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Research Letter

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Volatile phenols are produced by strains of *Dekkera bruxellensis* under Brazilian fuel ethanol industry-like conditions

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One sentence summary: Ethylphenol produced by *Dekkera bruxellensis* in sugarcane musts.
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ABSTRACT

Dekkera bruxellensis is a spoilage yeast in wine and fuel ethanol fermentations able to produce volatile phenols from hydroxycinnamic acids by the action of the enzymes cinnamate decarboxylase (CD) and vinyphenol reductase (VR) in wine. However, there is no information about this ability in the bioethanol industry. This work evaluated CD and VR activities and 4-ethylphenol production from *p*-coumaric acid by three strains of *D. bruxellensis* and PE-2, an industrial *Saccharomyces cerevisiae* strain. Single and multiple-cycle batch fermentations in molasses and sugarcane juice were carried out. *Dekkera bruxellensis* strains showed similar CD activity but differences in VR activity. No production of 4-ethylphenol by *S. cerevisiae* in any fermentation system or media was observed. The concentrations of 4-ethylphenol peaked during active growth of *D. bruxellensis* in single-cycle fermentation but they were lower than in multiple-cycle fermentation. Higher concentrations were observed in molasses with molar conversion (*p*-coumaric acid to 4-ethylphenol) ranging from 45% to 85%. As the first report on 4-ethylphenol production in sugarcane musts by *D. bruxellensis* in industry-like conditions, it opens up a new avenue to investigate its effect on the viability and fermentative capacity of *S. cerevisiae* as well as to understand the interaction between the yeasts in the bioethanol industry.

Keywords: *Dekkera*; 4-ethylphenol; 4-vinylphenol; *p*-coumaric acid; ethanol; fermentation

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INTRODUCTION

The species *Dekkera bruxellensis* (anamorph *Brettanomyces bruxellensis*) is considered the main contaminant yeast in the wine industry (Cocolin *et al.* 2004; Renouf, Claisse and Lonvaud-Funel 2007; Oelofse, Pretorius and du Toit 2008; Milheiro *et al.* 2017) and in the fuel ethanol fermentation, affecting the ethanol productivity and efficiency (Souza-Liberal *et al.* 2007; Basílio *et al.* 2008; Pereira *et al.* 2012; Meneghin *et al.* 2013; Bassi *et al.* 2018). In aerobic systems, *D. bruxellensis* produces high amounts of acetic acid (Freer 2002), which is an inhibitor of *Saccharomyces cerevisiae* growth. However, in anaerobic systems, as it occurs in the ethanolic fermentation in Brazil, the production of acetic acid by *D. bruxellensis* is fairly rare (Pereira *et al.* 2012). Meneghin *et al.* (2013) showed that a concentration as low as 1000 cells/mL of *D. bruxellensis* in co-culture with *S. cerevisiae* resulted in a drop of 9.2% in the fermentative efficiency after 14 fermentative cycles, with decrease in the medium pH. However, the mechanism behind the harmful effect on fermentation efficiency is not yet known.

The genus *Dekkera* is recognised by its ability to convert hydroxycinnamic acids such as *p*-coumaric acid and ferulic acid into volatile phenols. The reduced compounds, such as 4-vinylphenol, 4-ethylphenol, 4-vinylguaiacol and 4-ethylguaiacol, produced from the degradation of hydroxycinnamic acids, cause sensorial impact in the wines with their 'horsy,' 'medicinal,' 'animal,' 'smoky' and 'spicy' aromas (Suarez *et al.* 2007; Kheir *et al.* 2013). The volatile phenols arise due to the sequential activity of two enzymes, cinnamate decarboxylase (CD) and vinylphenol reductase (VR). CD decarboxylates free hydroxycinnamic acid precursors (*p*-coumaric, ferulic and caffeic acids) into hydroxystyrenes (4-vinylphenol, 4-vinylguaiacol and 4-vinylcatechol), which are reduced by VR to their corresponding ethyl derivatives, such as 4-ethylphenol, 4-ethylguaiacol and 4-ethylcatechol (Edlin *et al.* 1995; Dias *et al.* 2003). When reducing hydroxystyrenes to their ethyl derivatives, the enzyme VR uses NADH as cofactor, which contributes to maintain the cell redox balance. In oxygen-limiting conditions, the enhancement of VR activity and consequently the increase in NAD^+ availability corroborates this theory (Curtin *et al.* 2013).

Godoy *et al.* (2008) purified and characterised both CD and VR enzymes in *Brettanomyces bruxellensis* for the first time and demonstrated the stability of these enzymes at pH 3.4 and in concentrations of 1% and 2% of glucose. CD activity was more sensitive to ethanol than VR activity. Low pH, high glucose and ethanol concentrations are found along the ethanolic fermentations and can affect enzymatic activities.

The decarboxylase action and consequently the production of volatile phenols may be related to the toxicity of phenolic compounds to yeast (Goodey and Tubb 1982) but not exclusively. Hydroxycinammic acids interfere with the cytoplasmic membrane (Sabel *et al.* 2017) and the influx of *p*-coumaric acid elicits a stress response. The proton pump is expressed and consequently the efflux of toxic compounds is induced. These mechanisms are involved in the release of nitrogen compounds resulting in the expression of genes linked to the nitrogen metabolism (Godoy *et al.* 2016). Vinylphenols reduced the growth of *S. cerevisiae* at the end of fermentations but hydroxycinnamic acids did not (Kosel, Cadez and Raspor 2014). Bassi *et al.* (2018) postulated that the volatile phenols produced by *D. bruxellensis* may be the cause of the decrease in *S. cerevisiae* viability, when both yeasts were in co-culture with *Lactobacillus fermentum*. The role of the bacterium was to stimulate *D. bruxellensis* growth by mechanisms not completely identified by the authors. The fact is that if hydroxycinnamic acids are present in sugarcane musts (Duarte-Almeida *et al.* 2006), then volatile phenols are expected to be produced.

