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Metschnikowia pulcherrima in mono or co-fermentations in brewing

***Metschnikowia pulcherrima*, also known as *Candida pulcherrima* (anamorph), is a non-*Saccharomyces* yeast, well known from the wine industry. It is currently used in sequential or co-fermentation with *Saccharomyces cerevisiae* to enrich the wine with more complex and varietal aromas. In this study, we examined the potential application of a commercial *M. pulcherrima* strain in the brewing industry. First, the fermentation dynamics in glucose, fructose, and maltose, at 13 and 20 °C, were studied, and GC-MS analysis of the volatile compounds related to beer flavour was performed. After being assured that *M. pulcherrima* is capable of metabolizing maltose, the main wort sugar, a Pale Ale beer was produced at 13 and 20 °C. A pure *M. pulcherrima* and two mixed cultures of *S. cerevisiae* with *M. pulcherrim*a in ratios of 1:1 and 1:10 were used, and fermentation was monitored by specific gravity and free amino nitrogen measurements. The ethanol content at the end of fermentation varied within 4.0 – 5.5 % (v/v). The volatile profile analysis of the produced beers revealed the presence of higher alcohols, medium-chain fatty acids and their esters, with the obtained results presenting satisfactory complexity in flavour.**

Descriptors: *Metschnikowia pulcherrima*, beer, fermentation, volatile compounds, co-fermentation

1 Introduction

Saccharomyces cerevisiae is the main yeast traditionally used in brewing. Nowadays, due to the recent knowledge on the positive effects of non-*Saccharomyces* yeasts in the fermentation process, new species were investigated, either as the main fermenting yeasts or in co-fermentations, to improve the aromatic profile of the final product [1-3]. *Metschnikowia pulcherrima*, also known as *Candida pulcherrima* (anamorph), is currently used in the wine industry as it enhances the overall flavour of the product [4]. It has a unique “needle” shape, belongs to the *Ascomycota* Phylum and replicates by budding [5].

M. pulcherrima is present mostly in vineyards. It is known for the production of a red pigment (pulherrimine), through the irreversible reaction of its precursor pulcherriminic acid and iron [6,7]. This pigment is of great importance as it has antibacterial properties and suppresses the growth of other undesirable yeasts such as *Pichia*, *Candida*, and *Hanseniaspora* [6,7].

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In addition to its antimicrobial activity, this yeast is often selected for the production of low-alcohol wine [8]. This non-*Saccharomyces* yeast has the ability to ferment the must until the ethanol content reaches 5 %, v/v, due to its low tolerance to ethanol that prevents further metabolism. On the contrary, it shows resistance to high acidity (pH 3 – 4) and sugar concentration (> 100 g/L) [9], [10].

The secondary metabolites produced in wines fermented with *M. pulcherrima* indicate that it has the ability to produce volatile phenolic compounds in high concentrations (off-flavours, horse-, smoked-, and clove-like aromas). Additionally, it enhanced monoterpene alcohols biotransformation, namely terpineol, geraniol, and nerol, known for their contribution to floral aromas [11]. It has even been reported to provide wine with fruity, rose-like aromas due to an increased amount of 2-phenylethanol but also to increase acetic acid concentrations [12–14].

Although several non-*Saccharomyces* yeasts have been examined in beer production [15–16], *M. pulcherrima* that might be a potent, alternative yeast has not been tested. Therefore, the aim of the present work was to examine the ability of a commercial *M. pulcherrima* wine yeast to metabolise wort sugars, and produce beer with desirable characteristics. To this aim, the yeast’s ability to ferment the main wort sugars, i.e. maltose, glucose and fructose, was initially studied. A simple kinetic model was used to fit the experimental data and predict the yeast growth rates in each substrate, while the obtained ethanol yield was determined. Additionally, a mixture of maltose, glucose, and fructose, at a ratio of 86:9:5, w/w/w, that is representative of wort, was tested. The effect of each sugar on *M. pulcherrima*’s ability to produce volatile compounds with a flavour impact was estimated by GC-MS analysis at the end of fermentations. The fermentations were

performed at two different temperatures (13 and 20 °C) as, in addition to fermentation kinetics, temperature may affect the volatiles production. At the second part of this study *M. pulcherrima* was used for a Pale Ale production at 13 and 20 °C. Barley malt was used for the wort production, and the fermentation process was followed through specific gravity measurement. The aromatic profile, ethanol content, bitterness, and colour of the products were measured at the end of fermentation.

2 Materials and Methods

2.1 Yeast strains

One commercial strain of *Saccharomyces cerevisiae* US-05 (Fermentis by Lesaffre, Marcq-en-Baroeul, France) and one strain of *M. pulcherrima* Flavia 365 (Lallemand, Montreal, Canada) were used. The dried yeast strains were rehydrated in warm water (30 °C) before inoculation.

2.2 Preparation and fermentation of sugar solutions

The media used for the experiments consisted of 1 g/L yeast extract, 2 g/L $(NH_4)_2SO_4$, 0.2 g/L $MgSO_4$, 0.2 g/L $ZnSO_4$, 1 g/L KH_2PO_4, and 1 g/L $KHPO_4$ (Merck KGaA, Darmstadt, Germany). Glucose, fructose, or maltose (98 % purity, Sigma-Aldrich, Steimheim, Germany) were added at a concentration of 120 g/L. An additional experiment, with mixed sugars (120 g/L) composed of 86 % maltose, 9 % glucose, and 5 % fructose, so as to imitate wort, was performed. The pH was adjusted to 4.5, which is reported to be the optimum for ethanol production [18]. Batches (200 mL) of the media containing each sugar were autoclaved at 121 °C for 20 minutes, cooled, and inoculated with pure culture of 6 x 10^6 cells/mL in Duran flasks of 250 mL volume. The fermentations were performed at two temperatures, 13 and 20 °C, with airlock systems. All fermentation trials were run in duplicate. Samples of 1 mL were withdrawn every 24 h, until the end of the fermentation, and subjected to yeast cell counting, residual sugar and ethanol content determinations. The results are presented as mean values of the duplicate samples.

2.3 Yeast cell counting

The cell counting was performed by microscopy, using a microscope CX60 (Olympus Corporation, Center Valley, USA) and a Thomas type hemocytometer [2].

2.4 Determination of residual sugars

For the determination of the monosaccharides, namely glucose and fructose, the dinitrosalicylic acid (DNS) method was used [19]. Maltose was measured by using high pressure liquid chromatography (HPLC) analysis, according to Drosou et al. [2] on a HP 1100 Series gradient HPLC system (Agilent Technologies, Waldbronn, Germany) equipped with a Refraction Index detector (Hewlett-Packard 1047A, Japan). The mobile phase was isocratic and consisted of C_2H_3N:H_2O, 70:30, v/v, the injection volume was 8 µL and the flow rate was set to 1.3 mL/min. Quantification was based on a calibration curve constructed with maltose.

