A simple cultural method for the presumptive detection of the yeasts *Brettanomyces/Dekkera* in wines

J.A. Couto, A. Barbosa and T. Hogg

Escola Superior de Biotecnologia, Universidade Católica Portuguesa, R. Dr António Bernardino de Almeida, Porto, Portugal

ABSTRACT

J.A. COUTO, A. BARBOSA AND T. HOGG.

Aims: The development of a simple and reliable procedure, compatible with routine use in wineries, for the presumptive detection of *Brettanomyces/Dekkera* from wine and wine-environment samples.

Methods and Results: The method of detection of these yeasts employs a selective enrichment medium. The medium contains glucose (10 g l^{-1}) as carbon and energy source, cycloheximide (20 mg l^{-1}) to prevent growth of *Saccharomyces*, chloramphenicol (200 mg l^{-1}) to prevent growth of bacteria and *p*-coumaric acid (20 mg l^{-1}) as the precursor for the production of 4-ethyl-phenol. After the inoculation with wine, the medium is monitored by visual inspection of turbidity and by periodic olfactive analysis. Contaminated wines will develop visible turbidity in the medium and will produce the 4-ethyl-phenol off-odour, which can be easily detected by smelling.

Conclusions: A selective enrichment liquid medium was developed to differentially promote the growth and activity of *Brettanomyces/Dekkera*. The method is simple to execute, employing a simple-to-prepare medium and a periodic olfactive detection.

Significance and Impact of the Study: The characteristics of the procedure make it particularly applicable in a wine-making environment thus presenting important advantages to the wine industry.

Keywords: *Brettanomyces/Dekkera*, detection, volatile phenols, wine, wine industry.

INTRODUCTION

The volatile phenols, 4-ethyl-phenol and 4-ethyl-guaiacol, are aromatic compounds that affect wine quality and their presence in wine is currently an issue of great concern among wine producers. The precursors of these volatile phenols are natural constituents of grape juice and wine, namely the hydroxycinnamic acids *p*-coumaric and ferrulic acids (Heresztyn 1986; Chatonnet *et al.* 1995). Although both 4-ethyl-phenol and 4-ethyl-guaiacol present problems in wine the former is, by far, the major source of concern to the modern wine industry. The transformation of *p*-coumaric and ferulic acids into their undesirable volatile phenols is predominantly associated with the activity of the *Brettanomyces* yeast genus and its ascoporogenous sexual forms classified within *Dekkera* (Heresztyn 1986; Chatonnet *et al.* 1995; Edlin *et al.* 1995). The mechanism of conversion involves a sequence of two enzymatic reactions. In the first, a carboxylase decarboxylates the hydroxycinnamic acids into the corresponding vinyl derivative (4-vinyl-phenol from *p*-coumaric acid or 4-vinyl-guaiacol from ferrulic acid). In the second reaction a reductase converts the vinyl group into the corresponding ethyl compound (Heresztyn 1986; Chatonnet *et al.* 1995). The quality of wine may be affected by concentrations above 425 μg l^{-1} (1 : 10 ratio of 4-ethyl-guaiacol/4-ethyl-phenol in red wines), which is cited as the aroma threshold (Chatonnet *et al.* 1990). However, the limit of olfactory detection of this compound varies according to the individual (human) and the intrinsic aromatic quality and intensity of the wine in question. Once above this limit of detection, 4-ethyl-phenol imparts, amongst other descriptors, 'animal', 'leather' and 'horse sweat' notes to a wine.

Correspondence to: José António Couto, Escola Superior de Biotecnologia, Universidade Católica Portuguesa, R. Dr António Bernardino de Almeida, 4200-072 Porto, Portugal (e-mail: jagc@esb.ucp.pt).

