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ORIGINAL PAPER

Mutagenesis, screening and isolation of *Brettanomyces bruxellensis* mutants with reduced 4-ethylphenol production

Izmari Jasel Álvarez Gaona[1,3] · Mariela Vanesa Assof[1] · Viviana Patricia Jofré[1] · Mariana Combina[1,2] · Iván Francisco Ciklic[1,4]

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Abstract

The use of non-conventional yeast species to obtain interesting flavors and aromas has become a new trend in the fermented beverages industry. Among such species, *Brettanomyces bruxellensis* (*B. bruxellensis*) has been reported as capable of producing desirable or at least singular aromas in fermented beverages like beer and wine. However, this yeast can also produce an aromatic defect by producing high concentrations of phenolic compounds like, 4-ethylguaiacol and particularly 4-ethylphenol (4-EP). In the present study, we designed a mutant screening method to isolate *B. bruxellensis* mutants with reduced 4-EP production. More than 1000 mutants were screened with our olfactory screening method, and after further sensory and chemical analysis we were able to select a *B. bruxellensis* mutant strain with a significant reduction of 4-EP production (more than threefold) and less phenolic perception. Notably, the selected strain also showed higher diversity and concentration of ethyl esters, the most important group of odor active compounds produced by yeasts. Based on these results, we consider that our selected mutant strain is a good candidate to be tested as a non-conventional yeast starter (pure or in co-inoculation) to obtain wines and beers with novel aromatic properties.

Keywords *Brettanomyces bruxellensis* · Non-conventional yeasts · Yeast starter · 4-ethylphenol · Wine · Beer

Introduction

The yeast *Brettanomyces bruxellensis* (teleomorph: *Dekkera bruxellensis*), is usually associated with aromatic defects in wine, mainly because of the production of phenolic compounds like 4-ethylguaiacol (4-EG) and particularly 4-ethylphenol (4-EP) (Loureiro and Malfeito-Ferreira 2003). The aromas associated with this "Brett character" or "Bretty wines" are usually described as barnyard, horse sweat, wet animal or wet leather, to name a few. 4-EP has a low perception threshold and contaminated wines usually showed a 4-EP/4-EG ratio of 8:1 (Chatonnet et al. 1992). Thus, 4-EP is considered the principal contributor to this aromatic defect. However, at low concentrations, phenolic compounds can also add some aromatic complexity to the wine (Chatonnet et al. 1995; Fugelsang and Edwards 2007; Ribéreu-Gayon et al. 2000). In the case of beer, the formation of such phenolic compounds by *B. bruxellensis* is essential for the elaboration of some particular styles like the Belgian Lambic, Gueuze and Coolship Ale (Vanderhaegen et al. 2003). *B. bruxellensis* can be also involved in other fermentative processes like cider (Morrissey et al. 2004), kombucha (Teoh et al. 2004), kefir (Laureys and De Vuyst 2014) and bioethanol (de Souza Liberal et al. 2007).

Despite its important contribution to different fermented products, the fermentative attributes of *B. bruxellensis* have not been fully investigated and most of the studies are focused on its role as spoilage yeast. In the work of Blomqvist et al. (2010) some fermentative characteristics

✉ Iván Francisco Ciklic
ciklic.ivan@inta.gob.ar

[1] Estación Experimental Agropecuaria Mendoza, Instituto Nacional de Tecnología Agropecuaria (INTA), San Martín 3853, 5507 Luján de Cuyo, Mendoza, Argentina

[2] Consejo Nacional de Investigaciones Científicas y Tecnológicas. Godoy Cruz, 2290, C1425FQB CABA, Argentina

[3] Facultad de Ingeniería y Ciencias Agrarias, Pontificia Universidad Católica Argentina Santa María de Los Buenos Aires, Av. Alicia Moreau de Justo 1300, C1107AAZ Buenos Aires, Argentina

[4] Laboratorio de Biotecnología, EEA INTA, Mendoza San Martín 3853, Luján de Cuyo, Mendoza, Argentina

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of different *B. bruxellensis* strains were evaluated and compared with *Saccharomyces cerevisiae* commercial strains. *S. cerevisiae* strains grew up to fivefold faster than *B. bruxellensis*, but with lower ethanol yields. *S. cerevisiae* also produced higher amounts of glycerol (up to sixfold more). Regarding the biomass production, *B. bruxellensis* produced amounts 16–28% higher than *S. cerevisiae* strains. This study also showed the ability of *B. bruxellensis* to tolerate drastic changes in the pH and temperature.