The effect of volatile phenols on *S. cerevisiae* growth and fermentation is an interesting investigation because it could help to explain the mechanisms behind the negative effects of *D. bruxellensis* contamination in ethanolic fermentations. However, none is known about the CD and VR activities of *D. bruxellensis* strains isolated from the ethanol fuel fermentation and their ability to produce volatile phenols from sugarcane musts so far. Whether the peculiar characteristics of Brazilian fuel ethanol fermentation favour the production of volatile phenols in sugarcane juice and/or molasses is something to be investigated. The aim of the current study is to survey the CD and VR activities of three strains of *D. bruxellensis* isolated from the ethanolic fermentation and the 4-ethylphenol production by pure cultures of this yeast species. Fermentations simulating industrial conditions both in single-cycle and in multiple-cycle batch fermentations compared to fermentation carried out by an industrial strain of *S. cerevisiae* were carried out. The distinguished characteristic of 4-ethylphenol production by strains of *Dekkera* may have an impact on fuel ethanol fermentation that has not received attention yet. However it is crucial to survey first whether this specific metabolite is produced from sugarcane musts and in industry-like conditions.

MATERIAL AND METHODS

Microorganisms and culture conditions

Dekkera bruxellensis strains CCA059, CCA077 and CCA155 are maintained at the culture collection of the Laboratory of Molecular and Agricultural Microbiology of the Universidade Federal de São Carlos, Brazil and at the Coleção de Culturas Tropical—Fundação André Toselo, Campinas, São Paulo State, Brazil, under the accession codes CCT7786, CCT7785 and CCT7784, respectively. The industrial strain of *S. cerevisiae* PE-2 (Basso *et al.* 2008) was also utilised in the experiments. The yeast strains were maintained on YPD (10 g/L yeast extract, 20 g/L glucose, 20 g/L peptone, 20 g/L agar) slants at 4°C and continuously transferred to a new growth medium before the experiments.

Protein extraction and quantification

The protein extraction followed the method described by Sambrook, Fritsch and Maniatis (1989). Protein was quantified by the method of Bradford (1976) using bovine serum albumin as protein standard.

Enzyme assays

The activity of CD was determined by the method of Edlin *et al.* (1998) with modifications according to Godoy *et al.* (2009). The specific CD activity was defined as the amount of enzyme that consumes 1 μmol of *p*-coumaric acid per min per mg of protein (U/mg). The activity of VR was determined by the method of Kato *et al.* (1991) with modifications according to Godoy *et al.* (2009). The specific VR activity was defined as the amount of enzyme that consumes 1 μmol of NADPH per min per mg of protein (U/mg). Both *p*-coumaric acid and 4-vinylphenol were purchased from Sigma®. All other chemicals and reagents were of analytical grade.

Data from the enzymatic analysis (in duplicate) were subjected to analysis of variance and the mean values were compared by using the Tukey´s test at 5% of significance level.

Fermentative tests

The fermentative tests were carried out according to Reis *et al.* (2018) with modifications. For the single-cycle batch system, the yeast inoculum was prepared in sugarcane juice or molasses with approximately 4 g/100 mL of total reducing sugars, pH 4.5, as supplied by a local fuel ethanol-producing unit and sterilised at 120°C for 20 min. The media were stored at 4°C. The sugarcane juice was clarified with cationic polymers in the fuel ethanol-producing unit. Molasses medium was prepared by diluting it with water until the desired sugar concentration. No nutrients were added to the media. The inocula were incubated at 30°C, 160 rpm for 24 h. The yeast cell mass was separated from the liquid growth medium by centrifugation at 580 × g for 5 min. This procedure was repeated until the concentration of 10^9 cells/mL was achieved. The cell mass was centrifuged and resuspended in the fermentation medium (clarified sugarcane juice or diluted molasses with approximately 14–17 g/100 mL of total reducing sugars, pH 4.5, as supplied by a local fuel ethanol-producing unit and sterilised at 120°C for 20 min) and added to the fermentation flasks (cotton-capped flasks), in triplicate. The initial cell concentration was 10^8cell/mL and the flasks were incubated at 30°C without shaking for 72 h.

For the fermentative test in multiple-cycle batch system, the inoculum was prepared as described above. The fermentations were conducted in flasks containing the same fermentation media described above, which were incubated at 30°C without shaking for six fermentation cycles of 12 h each. All assays were conducted in triplicate. The cell culture was centrifuged aseptically, the cell pellet was washed with sterile saline solution (0.85% NaCl) and added to the medium to initiate the next fermentative cycle. The results were presented as means of two independent experiments, with triplicates for each fermentation.

Analysis

In the single-cycle batch experiment, a sample of 1 mL was taken out for determining optical density at 600 nm every 12 h. A sample of 3 mL was withdrawn every 24 h, centrifuged at 580 × g for 5 min, filtered through a 0.45 μm membrane and maintained in amber glass flasks with Teflon cap at −20°C for chromatographic analysis of phenolic compounds.

In the multiple-cycle batch experiment, at the end of each cycle a sample of 1 mL was taken out to determine the number of cells in the Neubauer chamber, utilizing a solution of citrate-methylene blue to estimate the number of viable cells. From the supernatant, two aliquots were taken out: a volume of 3 mL was filtered through a 0.45 μm membrane and maintained in amber glass flasks with Teflon cap at −20°C for chromatographic analysis of phenolic compounds; and a volume of 13 mL was maintained in Falcon tubes at −20°C for the analysis of pH, total reducing sugars and ethanol.

At the end of each cycle, pH was measured in a digital pH-meter and the concentration of total reducing sugars was determined by the methodology of dinitrosalicylic acid by Miller (1959), after hydrolysis of the samples with hydrochloric acid. Ethanol was measured by gas chromatography, in which the samples were first filtered using a 0.45 μm membrane and injected in a gas chromatograph (GC 2010 Plus, Shimadzu®, Kyoto-Japan) equipped with a flame ionisation detector (FID), using a Restek Stabilwax-DA column (30 mm × 0.25 mm × 0.25 mm) in the following conditions: injector temperature: 220°C; carrier gas: helium; linear rate of the carrier gas: 20 cm/sec; split rate: without split; FID temperature: 280°C; column temperature: 30°C for 5 min, increment of 12°C per min up to 100°C, following increment of 15°C per min up to 165°C and increment of 80°C up to 230°C for 6 min.

