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A new method for the detection of early contamination of red wine by *Brettanomyces bruxellensis* using *Pseudomonas putida* 4-ethylphenol methylene hydroxylase (4-EPMH)

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Abstract *Brettanomyces/Dekkera bruxellensis* is a cause of major concern for the winemaking industry worldwide. If a slight presence of this spoilage yeast in red wine adds a *Brett* character, a strong contamination has irreversible and detrimental effects on the organoleptic qualities due to the production of volatile phenols such as 4-ethylphenol. Time is a key factor in the treatment of *B. bruxellensis* contaminations. Nowadays, the diagnostic and quantification resources available are time consuming and too expensive, making them either inadequate or inaccessible to most of the winemakers. This study was focused on a new, easy to use, inexpensive method that could allow winemakers to directly detect *B. bruxellensis* contamination in red wine at an early stage, hence, reducing wine spoilage. In this work, the ability of *Pseudomonas putida* 4-ethylphenol methylene hydroxylase was tested in order to catabolize the 4-ethylphenol and to elaborate an enzymatic assay with the purpose of detecting early contaminations by *B. bruxellensis* in red wine. We have developed a colorimetric enzymatic assay, based on the redox state of the 4-ethylphenol methylene hydroxylase co-factor, cytochrome C, that can detect and quantify low concentrations of 4-ethylphenol. The range of concentrations detected is well below the level detectable by the human nose. Combined to an enrichment step, this method allows the detection of *B. bruxellensis* at an initial concentration of less than 10 cells per ml.

Keywords *Brettanomyces bruxellensis* · Wine contamination · 4-Ethylphenol · 4-Ethylphenol methylene hydroxylase

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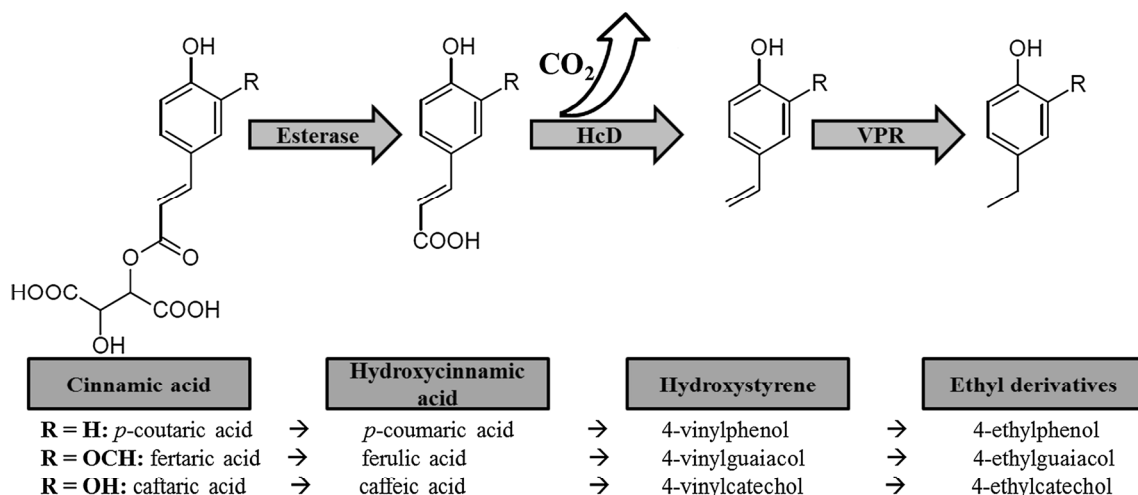   31320 Auzeville-Tolosane, France

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Introduction

The spoilage yeast *Brettanomyces bruxellensis* and its teleomorph, *Dekkera bruxellensis,* currently represent a major issue for winemakers around the world [1]. These yeasts are naturally present on the skin of grapes [2], but at low levels that would not be sufficient to cause organoleptic defects [3]. They can be easily transferred into the winemaking process and have detrimental effects on the wine quality by altering its odour, flavour and colour [4, 5]. Sometimes described as the "terroir" signature when the contamination is low, an elevated proliferation of *B. bruxellensis* in wine, especially in later stages of the vinification process and/or during storage [3], can lead to irreversible deterioration and therefore a substantial economic loss for winemakers.

