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*Full Length Research Paper*

# Identifying yeasts belonging to the *Brettanomyces/Dekkera* genera through the use of selective-differential media

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**The purpose of this work was to compare and optimise different selective and differential media to aid in isolating spoilage yeasts belonging to the *Brettanomyces/Dekkera* genera. Growth media containing selective and differential factors were employed. These were inoculated with strains of yeast representing Spanish oenological microbiota. Lastly, some of these isolation media were successfully applied in 24 types of wine with a high ethylphenol content, all of which were from the Haro Oenological Station (La Rioja, Spain). *p*-coumaric acid was determined using High performance liquid chromatography-photodiode-array detection-electrospray ionization mass spectrometry (HPLC-DAD-ESI/MS); 4-ethylphenol by using Solid phase micro extraction-gas chromatography-mass spectrometry (SPME-GC-MS); and the rest of the analysis was carried out using official OIV methodology. Actidione is the most effective selective factor for isolating *Brettanomyces/Dekkera* yeast genera. Other secondary selective factors (selective carbon sources, sorbic acid and ethanol as a microbicide agent) may be used successfully to eliminate potential false positivities; however, they slow growth and delay the time to obtain results.**

**Key words:** *Brettanomyces*/*Dekkera*, selective-differential medium, 4-ethylphenol, red wine.

## INTRODUCTION

Currently, the organoleptic deterioration of wine caused by the formation of volatile ethylphenols (metabolic products of *Brettanomyces* and *Dekkera* species) is a serious economic problem. Most studies describe the presence of *Dekkera/Brettanomyces* yeast genera in wine as significantly smaller than that of other genera (Loureiro and Malfeito-Ferreira, 2006). This may be due to it having a slower growth rate than other competing species. However, these yeasts appear in specific situations in which nutrients are scarce, which denotes them as nutritionally undemanding (Suárez et al., 2007). Isolating *Dekkera/Brettanomyces* by conventional media is nearly unviable because other competing species of bacteria, yeasts and fungi have a faster growth rate. This gives rise to the need for employing selective-differential media based on the ecophysiological characteristics of these species and of their main competitors.

The classical selective media employed for isolating yeasts are based on the use of bacteria-inhibiting

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antibiotics such as oxytetracycline, chlorotetracycline, gentamicin or chloramphenicol (Fugelsang and Edwards, 2007) and inhibitors of other yeast species such as actidione (Kurtzman and Fell, 1998; Couto et al., 2005).

Other culture media are based on selective nitrogen sources that can not be assimilated by *Saccharomyces cerevisiae* (Heard and Fleet, 1986). Other inhibitory agents such as sorbates and benzoates (Pitt and Hocking, 1985) are used to isolate spoilage species such as *Zygosaccharomyces bailii*. Described examples among the differential factors include bismuth sulphite, which indicates the presence of yeasts producing hydrogen sulphide (Jiranek et al., 1995; Rupela and Tauro, 1984). When isolating lactic bacteria, cyclohexadiene and spiramycine-type selective factors are used to inhibit yeast and fungi (Wibowo et al., 1985; Garijo et al., 2009). When isolating acetic bacteria, selective factors such as pimaricine are used to inhibit yeasts and fungi, whereas penicillin is used to inhibit lactic bacteria (Ruiz et al., 2000). Selective carbon sources such as ethanol (Bartowsky et al., 2003) or acetic acid and ethanol, are used as microbicide agents for competing species (Entani et al., 1985).

The selective media applied to detect *Dekkera/Brettanomyces* are fundamentally based on its high resistance to the antibiotic actidione (Van der Walt and Van Kerken, 1960; Chatonnet et al., 1992; Boulton et al., 1996; Alguacil et al., 1998; Mitrakul et al., 1999; Rodrigues et al., 2001; Fugelsang and Edwards, 2007; Garijo et al., 2008), sorbic acid, selective carbon sources such as maltose, trehalose and saccharose (Van der Walt and Van Kerken, 1960; Chatonnet et al., 1992) or ethanol (Rodrigues et al., 2001). Also ethanol is used as an antimicrobial agent (10%) in yeast isolation (Alguacil et al., 1998). The rest of the commercial media or other media in use are based on the above or similar factors. Their advantages and disadvantages are described in Table 1. The main disadvantages of these media are the false positives caused by actidione-resistant yeasts such as *Candida parapsilosis, C. tropicales, Kloeckera apiculata, Schizosaccharomyces pombe and Pichia guilliermondi* (Figure 1), contamination by opportunistic fungi in solid media, and the time to obtain results (Benito et al., 2009a). In order to mitigate these undesired effects, we used differential factors such as hydroxycinnamic acids, which indicate the presence of yeasts with the ability to generate ethylphenols and bromocresol green, which indicates the presence of yeasts that produce acetic acid (Rodrigues et al., 2001; Couto et al., 2005).