Table 1. Specific cinnamate decarboxylase (CD) and vinylphenol reductase (VR) activities (U/mg protein) of *D. bruxellensis* strains CCA059, CCA077 and CCA155.[1]

Yeast strain	CD activity[2]	VR activity[3]
CCA059	184.4[a]	94.8[a]
CCA077	215.5[a]	104.3[a,b]
CCA155	214.9[a]	154.3[b]

[1]Values are the mean of two replications. Means of the same column with the same superscript letters are not significantly different ($P < 0.05$);
[2]Coefficient of variation = 8.90%;
[3]Coefficient of variation = 25.62%.

Phenolic compounds (*p*-coumaric acid, 4-ethylphenol and 4-vinylphenol) were determined by High Performance Liquid Chromatography in a Prominence Shimadzu® (Kyoto-Japan) equipment with a GL Sciences column Inertsil ODS-4 (5 μm, 4.6 × 150 mm) in the following conditions: oven temperature: 40°C, wavelengths: 280 nm and 320 nm; gradient flux: 1 mL/min; mobile phase A (water:acetic acid 90:10) and B (methanol).

Molar conversion was calculated for each yeast strain, with the average results for 4-ethylphenol concentration at the end of the 6th fermentative cycle and *p*-coumaric acid concentration in the medium at the beginning of the cycle, as following:

$$\text{Molar conversion (\%)} = \text{(moles of 4-ethylphenol} \div \text{moles of } p\text{-coumaric acid)} \times 100$$

Production rate (4-EP/log cell number) was calculated for each yeast strain, with the average results for 4-ethylphenol concentration (in mg/L) and the log of cell number (number of cells/L) at the end of each fermentative cycle, as following:

$$\text{Production rate (4-EP/log cell number)} = \text{4-ethylphenol in mg/L} \div \text{log of cell number per L}$$

RESULTS AND DISCUSSION

No significant difference was observed among the strains for CD activity; however, the VR activity was significantly higher for the strain CCA155 in relation to the strain CCA059 (Table 1). Godoy *et al.* (2009) utilised the same methodology and observed that CD activity was common to all the 12 *Dekkera bruxellensis* wine strains evaluated but the VR activity was present only in some strains. In the case of CD activity, the range was 2.5–19.6 U/mg while for VR activity it was quite variable, varying from 0 (eight strains) to 3.7, 139.9, 152.6 and 275.4 U/mg. In the current study, the strains presented much higher CD activity (Table 1). Sturm *et al.* (2015) observed no significant differences in CD activity among five *D. bruxellensis* strains from wine, but VR activity was variable. Coronado *et al.* (2015) observed that 4-vinylphenol induces VR activity; however, as 4-vinylphenol is able to react with

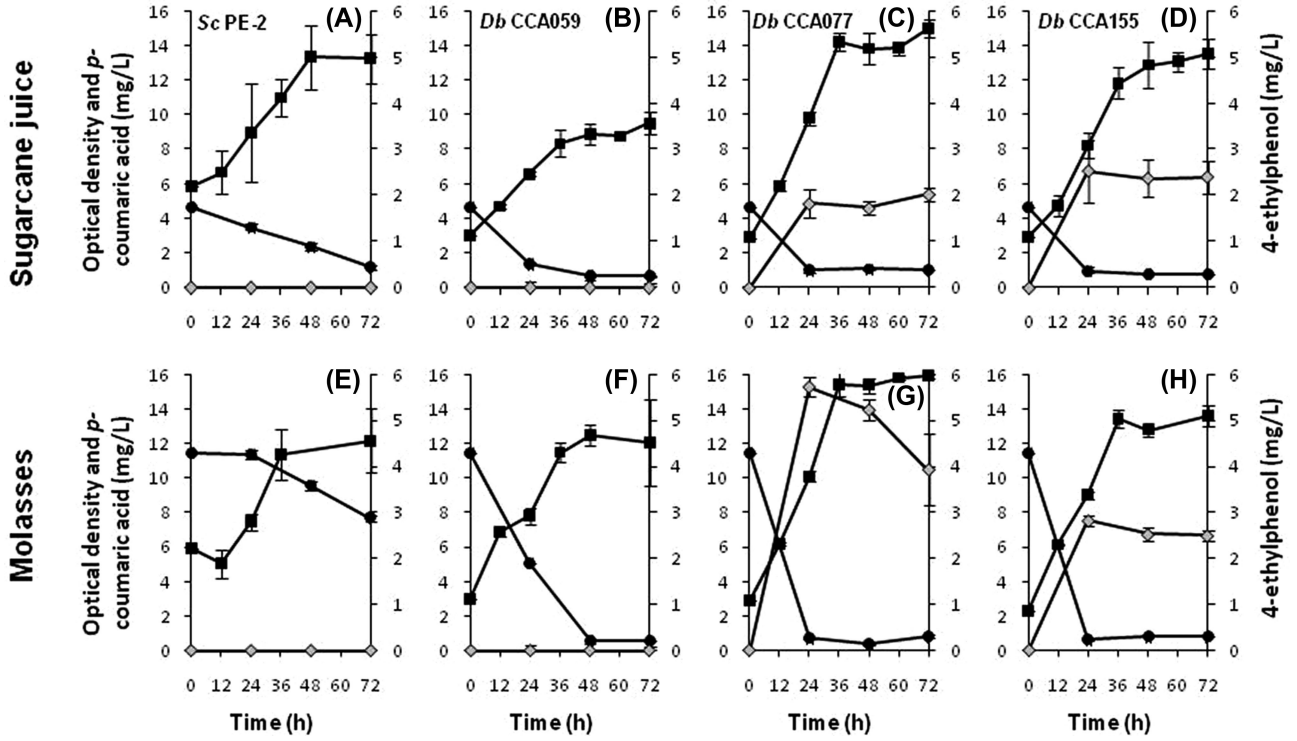

Figure 1. Optical density (■), *p*-coumaric acid (•, mg/L) and 4-ethylphenol (◆, mg/L) in single-cycle batch fermentation with strains of *S. cerevisiae* (Sc) and *D. bruxellensis* (Db) in sugarcane juice, at 30°C. The results are mean values of triplicates.

other wine compounds such as anthocyanins producing stable compounds, the induction of VR activity would be compromised, resulting in a lower enzymatic activity.