2.5 Ethanol determination

The ethanol content of the collected samples during the fermentation of single sugars was determined by means of a gas chromatography (GC 8500) Perkin-Elmer system (Massachusetts, USA) coupled to a flame ionization detector (FID) and equipped with a fused silica, megabore column (Agilent J&W Scientific, Agilent Technologies, Santa Clara, USA). The samples were filtered through a 0.22 µm pore size filter (Whatman, UNIFLO, Maidstone, UK) and 1 µL was injected splitless into the GC. The oven temperature program was set according to Mamma et al. [20]. Quantification was based on a calibration curve created with different concentrations of ethanol.

2.6 Kinetic model

Kinetic analysis was performed based on the integration of the logistic model, previously proposed by Wang et al. (2004). More specifically, the model is based on the assumption that the specific growth rate is proportional to $(X_\infty - X)$, and considering as initial conditions $X = X_0$ at $t = 0$ the integration results in equation 1 [21,22].

$$ln\frac{X}{X_0} = k * t - \ln\left[1 - \frac{X_0}{X_\infty} + \left(\frac{X_0}{X_\infty}\right) * e^{k*t}\right] \quad \text{(Eq. 1)}$$

where X is the cell concentration at time t, X_∞ the maximum cell concentration in infinite time, t the growth time and k the maximum specific growth rate.

Equation 1 was applied to fit the experimental data in all sugar substrates. The maximum specific growth rate and the maximum cell concentration were calculated by Microsoft Excel Solver 2007.

2.7 Cells and ethanol yields

Based on the produced cells and ethanol at the end of fermentation, the cells and ethanol yields were calculated, as follows:

a) Cells Yield: $Y_{X/S} = \frac{\text{cells produced} * 10^{-6}}{\text{g sugar utilized}}$, and

b) Ethanol Yield: $Y_{P/S} = \frac{\text{g ethanol produced}}{\text{g sugar utilized}}$

2.8 Wort production and fermentation

Barley malt (22 kg) was mixed with 100 L of water, heated up and maintained to 65 °C for 60 min, 72 °C for 15 min, 78 °C for 5 min, followed by the lautering process and finally boiled for 60 min. Magnum hop variety (100 g) was added at the beginning of the boiling process so as to avoid excessive transfer of aromatic compounds. The obtained wort had a specific gravity of 12.1 °P, and pH 5.1. The wort was divided in batches of 20 L, and each was inoculated with 11.5 g of dry yeast. Mono-cultures of *S. cerevisiae* and *M. pulcherrima* strains were used, plus mixed cultures of *S. cerevisiae* and *M. pulcherrima* strain, at ratios of 1:1 and 1:10. Fermentations were performed at 13 and 20 °C, and followed by specific gravity and Free Amino Nitrogen measurements at definite time intervals. Three successive equal specific gravity values denoted the end of fermentation. Subsequently, the products were transferred to bottles under asceptic conditions, sealed and stored at 13 °C.

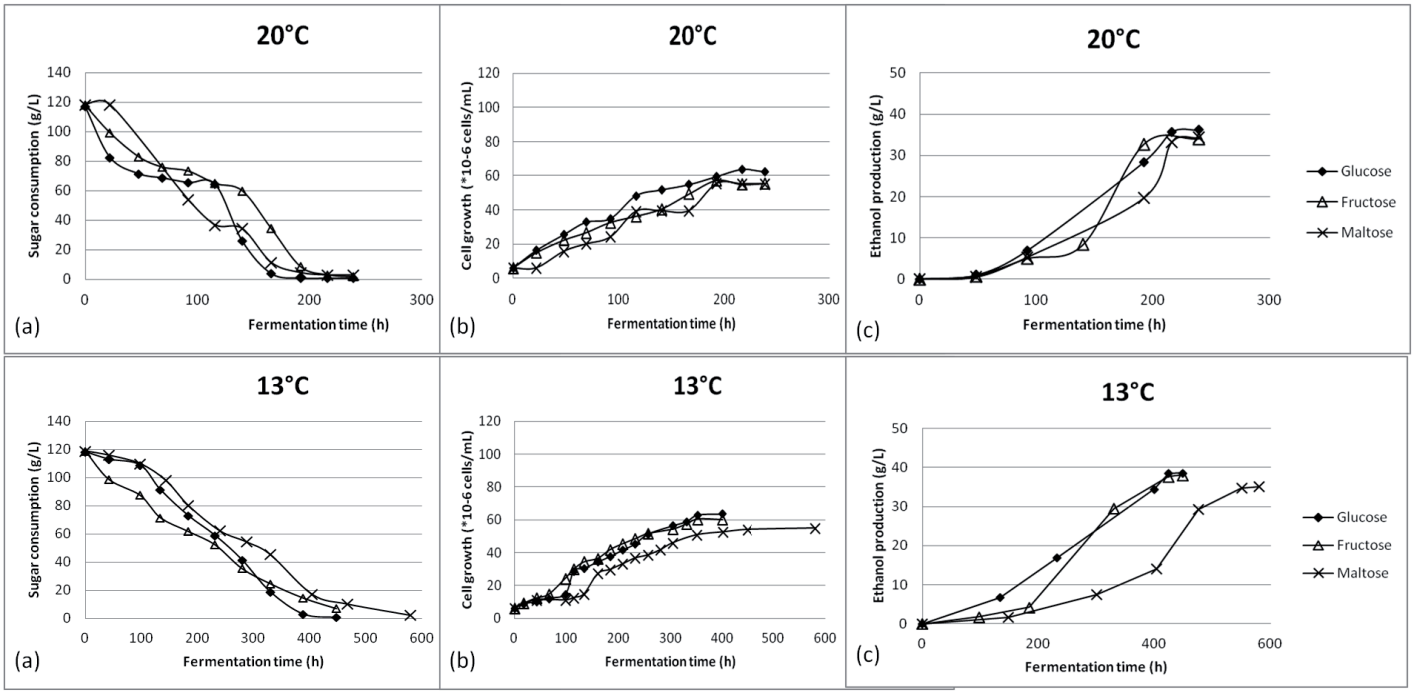

Fig. 1 Sugar consumption (a), cell growth (b), and ethanol production (c) during the three main sugars fermentation by *M. pulcherrima*, at 20 and 13 °C

The ethanol content, bitterness, and colour of the products were measured after 30 days of storage.

