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Factors Affecting Volatile Phenol Production During Fermentations with Pure and Mixed Cultures of *Dekkera bruxellensis* and *Saccharomyces cerevisiae*

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Summary

The paper examines the impact of ethanol, and hidroxycinnamic and vinylphenol precursors on the production of volatile phenols in fermentations of mixed and pure cultures of yeasts *Saccharomyces cerevisiae* and *Dekkera bruxellensis*. Three different *D. bruxellensis* strains were examined and they all showed a unique volatile phenol production pattern in the fermentations of pure and mixed cultures. Generally, the results showed that in mixed culture fermentations less vinylphenols and more ethylphenols were produced in comparison with *D. bruxellensis* pure culture fermentations. Vinylphenol precursors significantly inhibited the growth of *S. cerevisiae* and the production of ethylphenols. Nevertheless, it was found that *D. bruxellensis* genes encoding for enzymes coumaric acid decarboxylase (CAD) and vinylphenol reductase (VPR) are more responsive to vinylphenol precursors in comparison with hidroxycinnamic acids. Consequently, higher concentrations of vinylphenols in the cell were found to be more cytotoxic than hidroxycinnamic acids. In general, low ethanol concentrations induced higher production of volatile phenols by *S. cerevisiae* and *D. bruxellensis*. This was confirmed with the expression pattern of gene encoding for CAD of *D. bruxellensis*.

Key words: *Saccharomyces cerevisiae*, *Dekkera bruxellensis*, wine fermentation, volatile phenols

Introduction

Dekkera bruxellensis (anamorph: *Brettanomyces bruxellensis*) produces phenolic off-flavours that cause undesirable taints described as 'medicinal' and 'clove-like' in white wines (due to vinylphenols) and as 'leather', 'horse sweat' and 'barnyard' in red wines (due to ethylphenols) (*1*). These faults cause large economic losses (2) and are difficult to avoid, firstly because of the low hygiene level which can be reached in winemaking, and secondly because of the resistance and growth of this yeast in strict environmental conditions (*3*). Physiological tests of *D. bruxellensis* survival and development in such conditions (*4*) indicated that instead of avoiding *Dekkera bruxellensis* presence, it is better to reduce or prevent its production of volatile phenols.

The biosynthesis of volatile phenols is related to the sequential activity of two enzymes which decarboxylate hydroxycinnamic acids (ferulic, *p*–coumaric and caffeic acids) naturally present in grapes into vinylphenols, which are then reduced to ethylphenols (*5*). The decarboxylation step catalyzed by coumarate decarboxylase is common amongst wine yeasts and even *S. cerevisiae* has been reported to produce vinylphenols from hydroxycinnamic acids (HCAs) (*6*). However, the reduction step

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This paper is devoted to Professor Pavao Mildner for his insightful contributions to biochemistry and his important role in creating an open door to microbial biotechnology.

catalyzed by vinylphenol reductase is present in just a few yeast species including *D. bruxellensis* (*7*). In *S. cerevisiae*, genes encoding for phenyl acrylic acid decarboxylase (*PAD1*, YDR538W) and for the putative ferulic acid decarboxylase (*FDC1*, YDR539W) have been well studied (*8*). Moreover, a partial purification and identification of *Dekkera* spp. genes encoding for phenolic acid decarboxylase (PAD) (*9*) and vinylphenol reductase (VPR) (*10*) have been made. Even though volatile phenols are common products of *Dekkera bruxellensis*, their production is highly dependent on strain variability (*11*). Strains can grow fast and can produce low levels of volatile phenols or *vice versa* (*12*).

Wine fermentation is a complex process involving a microflora of wine microorganisms and their interactions including: neutralism, commensalism, mutualism/synergism, amensalism or antagonism and competition (*13*). Therefore, the uptake of specific compounds such as HCAs or vinylphenols by one species could influence the production of ethylphenols during wine fermentation.

The influence of different chemical factors and different temperatures on volatile phenol production has been well studied, but only in the case of *D. bruxellensis* in pure culture fermentations (*14,15*). The few existing studies of *D. bruxellensis* and *S. cerevisiae* mixed culture fermentations are limited to just a few experiments (*15–17*) and a physiological study should be conducted to assess the impact of microbial competition on the consumption of precursors (HCA and vinylphenols) and on the production of volatile phenols.

In order to study the impact of microbial competition on the metabolism of volatile phenols, we have made a series of fermentations with pure and mixed cultures of wine yeast *S. cerevisiae* and of three different isolates of *D. bruxellensis*. For physiological tests, the activities of coumarate decarboxylase and vinylphenol reductase were monitored by high-performance liquid chromatography (HPLC) and real-time polymerase chain reaction (RT-PCR) in a synthetic must supplemented with either precursor molecules or ethanol.

Materials and Methods

Yeast strains

Effects of precursors and ethanol were studied with pure and mixed cultures of *D. bruxellensis* strain ZIM 701 and *S. cerevisiae* strain EC-1118. Strain ZIM 701 was isolated from the spontaneous fermentation of must of Rebula wine from Slovenia (Wine cellar Goriška Brda) and EC-1118 is a commercial strain (Lallemand® SAS, Blagnac, France) used in winemaking. In order to study the effects of using different *D. bruxellensis* strains in fermentations of pure and mixed cultures with *S. cerevisiae* EC-1118, *D. bruxellensis* strains ZIM 2306 and ZIM 1762 were also applied. ZIM 2306 was isolated from soiled Rebula wine and ZIM 1762 from the sediment of lambic beer (*18*). All strains used in this study originate from the Collection of Industrial Microorganisms (ZIM) at the Biotechnical Faculty, Ljubljana, Slovenia.

Media

All yeast strains were maintained on YPD agar plates containing (in g/L): yeast extract 10 (BD Difco, BD Diagnostic Systems, Mississauga, ON, Canada), peptone 20 (BD Difco), glucose 20 (Sigma-Aldrich, St. Louis, MO, USA), and agar 15 (Difco), pH=6.0–6.2 at 28 °C. The inoculum was prepared after 27 (for *S. cerevisiae*) or 50 h (for *D. bruxellensis*) of incubation at 28 °C and 2.5×*g* in liquid YPD medium (without agar). Prior to inoculation, the suspension was centrifuged and resuspended in phosphate-buffered saline (PBS). For fermentation experiments synthetic must MS300 was used, which resembles in composition the natural must as described by Bely *et al*. (*19*). The medium contained 100 g/L of each glucose and fructose (both from Sigma-Aldrich), the pH was adjusted to 3.3 with HCl, and it was filter-sterilised through a 0.22-μm pore filter (Millipore, Billerica, MA, USA). The synthetic must MS300 containing 50 mg/L of each *p*-coumaric (Fluka, Sigma-Aldrich, Steinheim, Germany) and ferulic acids (100 mg/L of HCAs in total) was considered as a control medium for studying volatile phenol production, because the growth of *S. cerevisiae* and *D. bruxellensis* and CO_2 production remained unaffected by the addition of these two acids (data not shown).

The influence of vinylphenol precursors on volatile phenol production was studied in synthetic must MS300 supplemented with a combination of 4-vinylphenol and 4-vinylguaiacol (Sigma-Aldrich) in a total concentration of 100 mg/L. Both precursors were added at an equal concentration.