Barthelmebs, Divies and Cavin (2000) suggested that unknown mechanisms are involved in the metabolism of phenolic acids. The knockout of *PAD gene* in *Lactobacillus plantarum* induced two enzymes with CD activity. Schifferdecker *et al.* (2014) observed that the *PAD* gene of *Dekkera* yeasts is much like the *PAD* bacterial gene than the *PAD1* gene of *Saccharomyces cerevisiae*. Higher CD and VR activities observed in the strains of *D. bruxellensis* in this work may be related to the production of new enzymes or a higher gene expression in the strains isolated from the ethanolic fermentation.

The results in Fig. 1 revealed that the production of 4-ethylphenol occurred during active growth for the strains CCA077 and CCA155 of *D. bruxellensis*, both in sugarcane juice and in molasses (Fig. 1C, D, G and H). A decrease in the *p*-coumaric acid concentration was observed simultaneously to the increase in 4-ethylphenol concentration in the medium similarly to what is described by Sturm *et al.* (2015). The strain CCA059 and *S. cerevisiae* did not produce 4-ethylphenol along 72 h of cultivation in both substrates; however, a decrease in the concentration of *p*-coumaric acid was observed (Fig. 1A, B, E and F).

With *S. cerevisiae*, the decrease was slow especially in molasses and resulted in 4-vinylphenol, because a concentration of around 0.5–0.7 mg/L was detected in both media (data not shown). CD activity is commonly found in *Saccharomyces* strains but not VR activity, which is specific for *Dekkera/Brettanomyces* (Steensels *et al.* 2015).

With the strain *D. bruxellensis* CCA059 there was production of 4-vinylphenol only in molasses (approximately 1.6 mg/L, data not shown). Therefore, the decrease of *p*-coumaric acid concentration in sugarcane juice is not attributed only to 4-vinylphenol production (Fig. 1B). There are alternative pathways for conversion of *p*-coumaric acid than the formation of 4-ethylphenol (Kheir *et al.* 2013). Trihydroxystilbenes, flavonoids and flavan 3-ols may be produced from *p*-coumaric acid (Goldberg *et al.* 1998). Other explanation is the adsorption of *p*-coumaric acid on yeast cell walls, decreasing the concentration of free acid into the medium (Salameh *et al.* 2008). As a single-cycle batch fermentation, without cell recycle, it seems that the adsorption is a plausible hypothesis due to the long-time of fermentation, especially with the strain *D. bruxellensis* CCA059 and with *S. cerevisiae*, with no VR activity. However 4-ethylphenol was produced in multiple-cycle batch fermentation (shorter fermentation time compared to single-cycle fermentation) as we will demonstrate further for the strain CCA059. The adsorption of *p*-coumaric acid on yeast cell walls (*D. bruxellensis* and *S. cerevisiae* strains) deserves further investigation.

Considering only the ethylphenol-producing strains, the strain CCA077 produced three times more 4-ethylphenol in molasses because this substrate presents almost three times more *p*-coumaric acid (13.3 mg/L) than sugarcane juice (4.8 mg/L), Fig. 1C and G. Indeed, sugarcane musts presented higher *p*-coumaric acid concentration than wine musts (ranging from 1–8 mg/L to 3–9.5 mg/L for white and red wines, respectively, according to Avar *et al.* (2007)). With the strain CCA155, the 4-ethylphenol concentration was almost the same in molasses and sugarcane juice with high consumption of *p*-coumaric acid (Fig. 1D and H). A proportion of *p*-coumaric acid was converted to 4-vinylphenol in molasses (1.5 mg/L, data not shown).

There is no consensus about the growth phase in which 4-ethylphenol is produced in *Dekkera* yeasts. While some authors (Coronado *et al.* 2015; Šućur, Cadez and Kosmerl 2016) assert that the production of 4-ethylphenol takes place during the

