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FOOD MICROBIOLOGY

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Factors affecting the production of 4-ethylphenol by the yeast *Dekkera bruxellensis* in enological conditions

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Received 31 October 2002; accepted 12 February 2003

Abstract

The conversion of *p*-coumaric acid into 4-ethylphenol was studied in *Dekkera bruxellensis* ISA 1791 under defined conditions in synthetic media. The production of 4-ethylphenol occurred roughly between mid-exponential growth phase and the beginning of the stationary phase. This behaviour was observed when glucose was the only energy and carbon source, the conversion rate being close to 90%. Ethanol, as the single energy source, yielded conversion rates close to 80% while in the presence of trehalose and acetic acid conversion rates lower than 10% were obtained. The production of 4-ethylphenol was not observed when the cells were maintained in buffer solution without carbon and energy sources. The precursor of 4-ethylphenol, *p*-coumaric acid, was not utilized as energy and carbon source. Furthermore, it was shown that 4-vinylphenol may be used as a precursor of 4-ethylphenol in the absence of *p*-coumaric acid.

Growth and 4-ethylphenol production were inhibited by increasing concentrations of ethanol, being fully prevented at 13% (v/v) ethanol.

The cultivation of strain ISA 1791 in mixed culture with *Saccharomyces cerevisiae*, in synthetic medium, showed that the cell numbers of *D. bruxellensis* increased from 10^4 cfu/ml to 5×10^9 cfu/ml. Laboratory microvinifications of white and red juices inoculated with as low as 10 cfu/ml of *D. bruxellensis* and 10^7 cells/ml of *S. cerevisiae* showed growth of *D. bruxellensis* to levels of about 5×10^8 cfu/ml. In addition, 4-ethylphenol production by *D. bruxellensis* was observed only after complete fermentation of the grape juices.

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Keywords: *Dekkera bruxellensis*; Wine; Grape juice; 4-ethylphenol; *p*-Coumaric acid; Ethanol

1. Introduction

The yeasts of the genus *Brettanomyces*, or its teleomorph *Dekkera*, were first described by Claussen in 1903, in beer production (Gilliland, 1961). These genera are particularly known as spoiling agents in beer, wine, cider and soft drinks industries (Deak and Beuchat, 1996). In wines, the metabolic products responsible for spoilage by *Brettanomyces*/*Dekkera* sp. are mainly volatile phenols, tetrahydropyridines and acetic acid (Heresztyn, 1986; Larue et al., 1991; Ciani and Ferraro, 1997). Volatile phenols are the sources of off-flavours in beers (Steinke and Paulson, 1964). In wines they are responsible for the taints described as "medicinal" in white wines (due to vinylphenols) and as "leather", "horse sweat" and "stable" in red wines (due to ethylphenols) (Chatonnet et al., 1993b).

The origin of volatile phenols is related with the sequential activity of two enzymes which decarboxylate hydroxycinnamic acids (e.g. ferulic, *p*-coumaric and caffeic acids) into hydroxystyrenes (vinylphenols) which are then reduced to ethyl derivatives (Steinke and Paulson, 1964). The decarboxylation step is present in a large number of bacteria, fungi and yeast species (Degrassi et al., 1995; Edlin et al., 1995; Suezawa, 1995; Suezawa et al., 1998). However, the reduction step is much less frequent and has been reported as particularly effective in the species *Dekkera bruxellensis* (Chatonnet et al., 1995, 1997), *D. anomala* (Edlin et al., 1995), *Pichia guilliermondii* (Dias et al., 2003), *Candida versatilis*, *C. halophila* and *C. mannitofaciens* (Suezawa, 1995).

Heresztyn (1986) described for the first time the production of ethylphenols by *Dekkera*/*Brettanomyces* in grape juices but the contribution of these molecules to

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0740-0020/03/$ - see front matter © 2003 Published by Elsevier Science Ltd.
doi:10.1016/S0740-0020(03)00023-6