The effect of the addition of 2.5, 5 or 10 % ethanol (Merck, Darmstadt, Germany) on the production of volatile phenols was studied in synthetic must MS300 supplemented with 10 mg/L of each *p*-coumaric and ferulic acids (20 mg/L of HCAs in total). A lower concentration of HCAs was used because 100 mg/L of HCAs combined with 10 % ethanol severely hindered the growth of both yeasts (data not shown).

For the determination of *S. cerevisiae* and *D. bruxellensis* colony counts in pure cultures, YPD agar plates were used. In mixed cultures, *D. bruxellensis* colony counts were determined using YPD agar plates containing 0.01 % cycloheximide (Sigma-Aldrich). *D. bruxellensis* is exceptionally tolerant to this antibiotic, but *S. cerevisiae* is very sensitive to its presence. Colonies of *S. cerevisiae* in mixed cultures were determined on YPD agar plates on which they would rapidly outgrow the small colonies of *D. bruxellensis.*

Fermentation conditions

All fermentations were carried out under microaerobic conditions in 250-mL Erlenmeyer flasks filled with 200 mL of synthetic must MS300 and sealed with rubber stoppers with a CO_2 outlet. For pure culture fermentations, a single strain of *D. bruxellensis* or *S. cerevisiae* was inoculated into MS300 medium to a final concentration of 10^6 cells per mL. For mixed culture fermentations, a single strain of *D. bruxellensis* and a single strain of *S. cerevisiae* were inoculated together, each having a final concentration of 10^6 cells per mL. The inoculum concentrations were determined by counting cells under a light microscope. Both pure and mixed culture fermentations were performed in triplicate. The fermentation temperature was 22 °C and the inoculated synthetic must was under constant magnetic stirring. Fermentations followed

a time course of 210 h (8.7 days) and the fermentation flasks were weighed daily to assess the CO_2 release and the progress of alcoholic fermentation. Yeast growth during fermentations was evaluated by viable yeast enumeration on YPD agar plates using the successive dilution method.

Chemical analysis

Samples were taken periodically from culture medium and were centrifuged at 4000×*g* for 5 min. Supernatants were filtered through a 0.22-μm pore filter (Millipore) prior to analysis. For the purpose of measuring volatile phenols, samples were analyzed by Knauer (Berlin, Germany) high-performance liquid chromatography apparatus consisting of a Midas autosampler (Spark, Emmen, The Netherlands), Knauer K1001 WellChrom pumps with 10-mL pump heads, a Knauer dynamic mixing chamber and a Shimadzu RF-551 fluorescence detector (Shimadzu, Kyoto, Japan). Separation was carried out with an XBridge Phenyl column (130 Å, 3.5 μm, 4.6 mm×150 mm, 1/pkg; Waters, Milford, MA, USA) using a 27 % acetonitrile as the mobile phase at a flow rate of 1 mL/min. For quantification, 4-vinylguaiacol (4-VG), 4-vinylphenol (4-VP), 4-ethylguaiacol (4-EG) and 4-ethylphenol (4-EP) were used as external standards (Sigma-Aldrich). The detector was set to high sensitivity at an excitation wavelength of 280 nm and an emission wavelength of 333 nm.

Gene expression analysis

After 50 h of fermentation (the beginning of the exponential production of volatile phenols by *D. bruxellensis*), 10-mL cell suspension samples were collected and centrifuged for 5 min at 2558×*g*. The supernatants were discarded and total RNA extraction was performed using TRI Reagent® (Molecular Research Center, Cincinnati, OH, USA) as described by Remize *et al*. (*20*). RNA concentration was quantified spectrophotometrically with a LAMBDA Bio spectrophotometer (PerkinElmer, Santa Clara, CA, USA). RNA quality was tested by electrophoresis on 1.5 % agarose gels using 0.5× TAE buffer. cDNA was synthesized using SuperScript® VILO™ cDNA Synthesis Kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. For all reverse transcription reactions, 650 ng of RNA were used. Primers were designed by using the computer program PRIMER3 (*21*). For *D. bruxellensis* genes encoding for a coumaric acid decarboxylase (CAD) and a vinylphenol reductase (VPR), the primers were obtained from Ganga *et al*. (*22*) and Tchobanov *et al*. (*10*), respectively (see Table 1). All the PCR reactions were performed on an ABI 7500 sequence detection system (Applied Biosystems, Life Technologies). The cDNAs were used as template for the RT-PCR with forward and reverse primers in a total volume of 20 μL using the SYBR Green mixture (EXPRESS SYBR® GreenER™ qPCR Supermix Universal, Life Tech-

Table 1. PCR primers of genes selected for expression analysis

Primer	Primer sequence (5-3')	Melting temperature °C	G-C base pairs %	Gene Ontology terms	Accession no. or origin of sequence	Reaction efficiency %
***S. cerevisiae* primers**						
ACT1 Fsc	CAGGTATTGCCGAAAGAATG	58.9	45.0	cytoskeleton	NM_001179927.1	96
ACT1 Rsc	GTCAAAGAAGCCAAGATAGAACC	58.1	43.5			
TEF1 Fsc	TGTCACACTGCTCACATTGC	59.4	50.0	protein translation	NM_001184177.1	97
TEF1 Rsc	GGAACGAACTTGACCAAAGC	59.7	50.0			
18S rRNA Fsc	CTGCTTAATTGCGATAACGAAC	58.4	40.9	protein translation	emb|Z75578.1|	94
18S rRNA Rsc	CAGACCTGTTATTGCCTCAAAC	59.4	45.5			
PAD1 Fsc	CTTTATGCAGGGCAGGTGTT	60.1	50.0	carboxy-lyase activity	NM_001180846.1	97
PAD1 Rsc	GGATCCTGCCAACACTTTGT	59.9	50.0			
FDC1 Fsc	TTCCTGCAACGAGTGAGATG	59.9	50.0	cinnamic acid catabolic process	NM_001180847 1	96
FDC1 Rsc	GGATGACCTTGGCTTTTGAA	60.7	45.0			
***D. bruxellensis* primers**						
ACT1 Fdb	CGGTTCTGGTATGTGCAAAG	59.1	50.0	cytoskeleton	gb|EI020428.1|	94
ACT1 Rdb	TTGACCCATACCGACCATAA	58.7	45.0			
TEF1 Fdb	GACTGCCACACTGCTCACAT	59.9	55.0	protein translation	9b|EU014766.1|	96
TEF1 Rdb	AGCAGCATCACCTCTCTTGA	58.7	50.0			
18S rRNA Fdb	CAAGCCGAAGGAAGTTTGAG	59.9	50.0	protein translation	emb|X83814.1|	96
18S rRNA Rdb	GTACAAAGGGCAGGGACGTA	59.9	55.0			
CAD Fdb	TCTTCCAAGCAGGGATTTTG	58.2	45.0	unknown	translated from peptide sequence[a]	95
CAD Rdb	CATTCCGCCTCCACTTTTATC	59.6	47.6			
VPR Fdb	GGCTTTTTGACCATCCAGTA	57.6	45.0	unknown	translated from peptide sequence[b]	97
VPR Rdb	AAGTGAAGTCAATCCTAGGCAAG	58.9	43.4			

[a]primer sequences for gene encoding for CAD were designed by Ganga *et al.* (*22*) according to the partially sequenced protein from *D. anomala* showing a coumarate decarboxylase activity (*9*), [b]primer sequences for gene encoding for VPR were translated from peptide sequences GFLTIQYDSGK and NLPRIDFTLNLP of a 26-kDa protein showing a vinylphenol reductase activity (*10*)

nologies). The reaction conditions were: 2 min at 50 °C and 2 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Melting curve analysis was performed in 30 s at 72 °C. For each gene, a standard curve was determined using the genomic DNA of either *S. cerevisiae* or *D. bruxellensis.* The reaction efficiencies for all genes of both yeast species are presented in Table 1. Genomic DNA was isolated using the MasterPure™ Yeast DNA purification kit (Epicentre Biotechnologies, Madison, WI, USA) according to the manufacturer's instructions.