Although *Saccharomyces* spp have many advantages as universal yeast starters, its exclusive use also restricts the full potential of each fermentative process, as for example, the formation of new flavors and aromas in alcoholic beverages (Steensels et al. 2015). Many research groups have been trying to overcome such limitations by improving different metabolic properties of *S. cerevisiae* through genetic engineering or classical genetics. Alternatively, the use of non-*Saccharomyces* yeast starters as pure cultures or in co-inoculation with traditional yeast starters is getting increasingly popular (Steensels and Verstrepen 2014). Considering the potential of *B. bruxellensis* as yeast starter, sensory and biochemical analysis has shown that it can produce intense and diverse aromatic profile (Licker et al. 1999). Indeed, a growing number of authors are reporting that these yeasts can produce desirable or at least interesting aromas to obtain more complex or distinctive fermented beverages (Steensels et al. 2015). Global genomic analysis has also revealed some insights about the great aromatic potential of this yeast. Concretely, the analysis of the alcohol dehydrogenase genes of *B. bruxellensis* showed a duplication of the *S. cerevisiae* gene homologues *ADH6* and *ADH7* (Piškur et al. 2012). These genes play an essential role in the transformation of sugar to alcohol and in the synthesis of aromatic compounds like higher alcohols and esters precursors. There are already some examples in beer where *B. bruxellensis* has been used as a single yeast starter (Yacobson 2010) or in sequential inoculation with *S. cerevisiae* (Holt et al. 2018). In the case of wine, there are only some preliminary studies like that of Dashko et al. (2015) where they tried a sequential inoculation of *B. bruxellensis* and *S. cerevisiae* to improve the aroma complexity of white wines from Ribolla variety. Blind tasting of these wines showed acceptable evaluations, but the phenolic perception was still high. To counteract the strong phenolic perception in such *B. bruxellensis* fermentations, it would be convenient to have strains of *B. bruxellensis* that produce low 4-EP.

Chemical mutagenesis is achieved by putting into contact a chemical agent (ethyl methane sulphonate, methyl methane sulphonate, N-Methyl-N'-nitro-N-nitrosoguanidine or nitrous acid) with yeast cells, long enough to provoke a 50–95% killing, followed by removal of the mutagen; this way, the mutation rate is significantly increased (Peréz Torrado et al. 2015). It is important to remark, that mutants obtained by this technique could be used without restrictions since they were obtained with classical genetic methods and are not considered genetically modified organisms (GMOs). *B. bruxellensis* strains with low 4-EP production could be of great interest to obtain aromatic distinctive products in fermentations with non-conventional yeast starters, leading to wines and beers with higher aromatic complexity and a reduced impact of the phenolic perception.

Thus, the aim of the present study was to obtain *B. bruxellensis* mutants with reduced production of 4-EP by chemical mutagenesis and to develop a screening method for their isolation. Finally, we would like to point out that to our best knowledge this is the first mutant screening ever performed with *B. bruxellensis*.

Material and methods

Strains, media and growth conditions

The CH29 *B. bruxellensis* strain used in the present study was isolated from contaminated red wine from Argentina (GenBank Accession number: KF002710). CH29 was selected from other *B. bruxellensis* native isolates, because of its high conversion rate of *p*-coumaric acid into 4-ethylphenol (Sturm et al. 2015). The yeast strain *Saccharomyces cerevisiae* BY4741 was used as negative control. *B. bruxellensis* strains were grown in YEPD medium (yeast extract 1% w/v, peptone 2% w/v and glucose 2% w/v) at 28 °C with 150 rpm shaking (Multitron Standard Infors HT, Bottmingen, Switzerland). Selective medium for detection of *B. bruxellensis* in wine ($Brett_{selective}$ medium: glucose 1% w/v, yeast extract 1% w/v, chloramphenicol 0.05% w/v, cycloheximide 0.01% w/v, *p*-coumaric acid 0.4% w/v, ethanol 6.25% v/v, 1.5% w/v agar and pH 4.7 adjusted with 1 M HCl), or an optimized version of this medium ($Brett_{screening}$ medium) with reduced *p*-coumaric acid (0.016% w/v) were used for mutant selection experiments as well as sensory and chemical analysis. Growth was monitored by measuring the optical density (OD) at 640 nm with a spectrophotometer UV–Visible (T60 UV–Visible PG instruments, Leicestershire, UK).

