Growth and 4-ethylphenol production by the yeast *Pichia guilliermondii* in grape juices

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Abstract

The behavior of *Pichia guilliermondii* strains producing high levels of 4-ethylphenol in synthetic media was studied in wines and grape juices. These strains lost their viability and did not produce 4-ethylphenol after 24 hr of inoculation in red wines with ethanol adjusted to 10 or 12 % (v/v) and pH 3.5, in the absence of free sulphite. Under the same conditions, at 12 % (v/v) ethanol, growth of *Dekkera bruxellensis* was observed. When grown in single culture in grape juices, selected strains of *P. guilliermondii* produced high levels of 4-ethylphenol. In mixed grape juice fermentations with *Saccharomyces cerevisiae*, *P. guilliermondii* began to die after starter inoculation at 10^7 cfu/mL and did not produce 4-ethylphenol. Low starter inoculation rates (10^2 cfu/mL) added 72 hr after *P. guilliermondii* inoculation resulted in high production of 4-ethylphenol. In conditions mimicking cold pre-fermentative maceration processes, at 10ºC for 72 hr, *P. guilliermondii* did not grow, while at 25ºC growth attained a 10^4 fold increase. At this temperature, addition of 200 mg/L potassium metabisulfite after grape crushing did not eliminate *P. guilliermondii* inoculated at 10^4 cfu/mL in grape juice of pH 3.57. The possibility that high levels of 4-ethylphenol in wines are due to the activity of *P. guilliermondii* should be mostly related with uncontrolled growth in contaminated grape juices before starter inoculation. In wines, its ability to produce 4-ethylphenol seems to be much lower than that of *D. bruxellensis.*

Introduction

Yeasts of the species *Dekkera bruxellensis* are recognised as the sole agents of phenolic taint in wines and have been isolated from wines all over the world

(Loureiro and Malfeito-Ferreira, 2003). This taint is mainly due to 4-ethylphenol which is associated with aromatic notes of “burnt beans, band-aid®, leathery, wet dog, barnyard and horse sweat” (Licker et al. 1998, Coulter et al. 2003). This molecule is the result of the decarboxylation of *p*-coumaric acid into 4-vinylphenol and sequent reduction to 4-ethylphenol (Steinke and Paulson 1964, Heresztyn 1986). Yeast species such as *D. anomala*, *Candida halophila*, *C. mannitofaciens* and *C. versatilis* are known 4-ethylphenol producers (Suezawa 1995, Dias et al. 2003), but in wines, only *D. bruxellensis* is known to produce amounts of this volatile phenol high enough to affect wine flavour (Chatonnet et al. 1995, 1997). Recently, strains of other species sporadically isolated from wine-related environments (*C. wickerhamii*, *C. cantarellii*, *Kluyveromyces lactis*, *Debaromyces hansenii* and *Pichia guilliermondii*) have been reported as producing 4-ethylphenol, in synthetic media, with different efficiencies (Dias et al. 2003b, Martorell et al. 2006). In particular, these authors showed that some strains of the species *P. guilliermondii* were able to produce 4-ethylphenol in synthetic media with efficiencies close to that of *D. bruxellensis*. However, it was not assessed if these strains could produce high amounts of 4-ethylphenol in wines or grapes juices. If so, they could also be regarded as agents of the phenolic taint together with *D. bruxellensis*. Therefore, this work was aimed at the elucidation of the possible role of *P. guilliermondii* in the production of phenolic taint in wines and grape juices.

Material and Methods

Yeast strains and maintenance conditions

The strains of *P. guilliermondii* used in this study were selected from previous works (Dias et al. 2003b, Martorell et al. 2006), all showing high conversion rates of *p*-coumaric acid into 4-ethylphenol in synthetic media (Table 1). The strains were maintained in GYP agar with 20 g/L glucose (Merck, Darmstadt, Germany), 5 g/L yeast extract (Difco Laboratories, Detroit, USA), 5 g/L peptone (Difco) and 20 g/L agar, pH 6.0, at 4ºC. The strains *Saccharomyces cerevisiae* ISA 1000 and *D. bruxellensis* ISA 1791 were used for comparative purposes and in coculture experiments. *D. bruxellensis* was maintained in slants of GYP agar added of 5 g/L of calcium carbonate, at 4ºC.