Once yeasts belonging to *Dekkera/Brettanomyces* genera are isolated, the classical criteria for identifying a species according to its morphological and physiological characteristics (Phaff, 1984; Barnett et al., 2000; Kurtzman and Fell, 1998) often give rise to errors. For this reason, it is necessary to draw on other, more modern methods based on molecular biology, or include new identification tests such as the rates for *p*-coumaric acid conversion into 4-ethylphenol.

## MATERIALS AND METHODS

### Media preparation and microbiological growth conditions

In this study, we used various solid and liquid selective-differential media to isolate *Brettanomyces/Dekkera*. Culture media were sterilised in an autoclave during 15 min at 121ºC, and the bromocresol green, antibiotics, sorbic acid and p-coumaric acid were subsequently added in an ethanolic solution. Later each medium was placed in sterile glass Petri dishes in quantities of 20 ml for solid media, and for liquid media, 50 ml in flasks. Each medium was verified using cultures of different yeast strains streaked out on each dish using superficial inoculation. The tests were carried out in triplicate. After inoculation, the dishes were incubated isothermally at 25ºC during 15 days.

### Yeasts indicating efficacy of media

The yeast strains used as positive controls were obtained from collections of sample cultures of different Spanish organisms as listed in Table 2. These include genera and species which often produce false positives when detecting *Brettanomyces/Dekkera*.

### Media used to evaluate selective factors

Seventeen selective-differential media were used in this study; their composition is described in Table 3. Media pH was adjusted by adding orthophosphoric acid. Glucose, maltose, threhalose, saccharose (J. T. Baker Chemicals B.V., Denventer, Holland), nitrogenated base, bacteriological peptone, yeast extract (all from Pronadisa, Madrid, Spain), *p*-coumaric acid (Fluka, Steinheim, Switzerland), ethanol, potassium sorbate, orthophosphoric acid (all from Panreac, Barcelona, Spain), bromocresol green (Sigma-Aldrich, St. Louis, USA) and chloramphenicol (Sigma-Aldrich, St. Louis, USA).

### Media used in microbiological analysis of altered wine

The Yeast extract peptone dextrose (YEPD/ACT) medium (150 ml) (Table 4) was mixed with 150 ml of each altered wine from the Haro Oenological Station containing abnormally high ethylphenol levels. Another 150 ml wine was filtered through sterile 0.45 µm membranes (Millipore, Billerica, USA) under aseptic conditions and the filter paper deposited on the YEPD/ACT agar plate. Following that, 100 µl of each liquid medium considered to be positive (positive vinylphenol reductase activity) and 1000 µl of each negative liquid medium were inoculated into the solid Dekkera/Brettanomyces differential (DBDM) medium (Table 4) (considered to be most selective for isolating *Dekkera/Brettanomyces*) in a Petri dish.

### Identification of yeasts isolates by HPLC, GC-MS

The classical tests for sugar assimilation and fermentation were used (Phaff, 1984; Barnett et al., 2000; Kurtzman and Fell, 1998). In addition, we introduced two additional tests; resistance to actidione and bioconversion of *p*-coumaric acid to 4-ethylphenol. The performance of each 4-ethylphenol producing strain was assessed by testing for *p*-coumaric acid bioconversion in liquid

