groupements de Distillateurs d'Alcool (UNGDA), Paris, France.
[d] Strain supplied by Escola Superior de Biotecnologia (ESB), Porto, Portugal.
[e] Strains isolated and supplied by Universidade de Évora (UE), Évora, Portugal.

with negative PNA hybridization. Estimations of fragment lengths were obtained by comparison to 100-bp ladder (Gibco-BRL, Gaithersburg, Maryland, USA). Restriction patterns obtained were compared with those obtained by Esteve-Zarzoso et al. (1999) and Fernández-Espinar et al. (2000).

3. Results

3.1. Growth characteristics in DBDM and production of 4-ethylphenol

The results of yeast growth in DBDM are shown in Table 2. In this medium, *D. bruxellensis*, *D. anomala* and *D. naardenensis* colonies were yellow cream pinpoints,

Table 2
Growth characteristics in DBDM, 4-ethylphenol production and PNA hybridization of analysed strains

Species	Strains	DBDM growth[a]	4-ethylphenol production[b]	PNA hybridization
D. bruxellensis	1146, 1327, 1328, 1329, 1331, 1650, 1655	+	+	+
B. bruxellensis	1649	+	+	+
Dekkera sp.	1147, 1600, 1601, 1699, 1700, 1701, 1702, 1703, 1704, 1717, 1791, 1792, 1794	+	+	+
D. anomala	1521, 1653, 1654	+	+	–
B. anomalus	1652	+	+	–
Dekkera sp.	1793	+	+	–
B. naardenensis	1721, 1722, 1723	+	–	–
D. custersiana	1981	–	–	–
B. nanus	1985	–	–	–
C. halophila	1982	–	+	–
C. mannitofaciens	1984	–	+	–
C. versatilis	1983	–	+	–
S. cerevisiae	1631	–	nd[c]	–
Z. bailli	1307	–	nd[c]	–
Unidentified strains	2001, 2002, 2004, 2013, 2014, 2015, 2017, 2018, 2020, 2021, 2028, 2029, 2030, 2032, 2033	+	+	+
	2005, 2022, 2026	+	+	–
	2003, 2006, 2007, 2008, 2009, 2010, 2011, 2012, 2023, 2024, 2025	+	–	–

[a] DBDM response: +, presence of growth, medium colour change and production of phenolic smell; –, absence of growth or of phenolic smell.
[b] Production of 4-ethylphenol in synthetic medium added of 100 mg l^{-1} *p*-coumaric acid: +, more than 1 mg l^{-1} produced during growth; –, less than 1 mg l^{-1}.
[c] Not determined.

frequently changing to green with incubation time. In addition, media color changed from blue to yellow due to acid production and the production of 4-ethylphenol was detected by its phenolic smell. *D. custersiana* and *B. nanus* did not grow in this medium, in the same way as *C. halophila*, *C. mannitofaciens*, *C. versatilis*, *S. cerevisiae* and *Z. bailii*. The group of unidentified strains originating from natural environments and cellar equipment was tested in DBDM medium and yielded results similar to those of *D. bruxellensis* (Table 2).

The ability to produce 4-ethylphenol is listed in Table 2 for all analysed strains. Typical 4-ethylphenol production and growth kinetics are shown in Fig. 1 for *D. bruxellensis*. The species *D. anomala* presented similar growth and 4-ethylphenol production patterns (Fig. 1). Both species were able to convert *p*-coumaric acid into 4-ethylphenol with an efficiency of about 90% (100 mg l^{-1} of *p*-coumaric acid would yield a maximum of 74 mg l^{-1} of 4-ethylphenol). On the contrary, *D. naardenensis* (Fig. 1), *D. custersiana* and *B. nanus* (results not shown) only produced traces (less than 1 mg l^{-1}) of this volatile phenol and were regarded as weak ethylphenol producers. The species *C. halophila*, *C. mannitofaciens*, *C. versatilis* were able to produce high amounts of 4-ethylphenol (results not shown).