Chemical mutagenesis of *B. bruxellensis* CH29

Mutagenesis of the *B. bruxellensis* CH29 strain was performed with the alkylating agent ethylmetane sulfonate (EMS [Sigma-Aldrich, India]) which induces a high base pair substitution frequency with minimal mortality. The mutagenesis protocol was adapted from a previous work on *S. cerevisiae* (Cordente et al. 2009). In order to determine the right concentration of the mutagenic agent EMS for *B. bruxellensis*, the survival rate ($SR_{50\%}$) of *B. bruxellensis* CH29 was estimated for EMS concentrations of 1, 3,

6 and 8% v/v. Further, we performed a second experiment where we determined the $SR_{50\%}$ only for EMS concentrations of 1 an 2%. Aliquots containing around 5×10^7 CH29 yeast cells (DO_{640nm} $1.87 \cong 5 \times 10^7$ CFU/mL, experimentally determined) were transferred to 2 mL tubes for mutagenesis experiments. After centrifugation at 5000 rpm for 2 min, the pellets were washed and resuspended in 1.5 mL sterile sodium phosphate buffer (KH_2PO_4 50 mM and pH 7.0). Yeast suspensions were transferred to sterile glass test tubes and EMS was added to a final concentration of 1–8% v/v. All tubes were then incubated at 30 °C on an orbital shaker for 30 min. Mutagenesis was stopped by adding one volume of 10% w/v sodium thiosulphate sterile solution. EMS-mutagenized cells were centrifuged at 13,000 rpm during 5 min and washed with Mili-Q® water. After re-suspension in 1 mL sterile water 200 µl of 10^{-4} and 10^{-5} dilutions were spread onto YEPD plates. Cell viability was estimated by standard plate counting method, after 5 days of incubation at 28 °C.

Construction of the mutant library and olfactory screening method

Mutants cells were grown in $Brett_{selective}$ medium plates to preselect strains resistant to exogenous ethanol and other potential inhibitors (chloramphenicol, cycloheximide and *p*-coumaric acid). Plates were incubated at 28 °C for up to 5 days and the grown colonies were transferred to fresh solid YEPD medium for subculture and maintenance. Mutants were screened with an olfactory test, selecting those with a lower "Brett character". For the olfactory test, mutants were previously incubated 72 h at 28 °C without shaking in 1 mL of $Brett_{screening}$ medium to favor the development of the 4-EP aromatic signal. The initial cell density of the cultures was set at 5×10^7. Mutant samples were compared with a positive ($Brett_{screening}$ medium inoculated with CH29 strain) and a negative control ($Brett_{screening}$ medium inoculated with BY4741 strain) by a previously trained person, selecting those mutants perceived with lower Brett character as compared to the positive control. Rounds of five mutant samples were evaluated with one-minute break between them and to minimize olfactory saturation, ground coffee was smelled between rounds. The preselected mutants were subjected to multiple rounds of olfactory selection, reducing the number of samples to be used for further sensory and chemical analysis to a minimum. Samples with no thick sediment were discarded to prevent the selection of false negatives (slow growing mutants).

Sensory analysis

Mutants previously isolated in the olfactory screening were further characterized by sensory analysis. Two different sensory analyses were performed, a ranking test and a duo-trio test (Noble 1993). For the ranking test, 2.5×10^7 cells/mL of each strain were inoculated in 1.5 mL liquid $Brett_{screening}$ medium and incubated in 2 mL tubes at 28 °C for 4 days without shaking. After incubation, the samples were labeled with random numbers and presented to two trained judges from our institute sensory panel. Eleven samples consisting of six mutants, two positive controls ($Brett_{screening}$ medium inoculated with CH29 strain), two negative controls ($Brett_{screening}$ medium inoculated with BY4741 strain) and a sample containing only the $Brett_{screening}$ medium, were ranked according to their smell from low to high "Brett character". After performing eight independent ranking tests, two mutants were associated with lower "Brett character" and were selected for further analysis with a duo-trio test. Both mutants plus a positive (*B. bruxellensis* CH29) and a negative control (*S. cerevisiae* BY4741) were inoculated in 15 mL of liquid $Brett_{screening}$ medium and incubated at 28 °C for 4 days without shaking. After incubation, 5 mL of each culture were centrifuged and the supernatant transferred to centrifuge tubes codified with random numbers. A total of six individual duo-trio tests were solved by fifteen members of the sensory panel. Each individual test consisted of three samples, one of them being labeled with an R for "reference". The judge had to determine which sample was different from the reference. The results were evaluated according to the Roessler tables (Roessler et al. 1978) for a $p = 0.05$.