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the phenolic taint in wines has only been enlightened recently (Chatonnet et al., 1995, 1997). Earlier, their origin was related with bacterial activity (Steinke and Paulson, 1964; Baumes et al., 1986; Cavin et al., 1993). In fact, lactic acid bacteria may produce significative amounts of vinylphenols but produce only traces of ethylphenols under wine conditions (Chatonnet et al., 1995, 1997). The fermenting yeast *Saccharomyces cerevisiae* and other wine contaminants (e.g. *Pichia* sp., *Torulaspora* sp., *Zygosaccharomyces* sp.), may also produce 4-vinylphenol but are incapable of producing 4-ethylphenol (Chatonnet et al., 1993b, 1995; Rodrigues et al., 2001). In *D. bruxellensis* the enzymes cinnamate decarboxylase and vinylphenol reductase are active under wine conditions and so these yeasts should be regarded as the off-flavour producers (Chatonnet et al., 1995, 1997). However, the factors affecting the production of 4-ethylphenol, in particular those related with wine production, are not yet well established. Thus, the present work was aimed to a better understanding of the effect of some environmental factors on the production of 4-ethylphenol by *D. bruxellensis*. Some studies were performed in synthetic media, while the growth kinetics of mixed cultures with *S. cerevisiae* and *D. bruxellensis* were also studied in grape juices.

2. Material and methods

2.1. Yeast strains and maintenance conditions

The strain *D. bruxellensis* ISA 1791 was isolated in our laboratory from a phenolic tainted red wine (Rodrigues et al., 2001b) and was maintained in GYP medium (20 g/l glucose (Merck, Darmstadt, Germany), 5 g/l yeast extract (Difco Laboratories, Detroit, USA), 10 g/l peptone (Difco) and 20 g/l agar, pH 6.0), added of 5 g/l of calcium carbonate (Merck), at 4°C. The strain *S. cerevisiae* ISA 1000 was isolated from a commercial wine starter (Malfeito-Ferreira et al., 1997) and was stored in GYP medium at 4°C.

2.2. Inocula preparation

D. bruxellensis ISA 1791 inocula were prepared by previous growth in media identical to that used in the respective test media (see below) without *p*-coumaric acid or 4-vinylphenol and incubated in an orbital shaker at 25°C and 150 rpm. For the tests at different temperatures inocula were incubated with magnetic stirring at 16°C and 30°C. Growth was followed by turbidimetry at 640 nm. At an OD of about 0.5 about 10^6 cells/ml cells were inoculated in 250 ml of each medium (see below).

For mixed culture tests, *S. cerevisiae* and *D. bruxellensis* were grown in 6.7 g/l YNB with aminoacids (Difco), 0.1 g/l biotin (Merck), 1.6 g/l thiamine (Merck), 10 g/l glucose (Merck) and 10 g/l fructose (Merck). Culture media were inoculated with 10^7 cells/ml of *S. cerevisiae* and 10^4 or 10 cells/ml of *D. bruxellensis*.

2.3. Growth and 4-ethylphenol production in synthetic media

The effect of different carbon sources on the production of 4-ethylphenol was studied using a medium composed by 6.7 g/l YNB with aminoacids (Difco), 100 mg/l *p*-coumaric acid (Sigma), 0.1 g/l biotin (Merck), 1.6 g/l thiamine (Merck) added of: (i) 20 g/l glucose (Merck), (ii) 8 g/l ethanol (Merck), (iii) 20 g/l fructose (Merck), (iv) 20 g/l trehalose (Merck), (v) 1 g/l acetic acid (Merck).

Previous experiments with medium (i) and 10 mg/l *p*-coumaric acid showed that the increase to 100 mg/l did not affect growth and conversion to 4-ethylphenol and so all experiments were carried out with the higher concentration.

The effect of temperature on 4-ethylphenol production was studied in media (i) and (ii), incubated at 16°C and 30°C.

The influence of the precursor was studied using a medium composed by 6.7 g/l YNB with aminoacids, 75 mg/l 4-vinylphenol (Lancaster, UK), 0.1 g/l biotin and 1.6 g/l thiamine added of (i) 20 g/l glucose and (ii) 8 g/l ethanol.

The toxic effect of ethanol was studied in medium (i) added of 2 g/l glucose and of increasing concentrations of ethanol, as shown in results.

The evaluation of 4-ethylphenol production in the absence of carbon source was performed by adding 100 mg/l of *p*-coumaric acid to cells producing 4-ethylphenol in medium (i) which were washed and centrifuged twice at 4°C with a solution of 50 g/l KH_2PO_4 (Merck) at pH 3.50. The pellet was resuspended in a medium composed by 50 g/l KH_2PO_4 (Merck) and 100 mg/l *p*-coumaric acid, at pH 3.50.