As important economic losses may derive from wine contamination with *Brettanomyces/Dekkera*, efforts towards the monitoring and control of these organisms are imperative. Molecular methods have already been studied to detect and identify *Brettanomyces/Dekkera* in wine, e.g. the development of a PCR method using a genomic DNA fragment of an isolated *Dekkera* strain (Ibeas *et al.* 1996), the development of a fluorescence *in situ* hybridization method using a peptide nucleic acid probe (Stender *et al.* 2000), and the development of a method based on a polymorphism in the rRNA internal transcribed spacer region (Egli and Henick-Kling 2001). A chemical composition approach, based on the differentiation between fermenting and spoilage yeasts in wine, by total free fatty acid analysis was also studied (Rozès *et al.* 1992). These methods all represent advantages in specific applications but all require a level of laboratory sophistication which is rarely available to most wine producers, certainly in routine laboratories. Rodrigues *et al.* (2001) developed a selective and differential solid medium able to recover *Brettanomyces/Dekkera* from wine and related environments, thus giving an important step forward for the elaboration of simpler methods for the detection of these organisms. The proposal of the work presented here is the development of a methodology that can be easily adopted by the wine industry and that it can be used on a routine basis for the detection of *Brettanomyces/Dekkera* in wine and wine contact surfaces.

MATERIALS AND METHODS

Yeast strains

Dekkera bruxellensis PYCC 4801 (Portuguese Yeast Culture Collection, Costa da Caparica, Portugal) and *Dekkera anomala* PYCC 5153 were used in this study.

Medium development

Yeast Nitrogen Base (YNB; Difco) was used as the basis for the development of the culture medium. Several compounds were added to this medium at different final concentrations in order to find the most appropriate level. Glucose was tested at 2, 5 and 10 g l^{-1}, chloramphenicol was tested at 50 and 100 mg l^{-1} and cycloheximide was tested at 10 and 100 mg l^{-1} in agitated cultures by orbital shaking (150 rev min^{-1}) and in static cultures at 30°C. *p*-coumaric acid was added to a final concentration of 10 mg l^{-1}. The pH of the medium was adjusted to 5. The organisms used to inoculate the media were described above. The media were monitored by the visual inspection of turbidity and by daily screening for the odour of 4-ethyl-phenol by a trained panel of six 'tasters'. After determining the most suitable medium composition and incubation conditions, 20 ml of double strength medium were used in 100-ml flasks and were inoculated with 20 ml of the wine sample to be analysed.

Growth curves

Growth, at 30°C, was monitored spectrophotometrically by measuring the increase in the optical density at 650 nm (OD_{650}), against a medium blank, in a UV/VIS Unicam 8620 spectrophotometer (Unicam, Cambridge, UK) using optical cells of 1-cm path length. Samples showing an OD_{650} >0·6 were diluted with sterile medium before measurement to maintain linearity of absorbance and cell mass.

Analysis of volatile phenols in wines

The method used was developed by Bertrand (1981). A volume of 50 μl of 4-decanol (internal standard) at a concentration of 1·073 g l^{-1} was added to 50 ml of wine or culture medium. This mixture was successively extracted with 4, 2 and 2 ml of ether/hexane (1 : 1, v/v) by stirring for 5 min. The organic phases were collected, mixed, and 2 μl of the extract was injected into a Perkin Elmer GC-FID (Perkin Elmer, Shelton, CT, USA). The column employed was a FFAP type (BP 21, 50 m × 0·25 mm × 0·2 μm, SGE). The injector (split/splitless) was heated to 250°C with a splitless time of 0·3 min. The carrier gas flow was adjusted to 1 ml min^{-1}. The temperature of the oven was maintained at 40°C for 5 min after the injection, and was then increased at a rate of 2°C min^{-1} up to 220°C. This temperature was then maintained for 20 min. The error of the method is ±10 μg l^{-1}.

Detection limit and time response of the culture medium

A dense culture, *c.* 10^8 CFU ml^{-1} of *D. bruxellensis* PYCC 4801 grown in YM (Difco) was used to prepare, by dilution in Ringer solution (Lab M, Bury, UK), several different concentrations, from *c.* 10^6–10^0 cells ml^{-1}, of this organism. Viable cells were enumerated by spread plating decimal dilutions (in sterile 9 ml Ringer solution) on YM agar (Difco). Counts were made after incubation at 25°C for 72 h. The different cell concentration solutions were used to inoculate the YNB-based medium as described above. Inoculated media were followed by visual inspection of turbidity and by detecting the odour of 4-ethyl-phenol everyday. The number of days needed to obtain positive results was recorded.