Chemical analysis of aromas

Sample preparation

The aromatic profile of the wild type CH29 strain and a selected mutant with low Brett character was determined in 5 mL $Brett_{screening}$ medium. Media were inoculated with 2.5×10^7 cells/mL and incubated at 28 °C for 3 days with 150 rpm shaking. Afterwards, cultures were centrifuged at 13,000 rpm for 2 min and the supernatant was recovered. The final volume was raced to 10 mL with mili-Q® water and the pH was adjusted to 7 with 5 N NaOH. Samples were stored at − 20 °C until their analysis.

Chromatographic conditions in GC/MS analysis

Volatile profiles (esters, alcohols, ketones, terpenes) and free volatile phenols (ethyl phenols) were determined in triplicate according to Massera et al. (2012) and Sangorrín et al. (2008), respectively. In accordance with these methods Polydimethylsiloxane/Divinylbenzene (PDMS/DVB, Supelco, USA) and Poliacrilate (PA, Supelco, USA) fiber coating were used to extract and concentrate volatile compounds by Headspace-Solid Phase Microextraction (HS SPME).

Chromatographic separations were done using a Factor Four VF-5MS (30 m × 0.25 mm × 0.25 µm) capillary column in a CP-3800 Gas Chromatograph (GC) equipped with a mass spectrometer (MS) 2200 Saturn Ion Trap (Varian, USA). Volatile compounds were identified by comparing their mass spectra with those reported in the Nist Mass Spectral Search Program (Nist Version 2.0). The quantification was made using calibration curves of free volatile phenols and calculating relative areas respect to the internal standard (1-anisol).

Table 1 Detection time of 4-EP aromatic signal, for two values of cell density and three concentrations of *p*-coumaric acid in Brett medium

OD	Detection time of 4-EP aromatic signal (h)		
	[*p*-coumaric acid] mg/L		
	400	600	800
1.87	72	72	> 96
0.62	72	72	> 96

Results

Optimization of the mutagenesis and olfactory screening method

To our best knowledge, this is the first mutagenesis and mutant screening ever performed with *B. bruxellensis*. Therefore, it was necessary to optimize the conditions of the mutagenesis as well as the screening method. First, we determined the right concentration of the mutagenic agent EMS for *B. bruxellensis*. It was previously shown that the most suitable concentration, which produces a high rate of mutations with a moderate impact on cell viability, should result in a survival rate close to 50% ($SR_{50\%}$) (Lawrence 1991). In a preliminary experiment, the survival rate of *B. bruxellensis* CH29 was estimated for EMS concentrations of 1, 3, 6 and 8% v/v (data not shown). Although the variability was very high, it was clear that over 2% the survival rate was below the $SR_{50\%}$. To reduce the variability and obtain a better estimation of the $SR_{50\%}$, we performed a second experiment where we determined the survival rate only for EMS concentrations of 1 an 2%. In this case, six repetitions were made for each determination, and after fitting a linear regression to the data, the value of $SR_{50\%}$ was extrapolated from the curve. The EMS concentration found for a $SR_{50\%}$ was ≈ 0.75% v/v (Fig. 1).

Due to the lack of specificity of the chemical mutagenesis, a large number of mutants must be analyzed to find the desired phenotype, and consequently, it was essential to have a simple and reliable method for the screening and selection of the mutants. Olfactory tests are frequently used in wineries to detect the presence of *B. bruxellensis* because of its easy handling, low costs and fast results (Couto et al. 2005). Considering the advantages of such methods, we developed an olfactory screening for the isolation of mutants with low 4-EP production. As a starting point, we wanted to check if it was possible to accelerate the appearance of the 4-EP aromatic signal. For this purpose, we tested two different initial cell densities (ODs_{640nm} 0.62 and 1.87) and three increasing concentrations (400, 600 and 800 mg/L) of the precursor *p*-coumaric acid. After inoculation in the proper media, the cultures were olfactory evaluated every 24 h during an incubation time of 96 h at 28 °C. As seen in Table 1, the appearance time of the 4-EP aromatic signal