Relative quantification was carried out using the relative expression software tool (REST) (*23*), while the expression of *S. cerevisiae* genes *PAD1* and *FDC1*, and the expression of *D. bruxellensis* genes encoding for CAD and VPR were standardized by the simultaneous expression of three house-keeping genes: 18S rRNA, translation elongation factor EF-1α (*TEF1*) and actin (*Act1*) for each yeast species separately. Mean threshold cycle (Ct) values were determined with three separate reactions per condition.

Results

Effects of precursors

The growth of *S. cerevisiae* in both pure and mixed cultures, supplemented with 100 mg/L of HCAs, reached similar levels of around 8.6 log CFU/mL (Fig. 1). In pure culture, it released 83 g/L of CO_2. The maximum population density reached by *D. bruxellensis* ZIM 701 was 8.5 log CFU/mL in pure culture and 7.9 CFU/mL in mixed culture fermentations (Fig. 1). Its exponential growth phase was steeper in mixed cultures, achieving stationary phase later on. However, in pure cultures the stationary phase was reached only after 160 h of fermentation and 22.1 g/L of CO_2 were released.

Substitution of 100 mg/L of HCAs with the same amount of vinylphenols reduced the growth of *S. cerevisiae* by 1.0 log CFU/mL in pure and mixed culture fermentations (Figs. 1a and c). After 214 h, its growth dropped to only 6.5 log CFU/mL. Furthermore, exponential growth of *D. bruxellensis* ZIM 701 was also reduced; however, after 160 h its population reached a similar density as in the medium containing HCAs (Figs. 1b and c). Generally, vinylphenols delayed CO_2 production of *S. cerevisiae* and *D. bruxellensis* ZIM 701 by 50 and 47 h, respectively.

In pure culture fermentations containing 100 mg/L of HCAs, *S. cerevisiae* converted 12.3 % of ferulic acid and 25.3 % of *p*-coumaric acid to 6.1 mg/L of 4-VG and 12.6 mg/L of 4-VP, respectively (Fig. 2). *D. bruxellensis* in pure culture completely assimilated HCAs and intracellularly metabolised them to ethylphenols, thus only small concentrations of vinylphenols were released into the medium (Fig. 2). In mixed cultures, vinylphenol concentrations reached similar levels in comparison with *S. cerevisiae* pure culture fermentations; however, after 70 h of incubation, *D. bruxellensis* ZIM 701 completely assimilated vinylphenols that were accumulated in the medium by *S. cerevisiae.* In total 70 % of HCAs were converted to ethylphenols in the mixed culture fermentations (Fig. 2).

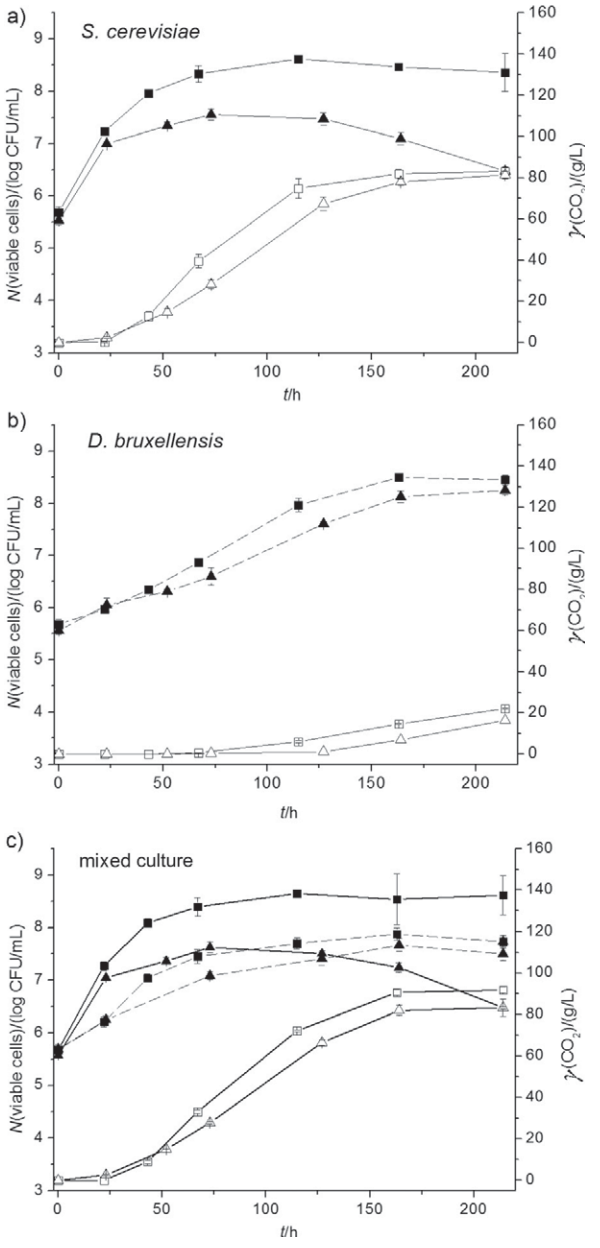

Fig. 1. Yeast growth (filled symbols) and CO_2 release (open symbols) in synthetic must MS300 containing 100 mg/L of hydroxycinnamic acids (■, □) or 100 mg/L of vinylphenols (▲, △): a) *S. cerevisiae* pure culture fermentations (solid line), b) *D. bruxellensis* ZIM 701 pure culture fermentations (dashed line), and c) mixed culture fermentations. Standard deviations of each point are represented by error bars

In the fermentation of *S. cerevisiae* pure culture, the concentration of externally added vinylphenols remained constant throughout the fermentation (Fig. 2a). *D. bruxellensis* ZIM 701 assimilated all of the vinylphenols, producing 32.2 mg/L of 4-EG and 32.8 mg/L of 4-EP in pure culture fermentations (65 % conversion to ethylphenols) (Fig. 2). Unexpectedly, in mixed culture fermentations the assimilation of vinylphenols by *D. bruxellensis* was not complete, and after 214 h of fermentation 31.8 mg/L of 4-VG and 39.2 mg/L of 4-VP remained in the fermented medium. Furthermore, only 8.2 mg/L of 4-EG and 0.8 mg/L of 4-EP were produced (Fig. 2).