Initially described in 1904 by Clausen, *B. bruxellensis* has been detected in wines since the 1950s [6]. The molecular mechanism by which *B. bruxellensis* causes organoleptic changes in wine has been characterized as the

**Fig. 1** Pathway of formation of volatile phenols via the decarboxylation of hydroxycinnamic acids [17]

production of volatile phenols [7]. Therefore, *Brettanomyces* can metabolize the hydroxycinnamic acids (HCAs) naturally present in the must into ethylphenols in two enzymatic steps (Fig. 1). The HCAs are first decarboxylated into vinyl derivatives (hydroxystyrenes) by hydroxycinnamate decarboxylase (HCDC) [8]. Subsequently, these hydroxystyrenes are reduced by vinylphenol reductase (VPhR) to their ethyl derivatives form [9]. These compounds, and mainly the 4-ethylphenol (4-EP) in red wine, are responsible for foul smells with descriptors such as sweaty leather, barnyard, mousiness, Band-Aid®, smoky and medicinal [4, 5]. They are only produced by a handful of organisms, such as *Brettanomyces bruxellensis* [4], *Brettanomyces anomalus* [8] and *Pichia guilliermondii* [10]. However, because of *B. bruxellensis'* tolerance to low pH and to higher concentrations of alcohol [9, 11], it is the only relevant organism to winemaking that produces significant amounts of these particular volatile phenols, which can cause considerable financial losses to a winery. It is much rarer for lactic and acetic bacteria isolated from wine to synthesize significant quantities of 4-EP [4].

The resiliency of *B. bruxellensis* during the whole winemaking process and its production of volatile phenols makes it necessary to have not only preventative measures but also control and curative actions, as *B. bruxellensis* contaminations can occur at many points in a cellar [12]. To fight against this type of contamination, a winemaker may use many strategies. As well as barrel sterilization, sulphur dioxide (SO_2) and/or dimethyl dicarbonate (DMDC) have routinely been used to control contaminations in the past decades [13, 14]. Recently, it has been discovered that the use of HCDC-expressing *Saccharomyces*

cerevisiae could reduce the production of 4-EP in contaminated wines [15]. Also, non-*Saccharomyces* killer toxins are proven to inhibit *B. bruxellensis* in wine [16]. Many other biological, chemical and physical methods, found in the literature, are used to prevent or control *Brettanomyces* contaminations [17]. In severe contamination cases, wine can also be treated with yeast lees [18] or by reverse osmosis [19]. However, this can alter the overall quality of the wine, by also reducing the overall concentration of anthocyanins [20].

Detecting *B. bruxellensis* in wine has proven to be difficult to do in a cost-effective, quantitative and timely manner. Indeed, most of the existing accurate detection methods are not accessible to many small producers. The traditional method of the plate counts on selective media, although the most accessible to winemakers, is also the least reliable due to *B. bruxellensis'* slow generation rate [21]. Also, the viable but non-cultivable state (VBNC), associated to *Brettanomyces*, makes it hard to quantify contamination rates [22, 23]. More accurate methods are available like qPCR [24–26] and in situ hybridization [27] to assess the presence of *B. bruxellensis,* as well as GC-MS [28, 29] and HPLC [30] to detect 4-EP; however, these also have their drawbacks. Firstly, they require high-tech materials and skilled operators, obliging winemakers to send their samples to expert laboratories. Secondly, for GC-MS and HPLC results, it is currently considered impossible to correlate a quantity of product (4-EP) measured to a cell concentration as specific VPhR activity can vary depending on the strain of *B. bruxellensis* [13]. Thirdly, these analyses take at least 5 days including shipment of the samples to the laboratory and return of the results to the winemaker.

Fig. 2 Pathway of 4-ethylphenol degradation via 4-EPMH isolated from *P. putida* [32]
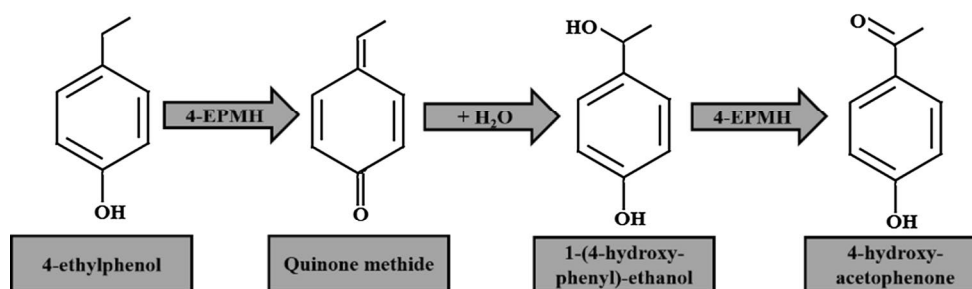
Therefore, there is an urgent need for microbiological detection tests that are simpler, quicker and more reliable.