2.9 Determination of wort specific gravity and Free Amino Nitrogen (FAN)

The specific gravity of the wort was determined according to the official method of the American Society of Brewing Chemists (ASBC), Beer-2A, rev. 2014 [23]. FAN was determined by the use of ninydrin, according to the official ASBC method, Wort- 12, rev. 2010 [23].

2.10 Ethanol, bitterness and colour determination

The ethanol content was determined in the final products by decarbonization and distillation according to ASBC method, Beer- 4A, rev. 2018 [23]. Similarly, the bitterness (expressed in IBU) and the colour were determined in the final products according to the official ASBC methods, Beer- 23A, rev. 2018, and Beer- 10A, rev. 2015, respectively [23].

2.11 Extraction and analysis of volatile compounds

The extraction and analysis of the volatile products was performed according to Drosou et al. [2]. Briefly, beer (50 mL) was mixed with 1-pentanol and diethyl ether (25 mL each). The mixture was stirred for 10 min at room temperature, centrifuged at 1370 g for 10 min, and the aqueous phase was subjected to a second extraction under the same conditions. The organic layers were mixed, washed with distilled water in a separation funnel, and the excess water was removed by anhydrous sodium sulphate. The obtained solution was filtered (filter paper Whatman No 42, Maidstone, UK), 10 µL of 3-octanol solution in chloroform (2500 ppm) was added as internal standard, condensed in a Vigreux column, and adjusted to a final volume of 100 µL. Analysis was performed by Gas Chromatography/ Mass Spectrometry (GC/MS), in an Agilent 6890 series GC System (Agilent Technologies, Santa Clara, USA), coupled to an 5975C MSD mass detector and equipped with a fused silica capillary column, 30 m × 0.32 mm i.d., 0.25 µm coating thickness (HP-5MS, Agilent Technologies). One µL of sample was injected using a split ratio of 100:1. The injector temperature was set at 250 °C , the carrier gas was helium at a flow rate of 1 mL/min, and the oven temperature program was set as previously mentioned in Drosou et al. [24]. All extractions and GC/MS analyses were done in duplicate.

2.12 Statistical analysis

All experiments were run in duplicate and their mean values and standard deviations are presented. Experimental data were subjected to analysis of variance in order to detect significant differences among the investigated factors (yeasts, sugars, fermentation temperature). Duncan's multiple-range test was applied in the cases of significant differences ($p < 0.05$). Analysis was performed using the software Statistica 7.0 (StatSoft, Tulsa, OK, USA).

3 Results and Discussion

3.1 Fermentation kinetics in simple sugars

Figure 1 presents the sugar consumption, cell growth, and ethanol content during the fermentation of glucose, fructose, or maltose, at 13 and 20 °C. From the results it can be noted that the studied *M. pulcherrima* strain was able to ferment all three simple sugars. Residual sugars amounted up to 2.5 g/L, contrary to other studies (3.5 g/L and 16.6 mg/L in total of glucose and fructose, accordingly) [15], [25], ascertaining that the strain used is potential for beer fermentation.

Table 1 **Maximum specific growth rate (k), maximum cell concentration (X_∞) and the correlation coefficient (R^2) obtained by fitting equation 1 to the experimental data. Superscript letters a and b present significant differences ($p < 0.05$) between different sugars at the same temperature, whereas superscript letters A and B present significant differences ($p < 0.05$) between different temperature in each sugar**

Model parameters	Fermentation temperature					
	13 °C			20 °C		
	Glucose	Fructose	Maltose	Glucose	Fructose	Maltose
k (h^{-1})	0.0142^{aA}	0.0139^{aA}	0.0103^{aA}	0.0363^{aB}	0.0315^{aB}	0.0209^{bB}
$X_\infty 10^{-6}$ (cells/mL)	64.92^{aA}	62.12^{aA}	61.04^{aA}	57.06^{aA}	51.07^{aB}	59.43^{aA}
R^2	0.951	0.975	0.936	0.970	0.973	0.969

As it is depicted in figure 1, noticeable differences in the fermentation duration were observed among the different temperatures and sugars. More specifically, the fermentation time increased at 13 °C, with the two monosaccharides consumed in shorter time than maltose. On the other hand maltose exhibited a slower consumption rate as well as a short lag phase at the fermentation beginning. A noticeable observation is that at 20 °C, after 100 h, a lag phase was observed in all sugars leading to a lag phase in the biomass production.

Table 1 demonstrates the yeast maximum specific growth rate (k) and maximum cell concentration (X_∞) obtained by fitting the experimental results to equation [1]. As indicated by the high correlation coefficients presented in table 1, the equation used is able to describe adequately the biomass growth during the fermentation of all sugars. The k values are higher at 20 °C in all sugars fermentations, demonstrating that *M. pulcherrima* grows more rapidly at the higher temperature. In glucose and fructose fermentations, similar growth rates were observed, at both temperatures. The maximum growth rate in maltose fermentation was lower, resulting, however, in a final cell concentration similar to the two monosaccharides, and verifying that *M. pulcherrima* is capable of metabolising maltose.

As can be seen in figure 1, the ethanol production at the end of the fermentation of the three sugars was close to each other, especially at 20 °C, amounting to 33 g/L. Higher values were detected at lower temperature (in the region of 39 g/L in monosaccharides, and 36 g/L in maltose fermentation).

Table 2 presents the cells yield and the ethanol yield obtained by all fermentations. It is obvious that temperature did not affect significantly the cells yield. On the contrary, ethanol yield was lower at 20 °C ($p < 0.05$). Concerning the differentiation of the type of sugars, the obtained values did not present any statistically significant difference.

3.2 Fermentation kinetics in mixed sugar fermentation

After validating that the studied strain of *M. pulcherrima* is able to metabolise maltose, a mixed sugar media, similar to wort, was fermented at 13 and 20 °C. As can be observed in figure 2, the cell growth starts from the beginning of fermentation at the higher temperature, whereas at 13 °C a lag phase was observed (until 100 h approximately). A similar lag phase was observed at 20 °C, between 80 and 100 h, as a result of the depletion of monosaccharides and adaptation to the maltose environment. Similar results with other non-*Saccharomyces* yeasts, namely Torulaspora delbrueckii strains, were observed in our previous study [2]. It is obvious that the small amounts of glucose and fructose are primarily used, with the strain trying to metabolise maltose into glucose before its consumption. This process gets slower at 13 °C, resulting in a slower growth rate that led to a final cell concentration of 60 x 10^6 cells/mL, whereas at 20 °C the final cell concentration exceeded 100 x 10^6 cells/mL, in half time duration (Fig. 2). Overall, it is evident that fermentation at low temperatures hinders the cell growth, thus resulting in a decreased sugars consumption.