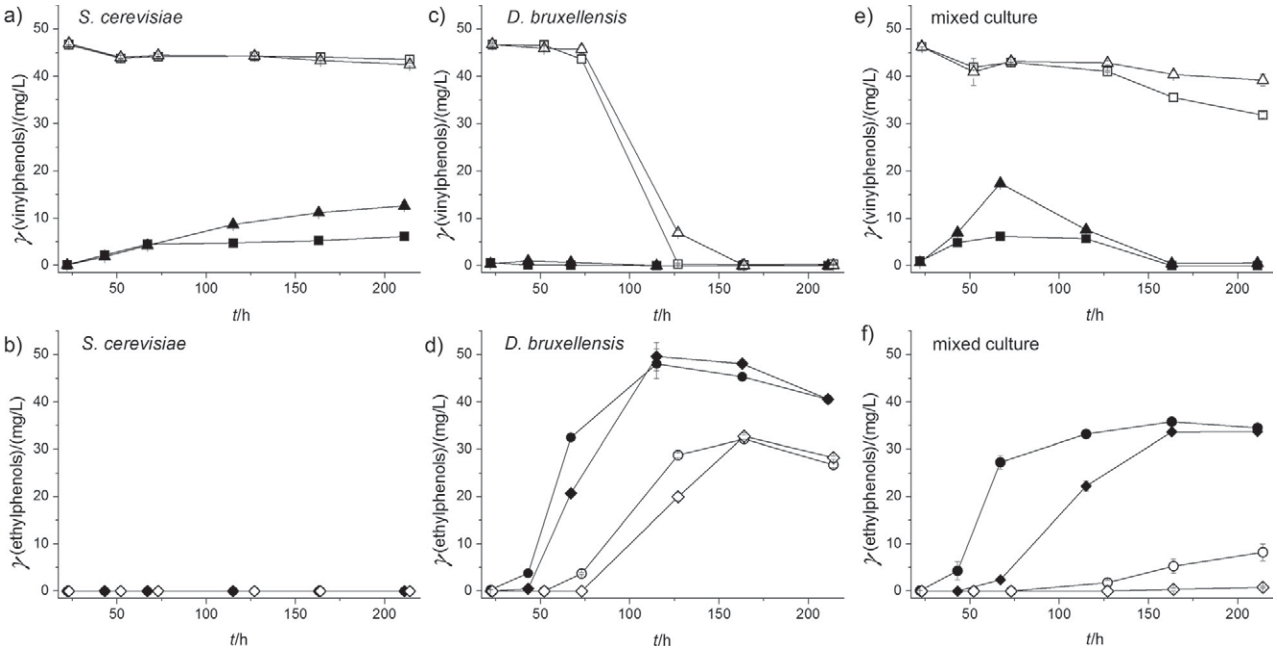

Fig. 2. Volatile phenol production in synthetic must MS300 supplemented with either 100 mg/L of hydroxycinnamic acids (filled symbols) or 100 mg/L of vinylphenols (open symbols): a) and b) *S. cerevisiae* pure culture fermentations, c) and d) *D. bruxellensis* ZIM 701 pure culture fermentations, and e) and f) mixed culture fermentations. Production of 4-vinylguaiacol (■, □) and 4-vinylphenol (▲, △) is presented in graphs a), c) and e) and production of 4-ethylguaiacol (●, ○) and 4-ethylphenol (◆, ◇) in graphs b), d) and f). Standard deviations of each point are represented by error bars

Gene expression values for fermentations conducted in 100 mg/L of HCAs are presented in Fig. 3. In comparison with pure cultures, *D. bruxellensis* genes encoding for CAD and VPR were down-regulated in mixed culture fermentations having a fold change of 0.80 and 0.69, respectively (Fig. 3). However, the fold change of *S. cerevisiae* genes was 1.04 for *PAD1* and 0.97 for *FDC1*.

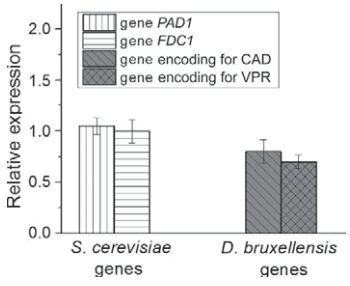

Fig. 3. Relative expression levels of genes *PAD1* and *FDC1*, and those encoding for CAD and VPR in *S. cerevisiae* (white bars) and *D. bruxellensis* (grey bars) after 50 h of fermentation in medium supplemented with 100 mg/L of HCAs. Fold change was calculated using pure cultures as controls and mixed cultures as test samples (relative expression level 1 means constant expression and level 2 means up-regulation in the mixed cultures)

In comparison with HCA precursors, the externally added vinylphenols up-regulated genes encoding for CAD and VPR in the pure (2.97-fold for CAD and 2.27-fold for VPR) and in the mixed cultures (1.79-fold for CAD and 1.42-fold for VPR) (Fig. 4). In contrast, genes *PAD1* and *FDC1* of yeast *S. cerevisiae* were down-regulated by vinylphenol precursors in the mixed (0.69-fold for *PAD1* and 0.57-fold for *FDC1*) and in the pure cultures (0.64--fold for *PAD1* and 0.62-fold for *FDC1*) (Fig. 4).

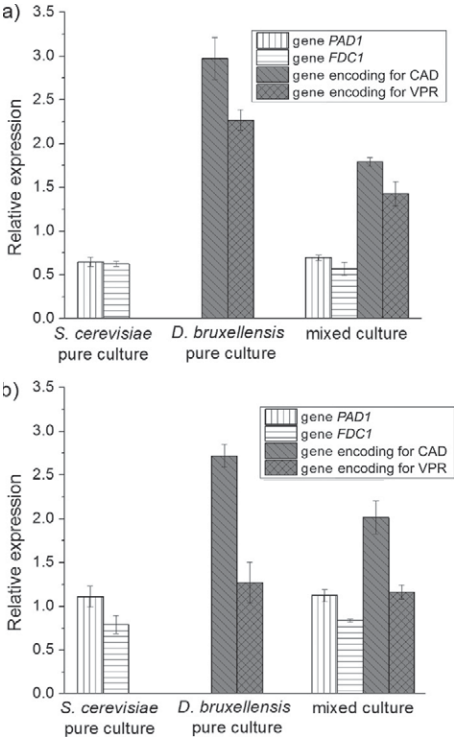

Fig. 4. Relative expression levels of genes *PAD1* and *FDC1*, and those encoding for CAD and VPR in *S. cerevisiae* (white bars) and *D. bruxellensis* (grey bars) after 50 h of fermentation: a) the substitution of 100 mg/L of HCAs with 100 mg/L of vinylphenols in pure and mixed cultures. Fold change was calculated using fermentations supplemented with HCAs as controls and fermentations supplemented with vinylphenols as test samples; b) the addition of ethanol to media containing 20 mg/L of HCAs. The controls were without ethanol and test samples were supplemented with 2.5 % ethanol

Effects of ethanol

In MS300 synthetic must supplemented with 20 mg/L of HCAs, raising volume fractions of ethanol from 2.5 to 10 % progressively reduced the growth of and CO_2 release by *S. cerevisiae* and *D. bruxellensis* ZIM 701 (Fig. 5). At 2.5 % the population density of *S. cerevisiae* was 0.75 log CFU/mL lower in comparison with MS300 control must (20 mg/L of HCAs, 0 % of ethanol). Furthermore, at 5 % it was reduced by 1.3 log CFU/mL and at 10 % by 1.7 log CFU/mL in pure and mixed culture fermentations, respectively. A 22-hour lag phase was present at 10 % of alcohol (Fig. 5).