Mixed cultures of *S. cerevisiae* and *D. bruxellensis* were cultivated in: (i) medium composed by 6.7 g/l YNB (Difco), 100 mg/l *p*-coumaric acid (Sigma), 0.1 g/l biotin (Merck), 1.6 g/l thiamine (Merck) added of 100 g/l glucose (Merck) and 100 g/l fructose (Merck); (ii) white grape juice (11.2° Brix, 5.4 g/l total acidity as tartaric acid, 49 mg/l total sulphite, 7 mg/l free sulphite, pH 3.39); (iii) red grape juice (13.2° Brix, 3.9 g/l total acidity as tartaric acid, 65 mg/l total sulphite, 10 mg/l free sulphite, pH 3.60).

All synthetic media were adjusted to pH 3.50 ± 0.01 and sterilized by filtration through membranes of 0.22 μm pore size (Millipore) and cultivation was carried out with orbital shaking at 25°C and 150 rpm or with magnetic stirring for tests at different temperatures. Growth was monitored by OD at 640 nm.

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Fermentations in grape juices were performed in two steps: (i) fermentation in 500 ml Erlenmeyer flasks with 250 ml grape juice, with magnetic stirring at 25°C; (ii) after the end of fermentation (sugar depletion followed by °Brix determinations) wine (30 ml) was transferred to 50 ml test tubes and maintained without shaking at 25°C.

Culture purity was regularly checked by streaking onto GYP plates. In mixed cultures, *S. cerevisiae* was counted by spreading onto GYP plates incubated at 25°C during 48 h while *D. bruxellensis* enumeration was carried out on GYP plates added of 10 mg/l cycloheximide (Sigma) incubated at 25°C during 5 days.

2.4. Chemical analysis

Samples were taken periodically from culture media, pH was determined (PHM 82, Radiometer, Copenhagen, Denmark) and samples were frozen in glass vials (4-ethylphenol determinations) and Eppendorfs (HPLC determinations) at the temperature of −18°C until used.

The 4-ethylphenol production was measured according to a protocol described by Bertrand (1981). Briefly, the volatile phenol was extracted by ether-hexan from a 10 ml sample with pH adjusted to 8 with NaOH. The 4-ethylphenol was separated by collecting the organic phase of the mixture. The quantification was achieved by gas chromatography using 3,4-dimethylphenol as internal standard.

The concentrations of glucose, fructose, ethanol and acetic acid were assessed by HPLC after thawing the samples and centrifuging for 10 min. The supernatant (1 ml) was transferred to another Eppendorf tube and samples were deproteinized by adding 34.5 µl perchloric acid (60% p/v) (Merck). The samples were kept on ice during 30 min and centrifuged. The supernatant was transferred (750 µl) to another Eppendorf tube and 750 µl of arabinose (10 g/l) (Merck) was added as internal standard. The samples were filtered by a membrane of 0.22 µm pore size (Millipore). An amount of 20 µl was injected in a HPLC pump (Waters 501, USA), at a flow rate of 1 ml/min of a solution of H_2SO_4 0.01 N. The separation was performed in a column Polyspher OAKC (Merck) at 70°C. The compounds were quantified in a refractometer (Waters R401) and the integration was done using the software Chrom-Card for Windows (Fisons, Rodano, Italy).

3. Results

3.1. Effect of carbon and energy sources on the production of 4-ethylphenol

The typical behaviour of *D. bruxellensis* ISA 1791 grown on glucose as single energy and carbon source and on 100 mg/l of *p*-coumaric acid is shown in Fig. 1. Glucose was consumed to produce ethanol during the exponential growth phase, the initial 20 g/l glucose being consumed and producing a maximum detected level of 7.6 g/l ethanol, which was consumed after glucose depletion. Growth was accompanied by medium acidification from pH 3.5 to about 2.2. Concerning volatile phenols, the onset of 4-ethylphenol production was about the mid-exponential phase and production was completed at the beginning of the stationary phase, with a conversion rate of 92.5% (Table 1). The precursor 4-vinylphenol was detected in the beginning of growth phase in low amounts because it was rapidly converted to 4-ethylphenol. The precursor *p*-coumaric acid was not utilized by strain *D. bruxellensis* ISA 1791 as single energy and carbon sources.

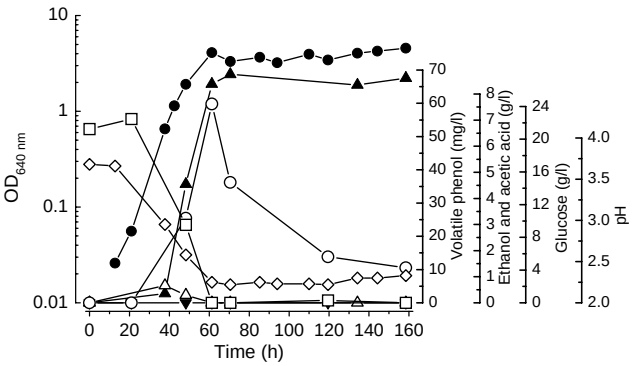

Fig. 1. Growth of *D. bruxellensis* ISA 1791 in YNB with aminoacids added of 100 mg/l *p*-coumaric acid and 20 g/l glucose, at 25°C. Symbols: ●, optical density; ▲, 4-ethylphenol; Δ, 4-vinylphenol; □, glucose; ○, ethanol; ▼,acetic acid; ▯, pH.