The production of 4-ethylphenol was also assessed for all unidentified strains (Table 2) and representative results of 4-ethylphenol production are shown in Fig. 1. In Table 3 the maximum amounts of 4-ethylphenol produced in synthetic media for these strains are shown. Some of them produced 4-ethylphenol with efficiencies close to those of *D. bruxellensis*. On the contrary, other weak producing strains, although being detected in DBDM by the release of a phenolic smell, were not able to produce significant amounts of 4-ethylphenol (see Table 3).

3.2. Yeast characterization and identification

The FAME compositions were determined for *Dekkera*/*Brettanomyces* and unidentified strains (data not shown). The utilization of PCA and cluster analysis enabled the allocation of identified strains of *Dekkera*/*Brettanomyces* into 4 different groups: (i) Group I, comprising *D. bruxellensis* (21 strains) and several unidentified strains; (ii) Group II, only composed of *B. nanus* (ISA 1985); Group III, comprising *D. anomala* (4

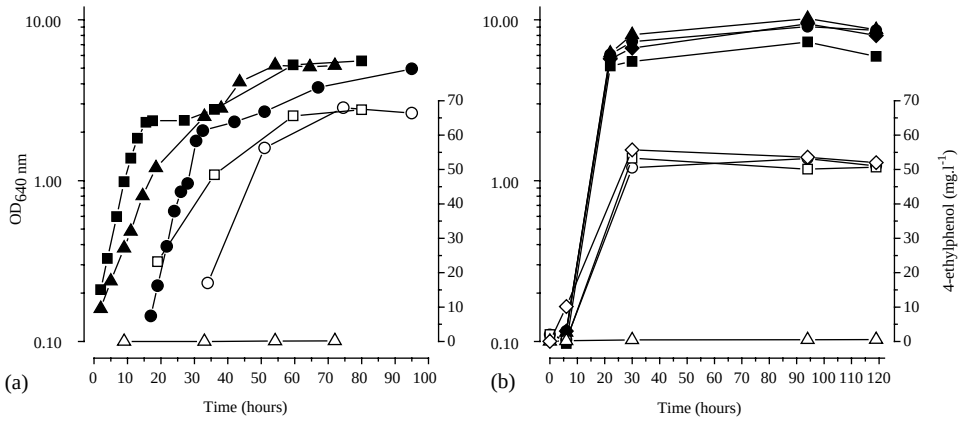

Fig. 1. Growth (filled symbols) and production of 4-ethylphenol (open symbols) of *Dekkera bruxellensis* ISA 1703 (circles), *D. anomala* ISA 1653 (squares) and *D. naardenensis* ISA 1723 (triangles) (A); and *P. guilliermondii* ISA 2005 (losangles), *P. guilliermondii* ISA 2010 (triangles), *P. guilliermondii* ISA 2022 (squares), and *P. guilliermondii* ISA 2026 (circles) (B). Representative results of duplicate experiments.

Table 3
Production of 4-ethylphenol of strains not hybridizing with *D. bruxellensis* species-specific PNA probes

Strains	Origin	Species[a]	Production of 4-ethylphenol ($mg\,l^{-1}$)[b]
2003	Grapes from winery 1	*C. cantarelli*	0.29±0.02 (2)
2005	Grapes from winery 5	*Pichia guilliermondii*	61.5±5.10 (3)
2006	Grapes from winery 6	*P. guilliermondii*	0.48±0.18 (2)
2007	Insect from winery 1	*P. guilliermondii*	0.48±0.11 (2)
2008	Insect from winery 1	*P. guilliermondii*	0.44±0.18 (2)
2009	Insect from winery 1	*P. guilliermondii*	0.35±0.18 (2)
2010	Wine from winery 4	*P. guilliermondii*	0.48±0.11 (3)
2011	Insect from winery 3	*C. cantarelli*	0.04±0.04 (2)
2012	Insect from winery 1	*Kluyveromyces lactis*	0.43±0.20 (2)
2022	Grapes from winery 5	*P. guilliermondii*	52.4±2.54 (2)
2023	Grapes from winery 6	*Debaryomyces hansenii*	0.23±0.19 (2)
2024	Grape juice from winery 5	*C. wickerhamii*	0.01±0.01 (2)
2025	Pump outlet from winery 5	*P. guilliermondii*	0.37±0.08 (2)
2026	Press roll from winery 5	*P. guilliermondii*	51.4±2.54 (2)