In the literature, researchers did not focus on the detection properties of enzymes in relation with *B. bruxellensis*. It has been shown that it is possible to spectrophotometrically follow the degradation of *p*-coumaric acid, a precursor to 4-EP, by *p*-coumaric acid decarboxylase [31]. However, this has never been done with the degradation of 4-vinylphenol (4-VP) or 4-EP.

Reeve et al. [32, 33] purified the 4-ethylphenol methylene hydroxylase (4-EPMH) from *Pseudomonas putida*. This enzyme degrades the 4-EP into two products: the major one corresponded to 4-hydroxyacetophenone in three steps (Fig. 2) and uses cytochrome C as a cofactor and the minor one is 1-(4′-hydroxyphenyl)ethanol. In a similar experiment as and when the substrate is 1-(4′-hydroxyphenyl)ethanol, the product isolated was 4-hydroxyacetophenone together with some unchanged substrate.

In their study, the substrate specificity of the hydroxylase was investigated with a number of aromatic compounds in the spectrophotometric assay. Those that gave activity were also used in the oxygen-monitor assay to measure the stoichiometry of the reaction, thus ensuring that a substantial proportion of the substrate was oxidized and that apparent activity was not due to small amounts of contaminants. The substrates and their relative activities with the hydroxylase in the standard assay were as follow: *p*-cresol (4-methylphenol), 4-*n*-propylphenol, 4-*n*-butylphenol, 4-*n*-pentylphenol, 4-*n*-heptylphenol, 4-*n*-nonylphenol, 1-(4-hydroxyphenyl)ethanol, 2,4-xylenol, *p*-hydroxybenzyl alcohol, 4-allyl-2-methoxyphenol (eugenol) and 5-indanol. Compounds not active with the enzyme included 2,3-xylenol, 2,5-xylenol, 2,6-xylenol, 3,4-xylenol, 3,5-xylenol, *o*-cresol, 4-methoxyphenol, 4-isopropylphenol and tyrosine. In the steady-state kinetic experiments, it was shown that this enzyme was inhibited by phenazine ethosulphate concentrations above 5 mM.

The aim of this work was to develop a direct enzymatic assay to detect the presence of 4-EP in wine. This assay is combined to an enrichment media that was optimized to detect very low concentrations of 4-EP produced by few initial cells, so that a simple, rapid and cost-effective assay can be performed to detect *B. bruxellensis* in red wine.

Materials and methods

Yeast strains and media

Three genetically different strains of *B. bruxellensis* have been used in this study. Strains V1 and V2 have been isolated from Madiran grape must by plating on selective media, as described by Nisiotou and Gibson [34]. Strain M has been isolated from a Lebanese wine. These strains were genetically identified and proven different [35].

Growth medium consisting of MYPG (3% (w/v) malt extract, 3% (w/v) yeast extract, 2% (w/v) peptone, 10% (w/v) glucose, ±15 g/l bacteriological agar, pH 5.0) was supplemented with chloramphenicol 25 μg/ml and cycloheximide 10 μg/ml. 4-vinylphenol (10% w/w in propylene glycol, Sigma–Aldrich) at different concentrations was added to the medium as a precursor to 4-EP.

Cell counting and inoculation

Strains were plated on MYPG agar and grown at 28 °C. A single colony per strain was then used to inoculate 100 ml of sterile grape juice, pH 3.5, 150 g/l of sugar. This culture was grown for 72 h at 28 °C and kept at 4 °C. Cell concentration in the liquid was quantified using a Thoma hemocytometer ($0.0025 \, mm^2$ with 0.1 mm depth), a Zeiss phase-contrast microscope and methylene blue dye to determine cell viability.