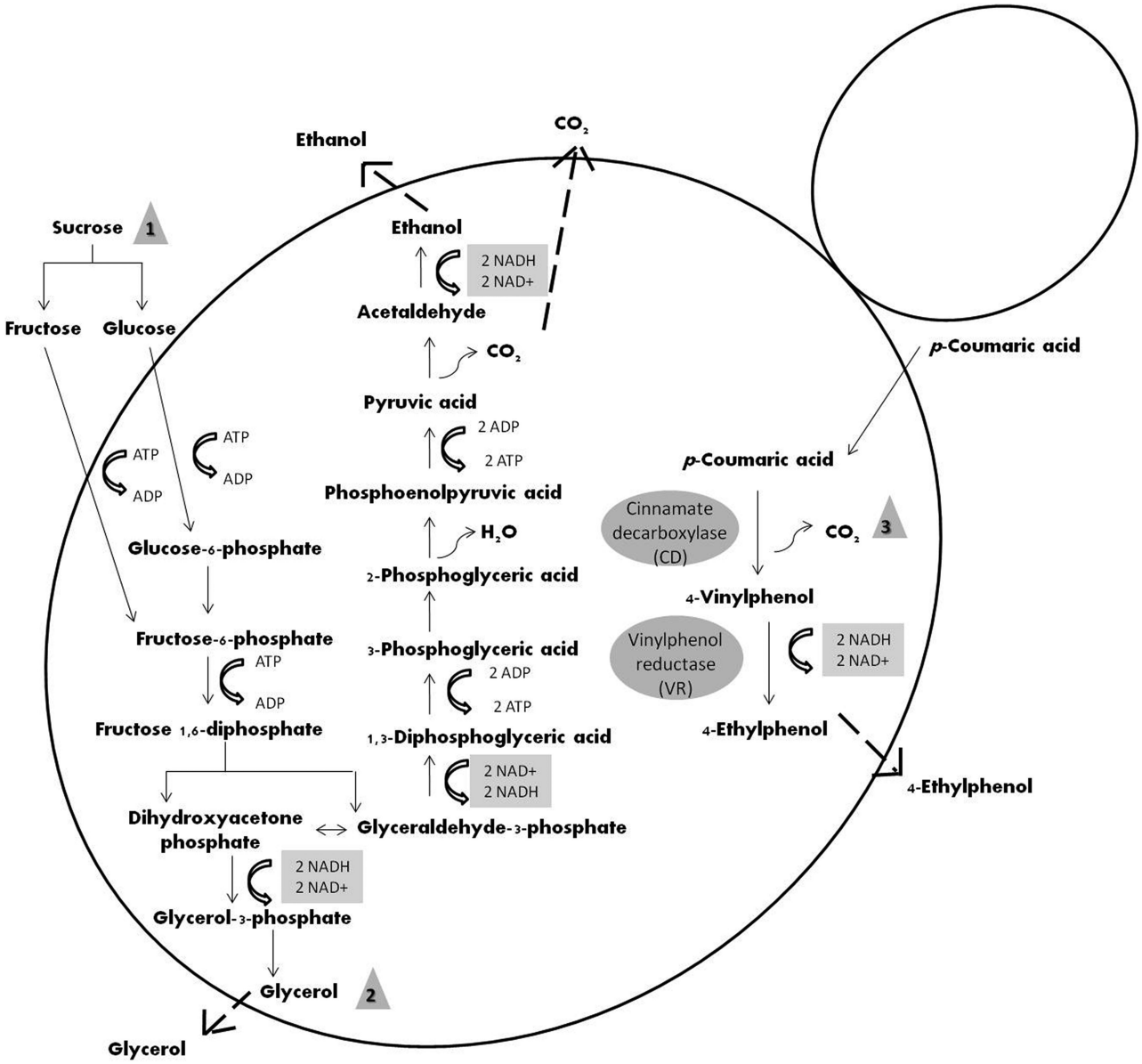

Figure 2. Schematic representation of yeast biochemical reactions to produce ethanol and volatile phenols. Sucrose is cleaved into fructose and glucose mostly extracellularly by *S. cerevisiae* and intracellularly by *D. bruxellensis* by the enzyme invertase (in 1). Glycerol is produced in very low amounts by *D. bruxellensis* (in 2). In 3, the metabolic route to produce 4-vinylphenol is common to both yeasts but 4-ethylphenol is produced only by *D. bruxellensis*. The enzymes of the volatile phenol routes are in the grey circles. NAD^+ and NADH cofactors are represented in grey squares.

stationary phase of growth, others (Chatonnet *et al.* 1992; Dias *et al.* 2003; Sturm *et al.* 2015) confirm that the major production occurs during the exponential phase of growth (mid or late exponential phase). CD and VR enzymes are intracellular and when the yeast reaches stationary phase of growth, the enzymes are released into the medium due to the cell lysis (Godoy *et al.* 2008). However, this observation does not explain the fact that 4-ethylphenol production is coincident with active growth as demonstrated in our work. The variation in optical density considering the peak of growth in relation to the initial inoculum was much higher for *D. bruxellensis* strains compared to *S. cerevisiae*. The optical density increased 3.0 or 4.0 times, 5.0 or 5.5 times, 4.6 or 6.0 times for the strains CCA059, CCA077 and CCA 155, respectively, compared to 2.3 or 2.1 for *S. cerevisiae*, in sugarcane juice or molasses. This result demonstrated the active growth of *D. bruxellensis* strains along with 4-ethylphenol formation in the sugarcane musts. If we consider that *D. bruxellensis* has very low production of glycerol (Blomqvist *et al.* 2010; 2012; Pereira *et al.* 2014; Tiukova 2014), its growth would be impacted under oxygen-limiting conditions due to the redox imbalance, since glycerol production may restore the redox imbalance during biomass production under oxygen limitation. As *D. bruxellensis* is able to grow actively under limitation of oxygen (Pereira *et al.* 2012; Meneghin *et al.* 2013), we may infer that the redox imbalance may be compensated by oxidation of NADH to NAD^+ through production of 4-ethylphenol (Fugelsang and Edwards 1997; Šućur, Cadez and Kosmerl 2016). Curtin *et al.* (2013) demonstrated that the production of 4-ethylphenol was stimulated by oxygen limitation. Figure 2 displays the biochemical reactions involving ethanol and volatile phenol production.