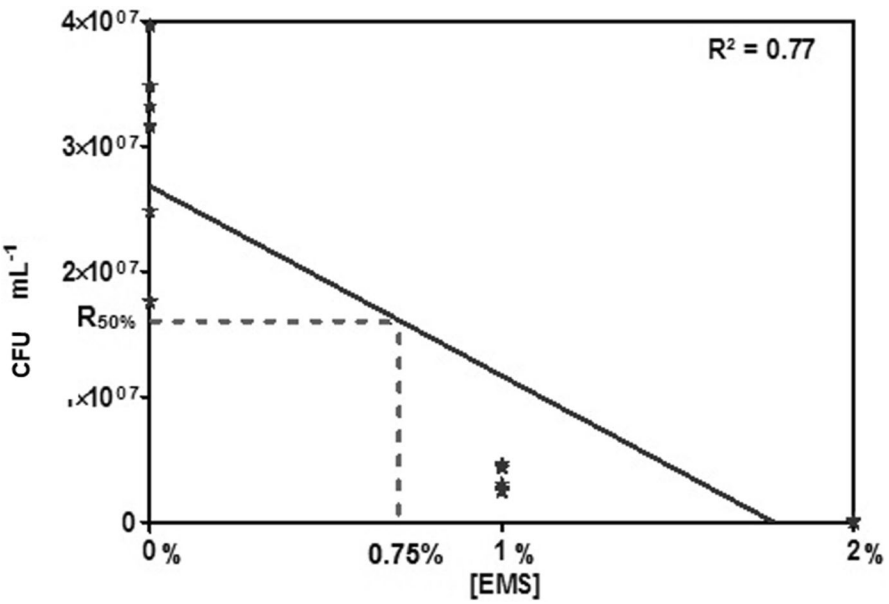

Fig. 1 Determination of the $SR_{50\%}$ for CH29 *B. bruxellensis* strain. The value was extrapolated from the fitted line, using concentrations of 1 and 2% of EMS. Each point represents the average of six independent measurements

was of 72 h for both initial cell densities at *p*-coumaric acid concentrations of 400 and 600 mg/L. Nevertheless, for the higher cell density (OD_{640nm} 1.87) the signals were stronger perceived. At the highest *p*-coumaric concentration of 800 mg/L, an inhibitory effect was observed. The appearance time increased to 96 h independently of the cell density and the signal intensities were even weaker as compared to the other tested concentrations. This inhibitory effect might be explained by the well-known antimicrobial properties of *p*-coumaric acid (Davidson and Branden 1981; Lou et al. 2012). In summary, the olfactory screening test was set with the highest cell density (OD_{640nm} 1.87), which gave stronger signals; but in contrast with the lowest concentration of the precursor in the media (400 mg/L *p*-coumaric acid) to avoid growth inhibition and an incubation time of 72 h. Using the newly selected conditions, we performed an aromatic test as a trial experiment, with a reduced number of samples. The 4-EP aromatic signals were detected within the expected time, but the 4-EP aroma was too strong (data not shown) causing a rapid saturation of the smelling. To reduce the observed smell saturation, we tried lower concentrations of *p*-coumaric acid (4, 8 and 16 mg/L) based on the perception threshold of 4-EP (440 µg/L in model medium [Chatonnet et al. 1992]). Each medium was inoculated by duplicate with *B. bruxellensis* CH29 (OD_{640nm} 1.87) and after 72 h incubation at 28 °C, the intensity of the 4-EP aroma was compared in a blind assay against a positive control (*B. bruxellensis* CH29 with 400 mg/L *p*-coumaric acid) and a negative control (*S. cerevisiae* BY4741 with 400 mg/L of *p*-coumaric acid). All samples could be distinguished from the positive and negative control and importantly, the sample's aromatic signals were perceived weaker than the positive control. In summary, reduction of the precursor concentration produces a moderate 4-EP aroma and helps to reduce the saturation effect on the smell. Based on these results, we decided to keep working with a precursor concentration of 16 mg/L and this media was designated as $Brett_{screening}$ media.

Olfactory screening of mutant variants with low 4-EP aroma

The mutants generated with the EMS treatment were grown in YEPD plates. Later, these mutant colonies were replicated as patches in $Brett_{selective}$ medium plates, incubated 48 h at 28 °C and stored at 4 °C. The growth on $Brett_{selective}$ medium plates served not only as a previous adaption to the selection media, but also as a preselection step. Only the mutants able to grow under such stressing conditions (6.25% v/v Ethanol, 0.05% w/v chloramphenicol and 0.01% w/v cycloheximide) would be further used for the olfactory screening. In order to avoid the selection of non-growing mutants, which could be wrongly associated with reduced 4-EP aroma production, a first selection criteria used during the test was the presence or absence of sediment in the incubated cultures. A visual inspection of the cultures allowed us to discard those with no sediment or a scarce one. The screening test was performed in several rounds of olfactory evaluations in order to reduce the number of false positives to a minimum. This means, that candidates preselected in previous rounds of independent tests, had to be put together for new evaluation rounds. A total of 1100 mutants were evaluated through the olfactory screening test and only six mutants with low 4-EP aroma were selected for further sensory and chemical aroma analysis.