The lag phase presented in the cell growth at lower temperature can also be observed in the production of ethanol, which started after 100 h of fermentation at 13 °C. Nevertheless, the ethanol production retained an almost steady rate until the end of fermentation at 13 °C, and finally exceeded the concentration of 40 mg/L. A higher production rate and a final concentration of 38 g/L, similar to the single sugars fermentations, was obtained in half time, at 20 °C.

3.3 Production of volatile compounds in simple sugars media

The main detected volatile compounds are presented in table 3, and comprise higher alcohols (isoamyl, active amyl, and phenylethyl alcohol), their acetate esters, medium-chain fatty acids (MCFA) (hexanoic, octanoic, and decanoic acid), and the corresponding ethyl esters.

Two of the main factors that may alter the final volatile compounds concentration, and the product flavour, are the fermentation temperature and media. It is well known, that as the temperature rises, the expression of the genes related to the formation of higher alcohols

Table 2 **Cells and ethanol yield obtained by the fermentation of glucose, fructose, or maltose, at 13 and 20 °C. Superscript letters a and b, in the same column present significant differences ($p < 0.05$) between different sugars at the same temperature, while A and B significant differences ($p < 0.05$) between the temperatures in each sugar**

	13 °C		20 °C	
	Cells yield	Ethanol yield	Cells yield	Ethanol yield
Glucose	0.474 ± 0.020^{aA}	0.320 ± 0.011^{aA}	0.489 ± 0.010^{aA}	0.298 ± 0.007^{aB}
Fructose	0.479 ± 0.014^{aA}	0.331 ± 0.013^{aA}	0.439 ± 0.022^{aA}	0.296 ± 0.005^{aB}
Maltose	0.412 ± 0.019^{aA}	0.328 ± 0.006^{aA}	0.435 ± 0.012^{aA}	0.301 ± 0.005^{aB}

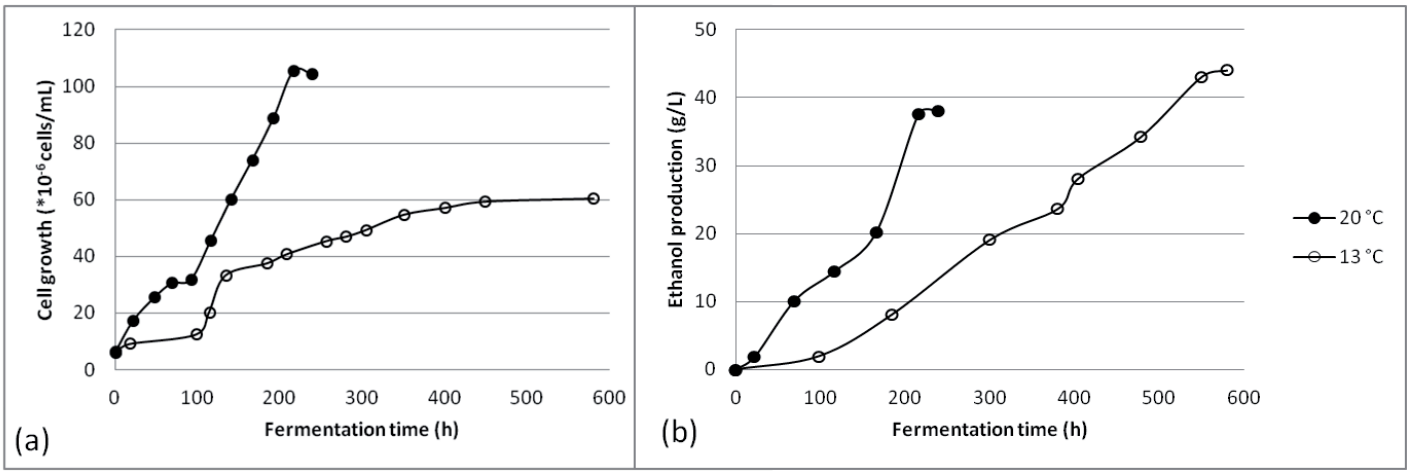

Fig. 2 Cell growth (a), and ethanol production (b) in mixed sugars medium fermentation by *M. pulcherrima*, at 20 and 13 °C

increases, leading to greater values [26, 27]. More specifically, table 3 shows that higher amounts of isoamyl, active amyl, and phenylethyl alcohol were produced at 20 °C ($p < 0.05$), with few exceptions. Comparing the fermentation substrate, high values of isoamyl and phenylethyl alcohol were detected in mixed sugars fermentation, at either temperature, promising rose-like notes in the final product.

Concerning the two acetate esters, our results partially agree with the previous study of Holt et al. [28], as their amounts augment (although with no significant differences in most cases) as the temperature rises. Known for providing with banana (isoamyl acetate), or rose and honey (phenylethyl acetate) flavour, they were highly produced in fructose substrate at 20 °C, presenting significant elevated amounts compared to the other sugars and at 13 °C.

Moreover, the MCFA produced are of great importance. The production of hexanoic acid at 20 °C in maltose substrate (0.43 ± 0.03 mg/L), was significantly higher than the concentration produced at 13 °C ($p < 0.05$). The results from the different sugars did not present any statistically significant difference between glucose and mixed sugars, at 13 °C, whereas at 20 °C the highest values were detected in glucose and maltose substrate ($p < 0.05$). Octanoic acid was also detected in higher amounts at 20 °C and more specifically in fructose and maltose fermentations. On the other hand, at 13 °C, in glucose and mixed sugars fermentations the presented values were significantly higher ($p < 0.05$), compared to fructose and maltose. Moreover, even though its concentration was lower than its threshold (10 mg/L, [1]), decanoic acid presented higher values at lower temperature in most sugars ($p < 0.05$).