Screening of ethanol and sulphite tolerances

The evaluation of the tolerance to ethanol and sulphite was performed according to the liquid medium assimilation tests described by Kurtzman and Fell (1998). A loopful of fresh culture (24-48hr) was dispersed in about 5 mL of Ringer solution in a 16 mm tube until the black lines approximately ¾ mm wide drawn on white cardboard become visible through the tube as dark bands. One drop of such suspension was delivered, with a sterile Pasteur pipette, to each of the test tubes containing 4 mL of YNB liquid medium (6.7 g/L) supplemented with glucose (20 g/L) and different levels of ethanol (Merck) (8, 10, 12, 13, 14, 14.5, 15, 15.5, 16, 16.5, 17 and 17.5 % v/v) or potassium metabisulfite (Merck) (40, 60, 70, 80, 90, 100, 120, 140, 160, 180, 200 and 210 mg/L). For each concentration, incubation was carried out untill the lines of the black cardboard became diffuse (value ++). Then 3 drops of yeast suspension were inoculated in the following higher ethanol or metabisulfite concentration. Growth was

considered negative if, after 3 weeks of incubation at 25 ºC, black lines were distinguishable but with indistinct edges visible through the test tube (value +).

Growth in wines

A loopful of fresh culture (24-48hr) was used to inoculate 100 mL of YNB broth (6.7 g/L YNB with amino-acids, 20 g/L glucose, adjusted to pH 3.50 ± 0.01 and sterilised by filtration through membrane of 0.22 μm pore size), incubated at 25ºC with orbital shaking (120 rpm). Growth was followed by measuring the absorbance at 640 nm and when about 0.5 units were reached, the tested wines were inoculated to give an initial population of about 10^4 cells/mL (single and mixed cultures). Experimental wines were obtained by blending several commercial red wines added with acetaldehyde to combine free sulphite and sterilised by filtration through membranes of 0.22 μm pore size. Incubation was carried out at 25ºC with orbital shaking (120 rpm) and cellular viability was measured by plating onto GYP agar. The effect of ethanol on viability of *P. guilliermondii* ISA 2126 was tested using a red wine at pH 3.50 and adjusted to 8%, 10% and 12% (v/v) ethanol. For mixed culture tests, *P. guilliermondii* ISA 2126 and *D. bruxellensis* ISA 1791 were inoculated in red wine with 10% (v/v) ethanol (pH 3.50) with 16 mg/L free sulfite and without free sulfite after removal with acetaldehyde. In order to test the influence of cellular adaptation in wine, strain *P. guilliermondii* ISA 2131 was inoculated in red wine with 12% (v/v) ethanol, pH 3.50, after previous growth in red wine (pH 3.50) with 6% (v/v) ethanol (adapted cells) or in YNB broth (6.7 g/L) with 20 g/L glucose (pH 3.50) (non-adapted cells).

The 4-ethylphenol and 4-vinylphenol production was measured according to a protocol already described by Rodrigues et al. (2001). 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. Samples were taken periodically from culture media and frozen in glass vials at -18 ºC until used.

Growth in grape juices

A loopful of fresh culture (24-48hr) of each yeast strain tested (*P. guilliermondii* ISA 2105 and ISA 2131 and *S. cerevisiae* ISA 1000) was used to inoculate 250 mL of YNB broth (6.7 g/L) supplemented with 20 g/L glucose, pH 3.50 and sterilised by filtration through membrane of 0.22 μm pore size. Incubation was carried out at 25ºC with orbital shaking (120 rpm).

The viability and 4-ethylphenol production of single cultures of *P. guilliermondii* (ISA 2105 and ISA 2131) were measured in white grape juice (23.2 Brix, pH 3.48) sterilized by autoclaving (121 ºC for 5 minutes) and supplemented with 20 mg/L *p*-coumaric acid. After inoculation with an initial population of about 10^4 cells/mL of each strain, the grape juices were incubated at 25ºC during 25 days, and cell viability was measured by plating onto GYP agar.