Sensory analysis of selected mutants with low 4-EP aroma

The six mutants selected for its low 4-EP aroma were used to perform a ranking test. This experiment allowed us to further reduce the number of mutants to be used in the following stages. Table 2 shows the median value of the positions assigned to each sample after eight independent tests. Mutants 6, 11 and 24 appeared on the first positions, in some cases even over one of the positive control replicas. Mutants 51 and 54 were placed in the middle positions (position 5–6), whereas mutant 27 (position 7) was closer to the non-inoculated sample (position 9) and the negative controls (position 10–11). According to their positions, the mutants could be separated into three different groups. One with higher 4-EP aroma (mutants 6, 11, 24), a second group with intermediate 4-EP aroma (mutants 51 and 54) and a third one with lower 4-EP (mutant 27). We selected mutant

Table 2 Ranking test results

Samples	Median[a] of the assigned positions
B. bruxellensis CH29 (R1)	3 ± 1.1
Mutant 11	3 ± 4.1
Mutant 24	3.5 ± 3.2
B. bruxellensis CH29 (R2)	4 ± 2.0
Mutant 6	4 ± 3.2
Mutant 51	5.5 ± 2.7
Mutant 54	6 ± 3.8
Mutant 27	7 ± 1.5
Liquid medium *Brett*	9 ± 2.0
S. cerevisiae BY4741 (R1)	10 ± 2.0
S. cerevisiae BY4741 (R2)	11 ± 1.2

Eight independent tests were performed with eleven samples including controls, and were ordered from high to low intensity of 4-EP aroma. The first position corresponds to the highest intensity and the eleventh to the lowest

[a]The median values are expressed with its corresponding standard deviation

54 from the second group and mutant 27 from the third group to perform additional sensory analysis. In this test, we wanted to check whether it was possible to discriminate the mutants from the positive control in a duo-trio olfactory test, which included all possible combinations. A total of six independent tests were performed by fifteen trained judges of our institute sensory panel. Table 3 shows the number of correct and wrong judgement obtained for each test, for which a minimum of twelve correct judgements were necessary to be considered statistically different for a $p = 0.05$ (Roessler et al. 1978). Interestingly, mutant 27 could be statistically discriminated from both positive (*B. bruxellensis* CH29) and negative (*S. cerevisiae* BY4741) controls. This result suggests that mutant 27 strain had a distinct aromatic profile associated with a weaker 4-EP aroma. With respect to mutant 54, it could be discriminated from the negative control but not from the positive one. Notably, the mutants could not be discriminated from each other. Apparently, both mutants have a similar aromatic profile, even when mutant 27 was perceived differently when compared with the positive control.

Aromatic profile of *B. bruxellesis* mutant 27 and control CH29 strain by HS SPME GC–MS

Mutant 27 showed the most consistent results during the sensory analysis and it was selected to perform a chemical analysis of its aromatic profile. This would allow us to quantify the concentration of 4-EP and to identify other compounds which may be contributing to its distinctive aromatic profile. The chemical scanning of aromatic compounds was carried out by HS SPME GC–MS, a method which has been widely used in wine for this purpose (Barbosa et al. 2009; Perestrelo et al. 2011; Pizarro et al. 2007; Robinson et al. 2010; Alberts et al. 2011; Tao et al. 2008). The mutant 27 and the control *B. bruxellesis* CH29 strains were grown in three independent cultures of $\text{Brett}_{\text{screening}}$ medium for 72 h at 28 °C and volatile compounds were extracted and identified. Table 4 shows the list of the identified aromatic compounds and the average concentrations of them. We detected a total of 26 different compounds which could be classified into 6 chemical groups: alkanes, esters, alcohols, phenols, aldehydes, ketones and terpenes. Of all found compounds, only five showed statistically differences between the mutant and the control strain. Remarkably, 4-EP was one of the most predominant compounds in terms of concentration, and mutant 27 showed statistically lower values compared to the control. The 4-EP reduction observed in the mutant is consistent with the sensory analysis previously performed. It is also interesting to compare the composition of the ethyl ester fraction of both strains. Seven out of eight detected ethyl ester compounds were present in the mutant 27 aromatic profile, whereas only five in the control strain. Furthermore, mutant 27 displayed a significant higher total concentration of ethyl esters (218.1 µg/L) compared to the wild type CH29 strain (158.2 µg/L) (Table 4). Considering the important contribution of the ethyl esters to the fruity character of wine (Tao et al. 2008), this result would suggest that mutant 27 has a more complex and better aromatic profile than the control strain.