Table 3 Volatile compounds obtained by the fermentation of glucose, fructose, maltose, or mix at 13 and 20 °C. Superscript letters a, b and c, in the same row, present significant differences ($p < 0.05$) between the volatiles produced by different sugar at the same temperature. Superscript letters A and B present significant differences ($p < 0.05$) between temperatures for the same sugar

	13 °C				20 °C			
	GLUCOSE	**FRUCTOSE**	**MALTOSE**	**MIX**	**GLUCOSE**	**FRUCTOSE**	**MALTOSE**	**MIX**
Isoamyl alcohol	1.54 ± 0.03^{aA}	0.68 ± 0.20^{aA}	1.01 ± 0.11^{aA}	3.06 ± 0.73^{bA}	4.40 ± 0.55^{abB}	5.10 ± 0.18^{bcB}	4.17 ± 0.20^{aB}	5.57 ± 0.18^{cB}
Active amyl alcohol	0.95 ± 0.02^{abA}	0.25 ± 0.06^{cA}	0.57 ± 0.18^{bcA}	1.11 ± 0.31^{aA}	1.53 ± 0.14^{abB}	2.07 ± 0.36^{aB}	1.31 ± 0.12^{bB}	1.60 ± 0.32^{abA}
Phenethyl alcohol	5.83 ± 0.39^{aA}	1.36 ± 0.14^{bA}	1.89 ± 0.31^{bA}	5.57 ± 0.82^{aA}	6.48 ± 0.42^{aA}	8.02 ± 0.64^{bB}	4.81 ± 0.27^{cB}	8.90 ± 0.23^{bB}
Tryptophol	0.05 ± 0.02^{a}	ND	0.26 ± 0.05^{bA}	0.40 ± 0.12^{bA}	ND	ND	0.07 ± 0.01^{aB}	0.11 ± 0.01^{bA}
Propanoic acid, 2-methyl-	ND	ND	ND	ND	0.13 ± 0.03^{ab}	0.12 ± 0.01^{ab}	0.24 ± 0.08^{a}	0.10 ± 0.02^{b}
Butanoic acid	ND	ND	ND	ND	0.13 ± 0.01^{a}	0.15 ± 0.01^{a}	0.13 ± 0.02^{a}	0.07 ± 0.01^{b}
Hexanoic acid	0.67 ± 0.17^{aA}	0.14 ± 0.02^{bA}	0.11 ± 0.03^{bA}	0.50 ± 0.15^{aA}	0.38 ± 0.05^{aA}	0.23 ± 0.04^{bA}	0.43 ± 0.03^{aB}	0.20 ± 0.04^{bA}
Octanoic acid	0.97 ± 0.19^{aA}	0.23 ± 0.02^{bA}	0.27 ± 0.10^{bA}	0.84 ± 0.26^{aA}	0.62 ± 0.05^{aA}	1.01 ± 0.09^{bB}	0.63 ± 0.08^{aB}	0.95 ± 0.10^{bA}
n-Decanoic acid	0.07 ± 0.02^{aA}	0.06 ± 0.03^{aA}	0.06 ± 0.05^{a}	0.09 ± 0.03^{aA}	0.02 ± 0.01^{abB}	0.01 ± 0.00^{aB}	ND	0.03 ± 0.01^{bB}
Isoamyl acetate	0.01 ± 0.01^{aA}	0.01 ± 0.01^{aA}	0.05 ± 0.02^{aA}	0.14 ± 0.07^{bA}	0.07 ± 0.01^{aA}	0.20 ± 0.03^{bB}	0.07 ± 0.01^{aA}	0.10 ± 0.01^{aA}
Phenylethyl acetate	0.08 ± 0.02^{aA}	0.02 ± 0.00^{bA}	0.02 ± 0.01^{bA}	0.10 ± 0.03^{aA}	0.14 ± 0.02^{abA}	0.17 ± 0.03^{aB}	0.01 ± 0.00^{cA}	0.10 ± 0.01^{bA}
Hexanoic acid, ethyl ester	ND	ND	0.03 ± 0.01	ND	0.02 ± 0.01^{a}	0.08 ± 0.01^{b}	ND	0.03 ± 0.01^{a}
Octanoic acid, ethyl ester	0.20 ± 0.02^{abA}	0.08 ± 0.04^{bcA}	0.02 ± 0.01^{c}	0.25 ± 0.08^{aA}	0.02 ± 0.01^{aB}	0.10 ± 0.03^{bA}	ND	0.10 ± 0.01^{bA}
Decanoic acid, ethyl ester	0.01 ± 0.01^{aA}	0.03 ± 0.00^{aA}	0.03 ± 0.01^{aA}	0.37 ± 0.09^{bA}	0.01 ± 0.00^{aA}	0.01 ± 0.00^{aB}	0.01 ± 0.00^{aA}	0.01 ± 0.00^{aB}

Likewise, MCFA esters provide essential flavor enhancement. Hexanoic acid ethyl ester was not detected in most fermentations, at low temperature, whereas at 20 °C it was detected in lower concentrations than the flavor threshold (0.23 mg/L) [1]. On the contrary, octanoic acid ethyl ester was produced in higher concentration at 13 °C ($p < 0.05$), with the greatest value (0.25 ±0.08 mg/L) obtained in the mixed sugars substrate, which is similar to wort. Despite the goaty aroma over the threshold of 15 mg/L [1], the high production of octanoic acid at both temperatures is a positive outcome, as it is the precursor of its ester, known for its fruity notes. According to Benito et al. [29] and Clemente-Jimenez et al. [30] *M. pulcherrima* is able to produce high amounts of this ester, surpassing *S. cerevisiae* by 25 %, thus enhancing the product with the aroma of sour apple [31]. Last of all, decanoic acid ethyl ester presented significantly higher amounts ($p < 0.05$) at lower temperature in mixed sugars fermentation (0.37 ± 0.09 mg/L), implying that in a wort fermented by pure culture of *M. pulcherrima*, the last would enrich the beer with apple-like flavour [1]. At 20 °C, it was detected in traces in all sugars fermentations.

In general, by comparing the two fermentation temperatures, the concentrations of MCFA esters were higher at 13°C, as at higher temperatures, the yeast strains use higher amounts acetyl-CoA for biomass production, thus, the available acetyl-CoA for ester formation is narrowed down [32]. It is evident that the type of sugar and the fermentation temperature affect the metabolism of the yeast, which differentiates the production of MCFA and especially their corresponding esters. This positive outcome needs further investigation in wort as in mixed sugars substrate the produced amount of these fruity esters are promising for the aroma and flavour enhancement.

3.4 Wort fermentation

Based on the aforementioned results, a production of a Pale Ale beer was decided with pure *M. pulcherrima* culture, and co-culture with *S. cerevisiae* (US-05), in two different ratios, at 13 and 20 °C. As can be observed in figure 3, at the higher temperature the fermentation rate was higher in all cases, in agreement with the results obtained by simple sugars fermentations with *M. pulcherrima* (Fig. 1). The pure culture of *M. pulcherrima* had a slower fermentation rate than the co-culture fermentation of 1:10 ratio, and even more than the 1:1 ratio, especially at 20 °C. The difference in the fermentation rate, especially in the early stages of fermentation (100 to 180 hours), is due to the participation of the conventional yeast, which assisted in the consumption of sugars. Moreover, greater amount of amino nitrogen was left unused in the pure culture fermentation (80 mg/L), consenting with earlier studies that in mixed culture fermentations amino nitrogen is highly needed [35]. The mixed fermentation of 1:1 ratio needed half the duration to finish, indicating that *S. cerevisiae* dominated at this ratio and completed the fermentation earlier. Additionally, the immediate sugar consumption was followed by a rapid FAN utilization. The higher residual sugars, at the end of all three fermentations at 13 °C specify the difficulty of the strains to complete the fermentation process due to lower temperature. According to literature review, researchers in the wine industry, usually use *M. pulcherrima* in co- or in sequential fermentation with the conventional yeast, as it sluggish as the ethanol rises [36–38].