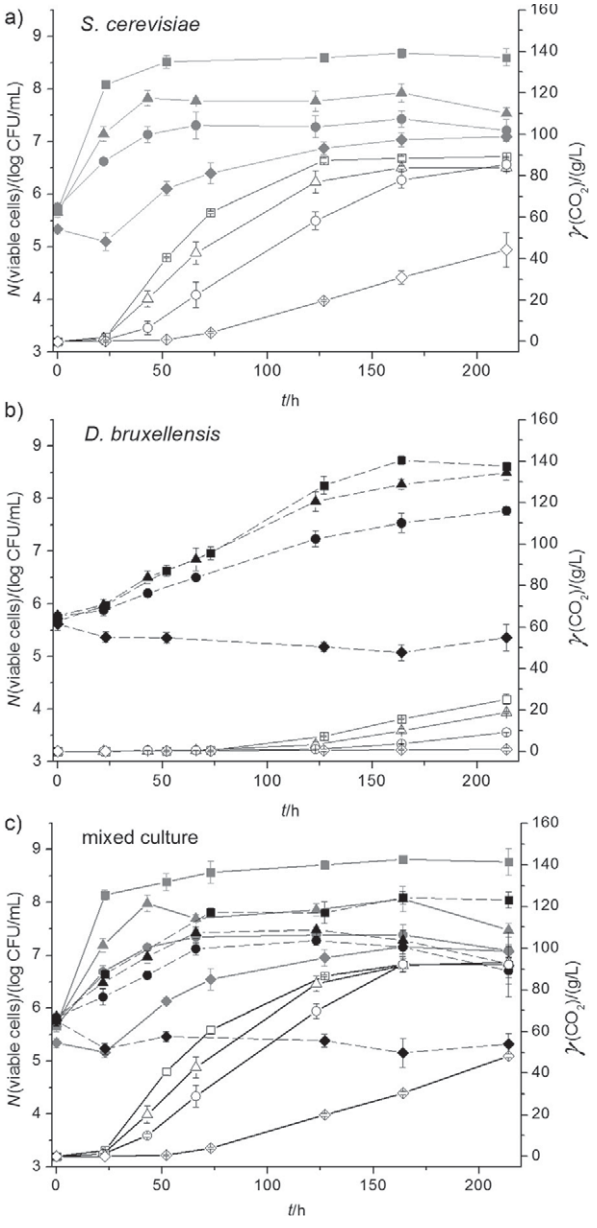

Fig. 5. Effect of 0 (■, □), 2.5 (▲, △), 5 (●, ○) and 10 % (◆, ◇) of ethanol on yeast growth (filled symbols) and CO_2 release (open symbols): a) *S. cerevisiae* pure culture fermentations (grey symbols), b) *D. bruxellensis* ZIM 701 pure culture fermentations (black symbols with dashed line), and c) mixed culture fermentations. Synthetic must MS300 was supplemented with 20 mg/L of hydroxycinnamic acids. Standard deviations of each point are represented by error bars

Only in mixed cultures supplemented with 2.5 or 5 % ethanol a steady decline was observed in *D. bruxellensis* ZIM 701 growth to a concentration of 6.7 log CFU/mL (Fig. 5). However, in pure cultures, the growth continued to increase until the end of fermentations (Fig. 5). In comparison with the control medium, 5 % of ethanol reduced the maximum population density by 0.8 log CFU/mL and at 10 % of ethanol a 160-hour lag phase was observed (Fig. 5).

In control medium, fermentations of pure culture of *S. cerevisiae* converted 13.8 % of ferulic acid to 4-VG (1.4 mg/L) and 48.1 % of *p*-coumaric acid to 4-VP (4.8 mg/L) (Figs. 6 and 7). An addition of 2.5 % ethanol increased the production of 4-VG (2.5 mg/L) and 4-VP (6.0 mg/L), each by 12.0 %. At 5 % ethanol, the conversion was slightly delayed, but 4-VG production was still 5.3 % higher than in control medium. Synthetic must containing 10 % alcohol strongly delayed 4-VP and 4-VG production by 112 and 160 h, respectively.

As in control medium, *D. bruxellensis* ZIM 701 did not accumulate vinylphenols in the presence of 2.5 or 5 % ethanol. However, with 10 % ethanol, a slow increase in concentration of 4-VG and 4-VP was observed towards the end of fermentations of pure culture, reaching 0.6 and 1.0 mg/L, respectively (Figs. 6 and 7). A volume fraction of 2.5 % of ethanol accelerated the production of 4-EG by 36 h and 4-EP by 19 h (Figs. 6 and 7). Furthermore, supplementation with 5 % ethanol delayed the production of 4-EG by 30 h and 4-EP by 21 h. At 10 % of ethanol, the conversion was reduced to only 0.3 mg/L of 4-EG and no 4-EP was measured.

In fermentations of the mixed culture, 2.5 % ethanol did not affect vinylphenol production (Figs. 6 and 7). At a volume fraction of 5 %, the accumulation of 4-VP was delayed by 20 h. It reached 2.9 mg/L before it was further assimilated by *D. bruxellensis* ZIM 701. Similarly to *S. cerevisiae* and *D. bruxellensis* ZIM 701 pure culture fermentations, a volume fraction of 10 % alcohol showed a slow increase in vinylphenol accumulation, reaching 0.7 mg/L of 4-VG and 2.1 mg/L of 4-VP. In comparison with the control medium, low volume fractions of ethanol induced the production of ethylphenols. The addition of 2.5 % alcohol increased the production of 4-EG and 4-EP by 16.2 and 7.5 %, respectively (Figs. 6 and 7). Additionally, at 5 % ethanol, the production of 4-EG and 4-EP was further increased by 11.4 and 9.7 %. However, at 10 % ethanol no ethylphenols were produced in the mixed culture fermentations.

Because the addition of 2.5 % of ethanol increased the production of volatile phenols in pure and mixed cultures, this volume fraction was used for gene expression studies. In comparison with the control medium, ethanol induced the expression of *D. bruxellensis* gene encoding for CAD in mixed (2.01-fold) and especially in pure cultures (2.71-fold) (Fig. 4). However, ethanol did not have a significant effect on the expression of *D. bruxellensis* gene encoding for VPR and *S. cerevisiae* gene *PAD1*. The expression of *FDC1* gene was slightly reduced by ethanol in pure (0.79-fold) and in mixed (0.83-fold) cultures.