Relationship between the microbiological analysis (detection of *Brettanomyces/Dekkera*) and the chemical analysis of volatile phenols in wines

A total of 62 wines from different wine companies were analysed both in terms of the presence of *Brettanomyces/*

Dekkera and of volatile phenols (4-ethyl-phenol and 4-ethyl-guaiacol) concentration. After a period of between 2 and 8 months, these compounds were again analysed in fresh samples of the same wines. The results were compared and correlated with those of the first analysis. The wines were kept in the wineries between the two sampling dates.

RESULTS

Various configurations of broth were tested, all having the YNB medium as the basis. Glucose was added to this medium as energy and carbon source. Among the concentrations tested (2, 5 and 10 g l^{-1}), the level of 10 g l^{-1} led to the formation of relatively high levels of acetic acid, via yeast sugar metabolism, as pointed out by the panel of tasters, and did not lead to a faster response than the level of 5 g l^{-1} [6 days for 5 and 10 g l^{-1} of glucose and 7 days for 2 g l^{-1} (assays performed using contaminated wines as inoculum)]. The concentration of 5 g l^{-1} of glucose was found to be a good compromise between the production of acetic acid, which may interfere with the olfactory detection of the 4-ethyl-phenol odour, and the growth kinetics of the organisms. Chloramphenicol was added to inhibit bacterial growth; the concentration of 100 mg l^{-1} being the most suitable as in some cases, 50 mg l^{-1} permitted bacterial growth, as revealed by the direct observation of media under the bright-field microscope. A very well-known characteristic of *Brettanomyces/Dekkera* is an elevated tolerance to cycloheximide (Barnett *et al.* 1990). Therefore, this compound is used in the medium in order to repress the growth of other yeasts, including *Saccharomyces cerevisiae*. It is known that *Hanseniaspora* is also relatively tolerant to cycloheximide, but this genus is more likely to be present in the initial stages of the alcoholic fermentation (Cianni and Picciotti 1995; Charoenchai *et al.* 1998), thus not being expected to be an important contaminant of the final product. Cycloheximide was tested at 10 and 100 mg l^{-1}, under static or agitated incubation at 30°C. In Fig. 1, showing the growth curves obtained for *D. bruxellensis* PYCC 4801, it can be observed that the growth is considerably faster and that a higher final biomass is achieved in the agitated medium containing 10 mg l^{-1} of cycloheximide than in static cultures with 100 mg l^{-1} of this compound. A similar behaviour was obtained for *D. anomala* PYCC 5153. As reported previously, oxygen stimulates the growth of *Brettanomyces/Dekkera* (Lonvaud-Funel 2000), and seems to be an important factor in accelerating the growth of these organism. The selectivity conferred by the addition of 10 mg l^{-1} of cycloheximide was found appropriate to reduce the interference of other yeasts (data not shown). This is corroborated by the studies of Rodrigues *et al.* (2001), who demonstrated that the concentration of 10 mg l^{-1} of cycloheximide permits the growth of *Brettanomyces/Dekkera* strains and inhibits growth and activity of most other wine yeasts. After these experiments it was established that the liquid culture medium would have the following composition: YNB (double strength), glucose (10 g l^{-1}), *p*-coumaric acid (20 mg l^{-1}), cloramphenicol (200 mg l^{-1}), cycloheximide (20 mg l^{-1}). The pH is adjusted to 5 and the cultures are incubated at 30°C under agitation. The medium is inoculated with an equal volume of the wine sample (1 : 1 dilution), e.g. 20 ml, which brings these concentrations to half their original values.