[a] See Table 4 for species assignment.
[b] Maximum amount produced during growth (see some experiments in Fig. 1) in culture medium supplemented with 100 $mg\,l^{-1}$ *p*-coumaric acid (results presented as mean±standard deviation, number of repetitions between brackets).

strains) and *D. naardenensis* (ISA 1723); (iv) Group IV, comprising *D. custersiana* (ISA 1981) and *D. naardenensis* (2 strains) (Fig. 2). The unidentified strains originating from wines were clustered in the group composed by *D. bruxellensis* except for strain ISA 2010. None of the strains originating from insects, grapes, grape juice or cellar equipment during harvesting showed FAME profiles similar to those of *D. bruxellensis* in cluster I (Fig. 2). FAME characterization results suggested that these latter strains should not belong to *Dekkera* sp. and further tests using molecular methods were carried out to set their identities.

The results of PNA FISH hybridizations directed to *D. bruxellensis* identifications are shown in Table 2 and Fig. 3 documents the microscopic observation of a positive outcome. All identified *D. bruxellensis* strains yielded positive results. The strains of *Dekkera* sp. showing positive results may then be assigned to *D. bruxellensis*. The unidentified strains clustered together with *D. bruxellensis* by FAME analysis (Group I) also produced positive responses and should be regarded as *D. bruxellensis*. All other identified species tested yielded negative results.

The unidentified strains which did not cluster with *D. bruxellensis* by FAME compositions also did not hybridize with the PNA probe and were identified by restriction analysis of the 5.8S rRNA gene and the internal transcribed spacers (ITS 1 and 2). The ITS1 and ITS4 primers were used to amplify this region as was described previously by Esteve-Zarzoso et al. (1999). In Table 4 are shown the size of PCR products and the restriction fragments obtained by using the different restriction endonucleases CfoI, HaeIII and HinfI. Fragments smaller than 50 bp could not be visualized reliably and are not included in this table. According to these results the unidentified strains were identified as *P.*