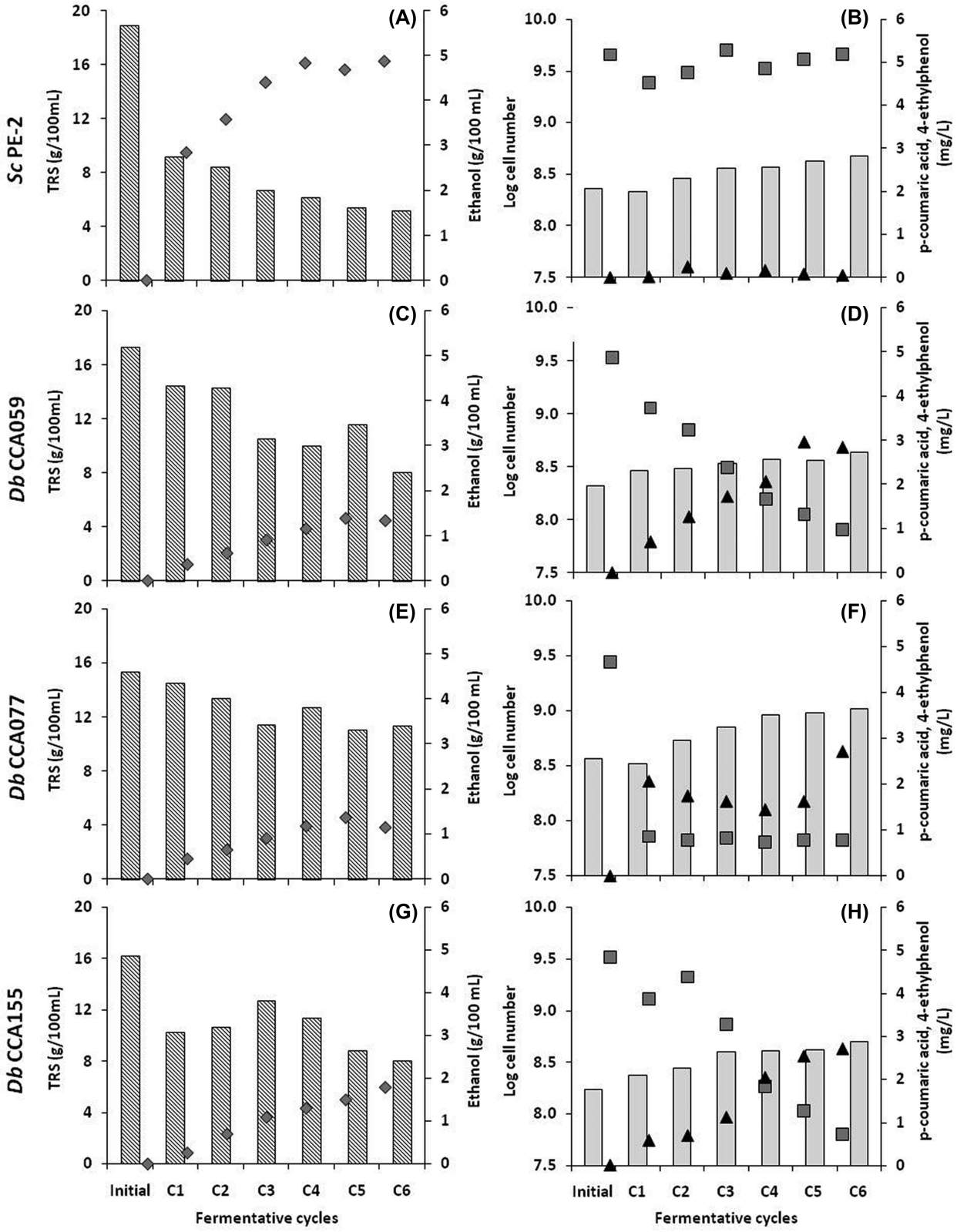

Figure 3. Total reducing sugars (striped bars, TRS, g/100 mL), ethanol (◆, g/100 mL), log of cell number (grey bars), *p*-coumaric acid (■, mg/L) and 4-ethylphenol (▲, mg/L) in multiple-cycle batch fermentation with strains of *S. cerevisiae* (Sc) and *D. bruxellensis* (Db) in sugarcane juice, at 30°C. The results are mean values of two independent experiments performed in triplicates.

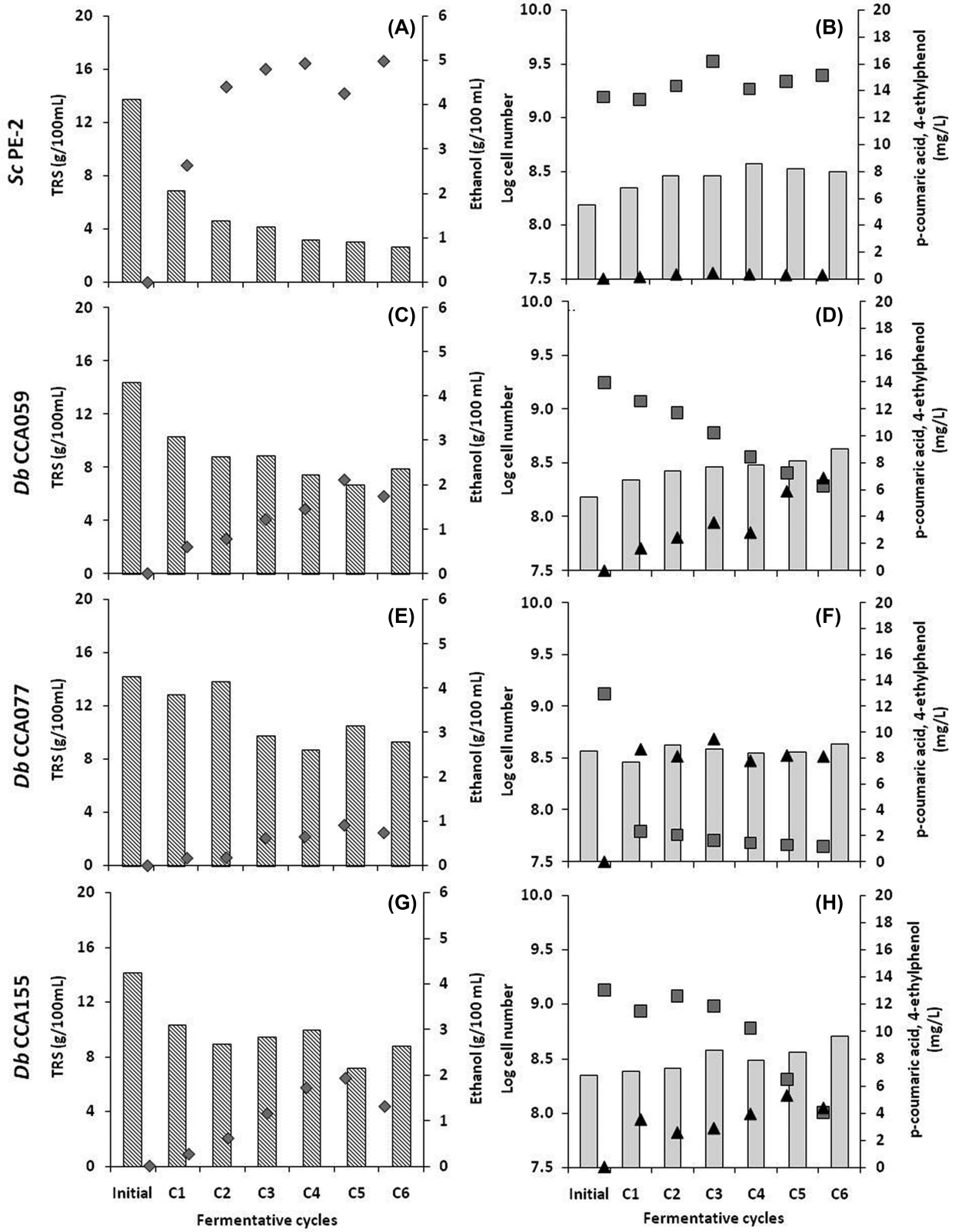

Figure 4. Total reducing sugars (striped bars, TRS, g/100 mL), ethanol (◆, g/100 mL), log of cell number (grey bars), *p*-coumaric acid (■, mg/L) and 4-ethylphenol (▲, mg/L) in multiple-cycle batch fermentation with strains of *S. cerevisiae* (Sc) and *D. bruxellensis* (Db) in molasses, at 30°C.The results are mean values of two independent experiments performed in triplicates.