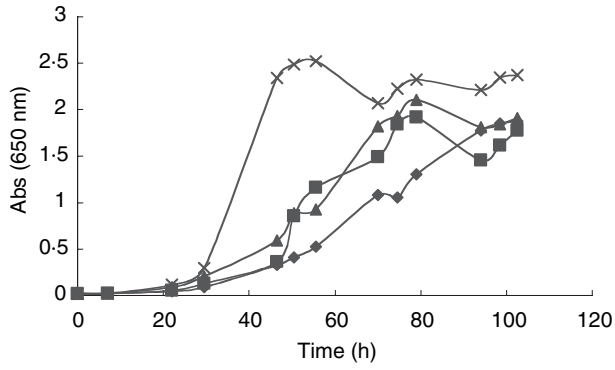

Fig. 1 Growth curves obtained for *Dekkera bruxellensis* PYCC 4801 grown under the following conditions: 10 mg l^{-1} of cycloheximide in agitated culture (150 rev min^{-1}) (×), 100 mg l^{-1} of cycloheximide in agitated culture (150 rev min^{-1}) (■), 10 mg l^{-1} of cycloheximide in static culture (▲), 100 mg l^{-1} of cycloheximide in static culture (◆)

The influence of the cell density of samples in the time needed to obtain a positive result was studied. Table 1 shows the results obtained from three separate assays in which different levels of *Dekkera* were used to inoculate the liquid culture medium described above. It is shown that the response time is greatly affected by the level of contamination of the solutions. Elevated cell densities (10^4–10^6 cells ml^{-1}) allowed the detection of positive results (visible turbidity and the detection of the 4-ethyl-phenol odour) in 2–3 days. Lower concentrations require more time. For low concentrations (1–10 cells ml^{-1}) positive results were detected after 7–10 days.

Having selected a formulation, the methodology proposed in this work was compared with that of Rodrigues *et al.* (2001) which is based on the inoculation of a solid differential medium, used as a reference method. The data obtained (Table 2) show that there is a good agreement between the two methodologies in terms of both positive and negative results.

The liquid medium was then trialled with a set of 62 red wines (barrel or tank samples) from various Portuguese viticultural regions and a number of separate companies. The relationship between the microbiological analysis (detection of *Brettanomyces/Dekkera*) and the chemical

Table 1 The influence of yeast concentration in the time needed to detect the production of 4-ethyl-phenol in the culture medium

D. bruxellensis PYCC 4801 (number of cells ml^{-1})	Time needed to obtain a positive result (days)
Assay 1	
$2{\cdot}8 \times 10^6$	≤3
$2{\cdot}8 \times 10^5$	≤3
$2{\cdot}8 \times 10^4$	3
$2{\cdot}2 \times 10^3$	4
$3{\cdot}3 \times 10^2$	5
25	7
2·7	7
0·5	10
Assay 2	
$4{\cdot}7 \times 10^6$	1
$4{\cdot}7 \times 10^5$	2
$4{\cdot}7 \times 10^4$	3
$4{\cdot}7 \times 10^3$	4
$6{\cdot}3 \times 10^2$	5
39	9
7	9
Assay 3	
27	6
7	6
1·4	10
0·2	ND*

Results of three separate assays with *Dekkera bruxellensis* PYCC 4801 are shown.
*Not detected.

Table 2 Comparison between the proposed liquid medium and the solid medium *Dekkera/Brettanomyces* differential medium (DBDM) of Rodrigues *et al.* (2001)