The final ethanol content (%ABV), bitterness (IBU) and colour (SRM) obtained by the fermentation at 13 and 20 °C are shown in table 4. According to the guidelines of Beer Judge Certification Program [37], the ABV of an American Pale Ale beer range between 4.5 – 6.2 %, the bitterness units should be 30 – 45 and the colour in SRM scale 5 – 14.

To begin with, the lowest ethanol content produced by pure culture at 13 °C fermentation. According to the literature data, available from wine fermentations, most strains of *M. pulcherrima* produce up to 4 %, v/v, [4], even though there are some reports of reaching up to 6 – 7 %, v/v, [36] . Despite the difference of the main sugars

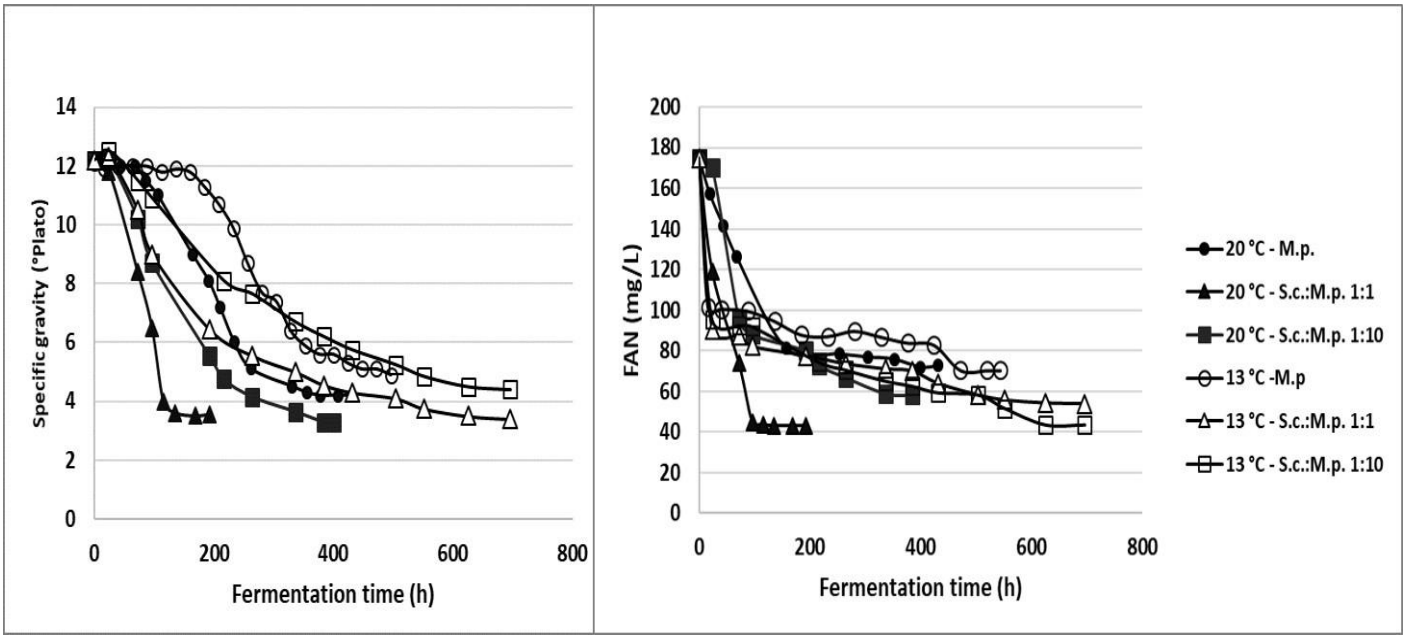

Fig. 3 Fermentation kinetics, expressed by specific gravity (Plato degrees, °P) decrease, and free amino acid (FAN) consumption, at 20 and 13 °C, in mono- and co-cultures of *M. pulcherrima* (M.p.) and *S. cerevisiae* (S.c.) in different ratios

Table 4 **The final ethanol content (%ABV), bitterness (IBU), and colour (SRM) obtained by the fermentation at 13 and 20 °C. Superscript letters, a, b, in the same column present significant differences (a>b>c>d, $p < 0.05$) between yeast strains. Superscript letters A and B present significant differences ($p < 0.05$) in each measurement between temperatures**

	Fermentation temperature					
	13 °C			20 °C		
Yeast strain	%ABV	IBU	SRM	%ABV	IBU	SRM
	Initial Values					
	–	29.0 ± 1.0	7.9 ± 0.0	–	29.0 ± 1.0	5.0 ± 0.0
M.p.	4.0 ± 0.0^{aA}	27.6 ± 0.1^{aA}	5.7 ± 0.1^{bA}	4.9 ± 0.0^{aB}	23.8 ± 0.5^{aB}	5.6 ± 0.0^{bA}
S.c.:M.p. 1:1	5.4 ± 0.1^{bA}	28.0 ± 0.0^{aA}	5.1 ± 0.4^{abA}	5.1 ± 0.2^{abA}	27.9 ± 0.0^{bA}	5.3 ± 0.1^{bA}
S.c.:M.p. 1:10	5.0 ± 0.0^{bA}	28.8 ± 0.0^{bA}	4.0 ± 0.2^{aA}	5.5 ± 0.0^{bA}	28.5 ± 0.2^{bA}	4.7 ± 0.1^{aA}

between wort and must, at 20 °C, the pure culture reached almost 5 %, v/v, similarly to the mix 1:1. By contrast, at 13 °C *M. pulcherrima* presented the lowest value signifying the low metabolic activity of this strain towards to low temperature. This outcome disagrees with the results obtained from the synthetic media, which showed higher ethanol production at lower temperature. This difference may be attributed to the residual sugars left unfermented (i.e. maltotriose or dextrins) that were not present in the synthetic media fermentations (> 4 °P). The mixed fermentations were not affected by the fermentation temperature. As far as it concerns the bitterness of the final beers (Table 4), in pure culture the IBU dropped, probably due to absorption of a small portion of hop acids by the yeast cells [36]. The colour at 13 °C was brighter (lower values), as the yeasts and other molecules can settle more easily. The increased value at 20 °C is probably due to oxidation reactions [39, 40] that may occur at higher temperatures.