**Table 1.** Comparative study on selective and differential media for yeasts of the *Dekkera/Brettanomyces* genera.

| Medium | Selective-differential factors | Advantages | Disadvantages | Possible Improvements | False positives |
|---|---|---|---|---|---|
| **BSM Solid** (Millipore) | Actidione<br>Chloramphenicol<br>Chlorotetracycline<br>Gentamicin | Relatively fast (4 to 6 days) | Not differential<br>False positives<br>(Resistance to actidione)<br>Fungal growth (solid) | Ethanol (microbicide)<br>*p*-coumaric acid<br>Bromocresol green<br>Liquid application | *Pichia guillermondii*<br>*Kloeckera apiculata*<br>*Schizosaccharomyces pombe*<br>*Candida parapsilosis* |
| **DBDM** Rodrigues (2001) | Actidione<br>p-coumaric acid<br>Bromocresol green<br>Ethanol (source of carbon)<br>No carbohydrates | Most selective medium for *Dekkera/Brettanomyces,*<br>Ecological importance (strain isolation) | Slow for industrial use (>15 days)<br>Fungi (solid) | Chloramphenicol<br>Carbohydrates(speed) | *Pichia guillermondii* |
| **Liquid** Couto et al. (2005) | Actidione<br>Chloramphenicol<br>*p*-coumaric acid | Relatively fast<br>(4 to 6 days *f*(CFU/ml)<br>Less important for false positives (industrial use) | No direct measurements<br>No isolation medium | Ethanol (microbicide)<br>Bromocresol green | |
| **DHSA** Chatonnet et al. (1992) | Actidione, penicillin, gentamicin<br>Ethanol (microbicide)<br>Trehalose, Saccharose<br>Sorbic acid<br>Bromocresol green<br>Nutrients | Relatively fast (5 to 8 days *f*(CFU/ml) | Fungi (solid)<br>Complex preparation (micronutrients) | Liquid application<br>p-coumaric acid | *Pichia guillermondii*<br>*Schizosaccharomyces pombe*<br>*Candida parapsilosis* |

YEPD containing 100 mg/l of *p*-coumaric acid using HPLC-DAD-ESI/MS.

**Detection of ethylphenols in altered wines from the haro oenological station (La Rioja, Spain)**

Ethylphenols was quantified by solid phase microextraction gas chromatography MS (SPME/GC/MS) with single ion monitoring (SIM) detection. An 85 µm polyacrylate film fibre in a solid phase microextraction holder (Supelco, Bellefonte, USA) was used for the extraction of the samples. Samples of 1900 µl were mixed with 100 µl of a 10000 µg/l 3,4-dimethylphenol internal standard (Merck, Hohenbrunn, Germany) to which 1 g NaCl was added and heated to 57ºC for 40 min with magnetic stirring. Agilent J and W scientific column (Folsom, California, USA) was used in gas chromatography, which involved an Agilent Technologies 6890N apparatus with an MSD-5973N mass spectrometry detector. Separations were performed in a DB-WAX column (30 m × 0.25 mm internal diameter × 0.25 µm film thickness) using splitless injection. The fibre was allowed a total 3 min desorption time, adhering to the following temperature program: 60ºC for 1 min, 10ºC/min ramp until 150ºC, followed by a 3ºC/min ramp to 210ºC for 20 min. The helium flow rate was 1 ml/min. Detection was performed using SIM mode mass spectrometry (4-ethylphenol ion target: 107; 4-ethylguaiacol ion target: 137). Calibration patterns were produced using Alfa Aesar products (Karlsruhe, Germany) at concentrations of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1 mg/l. The rest of the analyses were carried out according to the Compendium of International Methods of Analysis of Wines and Musts (OIV, 2005).

**Monitoring and detection of *p*-coumaric acid and 4-ethylphenol by HPLC-DAD-ESI/MS in liquid selective-differential media**

The phenols in the wines were analysed using an Agilent Technologies 1100 (Palo Alto, CA, USA) HPLC equipped

