

Effect of supplementation with pollen and brewer's yeast in the fermentation and in the physicochemical properties of honey spirits

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Abstract

The honey, rich in sugars, can be used in the preparation of the fermentation of must, however, because it is characterized as a raw material with low nitrogen content, there may be the need for supplementation of this nutrient. The objective of this study was determine the effects of different concentrations of pollen (P) and brewer's yeast (B) (1 g.L⁻¹, 10 g.L⁻¹ and 20 g.L⁻¹) as nitrogen supplements in the honey must fermentation and possible differences in the physicochemical characteristics of the honey spirit obtained. It was found that, compared to the experiment without supplementation (control-C), for all experiments supplemented, the time of the fermentation process was equal or lower. For the experiments C and P1, the total fermentation time was defined at 96 hours. For P10 and P20, the total fermentation time was defined at 72 hours. For B1, the total fermentation time may occur in 84 hours. B10 and B20 the time of the fermentation process may be in 48 hours. In relation to alcohol content, only the experiments C, P1 (1 g.L⁻¹ of pollen) and B1 (1 g.L⁻¹ of brewer's yeast) are in accordance with Brazilian legislation (38-54%), with values of 45.30%, 39.05% and 40.15%, respectively. For C, P1 and B1, the contents of aldehydes, esters, furfural and methanol were within the established for spirits. The supplementation with pollen or brewer's yeast in the honey must, can shorten the time of the fermentation process without negatively affecting the physicochemical parameters of honey spirit.

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Introduction

According to the Ministry of Agriculture, Livestock and Supply (Ministério de Agricultura, Pecuária e Abastecimento (MAPA), Decree 6.871 of 4 July 2009 (Brazil, 2005), spirit is a beverage with an alcohol content of 38-54% by volume at 20°C obtained from the lowering of the alcohol content of simple alcoholic distillation or by distillation of the must fermented. The spirits will have the name of the raw material of their origin.

Honey, one of the oldest and most traditional sweetening foods, has been reported to contain about 200 substances. Honey is a viscous, supersaturated sugar solution coming from nectar which has been collected and modified by the honey bee, *Apis* (Vandamme *et al.*, 2013). This natural product is essentially a concentrated aqueous solution of different carbohydrates, including fructose, glucose, maltose, sucrose, and other oligo- and polysaccharides (Escuredo *et al.*, 2013).

The quality of honey is determined by their

sensory, physical and chemical properties. Its physical and chemical properties depend on the nectar and pollen floral source, color, flavor, moisture and protein content and sugars (Azeredo *et al.*, 2003). All honeys share certain general characteristics, including a moisture content below 20%, a sugar content of 70-80%, an ash content ranging from 0.1% to 0.2%, and a pH between 3.8 and 4.7. Proteins, free amino acids (principally proline), organic acids, aromatics, and vitamins and mineral are minor components and several enzymes are important components of honey such as α -glucosidase, β -glucosidase, amylase and glucose oxidase (Won *et al.*, 2008; Roldán *et al.*, 2011). The specific percentages of all these different components may vary depending on the plant origin, the geographical location, the season in which the honey was collected, the treatment of honey since its harvesting, and its age (Vandamme *et al.*, 2013).

Honey is a substance that has been used for centuries to make beverages can be fermented to produce different types of mead and spirits that may have different flavors depending on the floral

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source of honey and additives and yeast used in fermentation (Gupta and Sharma, 2009). For contain low concentrations of protein and amino acids, in fermentation processes may be necessary the supplement these compounds. The nitrogenous compounds available for consumption by yeast are known as free amino nitrogen (FAN) which can be defined as the sum of the individual wort amino acids, ammonium ions and small peptides (di-, tripeptides) (Lei *et al.*, 2013).

Comparing the composition of musts of honey and wine, the sugar levels is >60% for honey and 20–25% for wine and 0.04% for nitrogen concentrations for honey and 4–5% for de wine (Pereira *et al.*, 2009). For contain low concentrations of protein and amino acids, in fermentation processes may be necessary the supplement these compounds. For supplementation of nitrogenous compounds content with the purpose to produce fermented honey can be used as pollen, which is collected from flowers as a source of proteins, lipids, vitamins and minerals for the survival of bees (Roldán *et al.*, 2011).