As ethanol was consumed by the selected strain during the stationary growth phase the possibility that this metabolite could be used as single carbon and energy source with production of 4-ethylphenol was assessed. During the consumption of ethanol the maximum production of 4-ethylphenol was about 62 mg/l yielding a conversion rate of 83.5% (Table 1). The plots of growth and 4-ethylphenol production when ethanol was added as single energy and carbon source are shown later in Fig. 5.

Growth and 4-ethylphenol production were also evaluated on glucose and fructose at concentrations similar to that found in grape juices (Fig. 2). Growth occurred at a rate of 0.12 h^{-1} while the pattern of 4-ethylphenol production was similar to that found on glucose (see Fig. 1). However, glucose and fructose were not completely fermented, remaining 11.5 and 53.4 g/l, respectively, at the end of the experiment. The final concentration of ethanol was 41.3 g/l. The onset of acetic acid production was observed after the end of the exponential growth phase and reached 7.1 g/l (Fig. 3).

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Table 1
Growth rate and maximum 4-ethylphenol production by *D. bruxellensis* ISA 1791 grown in culture media with 100 mg/l of *p*-coumaric acid and different carbon and energy sources, at 25°C

Carbon and energy source	Growth rate (h^{-1})[a]	Maximum 4-ethylphenol (mg/l)	Conversion rate (%)[b]
Glucose	0.15±0.01 (2)	68.9±2.7 (2)	92.5
Ethanol	0.04±0.01 (2)	62.2±1.9 (2)	83.5
Trehalose	0.11±0.02 (3)	4.7±1.5 (3)	6.3
Acetic acid	0.06±0.01 (3)	1.8±0.3 (3)	2.2

[a] Number of repetitions are shown in parentheses.
[b] Calculated by the ratio between the maximum concentration of 4-ethylphenol produced and the maximum theoretical concentration (74.5 mg/l) produced by the conversion of 100 mg/l of *p*-coumaric acid.

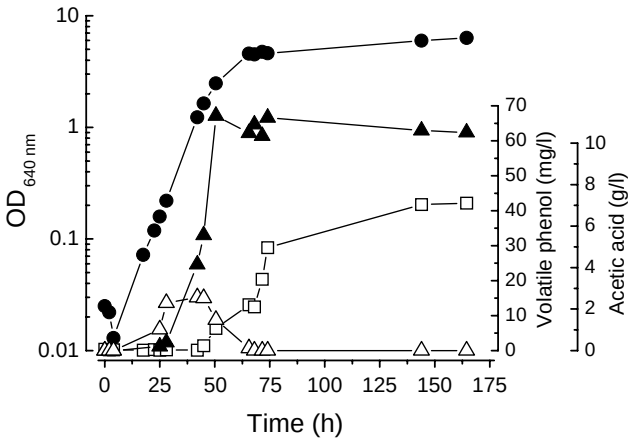

Fig. 2. Growth of *D. bruxellensis* ISA 1791 in YNB with aminoacids added of 100 mg/l *p*-coumaric acid, 100 g/l glucose and 100 g/l fructose incubated at 25°C. Symbols: ●, optical density; ▲, 4-ethylphenol; Δ, 4-vinylphenol; □, acetic acid.

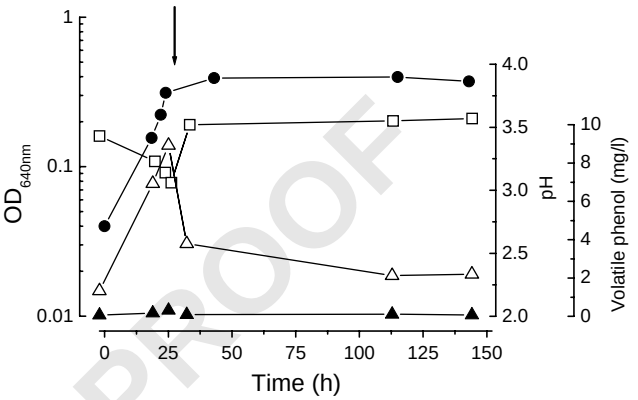

Fig. 3. Effect of the removal of glucose from actively growing cells of *D. bruxellensis* ISA 1791. The arrow indicates the time of glucose removal. Symbols: ●, optical density; ▲, 4-ethylphenol; Δ, 4-vinylphenol; □, pH.