Table 3 Correct and incorrect judgments for discriminatory duo-trio test

Duo–Trio test		Correct judgments	Incorrect judgments
B. bruxellensis CH29	*S. cerevisiae* BY4741	12*	3
B. bruxellensis CH29	Mutant 27	12*	3
B. bruxellensis CH29	Mutant 54	10	5
S. cerevisiae BY4741	Mutant 27	13*	2
S. cerevisiae BY4741	Mutant 54	14*	1
Mutant 27	Mutant 54	8	7

*At least 12 correct judgments for a statistically significant result (Roessler table with $p = 0.05$)

Discussion

The use of *B. bruxellensis* to obtain new interesting aromas has been explored in wine (Dashko et al. 2015) as well as in beer fermentations (Holt et al. 2018). However, the natural capability of this yeast to produce phenolic off-flavors like 4-EP, could have a negative impact on the overall aromatic profile. To counteract this negative effect, we developed a mutant screening to isolate *B. bruxellensis* mutants with reduced 4-EP.

Since there were no precedents for a mutant screening with *B. bruxellensis*, we had to set up the right parameters for the mutagenesis and for the olfactory screening method. Chemical mutagenesis has some disadvantages, since the improvement of some traits might be at expenses of other desirable characteristics (Akada 2002). Still, it is a valuable tool for genetic improvement of yeasts because it is considered a classic genetic method and the obtained strains can be used without legal restrictions (Peréz Torrado et al. 2015). The *B. bruxellensis* CH29 strain used in this work, showed a more sensitive EMS phenotype compared to the *S. cerevisiae* Maurivin PDM commercial strain from the work of Cordente et al. (2009). In our case 0.75% EMS was enough to achieve the $SR_{50\%}$, whereas in the referred work a concentration of 2% EMS was required. With respect to the olfactory screening method, we established a cell density of DO_{640nm} 1.87 (5×10^7 CFU/mL) and incubation time of 72 h for a reliable detection of the 4-EP aromatic signal. These values are in agreement with

Table 4 Comparative aromatic profile of control *B. bruxellensis* CH29 strain and mutant 27 grown in $Brett_{screening}$ medium

Compound group	Compound name	Description	*B. bruxellensis* CH29 (µg/L)	R.S.D. (%)	mutant 27 (µg/L)	R.S.D. (%)
Alcohols	1-Pentan-1-ol	Medicinal alcohol, fruity, balsamic	172.4	12	129.3*	8
	2-Butyl-1-octanol	–	57.3	18	67.4	4
	2-Hexyl-1-decanol	–	27.1	24	ND	ND
	Heptadecan-1-ol	–	1085.4	8	1108.8	26
Alkanes	10-Heneicosene	–	ND	ND	40.5	11
	Decane	–	27.1	13	30.1	12
Aldehydes	Acetaldehyde	Fruity	7.1	10	9	21
Esters	2,2,4-Trimethylpentanediol-1,3-diisobutyrate	Plastic	24.3	10	44.1*	12
	3-Hydroxy-2,4,4-trimethylpentyl 2-methylpropanoate	Fruity	4.3	23	4	25
	4-Tert-butylcyclohexyl acetate	Sweet creamy, woody with soft floral	ND	ND	2.3	16
	Ethyl 2-methylbutyrate	Green, fruit, apple	52.9	12	43	17
	Ethyl butyrate	Pineapple, lard, fruity	17.1	24	24.5	30
	Ethyl decanoate	Fruity, liqueur	25.5	24	23.6	25
	Ethyl hexanoate	Apple, slightly sweet, fruity, pineapple, banana	10	38	ND	ND
	Ethyl isobutyrate	Apple, slightly sweet	ND	ND	11.6	22
	Ethyl laurate	Greasy, slightly fruity or floral	ND	ND	12.8	17
	ethyl nonanoate	Fatty acids, fruity, perfumed	ND	ND	2.9	26
	Ethyl octanoate	Fruit, pineapple, apple, brandy	52.7	18	99.7*	14
	Isobornyl acetate	Camphor, pine, balsamic, herbal	8.4	23	7.8	17
	Isopropyl laurate	Alcoholic	3.4	38	1.9	30
Ketones	3-Isopentyl-2,4,4-trimethyl-2-cyclohexen-1-one-	Solvent, fruity	31.7	23	38.2	18
Phenols	2,4-Di-tert-butylphenol	–	100.5	29	187.8*	21
	4-Ethylphenol	Leather, stable	1130.7	14	323.4*	20
Terpenes	Cedrol	Cedar	2.7	16	ND	ND
	3-Butyl-2-ethyltetrahydrothiophene	Sulfurous, solvent, fruity burnt	15.1	29	20.2	23
	β- methyl-ionone	Woody floral	19.4	21	19	16