3.5 Production of volatile compounds in beer

The volatile compounds detected in sugar substrates showed potential of *M. pulcherrima* for the final aroma and flavour of beers. Hence, with the aid of gas chromatography, the aromatic

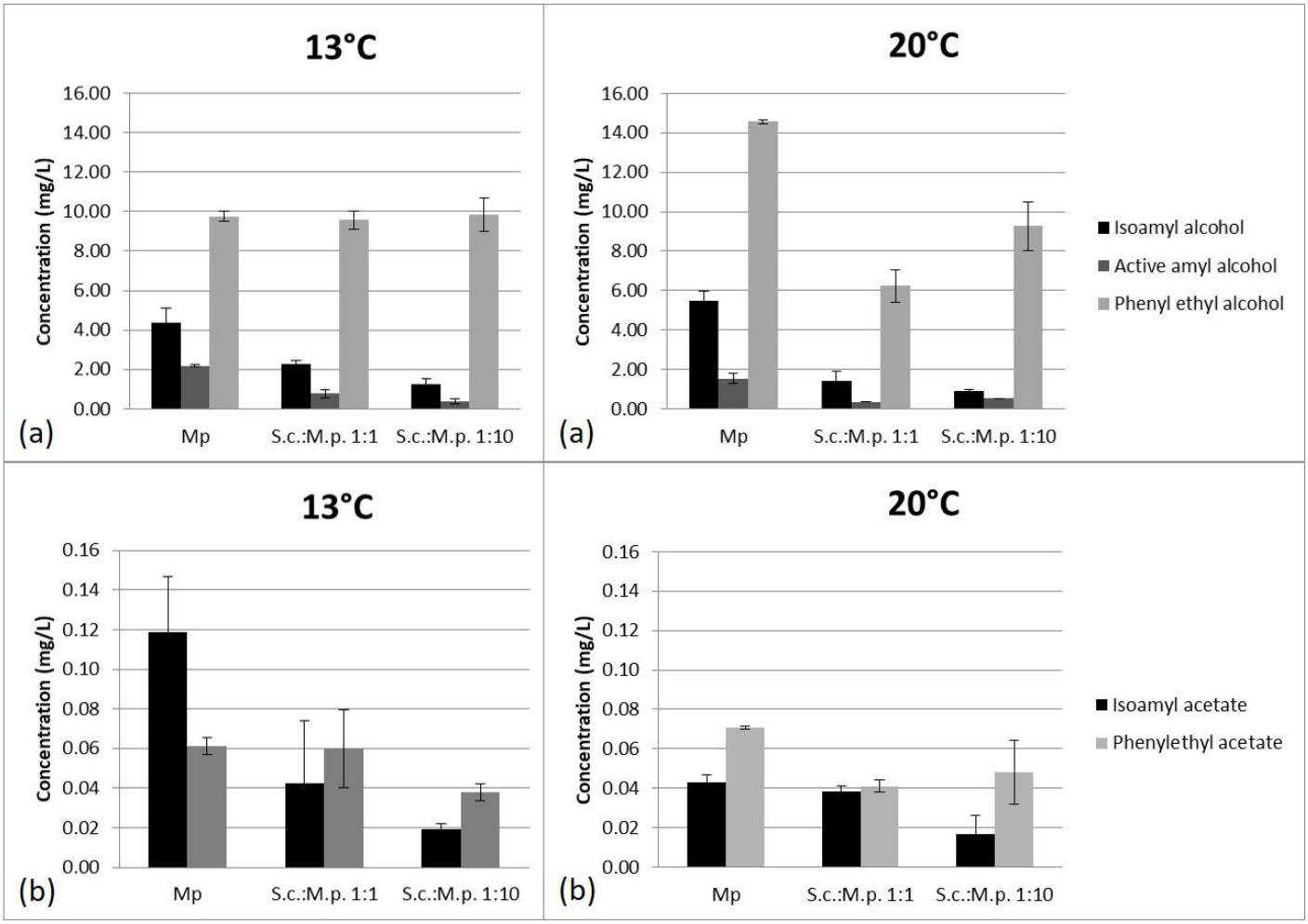

Fig.4 **Concentration of higher alcohols (a), and acetate esters (b) produced at 13 and 20 °C by mono- and co-cultures of *M. pulcherrima* (M.p.) and *S. cerevisiae* (S.c.) in different ratios**