The behavior and volatile phenols production of mixed cultures of *S. cerevisiae* and *P. guilliermondii* were measured in: (i) white grape juice (23.2 Brix, pH 3.48) added of 20 mg/L *p*-coumaric acid inoculated with 10^6 cells/mL of *S. cerevisiae* ISA 1000 and 10^4 cells/mL of *P. guilliermondii* ISA 2105 or 2131; (ii) red (24.2 Brix, pH 3.61) and white (21.6 Brix, pH 3.50) grape juices added of 20

mg/L *p*-coumaric acid inoculated with 10^2 cells/mL of *S. cerevisiae* ISA 1000 (after 72 hr of incubation) and 10^4 cells/mL of *P. guilliermondii* ISA 2105 or 2131; (iii) red (22.7 Brix, pH 3.57) and white (21.9 Brix, pH 3.50) grape juices added of 20 mg/L *p*-coumaric acid inoculated with 10^7 cells/mL of *S. cerevisiae* ISA 1000 (after 48 hr of incubation) and 10^4 cells/mL of *P. guilliermondii* ISA 2105 or 2131. During the fermentations, *S. cerevisiae* was counted by spreading onto GYP plates while *P. guilliermondii* enumeration was carried out onto GYP plates added of 100 mg/L cycloheximide (Sigma), incubated at 25 ºC during 48 hr. Volatile phenols were determined as previously described.

Effect of temperature and sulfite on growth in grape juices

The effect of cold pre-fermentative maceration and potassium metabisulfite on yeast growth of *P. guilliermondii* ISA 2105 and *D. bruxellensis* ISA 1791 was tested in 50 mL of red grape juice (22.7 Brix, pH 3.57) with the correspondent grape skins, sterilised by autoclaving (121 ºC for 5 minutes) and supplemented with 20 mg/L *p*-coumaric acid. On cold maceration tests, the grape juices were inoculated with an initial population of about 10^4 cells/mL of each strain (single culture) and incubated at 10 ºC during 72 hr. On sulfite resistance tests, the grape juices were added with 50, 100 and 200 mg/L of potassium metabisulfite and incubated at 25 ºC during 72 hours. Cell viability was measured by spreading onto GYP plates incubated at 25 ºC. All experiments were repeated at least twice and are shown results with the respective standard errors.

Results

Screening of ethanol and potassium metabisulfite tolerance

Two of the main factors affecting yeast growth in wines seem to be ethanol and sulfite contents and so their effects against yeast growth of *P. guilliermondii* strains isolated from the wine-related environments were evaluated. For comparative purposes one commercial starter of *S. cerevisiae* and one strain of *D. bruxellensis* were also used. The results presented in Table 1 regarding ethanol effect, showed that the most tolerant strain was *S. cerevisiae*, growing under 17% (v/v) ethanol, followed by *D. bruxellensis*, growing under 15.5% (v/v) ethanol. The strains of *P. guilliermondii* showed growth under maxium ethanol concentrations ranging from 14.0 up to 15.0 % (v/v).

The potassium metabisulfite effect is shown in table 1. The most resistant strain was *S. cerevisiae* ISA 1000, growing under 200 mg/L, while strains of *P. guilliermondii* grew from 70 to 140 mg/L. *D. bruxellensis* was among the most sensitive strains, being inhibited by levels higher than 70 mg/L of potassium metabisulfite, at initial pH 3.50.

Behavior in wines

Knowing that *P. guilliermondii* strains isolated from wine-related environments had the ability to produce high levels of 4-ethylphenol, our first concern was to elucidate if this ability was also present when inoculated in wines. A group of *P. guilliermondii* strains (ISA 2105, 2126, 2131, 2134, 2139 and 2143) was selected, according to their different origins (see table 1), for testing their survival after inoculation in red wines. The decrease in viability, as measured by plate counts, was variable among the strains tested but, after 24 hr of incubation, no viable cells were recovered in 0.1 mL of sample (results not shown). The analysis of 1 mL of wine after 3 days of incubation and of the

remaining 15 mL of wine used in experiments, after 17 days, did not show any viable cell for each of the strains. The levels of 4-ethylphenol remained constant during the experiments (results not shown). In addition, increasing wine pH to 4.0 did not result in viability recovery (results not shown). Therefore, the following tests were addressed to evaluate the behavior in wines with ethanol content adjusted to 8, 10 or 12 % (v/v). The strain *P. guilliermondii* ISA 2126 showed no viable cells after 24 hr of incubation for any of the concentrations tested. The repetition of these experiments using 3 other different wine blends confirmed the observed loss of viability (results not shown).