Fuel ethanol fermentations in Brazil are carried out with cell recycle in short fermentation periods of 6–12 h (Amorim *et al.* 2011). Confirming the 4-ethylphenol production by *D. bruxellensis* in sugarcane musts, our next hypothesis was to verify if the conditions mimicking industrial fermentation (cell recycle, 12-h fermentative cycle, high cell concentration) would allow the production of 4-ethylphenol by the strains of *D. bruxellensis* both in sugarcane juice and molasses compared to the fermentation with an industrial strain of *S. cerevisiae*, one of the most utilised industrially in Brazil, PE-2 (Basso *et al.* 2008; Amorim *et al.* 2011).

Considering the sugarcane juice as substrate for batch fermentation with cell recycle, the strain of *S. cerevisiae* presented the lowest concentration of residual reducing sugars and the highest ethanol production compared to *D. bruxellensis* strains. High residual sugar concentration in the fermentation broth associated with low ethanol production rates are characteristics of the contamination by *D. bruxellensis* in fuel bioethanol fermentation (Souza-Liberal *et al.* 2007; Pereira *et al.* 2012; Meneghin *et al.* 2013; Bassi *et al.* 2018).

The variation in the cell number was quite similar among the yeast strains. Regarding the concentrations of *p*-coumaric acid and 4-ethylphenol along the fermentative cycles, the yeasts displayed different patterns of consumption and production, respectively (Fig. 3).

The concentration of *p*-coumaric acid in sugarcane juice after each fermentative cycle lasting 12 h varied from 4.5 to 5.3 mg/L with *S. cerevisiae* with no significant production of 4-ethylphenol (Fig. 3B). With *D. bruxellensis* strains CCA059 and CCA155, the concentration of *p*-coumaric acid decreased slowly after each fermentative cycle, with increasing 4-ethylphenol production simultaneously, reaching approximately 3 mg/L at the end of the 6th fermentative cycle. The consumption of *p*-coumaric acid was around 80%–85% in this situation for both strains (Fig. 3D and F). With the strain CCA077, the highest *p*-coumaric acid consumption occurred soon at the end of the 1st fermentative cycle, around 84%, also reaching almost 3 mg/L of 4-ethylphenol at the end of the fermentative cycles (Fig. 3F).

The production of ethanol by the strains of *D. bruxellensis* increased along the fermentative cycles but it was almost three times lower than by *S. cerevisiae* (Fig. 3A, C, E and G). Ethanol is found to have inhibitory effect on the 4-ethylphenol production by *D. bruxellensis*. Concentrations ranging from 2.5% to 5% favour the production of 4-ethylphenol whilst 12.5 and 13.5% drastically affected the production (Kosel, Cadez and Raspor 2014; Šućur, Cadez and Kosmerl 2016). Such high ethanol concentration would hardly be obtained in the fuel ethanol fermentations so that ethanol would not be a limiting factor for 4-ethylphenol production. In the current study with pure cultures of *D. bruxellensis*, ethanol production was not higher than 2% (Fig. 3C, E and G). The pH of the fermentation medium varied within the range of 4.0–4.5 (data not shown), which would not impact the 4-ethylphenol production. Godoy *et al.* (2008) demonstrated that both CD and VR activities were stable in pH 3.4, but the production of 4-ethylphenol was inhibited in pH below 2.17 (Benito *et al.* 2009).

In molasses, the same results were obtained for *p*-coumaric acid, 4-ethylphenol and the fermentation parameters for *S. cerevisiae* compared to sugarcane juice (Fig. 4A and B). However, the residual sugar concentration into the medium was lower in molasses (Fig. 4A). For *D. bruxellensis* strains, ethanol production was lower in molasses only for CCA077 (Fig. 4A, C, E and G).

Regarding the concentrations of *p*-coumaric acid and 4-ethylphenol along the fermentative cycles, the yeasts also displayed different patterns of consumption and production, respectively, as in sugarcane juice (Fig. 4). The concentration of *p*-coumaric acid in the fermentation with *S. cerevisiae* varied from 13.3 to 16.2 mg/L (Fig. 4B) with no formation of 4-ethylphenol. Both CCA059 and CCA155 strains of *D. bruxellensis* displayed slow decrease of *p*-coumaric acid along the fermentative cycles with increasing 4-ethylphenol formation, reaching concentrations of 5 to 7 mg/L at the end of the 6th fermentative cycle (Fig. 4D and H). For CCA077, the highest consumption of *p*-coumaric acid and 4-ethylphenol formation was achieved at the start of the fermentation, peaking 9.5 mg/L of 4-ethylphenol (around 91% of consumption of *p*-coumaric acid, against 55% and 69% for CCA059 and CCA155, respectively), Fig. 4F.

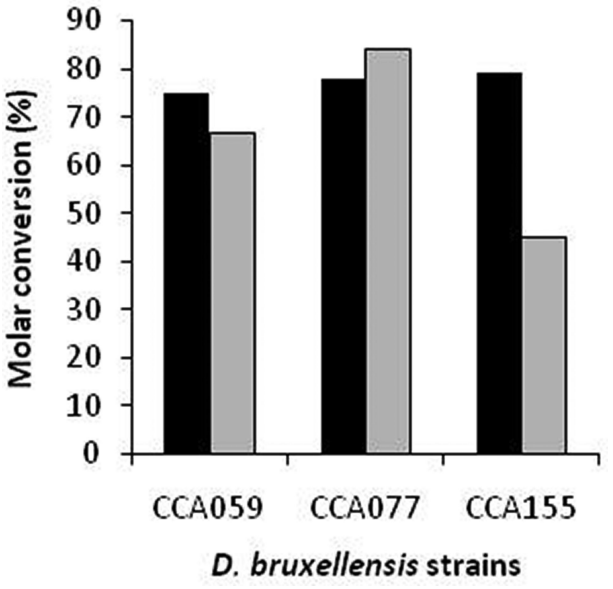

Figure 5. Molar conversion rate of *p*-coumaric acid into 4-ethylphenol (%) by strains of *D. bruxellensis* in sugarcane juice (black bar) and molasses (grey bar), at the end of the 6th fermentative cycle in multiple-cycle batch fermentations.