The effect of a wine residual sugar, trehalose, was also tested concerning the production of 4-ethylphenol. The results evidentiate a much lower conversion rate of *p*-coumaric acid to 4-ethylphenol when compared with the data for glucose or ethanol (Table 1).

The production of 4-ethylphenol under the presence of acetic acid as single carbon and energy source was also assessed. Growth proceeded at low rate while the conversion of *p*-coumaric acid to 4-ethylphenol was much lower when compared with that of glucose or ethanol (Table 1).

The effect of the absence of carbon and energy sources on growth and volatile phenol production by strain ISA 1791 is shown in Fig. 4. When glucose was removed, after the onset of growth and volatile phenol production, the initial slight increase in the concentrations of 4-vinylphenol and 4-ethylphenol was arrested.

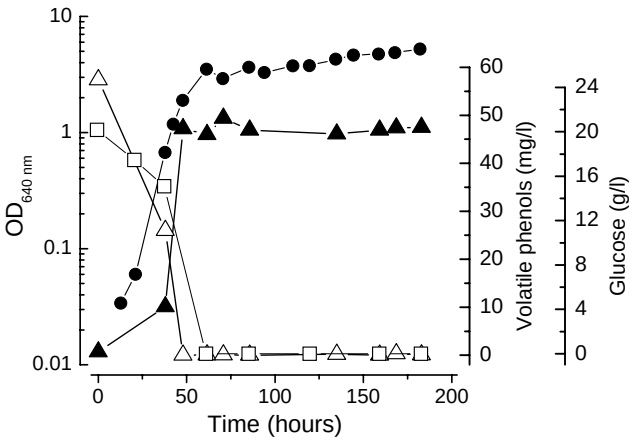

Fig. 4. Growth of *D. bruxellensis* ISA 1791 in YNB with aminoacids added of 75 mg/l 4-vinylphenol and 20 g/l glucose. Symbols: ●, optical density; ▲, 4-ethylphenol; Δ, 4-vinylphenol; □, glucose.

3.2. Effect of the precursor on the production of 4-ethylphenol

The production of 4-ethylphenol is the result of two consecutive reactions (see introduction) and we tested if the intermediary metabolite—4-vinylphenol—could be used as precursor of 4-ethylphenol in the absence of *p*-coumaric acid. In fact, when 4-vinylphenol was added to growth media, the production of 4-ethylphenol proceeded in the same way as when glucose was used as single energy and carbon source (Fig. 4). In addition,

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growth rate ($\mu = 0.14\,h^{-1}$), medium acidification (down to pH 2.2), ethanol production (up to 7.3 g/l) and subsequent consumption and absence of acetic acid production were similar to the results observed when *p*-coumaric acid was added to the medium (results not shown). This behaviour was also observed when ethanol was used as single carbon and energy source (results not shown).

3.3. Effect of temperature on the production of 4-ethylphenol

The effect of temperature on the growth and 4-ethylphenol production of *D. bruxellensis* ISA 1791 in medium with ethanol as single carbon and energy source is shown in Fig. 5, for two different incubation temperatures (16°C and 30°C). At 30°C, growth occurred at a growth rate of $0.07\,h^{-1}$ while at 16°C the growth rate was $0.02\,h^{-1}$. Maximum production of 4-ethylphenol was about 62 mg/l at both temperatures. The decrease in growth temperature from 30°C to 16°C induced lower production rates of 4-vinylphenol and 4-ethylphenol.