The fact that *P. guilliermondii* and *D. bruxellensis* grow at different rates on agar plates enables their differential counting and so it is possible to perform experiments in mixed cultures. In this way, the behavior of each species was evaluated under the same wine conditions. The viability evolution in wines with 10 % (v/v) ethanol, pH 3.50, demonstrated that the strain *P. guilliermondii* ISA 2126 lost viability while *D. bruxellensis* ISA 1791 maintained its viability during 48 hr and grew thereafter (results not shown).

The absence of growth in wines mentioned in the previous tests contrasts with the isolation of strain ISA 2131 from wines (see table 1). Thus, we hypothethised that cells had not been properly adapted to grow in wines. However, further tests with strain ISA 2131 previously grown in 6 % (v/v) red wine resulted in lower death rate but viability, as measured by plate counting, was not observed after 24 hr of incubation (results not shown).

In conclusion, none of the tested strains of *P. guilliermondii* was able to grow and produce volatile phenols in red wine blends under the common ethanol

contents (10-12 % v/v). These results led us to test if these yeasts could produce volatile phenols in grape juices.

Growth and 4-ethylphenol production in single and mixed cultures in grape juices

The strains *P. guilliermondii* ISA 2105 and 2131 grew in single culture in white grape juice and produced about 18 mg/L of 4-ethylphenol resulting from the conversion of natural and added (20 mg/L) *p*-coumaric acid. The situation mimicked in these experiments is not realistic in enological practice and so the ability to produce 4-ethylphenol was evaluated in mixed cultures with *S. cerevisiae*.

The behavior of strain *P. guilliermondii* ISA 2131 inoculated in white grape juices together with *S. cerevisiae* is shown in figure 1. Since after the inoculation time, the populations of *P. guilliermondii* started to die, until being not detected. The production of 4-vinylphenol, due to *p*-coumaric acid conversion by *S. cerevisiae*, was initiated at the begining of the experiment, while the conversion to 4-ethylphenol was not observed. The strain *P. guilliermondii* ISA 2105 behaved similarly to ISA 2131 (results not shown). These results indicate that the fermentation carried out as usual in wineries may prevent growth and 4-ethylphenol production by *P. guilliermondii*.

The following tests were devised to mimick situations where this species could be active in the absence of high inocula of *S. cerevisiae*. The first set of trials is depicted in figure 2 and shows the behavior of *P. guilliermondii* ISA 2105 in a hypothetical case of spontaneous fermentation where *S. cerevisiae* is present in low initial numbers (100 cfu/mL) added 72 hr after the beginning of the

experiment. In the absence of *S. cerevisiae*, *P. guilliermondii* grew exponentially and began to produce 4-ethylphenol. The decline in its population was observed under the presence of high *S. cerevisiae* populations but 4-ethylphenol had already been produced in high levels, up to 8 mg/L in red grapes juices or 12 mg/L in white grapes juices. This behavior was also displayed by ISA 2131 grown under the same conditions in white or red grape juices (results not shown). This situation covers the case of absence of starter inoculation but it is possible to have delayed starter additions in a technological process known as pre-fermentative maceration, either for red or white grape juices.

The set of trials depicted in figure 3 shows the case of a pre-fermentative maceration at 25 ºC, followed by *S. cerevisiae* addition (10^7 cfu/mL) after 48 hr. Up to 48 hr of incubation, *P. guilliermondii* ISA 2105 grew exponentially and lost their viability afterwards. The production of 4-ethylphenol ceased at levels of about 4 mg/L, either in red or white juices, lower than those described in the previous set of experiments. This behavior was also displayed by ISA 2131 grown under the same conditions in white or red grape juices (results not shown). The growth behavior of both strains of *P. guilliermondii* and *S. cerevisiae* was similar in both red and white grape juices but the production of 4-vinylphenol was different. In red grape juices, 4-vinylphenol production was rather low (0.8 mg/L) probably because the conversion of *p*-coumaric acid by *S. cerevisiae* was inhibited by the polyphenolic components of red wines, as described by Chatonnet et al. (1997). This inhibition did not occur in white grape juices and increasing levels of 4-vinylphenol were observed up to a maximum of about 7 mg/L at the end of the experiment (figure 3).