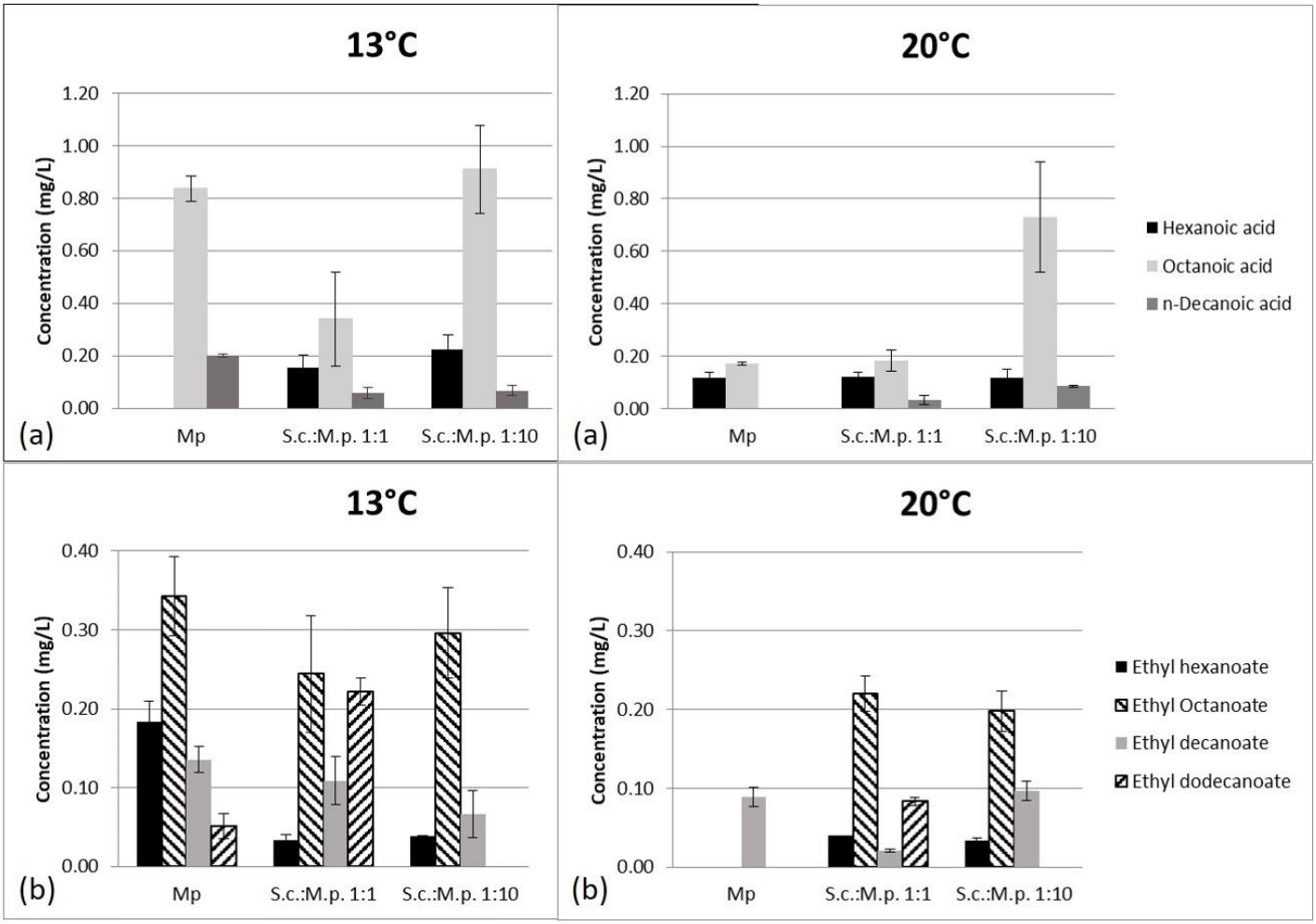

Fig. 5 Concentration of medium-chain fatty acids (MCFA) (a), and concentration of MCFA esters (b) produced at 13 and 20 °C by mono- and co-cultures of *M. pulcherrima* (M.p.) and *S. cerevisiae* (S.c.) in different ratios

profile of the beers produced either by pure culture or by the co-fermentations with *S. cerevisiae* and *M. pulcherrima* in ratios 1:1 and 1:10 is shown in figures 4 and 5. At first, the major higher alcohols detected were isoamyl, active amyl, and phenyl ethyl alcohol. By comparing the results from the three fermentations at 13 °C, it is noted that the pure culture produced higher isoamyl and active amyl alcohol ($p < 0.05$), while between the two mixed cultures significant differences were not detected (Fig. 4(a)). At 20 °C, the pure culture of *M. pulcherrima* produced the highest amounts in higher alcohols, and especially phenyl ethyl alcohol (over 14 mg/L). According to previous studies, *M. pulcherrima* is known for the high production of phenyl ethyl alcohol [36], particularly in sequential fermentation with *S. cerevisiae* reaching up to 19 mg/L [15].

The esterification of the abovementioned higher alcohols led to the detection of isoamyl and phenylethyl acetate esters, known for their banana and honey flavour [1]. As shown in figure 4(b), the temperature had no effect in the mixed cultures, whereas the pure *M. pulcherrima* culture produced higher amounts of isoamyl acetate at 13 °C (0.12 ± 0.03 mg/L), but still lower than the flavour threshold (1.6 mg/L) [1], [41]. These acetate esters were detected in a higher concentration in wine fermentations (2.44 and 0.8 mg/L of isoamyl and phenylethyl acetate esters, respectively) [15], signifying the influence of the type of fermented sugars.

Moreover, MCFA are of great importance for beer as they are precursors of the MCFA esters, albeit they affect foam stability. Figure 5(a) presents the major MCFA, i.e. hexanoic, octanoic and n-decanoic acid. The concentrations detected at both temperatures were well below their thresholds that impart unpleasant flavour. In particular, at 20 °C, all compounds were detected in the co-culture, with the greatest values observed in ratio 1:10. At 13 °C, octanoic acid, the precursor of the fruity ester, was vastly produced in pure culture and in the mixed culture of S.c:M.p. 1:10, with no significant difference between these two. Furthermore, the co-culture of 1:10 presented a great variety of MCFA.

As far as it concerns the MCFA corresponding esters (Fig. 5(b)), the highest concentration of ethyl octanoate was exhibited at 13 °C, in agreement with previous studies claiming that *M. pulcherrima* can produce high amounts of this particular ester [15, 32–34]. Another reason may be that at lower temperature not only the concentrations of the esters are greater but the variety of them as well, giving an advantage on the flavour of the produced beers, possibly due to synergistic effect between the compounds as mentioned by Lytra et al. [42].

In addition to the above discussed volatile compounds, the phenolic off flavour (POF) effect is well known in the brewing industry as specific styles (i.e. German wheat beers) require the contribution

of the phenolic character. 4-vinylguaiacol (4VG) coming from ferulic acid, found broadly in malt, is known for the clove-like notes and has a flavour threshold of 0.2 – 0.4 mg/L [43]. As reported by Scholtes et al. [44], the yeast strain may affect the production of 4VG. In our study, the highest production of 4VG was observed with the pure culture of *M. pulcherrima* at 13 °C, surpassing the flavour threshold (0.46 ± 0.01 mg/L), whereas with the co-cultures the concentrations remained lower than 0.1 mg/L. At 20 °C, the concentration detected was not significantly different among the three fermentations, with the average value equal to 0.1 mg/L. Thus, not only the yeast strain but the temperature as well plays an important role in the production of 4VG.

In conclusion, to enhance the aromatic profile of the produced beers, the selection of yeast (pure *M. pulcherrima* culture or co-cultures with *S. cerevisiae* at different ratios) should be combined with the selection of fermentation temperature. For the lower temperature, the pure culture of *M. pulcherrima* is suggested, as it can enhance the beer with more fruity, estery, and even phenolic notes. In that sense it might be a good alternative in the production of wheat beers as phenolic notes are required in their aroma. On the other hand, at 20 °C, the pure culture does not promote the production of MCFA and their esters, suggesting the co-fermentation, especially at the ratio of 1:10, as more capable for a bouquet of aromas.

4 Conclusion

In general, the results from the fermentations in synthetic media with different sugar substrates proved that the specific *M. pulcherrima* strain (Flavia 365) has the ability to ferment glucose, fructose, and maltose, as well as a mixed sugar substrate, similar to wort. In addition, it can produce adequate levels of ethanol, in pure culture, at either 13 or 20 °C. Thus, this strain can be used in monoculture fermentations. From the results derived from the GC-MS analysis, *M. pulcherrima* (Flavia 365) can enhance the final product with floral and fruity notes, thus it can be included in a beer production, either in mono- or co-culture fermentation with the conventional yeast.

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