Sample	Result obtained in the liquid medium*	Counts in DBDM (number of CFU)†
1	− (in 20 ml)	0/100 ml
2	− (in 20 ml)	0/100 ml
3	+ (5 days)‡	$5{\cdot}7 \times 10^3$ ml^{-1}
4	+ (5 days)	$2{\cdot}6 \times 10^2$ ml^{-1}
5	− (in 20 ml)	0/100 ml
6	− (in 20 ml)	0/100 ml
7	− (in 20 ml)	0/100 ml
8	− (in 20 ml)	0/100 ml
9	− (in 20 ml)	0/100 ml
10	− (in 20 ml)	0/100 ml
11	− (in 20 ml)	0/100 ml
12	− (in 20 ml)	0/100 ml
13	− (in 20 ml)	0/100 ml
14	+ (5 days)	$1{\cdot}5 \times 10^2$ ml^{-1}
15	− (in 20 ml)	0/100 ml
16	− (in 20 ml)	0/100 ml
17	+ (6 days)	12 ml^{-1}
18	− (in 20 ml)	0/100 ml
19	− (in 20 ml)	0/100 ml
20	+ (5 days)	$1{\cdot}1 \times 10^2$ ml^{-1}
21	+ (9 days)	0·9 ml^{-1}

*After 10 days of incubation.
†After 14 days of incubation.
‡Time of the detection of positive result.

analysis of volatile phenols was evaluated in these wines. Table 3 presents the levels of 4-ethyl-phenol and 4-ethyl-guaiacol found in wines that gave negative results in the microbiological analysis (i.e. not contaminated with *Brettanomyces/Dekkera*). The microbiological analysis was performed at the same time as the first chemical analysis. It can be seen that, from the 47 wines analysed, 45 kept stable in respect to the composition of volatile phenols in the period between the two analyses. Even in the wines containing high initial levels of volatile phenols, the amount of these compounds did not increase in the time elapsed until the second analysis. This observation indicates that the organisms responsible for the production of the detected levels of volatile phenols might be no longer viable. The wines 35 and 43 showed a considerable increase in the amount of the volatile phenols, as detected 4 months after the first analysis, in spite of the negative result of the microbiological analysis. Large volumes of wine (*c.* 10 000 l) in tanks or barrels will always represent a challenge in terms of microbiological sampling, low levels of contamination or localized pockets of unmixed wine might lead to the non-detection of viable cells even in wines which will eventually develop spoilage. It is also true that barrels and tanks were stored in company's facilities between samplings. Although all companies agreed to collaborate in the project, we cannot be certain if the cellar staff made unauthorized movements of wines, which might have led to the contamination. Table 4 shows the results obtained for the wines that gave a positive result in the microbiological analysis. Twelve of the 15 wines showed a considerable increase in the amount of the volatile phenols, even in those wines which contained very low levels of volatile phenols in the first analysis (samples 44 and 67), thus, presumably, carrying low levels of microbiological contamination at that time. The other three wines (samples 25, 53 and 59), in spite of the positive result in the microbiological analysis, did not show an increase in the volatile phenols. It is possible that certain wine and environmental parameters (pH, SO_2 and temperature) may impede the growth or inhibit the metabolic activity of the *Brettanomyces/Dekkera* cells present in these wines or the concentration of precursors may be limiting.

DISCUSSION

A selective enrichment broth was developed to differentially promote the growth and activity of *Brettanomyces/Dekkera*.

Table 3 Variation in the concentration of 4-ethyl-phenol (4EP) and 4-ethyl-guaicacol (4EG) in the wine samples in which the result of the microbiological analysis was negative