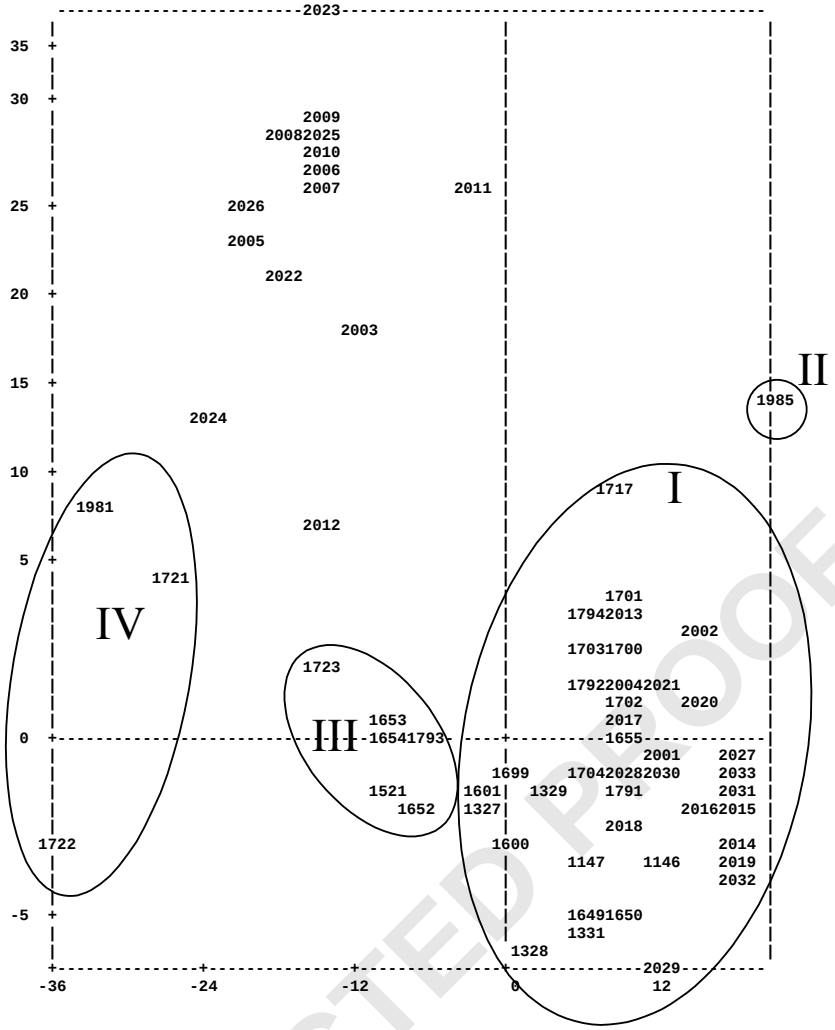

Fig. 2. Projection plan of the PCA of the long-chain fatty acid compositions of yeast strains studied. Illustrative strains are represented by numbers higher than 2000.

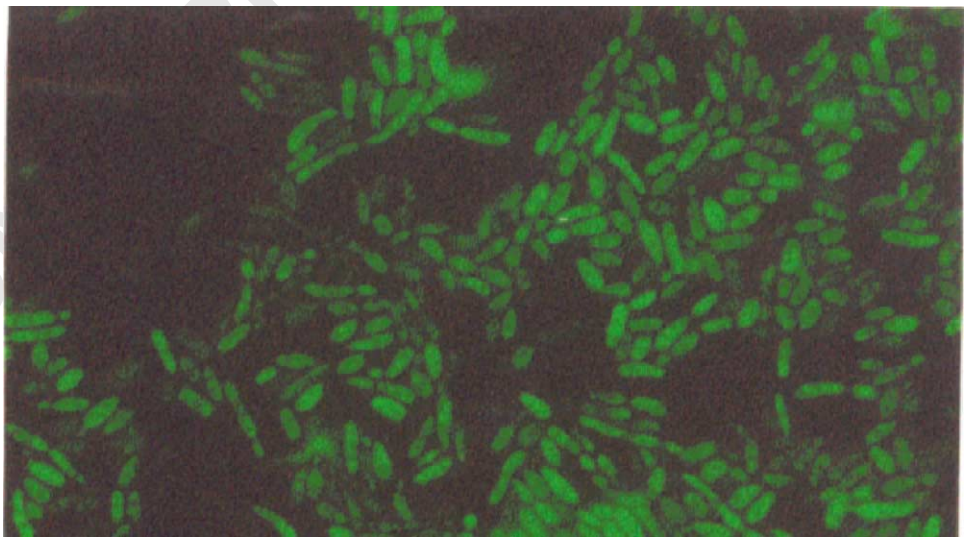

Fig. 3. Smear of pure culture of *D. bruxellensis* ISA 1146 specifically identified by PNA FISH using fluorescein-labelled PNA probes.

Table 4
Strain identification by RFLPs of the 5,8S-ITS region obtained by using the restriction endonucleases CfoI, HaeIII and HinfI