Effect of temperature and sulfite on growth in red grape juices

From the above described tests it arised that to avoid *P. guilliermondii* growth it is crucial to add yeast starter, but this is not possible when using pre-fermentative maceration operations. In red wines, the process is known as cold pre-maceration and is currently done at 8-10 ºC, during 48 to 72 hr. At this temperature *P. guilliermondii* ISA 2105 growth was significantly reduced and 4-ethylphenol was not produced (results not shown). The use of high concentrations of potassium metabisulfite (200 mg/L) did not prevent growth of *P. guilliermondii* ISA 2105 (results not shown).

Discussion

Dias et al. (2003b) reported the occurrence of strains of *C. wickerhamii*, *C. cantarellii*, *K. lactis*, *D. hansenii* and *P. guilliermondii* with positive results in DBDM plates addressed to the detection of *D. bruxellensis*. Given that a positive result depends on the release of a phenolic smell, some of these strains were first described as presumptive *D. bruxellensis* (Rodrigues et al. 2001), based on the assumption that this species was the sole 4-ethylphenol producer in wines and grape juices, according to Chatonnet et al. (1995, 1997). The production of 4-ethylphenol was checked for all those five species, but only some strains of *P. guilliermondii* converted *p*-coumaric acid into 4-ethylphenol, in synthetic media, with efficiencies close to that of *D. bruxellensis* (Dias et al. 2003b). The identification of further strains originated from wine-related environments confirmed the dissemination of *P. guilliermondii* through grapes, stems, equipment during harvesting, *Drosophila* spp. flies and, less frequently,

from wines and barrels' wood (Martorell et al. 2006). The analysis at infraespecific level by Martorell et al. (2006) enabled the establishment of one group of high 4-ethylphenol producers, gathering 2 different RAPD haplotypes, and another group of low 4-ethylphenol producers, gathering 4 different haplotypes.

Given that in synthetic media some strains of *P. guilliermondii* showed high efficiency in the production of 4-ethylphenol, an evaluation of this ability under wine conditions was essential to assess their spoilage potential. The first experiments using wines showed that, for the average ethanol content range of 10-12 % (v/v), those strains did not survive and did not produce 4-ethylphenol. The fact that from the 15 analysed strains only one (strain ISA 2131) was isolated from wine is consistent with this observation. However, we were not able to grow this strain in wine, even with cells adapted by previous growth in low ethanol wine. This suggests that *P. guilliermondii* in wines is probably in a non-proliferating state and/or that we did not manage to mimick the real conditions for yeast adaptation. Then we compared the behavior of *P. guilliermondii* ISA 2131 with that of *D. bruxellensis* ISA 1791 in mixed culture in wine. As expected, the results showed that the latter is comparatively better adapted to grow and produce 4-ethylphenol under wine conditions. The screening of ethanol tolerance showed that *D. bruxellensis* is more tolerant than *P. guilliermondii* but the small difference observed (see table 1) suggests the influence of other factors which, in a complex matrix like wines, are not easily determined (Loureiro and Malfeito-Ferreira, 2003). The effect of the matrix composition may also explain the expected higher tolerance to sulfite in

grape juices when compared with the tolerance values provided by growth in synthetic medium.

The above mentioned results led us to evaluate the growth and 4-ethylphenol production during grape juice fermentation. *P. guilliermondii* showed the ability to grow and produce 4-ethylphenol in grapes juices previous to the onset of fermentation by *S. cerevisiae*. Thus, *P. guilliermondii* may be particularly dangerous when using pre-fermentative maceration techniques. In these cases, the utilisation of high sulfite doses (aprox. 100 mg/L total sulfite) did not avoid its proliferation. Therefore, the use of such techniques at the advised low temperatures bears also the advantage of preventing the growth of this contaminant species.