Determination of sensorial detection threshold of 4-ethylphenol

The determination of the detection threshold levels was based on the standard method of the American Standard Test Manual (ASTM E 679-04) [36]. Eight groups of three solutions (ddH$_2$O or 4-EP in ddH$_2$O) were presented to a panel of judges. In each set of three solutions, only one

Table 1 Solutions of 4-EP in ddH$_2$O for the determination of sensorial threshold

Group	1	2	3	4	5	6	7	8
4-EP concentration (mg/l)	0.104	0.143	0.198	0.274	0.382	0.533	0.743	1.039

Each set of three solutions contained two vials of ddH$_2$O and one vial of 4-EP in ddH$_2$O at the following concentrations

contained 4-EP, and its concentration was different among the eight groups. Sample sets were presented in ascending order of 4-EP concentration to the judges (Table 1). The test was completed for each judge as soon as the spiked sample was correctly identified in two consecutive concentrations.

Elaboration of an enrichment medium by sensorial analysis

A commercial wine (Richaumont de Bordeaux 2008, 12% ethanol and pH 3.6) was contaminated purposefully with various cell concentrations of *B. bruxellensis* (1, 10, 100, 1×10^3, 1×10^4 and 1×10^5 cells/ml). These concentrations are obtained by sterile dilutions. Logically, it is not possible to accurately and reliably obtain cell concentrations of 1 and 10 cells/ml. It is the minimum number of cells obtained via dilution.

10 ml of infected wine at different concentrations (1, 10, 100, 1×10^3, 1×10^4 and 1×10^5 cells/ml) were added to 10 ml of MYPG sterile broth supplemented with 4-VP (0.1, 0.25 or 0.5 mM) as a precursor to 4-EP. (In mg/l, these latest concentrations are, respectively, 12, 30 and 60). These different samples were incubated at 28 °C. After 72 h, samples were sensorially analysed by a panel of judges each consecutive 24 h thereafter for a total of 168 h, or 7 days. For all tests, judges were blinded and had to rate the pungency level (from 0 to 4) of five different odours: leather (*L*), medicinal (*M*), Band-Aid® (*B*), sweat (*Sw*) and stable (*St*) (which means that there are no changes in the organoleptic properties); zero being undetectable and four being very strong.

Relative bad odour (RBO) per time point and per initial concentration of *B. bruxellensis* was calculated as follows: $\text{RBO} = \sum_0^n R_n$ where $R_n = [(XL_n + XM_n + XB_n + XSw_n + XSt_n)/N] \times n$

$N =$ number of judges $\times$ number of odours (25 in our case)

$n =$ pungency level (ranging from 0 to 4)

$X_n =$ number of judges who scored this odour
    (L, M, B, Sw or S) at pungency level n.

Cytochrome C electron transport linked assay

A linear regression curve has been drawn following the oxidation of cytochrome C in plastic cuvettes using a Thermo Scientific Genesys 10 UV scanning spectrophotometer at 550 nm. Different concentrations of cytochrome C were prepared by adding 2 ml of enrichment medium containing various concentrations of 4-EP (from 0 to 1 mg/l) to 0.2 ml Tris–HCl (1 M) pH 7.6, cytochrome C 13 mg/ml (Bovine, Sigma–Aldrich) and 4-EPMH 0.5 mg/ml.

To perform this assay on a contaminated wine sample, the level of 4-EP was first increased using the enrichment medium as follow: 20 ml of contaminated wine (at 100 and 1×10^3 cells/ml) were filtered using sterile 0.45-μm cellulose acetate filters. The filter, containing the cells, was then placed directly into a sterile polypropylene jar, fitted with a lid, containing 20 ml of sterile enrichment medium and grown at 28 °C for 24, 48 or 72 h before being tested as described for the standards.

Growth curve and 4-ethylphenol production kinetics

Different volumes of contaminated wine were filtered (0.45 μm) to obtain 1, 10, 100, 1×10^3 or 1×10^4 cells/ filter of each strain V1, V2 and M. The filters were then placed into 20 ml of sterile enrichment medium and incubated at 28 °C for 6 days. Six different initial concentrations of samples were thus obtained: 5×10^{-2}, 0.5, 5, 50, 500 and 5×10^3 cells/ml. Also here, 5×10^{-2} and 0.5 cells/ ml have no biological sense, it is the minimum number of cells obtained via dilution. Once a day, 1.5 ml were collected for both cell counting (pellet) and HPLC analysis (supernatant conserved at -20 °C).