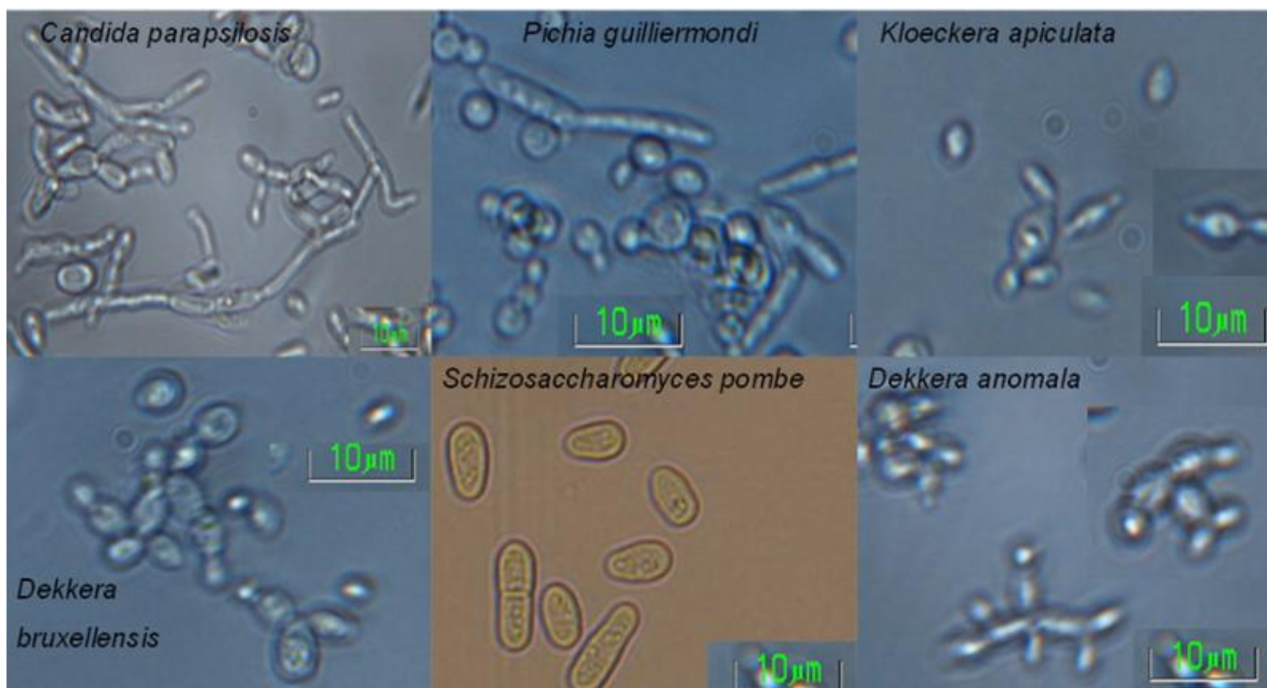


**Figure 1.** Actidione-resistant species belonging to different collections of model Spanish cultures.

with a quaternary pump, an autosampler and a photodiode-array detector. Gradients of solvent A (water/formic acid, 90 10, v/v) and B (methanol/formic acid, 90 10, v/v) were used in a reverse-phase Nova-Pak. C18 column (300 × 3.9 mm) as follows: 10 to 50% B linear (0.8 ml min$^{-1}$) from 0 to 25 min, 50 to 10% B linear (0.8 ml min$^{-1}$) from 25 to 30 min, re-equilibration of the column from 30 to 33 min. Detection was performed by scanning in the 200 to 400 nm range.

Quantification was performed by comparison against an external standard at 320, 280 and 260 nm and expressed as a function of the concentration of *p*-coumaric, 4-ethylphenol and 4-vinylphenol (all from Extrasynthe`se, Genay, France), respectively. Volumes of 10 µl previously filtered through an acetate methylester membrane (pore size 0.45 µm) (Teknokroma, Barcelona, Spain) were injected into the HPLC apparatus.

## RESULTS AND DISCUSSION

### YEPD medium enriched with actidione, ethanol, chloramphenicol and bromocresol green

After five days, all of the strains of *Dekkera* studied in a YEPD/ACT/BG medium (Table 2; *D. bruxellensis*, *D. anomala*, *K. apiculata*, *H. uvarum*, *P. guilliermondi*, *S. pombe)*, and two strains of *Candida parapsilosis* out of the six studied (Figure 1) had grown. Thereafter, the yeasts identified as belonging to the above species were cultured in a YEPD medium enriched with 10 and 100 mg/l of actidione as a sole selective factor. In this last medium, it was verified that the strains studied are resistant to the actidione selective factor in these concentrations. Actidione is employed in most commercial selective media (Chatonnet et al., 1992; Rodrigues et al., 2001; Couto et al., 2005), implying that the yeasts species identified during this study could give rise to false positives at the industrial level (Figure 1 and Table 1). The resistance to the selective factor depended on the strain. Despite its rapid growth in the control medium without actidione, *C. parapsilosis*, presented the slowest growth out of all the yeasts studied. We did not detect any growth in the rest of the strains that were studied under these conditions. This justifies the use of actidione as the principal selective agent for isolating yeasts belonging to the *Dekkera/Brettanomyces* genera, while also offering a more in-depth interpretation of the results and their reliability. This result is a call to optimise currently-employed media by searching for, combining and improving other secondary selective factors in order to inhibit these potential false positives.