The fermentation in mixed cultures showed that *P. guilliermondii* was quite sensitive to the presence of fermenting *S. cerevisiae*. The elucidation of the physiological basis for this behavior is behond the scope of this work. Research carried out by Nils and Arneborg (2003) suggests that the main factors for early death of species like *Kluyveromyces thermotolerans* and *Torulaspora delbrueckii*, are mediated by a cell-to-cell contact mechanism at high cell densities of *S. cerevisiae* and to a lesser ability of those species to compete for space. *D. bruxellensis* behaves differently, even at initial low concentrations (10 cfu/mL), it endured co-fermentation with *S. cerevisiae* and attained high cell densities (more than 10^7 cfu/mL) after the end of fermentation (Dias et al. 2003a).

The occurrence of *P. guilliermondii* in grapes and its ability to produce 4-ethylphenol in grape juices may explain the empirical observation of release phenolic odours in the vineyard by damaged grapes (Ribereau-Gayon, 1980,

cited by Donèche, 1992, Gadoury et al. 2002). In addition, this species has been used for post-harvest biocontrol in citrus, pears and grapes to prevent rotting by moulds (Lima et al. 1999). We are not aware of its utilisation in the vineyard, but any attempts to develop biocontrol products for wine grapes should be restricted to 4-ethylphenol non-producing strains.

Conclusions

Our results indicate that *P. guilliermondii* can cause the same problem as *D. bruxellensis*, but only in grape juices. In wines, its ability to grow and produce 4-ethylphenol is much lower than that of *D. bruxellensis* and any preventive measures against this species would result more effectively against *P. guilliermondii*. The possibility that high levels of 4-ethylphenol, already present in newly fermented wines (Rodrigues et al. 2001), are due to the activity of *P. guilliermondii*, should be related with uncontrolled growth in contaminated grape juices before starter inoculation. Therefore, adequate utilisation of pre-fermentative maceration techniques and sequent starter addition appears to be sufficient to avoid the risk associated with *P. guilliermondii* growth in grape juices.

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Table 1. Strain list, maximum ethanol concentration (% v/v) and maximum initial potassium metabisulfite concentration (mg/L) allowing growth in YNB broth with glucose (20 g/L) and initial pH 3.50, at 25ºC.

Species	ISA[a] number	Conversion rate[b]	Origin	Reference	Maximum concentration	
					Ethanol	P. metabisulfite
Saccharomyces cerevisiae	1000	0.0	Yeast starter Fermivin	Dias et al. (2003a)	17.0	200
Dekkera bruxellensis	1791	92.5	Red wine	Dias et al. (2003a)	15.5	70
Pichia guilliermondii	2105	82.6	Grapes, strain 2005	Dias *et al*. (2003b)	14.0	70
	2122	70.3	Grapes, strain 2022	Dias *et al*. (2003b)	15.0	140
	2126	69.0	Press roll, strain 2026	Dias *et al*. (2003b)	15.0	120
	2131	85.8	Red wine	Martorell et al. (2006)	15.0	140
	2134	87.7	Grapes	Martorell et al. (2006)	15.0	70
	2135	80.9	Grapes	Martorell et al. (2006)	15.0	120
	2136	86.4	Grapes	Martorell et al. (2006)	15.0	90
	2137	92.5	Stems	Martorell et al. (2006)	14.5	70
	2138	82.8	Stems	Martorell et al. (2006)	14.5	120
	2139	77.2	Stems	Martorell et al. (2006)	15.0	70
	2141	74.4	*Drosophila* spp.	Martorell et al. (2006)	15.0[c]	70
	2142	92.5	Grapes	Martorell et al. (2006)	15.0	140
	2143	87.7	*Drosophila* spp.	Martorell et al. (2006)	15.0	140
	2145	75.7	Stems	Martorell et al. (2006)	14.5	100
	2286	68.5	Barrel wood, strain 430b	Martorell et al. (2006)	15.0[c]	120

[a] ISA: Instituto Superior de Agronomia.