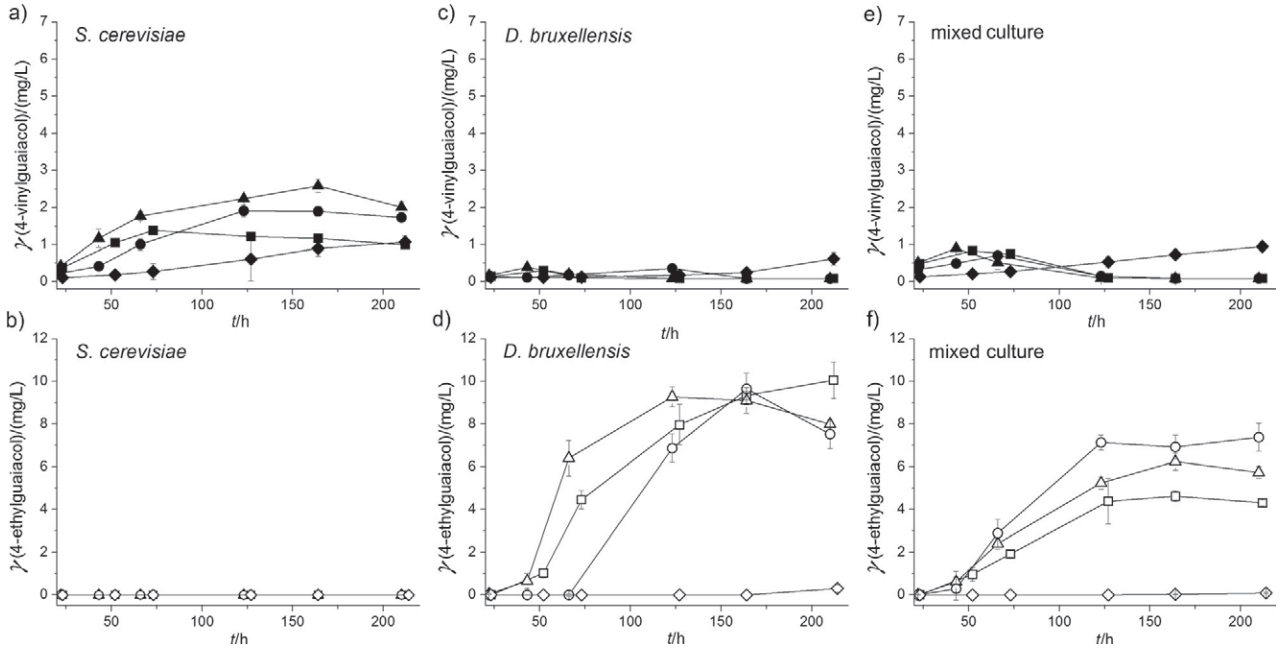

Fig. 6. Effect of 0 (■, □), 2.5 (▲, △), 5 (●, ○) and 10 % (◆, ◇) of ethanol on the production of 4-vinylguaiacol (filled symbols) and 4-ethylguaiacol (open symbols) in synthetic must MS300 supplemented with 20 mg/L of hydroxycinnamic acids: a) and b) *S. cerevisiae* pure culture fermentations, c) and d) *D. bruxellensis* ZIM 701 pure culture fermentations, and e) and f) mixed culture fermentations. Standard deviations of each point are represented by error bars

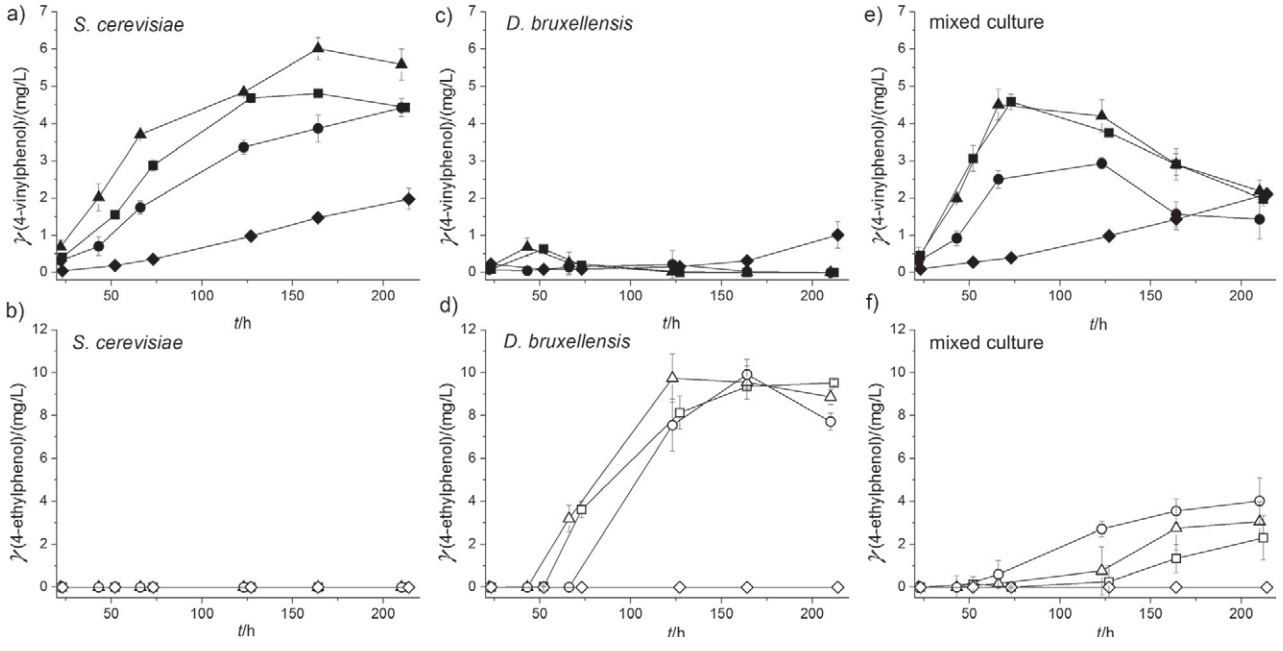

Fig. 7. Effect of 0 (■, □), 2.5 (▲, △), 5 (●, ○) and 10 % (◆, ◇) of ethanol on the production of 4-vinylphenol (filled symbols) and 4-ethylphenol (open symbols) in synthetic must MS300 supplemented with 20 mg/L of hydroxycinnamic acids: a) and b) *S. cerevisiae* pure culture fermentations, c) and d) *D. bruxellensis* ZIM 701 pure culture fermentations, and e) and f) mixed culture fermentations. Standard deviations of each point are represented by error bars

Effects of using different strains

In fermentations of pure and mixed cultures, *D. bruxellensis* ZIM 2306 reached 7.9 and 7.5 log CFU/mL, respectively, and exhibited a similar growth curve and CO_2 production as strain 701 in MS300 must containing 100 mg/L of HCAs (Fig. 8). Interestingly, a slight reduction in the growth of *S. cerevisiae* in mixed culture fermentations with *D. bruxellensis* ZIM 2306 was observed towards the end of fermentations (Fig. 8b).