### Media enriched with secondary selective factors

Actidione-resistant yeasts (Figure 1) were cultured in solid YEPD with increasing percentages of ethanol. They did present growth, but at a slower rate than in the control media without ethanol, which is probably due to aerobiosis conditions or ethanol evaporation. However, in a liquid medium, the *Kloeckera apiculata* and *Hanseniospora uvarum* strains present no growth for the 8% v/v ethanol volume. It is therefore valid in this case as

**Table 2.** Type strains used as positive controls.

| Species | Strains | Origin |
|---|---|---|
| *Candida krusei* | 1245, 1250, 1251, 1253, 1255 | IFI |
| *Candida parapsilosis* | 1341, 1342, 1338, 1355, 1339, 1337, 1336, 1341 | IFI |
| *Candida pulcherrima* | 1200, 1198, 1199, 1204, 1205, 1206, 1207, 1209, 1210. | IFI |
| *Dekkera anomala* | CB52, CB60, CB61 | IFI |
| *Dekkera bruxellensis* | D35, D36, D37, 2400, CB63, 6802, R3, 7801 | IFI |
| | 6802, R3, 7801 | ETSIA |
| *Hanseniospora uvarum* | 899, 898, 910 | IFI |
| *Hansenula anomala* | 925, 926, 927, 929, 932, 933, 934, 1114, 1115, 1117, 1118, 1119, 1120 | IFI |
| *Hansenula holstii* | 943, 944, 945. | IFI |
| *Hansenula polymorpha* | 1128 | IFI |
| *Hansenula saturnus* | 931 | IFI |
| *Hansenula subpelliculosa* | 1123, 1124, 1125. | IFI |
| *Kloeckera apiculata* | 1010, 1015, 1059, 1045, 1065 | IFI |
| *Pichia guilliermondi* | 962 | IFI |
| | 513 | ETSIA |
| *Pichia membranifaciens* | 946, 947, 948, 949, 950, 951, 952, 954, 956 | IFI |
| *Saccharomyces bayanus* | 697 | IFI |
| *Saccharomyces cerevisiae* | 87, 88, 89, 90, 211, 212, 213, 2202, 2203, 2205 | IFI |
| | 7V, 9CV, S6U | ETSIA |
| *Saccharomyces pastorianus* | 556 | IFI |
| *Saccharomyces veronae* | 1145, 602, 615, 617, 1135 | IFI |
| *Saccharomycodes ludwigii* | 974, 975, 976, 979, 980, 981 | IFI |
| *Schizosaccharomyces pombe* | 935, 936, 938, 939, 2139 | IFI |
| *Torulopsisstellata* | 1303 | IFI |
| *Torulosporarosei* | 717, 718, 719, 720, 722, 723, 724, 725, 726, 727, 728, 730, 731, 732, 733, 742. | IFI |
| *Zygosaccharomyces veronae* | 1148, 615 | IFI |
| *Zygosaccharomyces veronae* | | |

**IFI** (CSIC): *Instituto de fermentaciones industriales* (Institute of industrial fermentations). **ETSIA:** *Escuela Técnica Superior de Ingenieros Agrónomos* (Technical Superior School of Agricultural Engineering).

an inhibitory secondary selective factor in a liquid medium which accelerates determinations with respect to other ethanol-based media as a sole source of selective carbon, although it decreases the selectivity of those media. At an industrial level, these media are highly useful for performing tests in order to obtain qualitative results for detecting the presence of *Brettanomyces/Dekkera* in an economical way. Media based on ethanol as a single carbon source make it impossible for *H. uvarum*, *K. apiculata* and *S. pombe* to grow.

However, for the rest of the strains, growth was merely slowed with respect to the control in the YEPD medium. Sorbic acid was useful against actidione-resistant yeasts for 300 or 700 mg/l in the specific cases of *P. guillermondii* and *S. pombe* because of its special resistance to that compound (Warth, 1985). However, that resistance is inferior to that described by *D.*