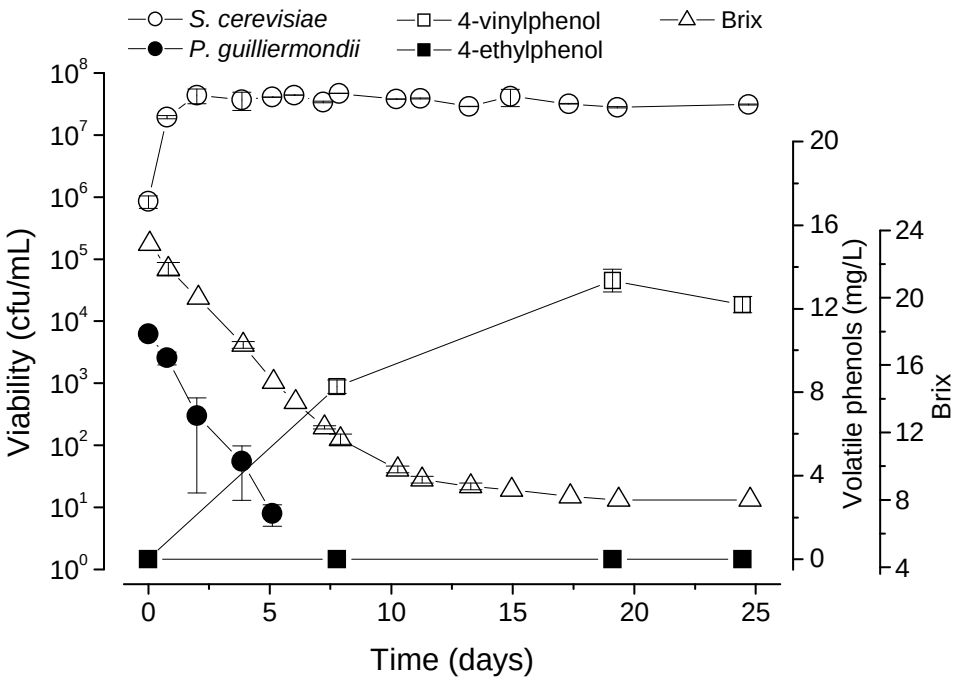
[b] Calculated by the ratio between the maximum concentration of 4-ethylphenol produced and the maximum theoretical concentration (74.5 mg/L) produced by the conversion of 100 mg/L of p-coumaric acid.

[c] When the duplicates did not show the same results, the highest score is presented. The difference between the duplicates was not higher than 0.5 % (v/v).

Figure 1. Viability and volatile phenol production of mixed cultures of *Saccharomyces cerevisiae* ISA 1000 and *Pichia guilliermondii* ISA 2131 grown in white grape juice supplemented with 20 mg/L *p*-coumaric acid, at 25 ºC. Inocula sizes were 10^6 cfu/mL for *S. cerevisiae* and 10^4 cfu/mL for *P. guilliermondii*.

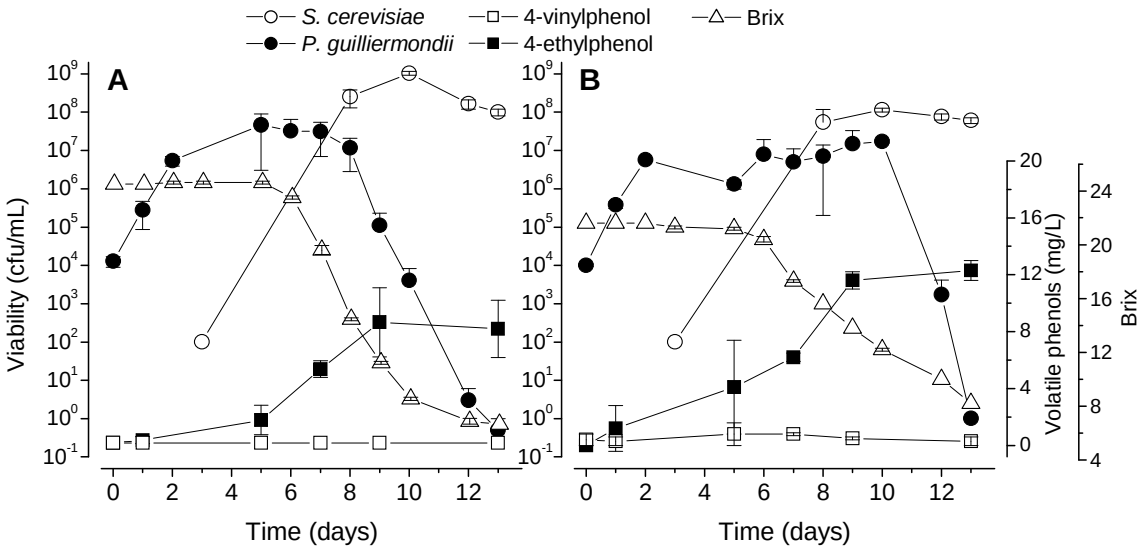
Figure 2. Viability and volatile phenol production of mixed cultures of *Saccharomyces cerevisiae* ISA 1000 and *Pichia guilliermondii* ISA 2105 grown in red (A) and white (B) grape juices added of 20 mg/L *p*-coumaric acid, at 25 ºC. Inocula size were 10^2 cfu/mL for *S. cerevisiae* (added at 72 hr of incubation) and 10^4 cfu/mL for *P. guilliermondii*.