Strain	Length PCR product (bp)	CfoI	HaeIII	HinfI	Species
2003	590	290, 220, 80	500, 90	300, 280	*C. cantarelli*
2005	625	300, 265, 60	400, 115, 90	320, 300	*P. guillermondii*
2006	625	300, 265, 60	400, 115, 90	320, 300	*P. guillermondii*
2007	625	300, 265, 60	400, 115, 90	320, 300	*P. guillermondii*
2008	625	300, 265, 60	400, 115, 90	320, 300	*P. guillermondii*
2009	625	300, 265, 60	400, 115, 90	320, 300	*P. guillermondii*
2010	625	300, 265, 60	400, 115, 90	320, 300	*P. guillermondii*
2011	590	290, 220, 80	500, 90	300, 280	*C. cantarelli*
2012	740	285, 190, 165, 90	655, 80	290, 180, 120, 80, 65	*K. lactis*
2022	625	300, 265, 60	400, 115, 90	320, 300	*P. guillermondii*
2023	650	310, 300	420, 150, 90	330, 330	*Debaryomyces hansenii*
2024	660	600	560, 100	325, 310	*C. wikerhamii*
2025	625	300, 265, 60	400, 115, 90	320, 300	*P. guillermondii*
2026	625	300, 265, 60	400, 115, 90	320, 300	*P. guillermondii*

guilliermondii, C. cantarelli, *C. wickerhamii*, *D. hansenii* and *K. lactis* (see Table 4). The first species was able to convert *p*-coumaric acid into 4-ethylphenol with efficiencies close to those of *D. bruxellensis* unlike the latter species. However, the conversion rates were variable within strains of *P. guilliermondii*.

4. Discussion

The results obtained in this work demonstrated that strains of the species *P. guilliermondii*, *C. cantarelli*, *C. wickerhamii*, *D. hansenii* and *K. lactis* showed the same cultural characteristics as *D. bruxellensis* and *D. anomala* when grown in DBDM. However, some of them differed from these *Dekkera* species concerning the conversion rate of *p*-coumaric acid into 4-ethylphenol. These differences could not be detected by the sensorial smell on DBDM agar because the perception threshold of this compound is rather low. While some strains of *P. guilliermondii* presented high conversion rates, *C. cantarelli*, *C. wickerhamii*, *D. hansenii*, *K. lactis* and some strains of *P. guilliermondii* showed to be weak ethylphenol producers. As far as we are aware this is the first report of *P. guilliermondii* as strong 4-ethylphenol producer. In fact, Chatonnet et al. (1992) did not test this species while *C. halophila*, *C. mannitofaciens* and *C. versatilis* were reported, and confirmed by our results, as 4-ethylphenol producers but were associated with soy sauce making (Suezawa, 1995). These *Candida* species did not grow in DBDM. Therefore, this medium should be regarded as selective and differential either for *Dekkera*/*Brettanomyces* sp. and other yeast species capable of 4-ethylphenol production and associated with wine environments.

The 4-ethylphenol producing strains of *P. guilliermondii*, were recovered from grapes, grape juice and from winery equipment in contact with grape juice, but were not recovered from wines (strains supplied by Universidade de Évora as described in Table 1). Therefore, according to our data and in agreement with data published by Stender et al. (2001), *D. bruxellensis* remains as the sole agents of "phenolic off-flavours" in wines. Further studies should be carried out to elucidate this matter for which combining DBDM and typing or identification techniques such as FAME compositions and/or PNA FISH hybridizations proved to be an adequate strategy for the rapid identification of *D. bruxellensis*. This strategy followed the concept of zymological indicators evaluation (Sancho et al., 2000) and would be rather useful for the wine industry if PNA probes targeting other contaminating species are developed in the future. Then, the role of FAME compositions would be to provide an overall characterization of the contaminant yeast flora, which would lead to the selection of a few species-specific molecular probes to confirm presumptive identifications.

Acknowledgements

This work was supported by Portuguese national projects PAMAF-IED 6043 and AGRO 96. L. Dias received a scholarship under the program PRODEP III. We are grateful to Dr. A. Martini (DVBPG), Dr. I. Spencer (IGC), Dr. J. Jiménez (US), Dr. M. Miniac (UNGA), Dr. P. Laureano (UE), Dr. R. Kunkee (UCD), Dr. T. Hogg (ESB) and Dr. B. Viljoen (UOFS) for kindly supplying yeast strains.

References

Anonymous, 1993. Système portable d'analyse des données numériques (SPAD.N, version 2.5 P.C.). Centre International de Statistique et d'Informatique Appliquées, Saint-Mandé, France.