Wine	First analysis 4EP–4EG ($\mu g\ l^{-1}$)	Second analysis 4EF–4EG ($\mu g\ l^{-1}$)	Time between the two analysis (months)
10	ND–ND	ND–ND	4
11	ND–ND	ND–ND	4
12	ND–ND	ND–ND	4
54	ND–ND	ND–ND	8
59	31·6–ND	25·9–ND	8
21	11·9–ND	14·1–ND	4
22	ND–ND	ND–ND	4
23	ND–ND	ND–ND	4
24	ND–ND	ND–ND	4
27	24·1–ND	25·4–ND	4
28	41·3–ND	50·1–ND	4
29	19·6–ND	19·2–ND	4
30	28·3–ND	52·3–ND	4
31	ND–ND	ND–ND	4
32	ND–ND	27·9–ND	4
33	ND–ND	33·6–ND	4
34	11·7–ND	31·7–ND	4
35	ND–ND	100·3–14·5	4
36	ND–ND	ND–ND	4
37	ND–ND	40·1–ND	4
38	ND–ND	17·1–ND	4
39	ND–ND	ND–ND	4
40	ND–ND	ND–ND	4
41	ND–ND	ND–ND	4
42	ND–ND	ND–ND	4
43	ND–ND	836·7–142·6	4
CA40	ND–ND	ND–ND	2
CA41	ND–ND	ND–ND	2
CA48	10·6–ND	19·4–ND	2
CA50	11·6–ND	16·9–ND	2
CAL37	411·0–90·2	428·0–96·2	2
S4	ND–ND	ND–ND	4
57	41·6–ND	27·0–ND	8
60	1799·9–176·5	1344·6–101·7	8
62	3008·0–318·8	2143·5–259·2	8
63	ND–ND	ND–ND	8
64	256·3–32·5	192·5–22·0	8
77	250·4–24·2	44·4–ND	8
78	206·2–23·3	59·8–ND	8
79	167·2–20·2	55·5–ND	8
80	203·7–27·2	82·2.– ND	8
81	246·3–29·4	145·4–11·5	8
82	278·1–27·9	164·2–26·9	8
65	82·8–23·5	62·4–19·4	8
68	56·9–17·5	35·2–ND	8
QT	ND–ND	ND–ND	3
CT	ND–ND	ND–ND	3

ND, not detected.

Table 4 Variation in the concentration of 4-ethyl-phenol (4EP) and 4-ethyl-guaicacol (4EG) in the wine samples in which the result of the microbiological analysis was positive

Wine	First analysis 4EP–4EG ($\mu g\ l^{-1}$)	Second analysis 4EF–4EG ($\mu g\ l^{-1}$)	Time between the two analysis (months)
53	54·5–ND	33·5–ND	4
55	353·5–47·2	376·2–50·6	4
60	34·2–ND	3158·8–302·1	4
25	ND–ND	ND–ND	4
44	ND–ND	555·2–120·1	4
45	856·1–182·9	2039·3–435·8	2
46	113·2–32·7	562·2–93·1	2
S5	107·8–32·9	225·9–73·7	4
47	2755·7–468·9	4496·0–619·7	4
48	1537·9–223·4	3025·0–442·7	4
58	66·2–24·6	821·42–117·1	8
59	671·2–93·6	648·0–101·7	8
61	1367·9–246·5	1676·6–330·9	8
66	73·1–18·7	261·5–44·9	8
67	21·5–ND	643·6–159·6	8

ND, not detected.

The precursor of 4-ethyl-phenol was incorporated in a concentration high enough to favour as strong an aromatic signal as possible. The use of sensory detection, as the primary method of attributing positive results, is the major innovation, this being particularly compatible with the practices of wine-making, even in those cases which do not have microbiology laboratory facilities. A validation test was made by comparing the performance of the methodology proposed in this work with that of Rodrigues *et al.* (2001) assumed for this purpose, as a reference method. A good agreement was found between the two methodologies. The reason for the apparent false-negative result obtained in two wines may be related to problems with sampling. Wine contamination, because of the eventual manipulation during the interval between samplings, can also be considered as an explanation. The decrease in the concentration of the volatile phenols noticed in some wines could be due to a dilution effect with wine containing less of these compounds, such as might occur in barrel topping or blending.

In comparison with other published methods, the one presented here presents certain characteristics which make it particularly applicable in a hygiene management role in a wine-making environment. In the first place, the method is very simple to execute, employing a simple-to-prepare medium and a periodic olfactive detection. Another important aspect is that relatively large volumes of wine can be used as inoculum, so aiding sensitivity. As an aid in stop/go decisions in cellar hygiene, the lack of rapidity is counterbalanced by the low cost and simplicity. It should be noted

that *Brettanomyces/Dekkera* activity would not be expected to change wines from unspoiled to a spoiled status in a period of days.

ACKNOWLEDGEMENT

Patrícia Peixoto is thanked for helpful technical assistance.

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