**Table 3.** Composition of media used to evaluate different selective factors.

| Medium | Yeast extract (g/L) | Glucose (g/L) | Maltose (g/L) | Trehalose (g/L) | Saccharose (g/L) | Peptone (g/L) | Agar (g/L) | Ethanol (% volume) | Actidione (mg/l) | *p*-coumaric acid (mg/l) | Bromocresol green (mg/l) | Chloramphenicol (mg/l) | Nitrogen base (g/l) | Sorbic acid (mg/l) | pH |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| YEPD/ACT/BG | 2 | 10 | - | - | - | 2 | 24 | 6 | 15 | 100 | 22 | 200 | - | - | 6.2 |
| DBDM | - | - | - | - | - | - | 24 | 6 | 10 | - | - | - | 7 | - | 6.2 |
| YEPD/ET | 2 | 10 | - | - | - | 2 | 24 | 6 | - | - | - | - | - | - | 6.2 |
| YEPD/ET | 2 | 10 | - | - | - | 2 | 24 | 8 | - | - | - | - | - | - | 6.2 |
| YEPD/ET | 2 | 10 | - | - | - | 2 | 24 | 10 | - | - | - | - | - | - | 6.2 |
| YEPD/ET | 2 | 10 | - | - | - | 2 | - | 6 | - | - | - | - | - | - | 6.2 |
| YEPD/ET | 2 | 10 | - | - | - | 2 | - | 8 | - | - | - | - | - | - | 6.2 |
| YEPD/ET | 2 | 10 | - | - | - | 2 | - | 10 | - | - | - | - | - | - | 6.2 |
| YEPD/SB | 2 | 10 | - | - | - | 2 | - | - | - | - | - | - | - | 300 | 3.5 |
| YEPD/SB | 2 | 10 | - | - | - | 2 | - | - | - | - | - | - | - | 700 | 3.5 |
| YEPD | 2 | 10 | - | - | - | 2 | 24 | - | - | - | - | - | - | - | 6.2 |
| BND | - | 10 | - | - | - | - | 24 | | - | - | - | - | 7 | - | 6,2 |
| BNM | - | - | 10 | - | - | - | 24 | | - | - | - | - | 7 | - | 6,2 |
| BNT | - | - | - | 10 | - | - | 24 | | - | - | - | - | 7 | - | 6,2 |
| BNS | - | - | - | - | 10 | - | 24 | | - | - | - | - | 7 | - | 6,2 |
| YEPD/ACT10 | 2 | 10 | - | - | - | 2 | 24 | - | 10 | - | - | - | - | - | 6.2 |
| YEPD/ACT100 | 2 | 10 | - | - | - | 2 | 24 | - | 100 | - | - | - | - | - | 6.2 |

**ET:** ethanol; **ACT:** actidione; **BG:** bromocresol green.

**Table 4.** Composition of media used in microbiological analysis of altered wine.

| Medium | Yeast extract (g/L) | Glucose (g/l) | Peptone (g/l) | Agar (g/l) | Ethanol (% volume) | Actidione (mg/l) | *p*-coumaric acid (mg/l) | Bromocresol green (mg/l) | Chloramphenicol (mg/l) | Nitrogen base (g/l) |
|---|---|---|---|---|---|---|---|---|---|---|
| YEPD/ACT (Liquid) | 4 | 20 | 4 | - | 6 | 40 | 200 | - | 400 | - |
| YEPD/ACT (Solid) | 4 | 20 | 4 | 24 | 6 | 40 | 200 | - | 400 | - |
| DBDM (Solid) | - | - | - | 24 | 6 | 10 | - | 22 | - | 7 |

**ACT:** Actidione.

**Table 5**. Secondary selective factors applied to actidione-resistant yeasts.

| Species (strain) | Tolerance of actidione | Ethanol assimilation | Ethanol 8% volume solid medium | Ethanol 8% volume liquid medium | Sorbic acid (300 mg/l) pH=3.55 | Sorbic acid (700 mg/l) pH=3.55 | Maltose assimilation | Trehalose assimilation | Saccharose assimilation |
|---|---|---|---|---|---|---|---|---|---|
| *Dekkera bruxellensis* | + | + | + | + | + | + | + | + | + |
| *Dekkera anomala* | + | + | + | + | + | + | + | + | + |
| *Schizosaccharomyces pombe* | + | - | + | + | + | - | + | + | + |
| *Pichia guilliermondi* | + | + | + | + | + | - | + | + | + |
| *Kloeckera apiculata* | + | - | + | - | - | - | - | - | - |
| *Hanseniospora uvarum* | + | - | + | - | - | - | - | - | - |
| *Candida parapsilosis* (strains 1336 and 1355) | + | + | + | + | - | - | + | + | + |

+: positive growth -: absence of yeast growth.