Chatonnet, P., Dubourdieu, D., Boidron, J., Pons, M., 1992. The origin of ethylphenols in wines. J. Sci. Food Agric. 60, 165–178.

Chatonnet, P., Dubourdieu, D., Boidron, J., 1995. The influence of *Dekkera*/*Brettanomyces* sp. yeasts and lactic acid bacteria on the ethylphenol content of red wines. Am. J. Enol. Vitic. 46, 463–468.

Chatonnet, P., Viala, C., Dubourdieu, D., 1997. Influence of polyphenolic components of red wines on the microbial synthesis of volatile phenols. Am. J. Enol. Vitic. 48, 443–448.

Egholm, M., Buchard, O., Christensen, L., Behrens, C., Freier, S., Driver, D., Berg, R., Kim, S., Norden, B., Nielsen, P., 1993. PNA hybridizes to complementary oligonucleotides obeying the Watson–Crick hydrogen bonding rules. Nature 365, 566–568.

Esteve-Zarzoso, B., Belloch, C., Uruburu, F., Querol, A., 1999. Identification of yeasts by RFLP analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers. Int. J. Syst. Bacteriol. 49, 329–337.

Fernández-Espinar, M., Esteve-Zarzoso, B., Querol, A., Barrio, E., 2000. RFLP analysis of the ribosomal internal transcribed spacer and the 5.8S rRNA gene region of the genus *Saccharomyces*: a fast method for species identification and the differentiation of flor yeasts. Antonnie van Leeuwenhoek 78, 87–97.

Fugelsang, K., 1997. Wine Microbiology. The Chapman & Hall Enology Library, New York.

Ibeas, J., Lozano, J., Perdigones, F., Jimenez, J., 1996. Detection of *Dekkera*/*Brettanomyces* strains in sherry by a nested PCR method. Appl. Environ. Microbiol. 62, 998–1003.

Kunkee, R., Bisson, L., 1993. Wine-making yeasts. In: Rose, A., Harrison, J. (Eds.), The Yeasts, Vol. 5, 2nd Edition. Academic Press, London, pp. 69–127.

Loureiro, V., Querol, A., 1999. The prevalence and control of spoilage yeasts in foods and beverages. Trends Food Sci. Technol. 10-11, 356–365.

Mitrakul, C., Henick-Kling, T., Egli, C., 1999. Discrimination of *Dekkera*/*Brettanomyces* yeast isolates from wine by using various DNA fingerprinting methods. Food Microbiol. 16, 3–14.

Rodrigues, N., Gonçalves, G., Pereira-da-Silva, S., Malfeito-Ferreira, M., Loureiro, V., 2001. Development and use of a new medium to detect yeasts of the genera *Dekkera*/*Brettanomyces* sp. J. Appl. Microbiol. 90, 588–599.

Sancho, T., Giménez-Jurado, G., Malfeito-Ferreira, M., Loureiro, V., 2000. Zymological indicators: a new concept applied to the detection of potential spoilage yeast species associated with fruit pulps and concentrates. Food Microbiol. 17, 613–624 doi:10.1006/fmic.200.0360.

Stender, H., Kurtzman, C., Hyldig-Nielsen, J., Sorensen, D., Broomer, A., Oliveira, K., Perry-O'Keefe, H., Sage, A., Young, B., Coull, J., 2001. Identification of *Dekkera bruxellensis* (*Brettanomyces*) from wine by fluorescence in situ hybridization using peptide nucleic acid probes. Appl. Environ. Microbiol. 67, 938–941.

Stender, H., Fiandaca, M., Hyldig-Nielsen, J., Coull, J., 2002. PNA for rapid microbiology. J. Microbiol. Methods 48, 1–17.

Suezawa, Y., 1995. Studies of halotolerant yeasts in soy sauce making. I. Bioconversions of ferulic and *p*-coumaric acid to volatile phenols by halotolerant yeasts. Nippon Nõgei Kagaku Kaishi 69, 1587–1596.