HPLC detection of 4-ethylphenol and 4-vinylphenol

The method used is based on the one described by Nikfardjam et al. [30] with some modifications. HPLC: Shimadzu; Flow: 1.2 ml/min; Wavelength: 230 nm; Equilibrium time: 5 min; elution gradient: 5 min 20% acetonitrile, 10 min 40% and 10 min 60%; Re-equilibration : 5 min; Column isocratic 5 μ ODS-2 TM (Waters®, 4.4 $\times$ 250 mm) equipped with the ODS-2 pre-column Spherimarge™. The system is equipped with a 150 UV detector (Spectra series) scanning. An automatic sample changer controlled temperature (autosampler AS 100) was used. A linear calibration curve was obtained with standard concentrations between 0.02 and 2 mg/l. ChromQuest 5.0 software was used for the acquisition and provided for analysis and integration of peaks.

Limits of detection and quantification were calculated for each component according to the method describes in ACS, 1980 [37]. For both molecules, the detection limit was 0.01 mg/l and the quantification limit 0.03 mg/l.

Results

Early olfactory detection of 4-ethylphenol through an enrichment medium supplemented with 4-vinylphenol

In order to optimize the detection of *B. bruxellensis* in wine, even at very low concentrations, we improved the composition of a liquid medium which allows rapid growth of *B. bruxellensis* and enhances olfactory detection of 4-EP.

In order to determine the best enrichment medium, 21 sterile jars containing enrichment media supplemented with various concentration of 4-VP were inoculated with various cell concentrations ($1–1 \times 10^5$ cells/ml) of strain V1 and then grown for 72 h. Then, each judge proceeded to evaluate the presence of 4-EP every 24 h. As shown in Fig. 3a, the judges could subjectively detect the presence of 4-EP created by one initial *B. bruxellensis* cell after only 6 days (144 h) in a medium supplemented with 0.1 mM of 4-VP.

It should be noted that 4-EP smell weakens after a peak in detection (Fig. 3). We also noticed a degradation of the wine colour as cell concentration increased with time.

To confirm the validity of the olfactory test, the level of 4-EP in samples has been analysed by HPLC. As shown in Fig. 3b, both contaminated samples showed significant increases in 4-EP concentration after 72 h (5.6 and 7 mg/l for 10 and 1×10^3 cells/ml of initial wine, respectively) compared to the blank (1.8 mg/l). It should be noted that we tested the lowest three initial concentrations because the method aims to determine early contaminations related to the lowest population. After a week (168 h), the presence of 4-EP decreased over time, especially in the samples that were spiked with large numbers of cells. Both results agree with our previous findings. It should be noted that 4-EP levels were already elevated in the blank sample (1.8 mg/l). This could be explained by a prior contamination by *B. bruxellensis*. However, in order to make sure that it would not interfere with our values, the wine had been autoclaved before additional cells were added.

4-Ethylphenol detection using 4-EPMH electron transport linked assay and cytochrome C

Due to the difficult direct detection of 4-EP or its derivative 4-hydroxyacetophenone in the UV range (data not shown), an assay using an electron transporter whose redox state would be followed using spectroscopy was developed. Reeve et al. [32] were the first to use cytochrome C with 4-EPMH, which electron transport chain schematic is seen
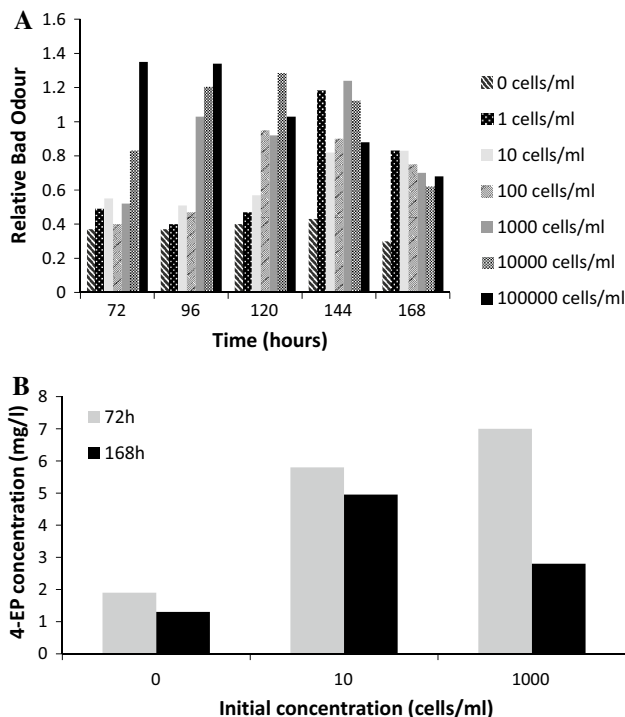
Fig. 3 Evolution of 4-EP production over time in enrichment medium. **a** Olfactory test. Results shown are an average for each judge on each criterion and then multiplied by the pungency level. **b** HPLC analysis of six samples of the olfactory test
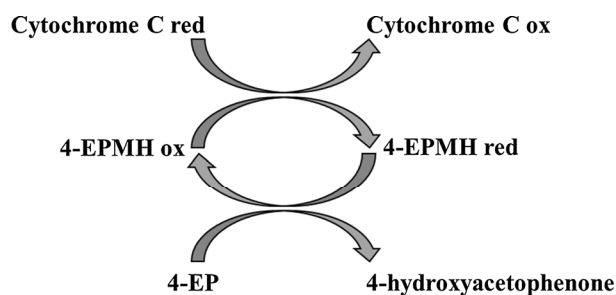