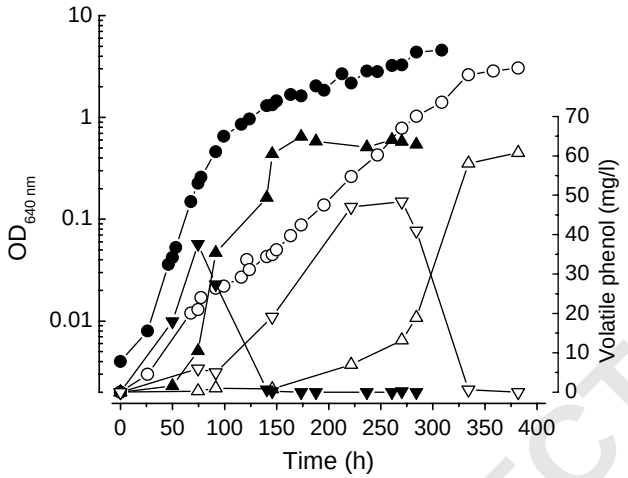

Fig. 5. Growth of the strain *D. bruxellensis* ISA 1791 in YNB with aminoacids added of 100 mg/l *p*-coumaric acid and 20 g/l ethanol incubated at 16°C (open symbols) and 30°C (closed symbols). Symbols: ●,○, optical density; ▲, Δ, 4-ethylphenol; ▼, ▽, 4-vinylphenol.

The effect of temperature on growth and volatile phenol production was similar when glucose was used as single energy and carbon source (results not shown). Therefore, the conversion rates observed in the presence of glucose or ethanol as single carbon and energy sources (see Table 1) were not affected by temperature.

3.4. Effect of ethanol on the production of 4-ethylphenol

The inhibitory effect of ethanol on growth and 4-ethylphenol production was assessed for 4%, 6%, 8%, 10%, 12% and 13% (v/v). Increasing ethanol concentrations up to 10% (v/v) resulted in lower growth rates but the maximum amount of 4-ethylphenol produced was not considerably affected (Fig. 6). Growth and 4-ethylphenol production were noticeably reduced at 12% (v/v) and were not observed at concentrations equal or higher than 13% (v/v) ethanol.

3.5. Growth and 4-ethylphenol production in mixed cultures in synthetic medium and grape juices

The growth of *S. cerevisiae* and *D. bruxellensis* in mixed cultures in synthetic medium is shown in Fig. 7. The population of *S. cerevisiae* increased to 3×10^9 cfu/ml and began to lose viability after the end of fermentation. Cell numbers of *D. bruxellensis* increased from 10^4 cfu/ml to about 5×10^9 cfu/ml which was similar to the maximum concentration reached by *S. cerevisiae*. The production of 4-vinylphenol was initiated at the beginning of the experiment, while the conversion to 4-ethylphenol was observed when *D. bruxellensis* reached the stationary phase.

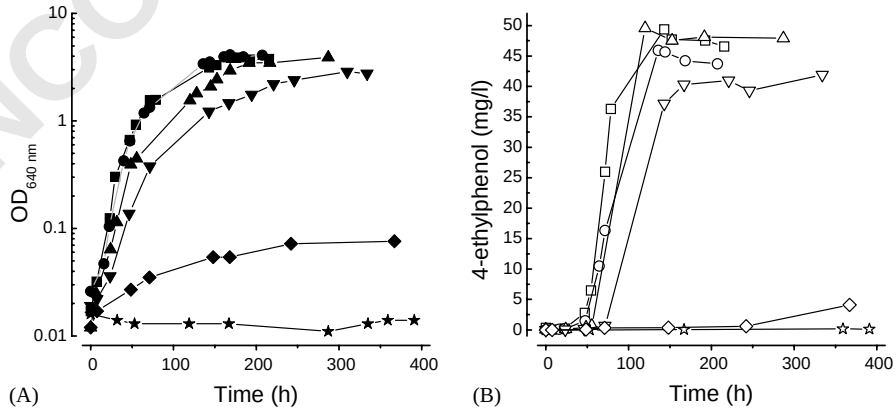

Fig. 6. Growth (A) and 4-ethylphenol (B) of the strain *D. bruxellensis* ISA 1791 in YNB plus 2% (w/v) of glucose added of 100 mg/l *p*-coumaric acid and increasing concentrations of ethanol (% v/v): (□,■) 4; (○,●) 6; (Δ, ▲) 8; (▽,▼) 10; () 11 and (☆, ★) 13.

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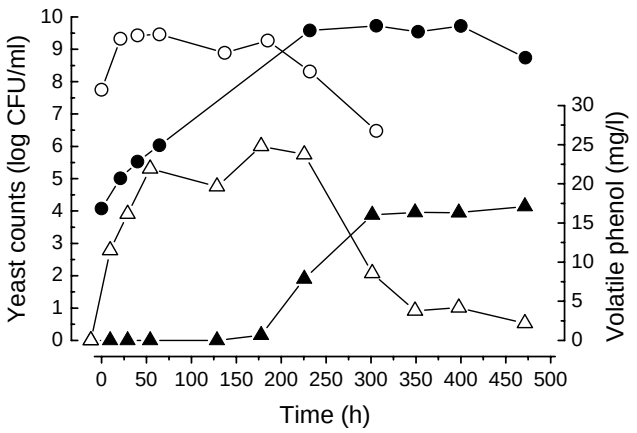