Comparing the sugarcane musts, the production of 4-ethylphenol was higher in molasses, but the concentration of *p*-coumaric acid is three times higher in this substrate comparing to sugarcane juice. The molar conversion of *p*-coumaric acid into 4-ethylphenol was lower in molasses with the strains CCA059 and CCA155 (Fig. 5). An inhibitory effect of the molasses was evident for these strains. Excess of substrate (*p*-coumaric acid), feedback inhibition by the 4-ethylphenol formed (Kheir *et al.* 2013) or other characteristic of the molasses may be the causes for the inhibitory effect of molasses. But this result did not occur with the strain CCA077 that displayed the same pattern of consumption of *p*-coumaric acid and formation of 4-ethylphenol in both sugarcane musts, with slightly superior molar conversion in molasses (Fig. 5). Molar conversions as high as 90% were found for *D. bruxellensis*, *Dekkera anomala* and *Pichia guilliermondii* (Suárez *et al.* 2007). In the current study the highest molar conversions ranged from 75% to 79% in sugarcane juice, and from 45% to 84% in molasses (Fig. 5).

Comparing the results obtained with the single-cycle batch fermentation and multiple-cycle batch fermentation, it is clear that the formation of 4-ethylphenol in *D. bruxellensis* strains is a process that depends on the adaptation of the yeasts to the sugarcane must and to the fermentation conditions. The cell recycled fermentation allowed a higher 4-ethylphenol formation than the long single-cycle fermentation. The most illustrative

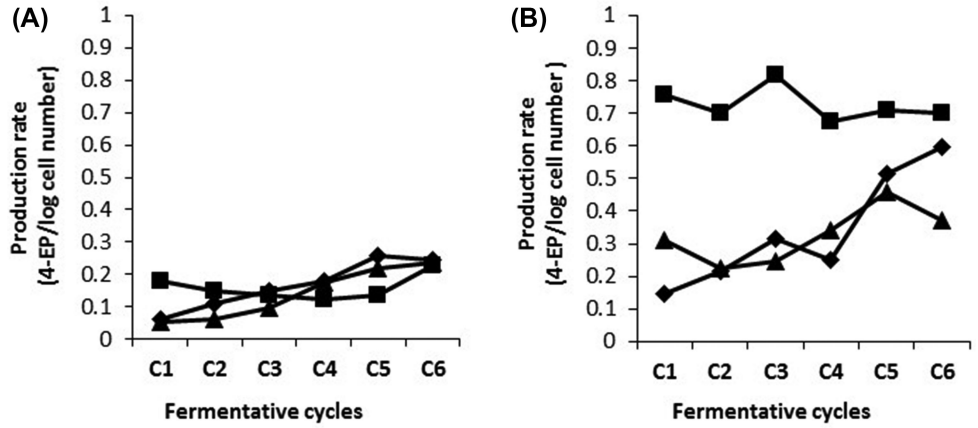

Figure 6. Production rate of 4-ethylphenol calculated as a function of the cellular counts (4-EP/log of cell number) in multiple-cycle batch fermentation with three strains of *D. bruxellensis* (◆CCA059; ■CCA077;▲CCA155) in sugarcane juice (**A**) and molasses (**B**), at 30°C.

example is the fact that the strain CCA059 did not present 4-ethylphenol formation in single-cycle batch fermentation but it did in multiple-cycle batch fermentation. Except for the strain CCA077, in which this adaptation was not necessary, for the other strains the 4-ethylphenol peaked in the 5th and 6th fermentative cycles, and considering that the molar conversion was relatively low (around 50%–60% in molasses) at these cycles, it is possible that the molar conversion would increase if the fermentation proceeded.

To demonstrate that the production rate of 4-ethylphenol is independent from the microbial growth rate in the sugarcane musts, we calculated the 4-ethylphenol production rate as a function of the log of the cell number for each fermentative cycle both in sugarcane juice and molasses (Fig. 6). In sugarcane juice, a rate of 0.2 mg/L/log cell number was obtained at the end of the 6th fermentative cycle for the three strains; however, the rate increased at each cycle for CCA059 and CCA155 and it was constant for CCA077 (Fig. 6A). In molasses the distinction among the strains was striking, reaching 0.6, 0.7 and 0.3 mg/L/log cell number for CCA059, CCA077 and CCA155, respectively, at the end of the 6th fermentative cycle (Fig. 6B). These results indicate that the production rate of 4-ethylphenol is dependent on the yeast strain, on the fermentation substrate and on the adaptation of the yeast to the fermentation conditions, i.e. the fact that the rate increases with the cell recycle. Barata *et al.* (2008) observed that 4-ethylphenol production rate depended on the yeast strain, incubation conditions and wine characteristics.

The concentrations observed in the current study were much higher than the ones allowed in wines, in which the sensory thresholds for volatile phenols are estimated to be 0.42 mg/L (Šućur, Cadez and Kosmerl 2016). Ávila and Ayub (2013) observed that 46% of 126 wine samples presented 4-ethylphenol concentration (average of 593.40 μg/L) above the threshold limit. When a strain of *D. bruxellensis* (ISA791) was added to a synthetic medium with 10 mg/L of *p*-coumaric acid, 4-ethylphenol was detected 2 days after the yeast inoculation, reaching a concentration of 7.69 mg/L after 8 days (Cabrita *et al.* 2011). In our work, even considering the difference in the substrates, the strain CCA077 stands out by producing 8.69 mg/L of 4-ethylphenol after the first fermentative cycle that lasted 12 h in molasses with 13 mg/L of *p*-coumaric acid.

The current study is the first to demonstrate the formation of 4-ethylphenol by strains of *D. bruxellensis*, isolated from the ethanolic fermentation, in sugarcane musts in conditions similar to the ones utilised in the industry. The effect of 4-ethylphenol produced in the fermentation parameters and on the viability of *S. cerevisiae* deserves special attention and future research.

CONCLUSIONS

The concentrations of 4-ethylphenol peak during active growth of *Dekkera bruxellensis* in single-cycle fermentation but they are lower than in multiple-cycle fermentation. The production rate of 4-ethylphenol is dependent on the yeast strain, on the fermentation substrate and on the adaptation of the yeast to the fermentation conditions. The production of 4-ethylphenol was higher in molasses than in sugarcane juice, and molar conversion (*p*-coumaric acid to 4-ethylphenol) ranged from 45% to 85%. This is the first report on 4-ethylphenol production in sugarcane musts by *D. bruxellensis* in industry-like conditions.

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Conflict of interest. None declared.

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