Fig. 4 Electron transport chain between 4-EPMH and cytochrome C

in Fig. 4. However, it was McIntire et al. [38] who laid the groundwork for cytochrome C as an electron acceptor in the reaction of *p*-cresol methylene hydroxylase, which also comes from *P. putida* and catalyses the conversion of *p*-cresol (very similar to 4-EP) into *p*-hydroxybenzylalcohol.

Figure 5a shows that it is possible to produce a linear regression curve using very small concentrations of 4-EP diluted in sterilized enrichment medium in order to obtain the desired detection limits (0.25–1.0 mg/l of 4-EP).

For the 4-EPMH/CytC assay, two known quantities of V1 cells extracted by filtration from contaminated wine were grown in the enrichment medium at 28 °C. Then, 4-EP concentrations were tested after 24, 48 and 72 h of incubation.
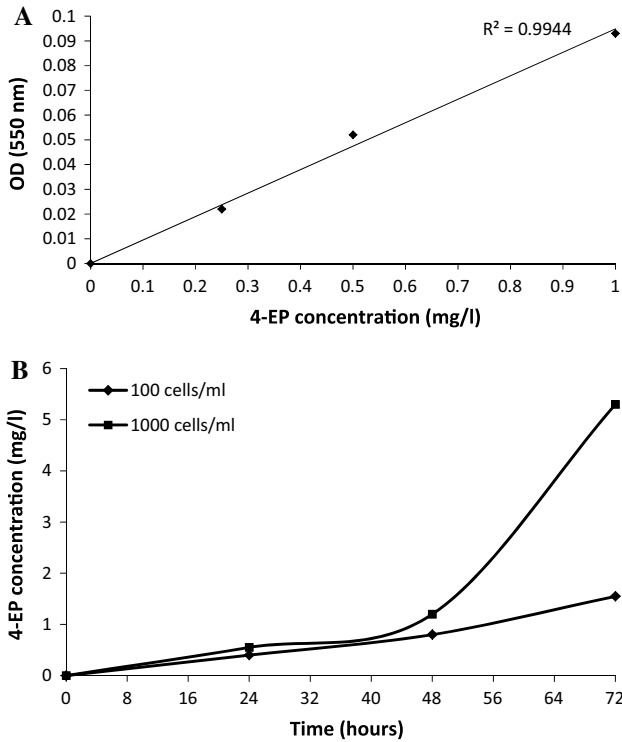
Fig. 5 Detection of 4-EP production in enrichment medium using 4-EPMH/CytC assay. **a** Normalized curve for pure 4-EP in enrichment medium. **b** Production of 4-EP over time by *B. bruxellensis* in contaminated wines. Each sample has been tested in duplicate

**Table 2** Levels of 4-EP detected by HPLC analysis for the two 72 h's samples

Method	100 cells/ml	10^3 cells/ml
HPLC	1.85 mg/l $\pm$ 0.5%	5.69 mg/l $\pm$ 0.5%
4-EPMH/CytC	1.5 mg/l $\pm$ 0.5%	5.2 mg/l $\pm$ 0.5%

Three samples for each concentration were tested

As shown in Fig. 5b, 4-EP production after 24 h was hardly detectable (0.4 and 0.5 mg/l for the initial inoculation of 100 and 10^3 cells/ml, respectively). However, after 48 h, 4-EP levels started to rise (0.8 and 1.2 mg/l) and drastically increased after 72 h (1.5 and 5.2 mg/l). These latest samples have been further analysed by HPLC, which confirmed these results (Table 2).