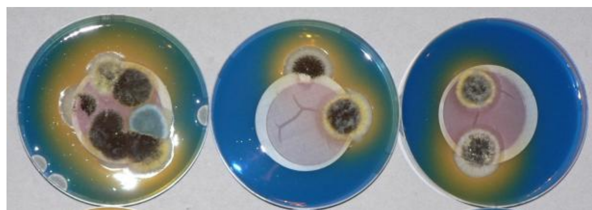

**Figure 2.** Contamination by opportunistic fungi making isolation and microbial count more difficult.

bruxellensis (Ribéreau-Gayon et al., 1972; Loureiro and Malfeito-Ferreira, 2006; Benito et al., 2009b). The above factors slow the development of *D. bruxellensis and D. anomala* with respect to the control in YEPD media, but they do not prevent development. The use of other selective carbon sources (maltose, trehalose and saccharose) made it impossible for the *K. apiculata* and *H. uvarum* strains to develop. The results of this section are summarised in Table 5. These results contribute to optimising selective media, and also enable us to choose between the speed required by the oenologist and the strict selectiveness needed by the microbiologist. According to the results we obtained, we conclude that using secondary factors (which are less selective than the antibiotic actidione) such as sorbic acid (> 300mg/l), ethanol (8% vol.) or sugars other than glucose (maltose, trehalose or saccharose) may be used as selective complements to actidione. This prevents the development of a significant number of the potential false positives caused by species and strains that are resistant to that antibiotic. It also enables us to obtain results in a shorter time than for media whose only carbon source is ethanol, although it somewhat reduces the selectivity of those media.

**Application of selective-differential media in wines with high ethylphenol content**

Initially, 150 ml volumes of altered wine were concentrated through microfiltration. Filters with a pore size of 0.45 μm and a diameter of 40 mm were placed in Petri dishes each containing an agarised selective-differential YEPD/ACT/BG medium. However, performing recounts was not viable due to contamination by opportunistic fungi (Figure 2). When 150 ml of the altered wines (Table 6) was mixed with 150 ml of the selective-differential YEPD/ACT/BG medium in liquid form, it caused the formation of 4-ethylphenol in some cases beginning at 5 to 7 days in those media that were highly enriched with hydroxycinnamic acids. In some cases, we were able to verify the presence of yeasts with vinylphenol reductase activities which were capable of generating 4-ethylphenol from the exogenous *p*-coumaric acid added to the selective-differential medium being used.

This procedure is sufficient to determine the qualitative presence of yeasts with this enzymatic activity at an industrial level, and even offer an approximate measurement of the problem according to the time at which the 4-ethylphenol

**Table 6.** Analysis of altered wines subjected to a test detecting *p*-cumaric acid to 4-ethylphenol bioconversion in a *p*-cumarica cid enriched liquid medium.

| Sample | 4-ethylguaiacol (mg/l) | 4-ethylphenol (mg/l) | Volatile acidity (g/l) | Degree of alcohol | pH | Free $SO_2$ (mg/l) | Total $SO_2$ (mg/l) | *p*-cumaric acid to 4-ethylphenol bioconversion >90% |
|---|---|---|---|---|---|---|---|---|
| 614282 | 0.112 | 1.3714 | 0.819 | 12.6 | 3.76 | 12.8 | 38.4 | - |
| 614516 | 0.1496 | 1.8526 | 0.828 | 11.8 | 3.8 | 6.4 | 19.2 | + |
| 608823 | 0.2281 | 3.8661 | 1.062 | 13.4 | 3.68 | 6.4 | 19.2 | + |
| 608821 | 0.683 | 1.7071 | 0.828 | 12.5 | 3.66 | 6.4 | 19.2 | + |
| 614277 | 0.1052 | 1.2526 | 0.954 | 13.1 | 3.82 | 12.8 | 38.4 | - |
| 608680 | 0.1065 | 1.3198 | 1.224 | 11.5 | 3.75 | 6.4 | 19.2 | + |
| 614279 | 0.1283 | 1.4197 | 1.116 | 12.3 | 3.7 | 6.4 | 19.2 | + |
| 610044 | 0.3049 | 2.2884 | 0.882 | 13.2 | 3.76 | 6.4 | 19.2 | + |
| 614741 | 0.3113 | 3.4437 | 0.756 | 14.4 | 3.82 | 6.4 | 19.2 | + |
| 614278 | 0.1362 | 1.4281 | 0.45 | 12.6 | 3.66 | 12.8 | 38.4 | - |
| 609915 | 0.0884 | 1.0608 | 0.936 | 13.35 | 3.2 | 6.4 | 19.2 | + |
| 609001 | 0.1101 | 1.2342 | 0.54 | 12.8 | 3.36 | 12.8 | 38.4 | - |
| 614280 | 0.1268 | 1.0189 | 0.936 | 13.15 | 3.69 | 6.4 | 19.2 | + |
| 614517 | 0.067 | 1.2946 | 0.378 | 13.9 | 3.81 | 6.4 | 19.2 | + |
| 609065 | 0.1458 | 0.6782 | 1.25 | 13.1 | 3.72 | 19.2 | 57.6 | - |
| 609950 | 0.0709 | 0.7429 | 0.69 | 12.8 | 3.55 | 6.4 | 19.2 | + |
| 614143 | 0.1596 | 0.9277 | 1.21 | 12.9 | 3.72 | 6.4 | 19.2 | + |
| 609002 | 0.0549 | 0.4361 | 1.06 | 13 | 3.75 | 19.2 | 57.6 | - |
| 614281 | 0.0611 | 0.7064 | 1.26 | 12.6 | 3.66 | 6.4 | 19.2 | + |
| 609951 | 0.0644 | 0.5696 | 0.68 | 12.95 | 3.54 | 12.8 | 38.4 | - |
| 608692 | 0.059 | 0.5791 | 0.49 | 14.45 | 3.45 | 12.8 | 38.4 | + |
| 609953 | 0.0571 | 0.5893 | 0.66 | 12.85 | 3.61 | 6.4 | 19.2 | + |
| 609913 | 0.0358 | 1.063 | 0.83 | 12.9 | 3.63 | 6.4 | 19.2 | + |
| 610043 | 0.0884 | 0.6286 | 0.53 | 14.45 | 3.43 | 12.8 | 38.4 | - |