Strain ZIM 2306 accumulated minimal amounts of vinylphenols and the conversion of HCAs to ethylphenols was 81.1 % for 4-EG (40.6 mg/L) and 87.3 % for 4-EP (43.7 mg/L) (Figs. 9a and b). Interestingly, in fermentations of mixed cutures the production shifted in

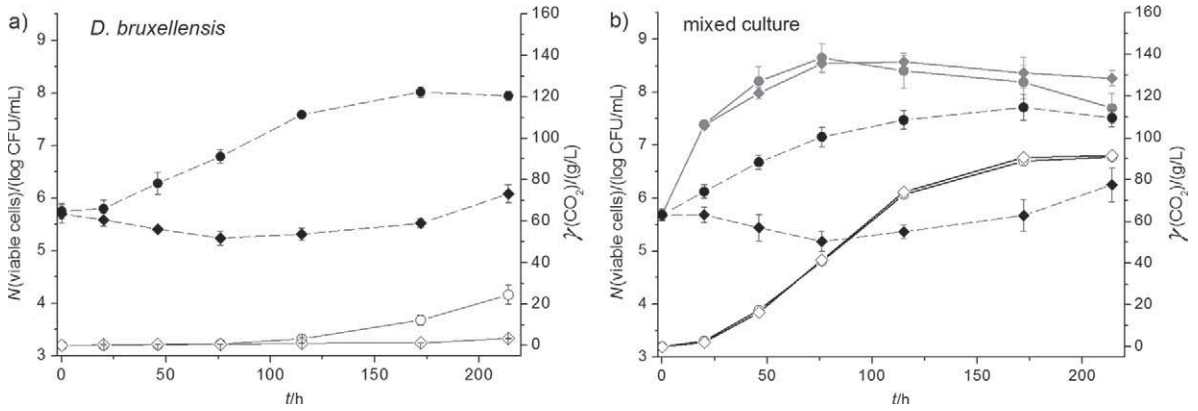

Fig. 8. Yeast growth (filled symbols) and CO_2 release (open symbols) of *D. bruxellensis* strain ZIM 2306 (● with dashed line) or strain ZIM 1762 (◆ with dashed line) in a) pure or b) mixed culture fermentations with *S. cerevisiae* (grey symbols). Grey circle (●) represents the growth of *S. cerevisiae* in mixed cultures with *D. bruxellensis* strain ZIM 2306 and grey diamond (◆) represents its growth with strain ZIM 1762. Synthetic must MS300 was supplemented with 100 mg/L of hydroxycinnamic acids. Standard deviations of each point are represented by error bars

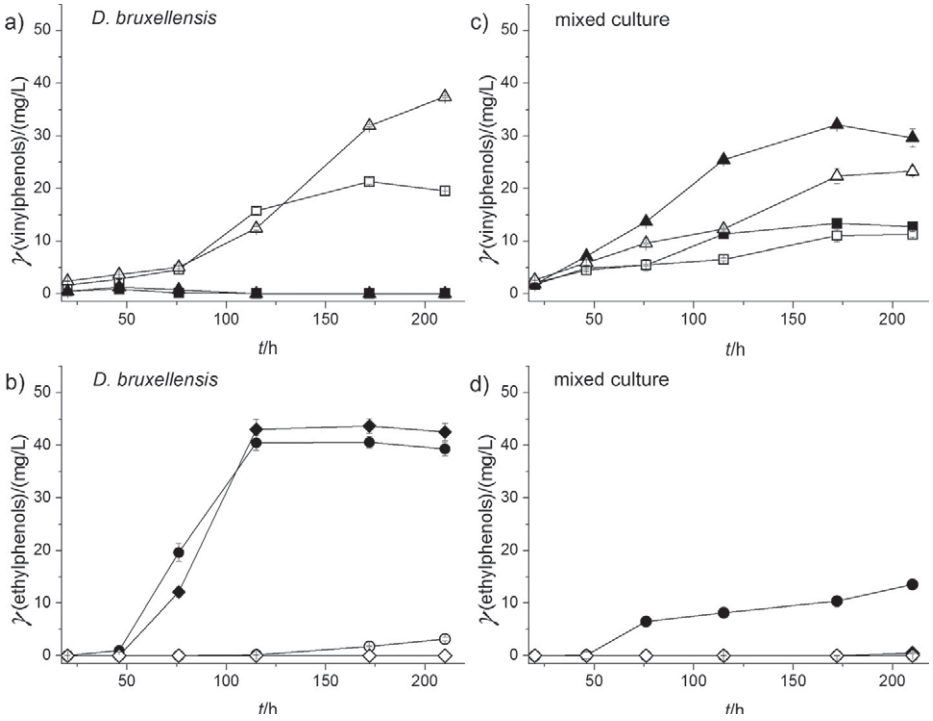

Fig. 9. Volatile phenol production by *D. bruxellensis* strain ZIM 2306 (filled symbols) or strain ZIM 1762 (open symbols) in pure (a and b) or mixed culture (c and d) fermentations with *S. cerevisiae*: production of 4-vinylguaiacol (■, □) and 4-vinylphenol (▲, △) is presented in graphs a) and c), and production of 4-ethylguaiacol (●, ○) and 4-ethylphenol (◆, ◇) in graphs b) and d). Fermentations were carried out in synthetic must MS300 supplemented with 100 mg/L of hydroxycinnamic acids. Standard deviations of each point are represented by error bars

favour of vinylphenols reaching 13.4 mg/L of 4-VG (26.8 % of ferulic acid) and 32.1 mg/L of 4-VP (64.3 % of *p*-coumaric acid) and the production of 4-EG and 4-EP was reduced by 54.0 and 86.3 %, respectively (Figs. 9c and d).

In contrast to strains ZIM 701 and ZIM 2306, *D. bruxellensis* strain ZIM 1762 exhibited a long lag phase, lasting 76 h during which a reduction in biomass was observed to around 5.4 log CFU/mL. Afterwards, its growth slowly increased to 6.3 log CFU/mL in mixed and to 6.0 log CFU/mL in pure culture fermentations (Fig. 8). Also, its CO_2 production rates were minimal in comparison with strain ZIM 701.

In volatile phenol production, strain ZIM 1762 differed from other *D. bruxellensis* strains in its ability to accumulate high concentrations of vinylphenols in pure culture fermentations (Fig. 9a). Its conversion rates were the highest, ranging from 42.7 % for 4-VG (21.4 mg/L) to almost 75.0 % for 4-VP (37.4 mg/L). Furthermore, it only produced 3.2 mg/L of 4-EG (6.4 % of ferulic acid) and no 4-EP was detected (Fig. 9b). Although *S. cere-*

visiae and *D. bruxellensis* ZIM 1762 both produced considerable amounts of vinylphenols, the concentration obtained in mixed cultures did not exceed that of *D. bruxellensis* ZIM 1762 pure culture fermentations (Fig. 9c). Additionally, ethylphenol production was completely absent from the mixed culture fermentations (Fig. 9d).

Discussion

Our study has confirmed the observations by Sáez *et al.* (*17*) that mixed cultures with *D. bruxellensis* do not have an impact on the growth of *S. cerevisiae* (Figs. 1a and c). However, in the wine fermentation by *S. cerevisiae*, the exponential phase of *D. bruxellensis* ZIM 701 was steeper in comparison with the pure culture fermentations (Figs. 1b and c). This could be explained by a rapid metabolism of glucose by *S. cerevisiae* in mixed culture fermentations. It is known that *D. bruxellensis* grown in the presence of high concentrations of glucose shows a relatively low growth rate in comparison with other wine yeasts (*15*). Therefore, in mixed culture fermentations glucose levels dropped more rapidly in comparison with *D. bruxellensis* ZIM 701 pure culture fermentations, allowing it to grow faster.