4-Ethylphenol production over time for three different strains of *B. bruxellensis*

Several identified quantities of cells from each strain were extracted by filtration from contaminated wines and grown in 20 ml of enrichment medium at 28 °C giving the following concentrations: 5×10^{-2}, 0.5, 5, 50, 500, 5×10^3 cells/
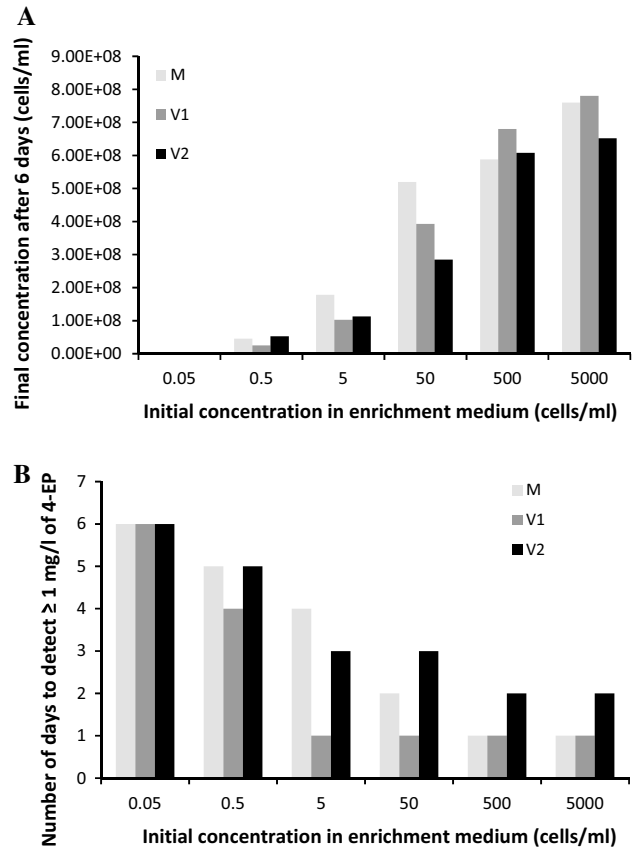
Fig. 6 Growth and 4-EP production kinetics of strains V1, V2 and M by HPLC. **a** Final cell concentration after 6 days depending on the initial concentration in enrichment medium. **b** Number of days required for each initial cell concentration of each strain in enrichment medium to produce ≥ 1 mg/l of 4-EP

ml of wine. Every day and during 6 days, cells were numbered and 4-EP levels were tested by HPLC.

As shown in Fig. 6a and after 6 days of incubation, the final number of cells for each initial inoculate is approximately similar among the three strains used in this study, suggesting a growth at the same rate. Regarding the 4-EP production and as expected, 4-EP is produced earlier when initial cells concentration is important (Fig. 6b).

It is to notice that Fig. 6a shows a number of 0 cell/ml for the 0.05 sample after 6 days but Fig. 6b shows that 6 days are needed to get a concentration higher than 1 mg/l of 4-EP. This can be explained by the fact that when counting on a hemocytometer, it is possible to estimate the number of cells in a specific volume of solution and not for all the volume.

Discussion

Brettanomyces bruxellensis is a current major issue for winemakers worldwide. It is responsible for the spoilage

of wines due to its ability to produce volatile phenols conferring off-odours and off-flavours and losses of fruity sensorial qualities [7]. This type of yeasts can adapt to the wine-cellar environment, under high ethanol concentrations [39], nutrient deprivation [40], thermal treatment [41] and winery disinfection [42]. Tools for fighting contamination are limited and inefficient on late-stage contaminations, illustrating that any contamination should be detected on early stages of winemaking to avoid serious consequences. Unfortunately, recent and sophisticated detection methods are not affordable to small wine producers in terms of budget and obtaining quantitative results remain too long. Thus, the importance of developing a new, easy to use, rapid and cost-effective test to detect early contamination of wines by *B. bruxellensis*.

According to Couto et al. [43], the present work shows that rapid olfactory detection of *B. bruxellensis* is possible with a specific enrichment broth supplemented with 4-VP, a precursor to 4-EP.