appears (Benito et al., 2009a). The formation of 4-ethylphenol can be considered as an indicator for *Brettanomyces/Dekkera* presence, since the yeasts that originate false positives cannot show vinylphenol reductase activity (Table 6).

**Classification of the yeasts isolated in wines having tested positive**

For ecological purposes, small volumes of the abovementioned liquid medium were inoculated by immersion once we detected the presence of yeasts capable of generating 4-ethylphenol in dishes with a solid DBDM medium (the most selective existing medium). Nine random strains that produce ethylphenols were then isolated.

**Table 7.** Classification of yeasts isolated in a DBDM medium using classical sugar fermentation and assimilation techniques. The last column evaluates the vinylphenolreductase activity in a model medium.

| Strain | Assimilation | | | | | | | Fermentation | | | | | | | Vinylphenol reductase activity |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Galactose | Glucose | Lactose | Maltose | Raffinose | Saccharose | Trehalose | Galactose | Glucose | Lactose | Maltose | Raffinose | Saccharose | Trehalose | |
| 741 | - | + | - | + | - | + | - | - | + | - | + | + | + | + | + |
| 143 | - | + | - | + | - | + | - | - | + | - | + | - | + | + | + |
| 280 | - | + | - | + | - | + | + | - | + | - | + | + | + | + | + |
| 515 | - | + | - | + | - | + | - | - | + | - | + | - | + | + | + |
| 692 | - | + | - | + | - | + | - | - | + | - | + | - | + | + | + |
| 143 | - | + | - | + | - | + | - | - | + | - | + | - | + | + | + |
| 517 | - | + | - | + | - | + | + | - | + | - | + | + | + | + | + |
| 279 | - | + | - | + | - | + | - | - | + | - | + | - | + | + | + |
| 823 | - | + | - | + | - | + | + | - | + | - | + | + | + | + | + |

Kurtzman and Fell, 1998) in order to check for a smaller or null presence of these species compared with D. bruxellensis, as proposed by other authors (Phister and Mills, 2003; Dias et al., 2003; Cocolin et al., 2004; Martorell et al., 2006). This is attributed to its lower resistance to ethanol (Benito et al., 2009b). However, from a strictly industrial viewpoint, the problem is to detect the presence of yeasts with great capacity to produce ethylphenols, not the classification of those yeasts (Table 7). These results allow us to classify yeasts capable of generating ethylphenols without having to recur to molecular biology techniques.

## Conclusion

The main drawbacks of the existing selective-differential media are the false positives caused by yeasts which have no vinylphenol reductase activity (but are resistant to different selective factors) and the contamination caused by opportunistic fungi in solid media. Actidione is the most effective selective factor for the major yeast strains present in the wine-making process, since sole *D. bruxellensis*, *D. anomala*, *K. apiculata*, *H. uvarum*, *P. guilliermondii, S. pombe* and some strains of *C. parapsilosis* were capable of developing in the presence of 10 mg/l. Other secondary selective factors (selective carbon sources, sorbic acid and ethanol as a microbicide agent) may be used successfully to eliminate potential false positives, although some of them added to the time to obtain results. For highly selective isolations, it would be interesting to use media based on actidione as an antimycotic and ethanol as the sole source of carbon. However, on the industrial level, it would be more interesting to obtain results as quickly as possible based on differential factors such as ethylphenol production and secondary inhibitory factors complementing actidione which do not cause excessively slow microbial growth in liquid media.

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