In *D. bruxellensis* ZIM 701 pure culture fermentations, HCAs were rapidly metabolized to vinylphenols in an intracellular fashion and a complete conversion to ethylphenols was observed. On the other hand, in mixed cultures the conversion was delayed and ethylphenol production was reduced by 30 % (Fig. 2). It therefore seems that for ethylphenol production, the assimilation of HCAs and intracellular decarboxylation to vinylphenols is the preferable metabolic pathway in comparison with the direct assimilation of vinylphenols accumulated by *S. cerevisiae*. Additionally, in the mixed cultures, a slight reduction in the expression of gene encoding for VPR (0.69--fold) was observed (Fig. 3). This reduction could be a consequence of a lower level of intracellular substrate availability in the mixed cultures. Under these conditions, *D. bruxellensis* ZIM 701 started to assimilate vinylphenols only after 70 h of fermentation, when vinylphenol concentrations in the medium had already reached or exceeded those measured in *S. cerevisiae* pure culture fermentations. Since most of ethylphenols were produced during the exponential growth phase of *D. bruxellensis* (starts from 50 h on), a certain amount of HCAs that was transformed to vinylphenols by *S. cerevisiae* was unavailable for assimilation by *D. bruxellensis*. Consequently, the levels of intracellular vinylphenols dropped and the production of ethylphenols stagnated. Accordingly, some studies have stated that ethylphenol production is closely linked to the exponential growth rate of *D. bruxellensis* and that it does not proceed if the cells reach the stationary phase (*4*,*12*).

The substitution of HCAs with 100 mg/L of vinylphenols significantly reduced the growth of *S. cerevisiae;* however, *D. bruxellensis* ZIM 701 was only slightly affected (Fig. 1). Therefore, *D. bruxellensis* could be more resistant to higher levels of vinylphenols in the fermentation medium. It has previously been suggested that microorganisms decarboxylate HCAs in order to produce less toxic compounds (*24*). However, Hammond *et al.* (*25*) observed that in beer brewing 4-VG inhibited the growth of *Saccharomyces* and *Dekkera* spp. to a greater extent than ferulic acid. They further stated that microorganisms which possess the enzyme coumarate decarboxylase are particularly susceptible to the antimicrobial effects of ferulic acid, especially because 4-VG is the end product.

In pure culture fermentations, *D. bruxellensis* completely assimilated the externally added vinylphenols from chemically defined medium; however, the conversion to ethylphenols was not complete, reaching 65 % (Figs. 2c and d). Substitution of HCAs with vinylphenols induced the expression of *D. bruxellensis* genes encoding for CAD and VPR (Fig. 4a). This is interesting, as it indicates that decarboxylase and reductase enzymes could be more responsive to vinylphenols in comparison with HCAs. This could imply the greater cytotoxicity of vinylphenols observed in our study and the need to metabolize them to less toxic compounds.

The responsiveness of coumarate decarboxylase to vinylphenols is not surprising as Godoy *et al.* (*26*) reported that apart from *p*-coumaric acid, the externally added 4-vinylphenol could also induce its activity. Nevertheless, the very low ethylphenol levels obtained from vinylphenol precursors could therefore only be explained by a significantly decreased growth rate of *D. bruxellensis*.

Furthermore, in the mixed cultures the assimilation of vinylphenols by *D. bruxellensis* was strongly reduced (Fig. 2e) and consequently ethylphenol production greatly decreased in comparison with *D. bruxellensis* ZIM 701 pure culture fermentations (Fig. 2d). Interestingly, the induction of genes encoding for CAD and VPR was proportionally smaller than the induction observed in *D. bruxellensis* pure cultures (Fig. 4a).

An addition of 10 % of ethanol strongly reduced the growth and volatile phenol production of yeasts *D. bruxellensis* ZIM 701 and *S. cerevisiae*, the former having a longer lag phase compared to the latter (Figs. 5–7). In correlation with our findings, Barata *et al.* (*4*) reported that *S. cerevisiae* is relatively more tolerant to ethanol than *D. bruxellensis*. Additionally, Barbin *et al.* (*27*) observed a long lag phase for nine *D. bruxellensis* strains in synthetic wine supplemented with 10 % of ethanol. Furthermore, we noticed that in mixed cultures containing low volume fractions of ethanol, the growth of *D. bruxellensis* was reduced towards the end of fermentations (Fig. 5c). This was expected as *S. cerevisiae* produced around 9.5 % of ethanol from sugar fermentation (estimate from CO_2 release) and together with externally added ethanol the fraction could exceed that of 10 %.

A volume fraction of 2.5 % of ethanol increased vinylphenol production by *S. cerevisiae* and ethylphenol production by *D. bruxellensis* in mixed and in pure culture fermentations (Figs. 6 and 7). With the exception of 4-VG, a volume fraction of 5 % of ethanol delayed volatile phenol production by both yeasts in pure culture fermentation. However, during mixed culture fermentations, further acceleration of ethylphenol production was observed reaching higher levels than in control medium or medium supplemented with 2.5 % of ethanol. Our results show that the expression of *D. bruxellensis* gene encoding for CAD was induced in the presence of 2.5 % ethanol, especially in pure culture fermentations (Fig.

4b). Correspondingly, Ganga *et al*. (*22*) reported that in comparison with the control medium, a fraction of 3 % of alcohol increased the activity of coumarate decarboxylase and the metabolization of *p*-coumaric acid by yeast *D. bruxellensis*. In addition, Benito *et al.* (*14*) observed that in the absence of ethanol, 4-EP production was delayed in comparison with the fermentation with 5 % ethanol.

Each *D. bruxellensis* strain had a unique volatile phenol production pattern. Strain ZIM 1762 accumulated the highest concentrations of vinylphenols (Fig. 9a), strain ZIM 2306 exhibited a strong shift from ethylphenol to vinylphenol production in the mixed culture fermentations (Figs. 9c and d), while strain ZIM 701 completely transformed all of the HCAs to ethylphenols.

Experiments with strain ZIM 701 gave the impression that an increase in volatile phenol production is directly influenced by yeast growth, because the majority of volatile phenols were produced during the exponential growth phase. However, the results obtained from strain ZIM 1762 refute this assumption, because this strain was capable of producing high concentrations of vinylphenols (Fig. 9a) while maintaining minimal yeast growth (Fig. 8).

Lastly, the inhibitory effects of vinylphenols were also visible in the mixed cultures with strain ZIM 2306. In this instance, high concentrations of 4-VP were accumulated (Fig. 9c), which consequently reduced the growth of *S. cerevisiae* towards the end of fermentations (Fig. 8b).

Conclusions

In mixed cultures of *D. bruxellensis* and *S. cerevisiae* supplemented with HCAs, the accumulation of ethylphenols was lower in comparison with fermentations using pure cultures. This could be partially explained by the lower availability of intracellular vinylphenol precursors. Although the substitution of HCAs with vinylphenols induced the genes encoding for coumarate decarboxylase and vinylphenol reductase, their effect strongly reduced yeast growth and the production of ethylphenols. Low volume fractions of ethanol showed a positive effect on vinylphenol and ethylphenol production in the fermentations of pure and mixed cultures. Our expression studies showed that ethanol up-regulated the expression of *D. bruxellensis* gene encoding for CAD. Finally, it was found that volatile phenol production by *D. bruxellensis* is strictly strain dependent showing different accumulation rates in pure and in mixed cultures.

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