The most convincing results were obtained with 0.1 mM of 4-VP which is known to have a weaken smell comparing to 4-EP but the smell of this latest could be hidden as these molecules will possibly be adsorbed on the yeast cell walls [44]. Concerning the degradation of the wine colour, our results agree with the observations made by Morata et al. [45].

Nevertheless, this test is not entirely satisfactory for several reasons. (1) Six days even for a low contamination (against 10 days with a commercialized kit found in the market or by plate culture) are still too long, (2) Olfactory detection of 4-EP is too subjective and can vary with people sensitivity or training, (3) VPhR activity varies among different strains of *B. bruxellensis* [13], so it seems impossible to establish a general correlation between the pungent smell of 4-EP and the number of cells present in a given quantity of wine.

To assess the first two matters, the enrichment medium was combined with an enzymatic assay able to specifically detect 4-EP with a low detection threshold. The idea of combining the enrichment step with a simple enzymatic assay came from Reeve's work on 4-EPMH [33]. Even though it has been known since 1989 that *P. putida,* through 4-EPMH, is capable of degrading 4-EP, the literature does not show recent studies related to this subject in oenology.

In the present work, we revealed that 4-EPMH can degrade 4-EP produced by *B. bruxellensis* and that the amount of 4-EP can be accurately determined using a colorimetric enzymatic assay based on the oxidation of cytochrome C which is the 4-EPMH cofactor. Using this innovative technique associated with an enrichment step, we showed that it is possible to detect low levels of 4-EP after only 72 h, allowing winemakers to tackle their contamination promptly, before any irreversible organoleptic

damage occurs. Even though this assay may take about the same time needed to send samples and receive the results from a laboratory using GC-MS, qPCR or HPLC, the total cost of the analysis is divided by 30 (in France, submitting samples for analysis costs in average 60 euros/sample, whereas 4-EPMH-cyt C assay costs around 2 euros). In addition, compared to qPCR which has a detection limit varying between 1 and 10 cells/ml [25, 46], our method can be used to detect even lower concentrations of *B. bruxellensis*. As shown in this work, the 4-EPMH-cytC assay allows the detection of 4-EP production from a 20 ml sample of wine containing 5×10^{-2} cells/ml. This limit could be lowered again by filtrating an extended volume of wine (e.g. by filtrating a litre of wine, the detection limit drops down to 1×10^{-2} cells/ml). However, and as we argued previously, the VPhR activity seems to be strain-dependant, which makes it impossible to correlate a concentration of 4-EP with an initial cells concentration. Therefore, a kinetic of 4-EP production by the three different strains of *B. bruxellensis* in the enrichment medium was realized.

Our results (Fig. 6b) showed that it might be possible to correlate 4-EP levels with initial cells concentration in our test.

Along with the work of Oelofse [31], this is the only method of detection of *B. bruxellensis* in wine based on biotic degradation. To date, only Rayne and Eggers reported that very high levels of 4-EP, 3.9 mg/l, found in wines kept in barrels in the Okanagan Valley of Canada during the summer, in July, were found to decrease to 0.005 mg/l by September of the same year [47]. While this degradation could have been due partially to lees adsorption, it is most likely that part of the degradation is due to other factors like biotic degradation, which has economic importance and physiological interest [15, 48]. As we demonstrated in our study, this biotic degradation can be used to determine the presence of a wine contamination but it could also have a curative application. The 4-EPMH degrades 4-EP into 4-hydroxyacetophenone, which has a light floral aroma (www.flavornet.org).

Conclusion

In this study, we described a simple, fast and non-expensive method, which is able to identify *Brettanomyces* contamination in wine. This method relies on the enzyme 4-EPMH that degrades 4-EP.

The amount of 4-EP can be accurately determined using a colorimetric enzymatic assay based on the oxidation of cytochrome C which is the 4-EPMH cofactor. The enrichment medium is a required preliminary step because it accelerates the production of 4-EP. Our test is a sensitive method that allows the determination before the

contamination reaches a significant level. The aim is to link the concentration of 4-EP with an initial concentration of yeasts. This method could replace the HPLC technique that does not provide information about the initial concentration of yeasts present in wine. It also replaces the panel of judges who often give a subjective opinion about the wine quality. Our enzymatic assay could replace the kits already found in the market and which are mostly based on the sniffing technique.

Compliance with ethical standards

Conflict of interest None.

Compliance with ethics requirements This article does not contain any studies with human or animal subjects.

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