Fig. 7. Growth and volatile phenol production of mixed cultures of *S. cerevisiae* ISA 1000 and *D. bruxellensis* ISA 1791 grown in YNB added of 100 g/l *p*-coumaric acid, 100 g/l glucose and 100 g/l fructose. Symbols: ○, colony counts of *S. cerevisiae*; ●, colony counts of *D. bruxellensis*; Δ, 4-vinylphenol; ▲, 4-ethylphenol.

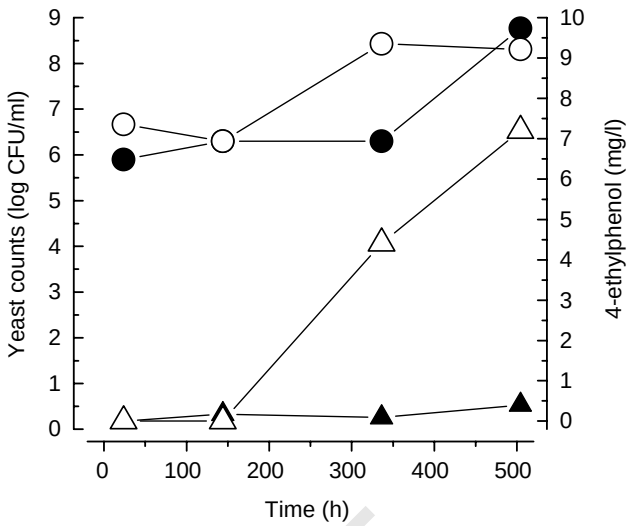

Fig. 8. Growth and volatile phenol production of *D. bruxellensis* ISA 1791 in post-fermented red (filled symbols) and white grape juices (open symbols) added of 10 mg/l *p*-coumaric acid after the end of fermentation. Grape juices were inoculated with 10^7 cells/ml of *S. cerevisiae* and 10 cells/ml of *D. bruxellensis* ISA 1791. Symbols: ○, ●, colony counts of *D. bruxellensis*; Δ, ▲, 4-ethylphenol.

The experiments carried out in white or red grape juices, inoculated with 10^7 cells/ml of *S. cerevisiae*, were performed in two steps: (i) fermentation in mixed cultures with two levels of *D. bruxellensis* (10^4 and 10 cells/ml); (ii) incubation of the resulting fermented juice added of 10 mg/l *p*-coumaric acid. The addition of *p*-coumaric acid did not affect yeast growth and was necessary because only a maximum of 0.3 mg/l of 4-ethylphenol were produced in unsupplemented juices during the same incubation time (results not shown). Fermentations were complete after 96 h for both white and red juices (results not shown). The changes in cell counts and volatile phenols in the post-fermentation period (ii) for the lower inoculation rate of *D. bruxellensis* are shown in Fig. 8. In both red and white juices, *D. bruxellensis* cell counts correspondent to the lower inoculum size increased and attained levels similar to the higher inoculum size. In addition, 4-ethylphenol production was similar for both inoculum sizes while the production of 4-ethylphenol was higher on white than on red juices at the end of the experiments.

4. Discussion

D. bruxellensis grown in the presence of glucose showed a relatively low growth rate when compared with the values reported for other wine-related yeasts like *S. cerevisiae* and *Z. bailii* (Rodrigues et al., 2001a). This is a well-known characteristic of the genera *Dekkera*/*Brettanomyces* (Deak and Beuchat, 1996) that explains the longer incubation periods required for their isolation on plating media (Rodrigues et al., 2001b).

In media with glucose as carbon and energy source, the conversion of *p*-coumaric acid to 4-vinylphenol and 4-ethylphenol occurred in a way similar to that presented by Edlin et al. (1995) in *B. anomalus* (anamorph of *D. anomala*). However, the reduction step was dependent on the carbon and energy source. High conversion rates of *p*-coumaric acid to 4-ethylphenol were only attained when glucose or ethanol were the substrate. Chatonnet et al. (1995) observed a considerable reduction in trehalose concentrations after growth of *Dekkera* sp. in wines suggesting that this residual sugar may allow the synthesis of high levels of 4-ethylphenol but their work did not discriminate trehalose from other residual sugars like glucose and fructose. According to our results, the conversion of *p*-coumaric acid to 4-ethylphenol in the presence of trehalose as substrate occurs at rather low conversion rates and so it is conceivable that the contribution of this sugar to the overall production of 4-ethylphenol is not relevant in wine.