Figure 3. Viability and volatile phenol production of mixed cultures of *Saccharomyces cerevisiae* ISA 1000 and *Pichia guilliermondii* ISA 2131 grown in red (A) and white (B) grape juices added of 20 mg/L *p*-coumaric acid, at 25 ºC. Inocula size were 10^7 cfu/mL for *S. cerevisiae* (added at 48 hr of incubation) and 10^4 cfu/mL for *P. guilliermondii*.


S. cerevisiae
P. guilliermondii
4-vinylphenol
4-ethylphenol
Brix
Viability (cfu/mL)
Volatile phenols (mg/L)
Brix
Time (days)

1

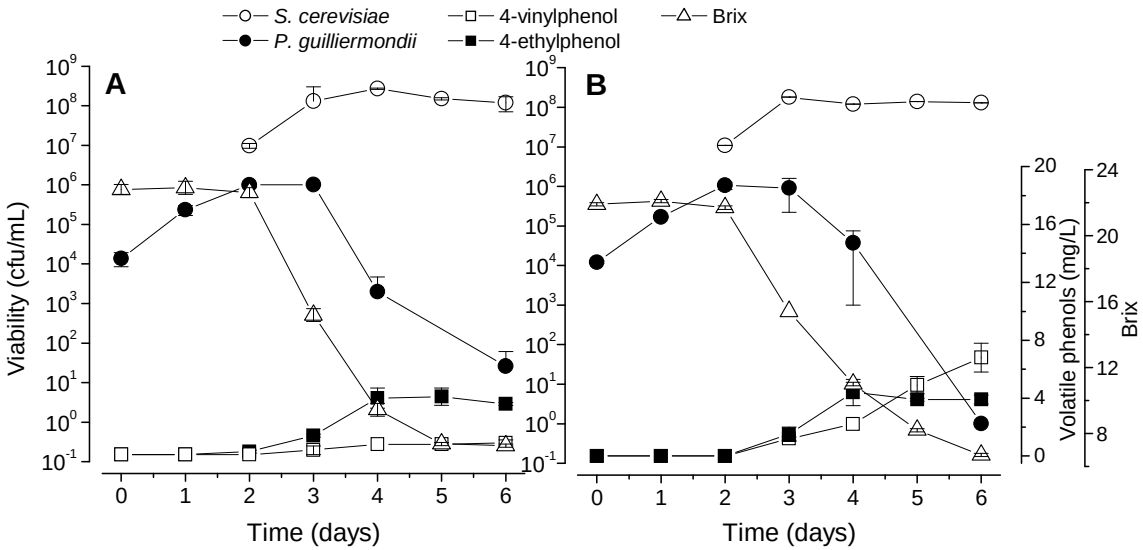
1


S. cerevisiae
P. guilliermondii
4-vinylphenol
4-ethylphenol
Brix
A
B
Viability (cfu/mL)
Volatile phenols (mg/L)
Brix
Time (days)
Time (days)

2
3

4

1


S. cerevisiae
P. guilliermondii
4-vinylphenol
4-ethylphenol
Brix
A
B
Viability (cfu/mL)
Volatile phenols (mg/L)
Brix
Time (days)
Time (days)

2
3