Values are the average of three replicates and their corresponding relative standard deviation RSD (coefficient of variation)

*Statistically significant differences for a t- Student test with $p = 0.05$. ND: not detected

previous results where the formation of volatile phenols was dependent not only on the concentration of *p*-coumaric acid but also of the initial size of the cell population (Baranowski et al. 1980; Goodey and Tubb 1982). A previous study has shown that the highest accumulation of volatile phenols occurs during the exponential growth phase and that the production of 4-EP overlaps with the beginning of the cellular growth (Sturm et al. 2015). Other studies have found that cell populations in the order of 10^4–10^6 UFC/mL favor the detection of the 4-EP aroma in a time period of 48–72 h (Couto et al. 2005; Dias et al. 2003; Fugelsang and Zoecklein 2003).

Our developed olfactory screening method, turned out to be a good instrument to reduce the number of mutants to perform further sensory and chemical analysis. After the evaluation of 1100 samples the number of preselected mutants was reduced to only six. Two different tests of sensory analysis were performed with these six mutants which allowed us to further characterize them. With the ranking test, mutants could be roughly separated into three different groups with higher and lower 4-EP perception. After this experiment, a duo-trio test was performed with two mutants (54 and 27) from the lower 4-EP perception group. Interestingly, mutant 27 could be discriminated

from both the positive and negative control with statistically significant differences. This would suggest that mutant 27 was perceived with a lower 4-EP aroma but still at detectable concentrations, or that it just possesses a different aromatic profile. At last, the aromatic profile of mutant 27 was determined by HS SPME GC–MS and compared with the CH29 control strain. Remarkably, we observed an important reduction of more than three folds in the 4-EP production of mutant 27 and its concentration was below the perception threshold (440 µg/L) as determined in model medium (Chatonnet et al. 1992). This is already a great improvement compared to the control and suggests that the mutagenesis has affected, at least in part, the metabolism involved in the 4-EP biosynthesis. Such proposition is also supported by the fact that the growth ability of mutant 27 was unaffected, and thus the 4-EP reduction can´t be attributed to a lack of growth.

Notably, mutant 27 also showed higher diversity and concentration of ethyl esters. Volatile esters are synthetized by yeasts during fermentation and only traces of them can be found in fermented beverages. However, they represent the most important group of odor active compounds produced by yeasts and are responsible for the valuable fruity, candy and perfume-like aroma character of beer, wine and sake (Saerens et al. 2010). For the mutant as well as the control *B. bruxellensis* strain, ethyl octanoate (ethyl caprylate) was the main contributor to the ethyl ester fraction. Ethyl octanoate is an important odor-active compound, and together with ethyl hexanoate is considered as the main contributor to tropical fruit and pineapple-like aromas of beer purely fermented with *B. bruxellensis* (Serra Colomer et al. 2019). Interestingly, mutant 27 was able to produce considerable higher amounts of ethyl octanoate than the control (99.7 µl/L vs. 52.7 µl/L), and the concentration found was well above the odor threshold as determined in a 10% w/w ethanol solution (2 µl/L [Guth 1997]). Thus, it is possible that the distinctive aromatic profile of mutant 27 derives from a smaller contribution of 4-EP combined with a higher impact of an enriched volatile esters fraction. Based on these results, we believe that our selected *B. bruxellensis* mutant 27 is a great candidate to be tested as a non-conventional yeast starter (pure or in co-inoculation) in the elaboration of wines and beers with high aromatic complexity.

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Author contributions IJÁG participated in the design and coordination of the study, performed the experiments and interpreted the data. MVA performed the experiments and participated in data analysis. VPJ contributed to experiment design and data analysis. MC participated in data interpretation and analysis, and helped to draft the manuscript. IFC conceived of the study, participated in its design and coordination, interpreted the data, and drafted the manuscript. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interests.

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