The absence of 4-ethylphenol and 4-vinylphenol production in resting cells indicates that the enzymes responsible for the reduction and decarboxylation steps depend on the availability of substrate for growth. In practical terms, wine is regarded as "dry" when the fermentable sugars are less than 2 g/l but this amount of sugar was not a limitation to the production of high levels of 4-ethylphenol by *D. bruxellensis*, as observed in the tests with increasing levels of ethanol. In addition, during wine maturation in barriques or during wine rackings, oxygen is dissolved in wine promoting yeast growth (Malfeito-Ferreira et al., 2001) and enabling the assimilation of substrates like ethanol with production of high levels of 4-ethylphenol. These results demon-

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strate that the conversion of *p*-coumaric acid to 4-ethylphenol in wines, in which the above-mentioned carbon sources are present in different concentrations or in which different levels of oxygen are dissolved, is a process in which it is difficult to individuate the contribution of each substrate to the overall production of 4-ethylphenol under winery conditions.

The production of 4-ethylphenol was not subjected to catabolic repression by glucose (Malfeito-Ferreira et al., 2001) and so this metabolite may be produced during grape juice fermentation. However, our results from growth in synthetic media or grape juice indicate that this production occurred after the end of the alcoholic fermentation by *S. cerevisiae*. *D. bruxellensis* populations survive during this phase even when the initial inoculum size was as low as 10 cells/ml and are able to convert *p*-coumaric acid into 4-ethylphenol in the post-fermentation period. This observation may explain why wines after fermentation show occasionally high levels of this compound (Rodrigues et al., 2001b) and evidentiate the need for accurate plant sanitation and grape sulphitation during harvesting. The observation of lower production of 4-ethylphenol in red wines as compared with white wines is probably related with experiment duration. In fact, regarding red wine, *D. bruxellensis* shows a bell-shaped growth curve accompanied by high production of 4-ethylphenol (Malfeito-Ferreira et al., 2001).

Chatonnet et al. (1995, 1997) established that yeasts of the genus *Dekkera* were the agents of "horse sweat" taints leading to wine spoilage, demonstrating that *S. cerevisiae* and lactic bacteria only could produce high amounts of 4-vinylphenol which did not result in significative amounts of 4-ethylphenol in wines. However, according to our results, this 4-vinylphenol may be converted by *D. bruxellensis* into 4-ethylphenol. This fact, and the difficulty in the isolation of *D. bruxellensis* from wines (Rodrigues et al., 2001b), may explain, at least partially, the former belief that the "horse sweat" taint was due to bacterial activity (Baumes et al., 1986).

Chatonnet et al. (1993a) stated that wines are more susceptible to the phenolic taint in warmer months. In fact, the effect of temperature on 4-ethylphenol production was related with the production rate, and not with the total amount produced. Thus, keeping wines at low cellar temperatures only delays the process, being an efficient prevention measure if cell growth is fully inhibited.

The inhibition of growth and 4-ethylphenol production by 13% (v/v) of ethanol correlates fairly well with the observation that wines with high level of ethanol did not show high concentrations of this phenol (Rodrigues et al., 2001b). In addition, these authors showed that 4-ethylphenol levels in wines were not correlated with acetic acid concentrations which is in agreement with our results in synthetic media where volatile phenol production was not accompanied by acetic acid production, except in media with high sugar content, as also observed by Gerós et al. (2000) in *D. anomala*. This observation indicates that these two *D. bruxellensis* spoiling features (acetic acid and 4-ethylphenol production) are independent. The influence of other factors that stimulate acetic acid production, like oxygen (Ciani and Ferraro, 1997) and ammonium sulphate (Uscanga et al., 2000) should not be relevant during wine production. Moreover, acetic acid production is variable among strains of the genera *Dekkera*/*Brettanomyces* cultivated under the same conditions (Freer, 2002).

In conclusion, this work enlightened the effect of a variety of factors on the production of 4-ethylphenol by *D. bruxellensis*, in synthetic media and grape juices, enabling the understanding of some empirical observations related with the development of the "phenolic taint" under winery conditions. Further experiments are being carried out to evaluate the factors affecting the behaviour of *D. bruxellensis* in wines.

Acknowledgements

We are indebted to Professor M. Loureiro-Dias for critically revising the manuscript. The research work was supported by national projects PAMAF/6043 and AGRO/96.

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