Pollen has been used as a “perfect health food” for many centuries due to its abundance of nutrimental constituents and bioactive compounds (Zilic *et al.*, 2014). The most important groups of chemical compounds in bee pollen are the following: proteins and amino acids, carbohydrates, lipids and fatty acids, phenols, enzymes, vitamins and bioelements (Rzepecka-Stojko *et al.*, 2012), as antioxidants. Such exogenous antioxidants are commonly obtained from food and include vitamins C and E, β -carotene, and a variety of phenolic compounds including flavonoids (3–5% dry weight). The proportions of these nutrients can vary widely among pollens of different plant species (Human & Nicolson, 2006).

Besides the pollen that can be used as a possible source of protein supplementation on fermentation, the brewer’s yeast is also presented as an option. Yeast cells contain plenty of protein, lipid, RNA, vitamins, and minerals. The brewer’s yeast is an inexpensive nitrogen source and generally recognized as safe (GRAS) and has good nutritional characteristics (Chae *et al.*, 2001). The brewer’s yeast cells, its autolysates and hydrolysates might be used as a nutrient source for the growth of fastidious microorganisms or related product formation (Ferreira *et al.*, 2010).

Amongst many industrial wastes and by-products rich in amino acids, vitamins, minerals and fatty acids, a special attention should be given to spent brewer’s yeast cultures resulting from the production of beer. During production of beer, yeast is used several times for fermentation but when cell viability is decreasing and concentration of autolysis products

rises, it must be replaced. The production of spent biomass can reach 2.5 kg.m^{-3} of final product (Kawarygielska and Pietrzak, 2014).

A functional food additive based on beetroot juice (*Beta vulgaris* L.) using brewer’s yeast and fermented by *Lactobacillus plantarum* A112, *L. acidophilus* BGSJ15-3 and *L. acidophilus* NCDO1748 is described brewer’s yeast contributes to the increase of the number of viable cells of lactic acid bacteria during the fermentation (Ferreira *et al.*, 2010). Therefore, the aim of this study was to determine the influence of supplementation with different concentrations of pollen and brewer’s yeast in the fermentation of honey must, aiming at the production of honey spirit, and compare with the fermented must without supplementation. Also, it was aimed to verify the influence of supplementation on the physicochemical characteristics of the honey spirits produced.

Materials and Method

The experiments the fermentation/production of the honey spirits were conducted at the Laboratory of Bioprocess of the Department of Food Engineering, State University of Santa Catarina (UDESC-Pinhalzinho, SC, Brazil). The honey, for the production of the must, was obtained from farmers in the western region of Santa Catarina, Brazil, and is characterized as wild honey. The pollen (Chalé do Mel), the brewer’s yeast (Nutri and Wieder) and the mineral water (Danferrana) were purchased in local trade.

Elemental analysis of the pollen and brewer’s yeast

The Elemental Analysis (Elementar CHNS) was performed by combustion in the Laboratory of Soil and Sustainability of the Department of Animal Science - State University of Santa Catarina. The method is based on complete combustion of a sample (1g) of known mass of organic material, which contains mainly carbon (C), hydrogen (H), nitrogen (N), sulfur (S) and oxygen (O) and subsequent analysis of gas resulting from the combustion process, essentially carbon dioxide (CO_2), water (H_2O), nitrogen oxides (NO_x) and sulfur dioxide (SO_2). For obtain the values of proteins, we multiplied the value of the percentage of nitrogen by 6.25 (reference value).

Physicochemical characterization of honey

For the physicochemical characterization of the honey, the following parameters were determined: reducing sugars (glucose), presence of albuminoid (Lund reaction), honey overheating or adding sugar

syrups (Fiehe reaction), presence of starch and dextrans (Lugol's reaction), dry matter, protein and pH. For the determination of reducing sugars, was used the Lane-Eynon method employing standardized Fehling's solution with 0.5% glucose, using 2g of sample of honey (IAL, 2005).

For the determination of the presence of albuminoid (its absence indicates adulteration), was weighed 2 g of the sample and transferred into a 50 ml tube with the aid of 20 mL of water. Was added 5 mL of tannic acid solution (0.5%) and was completed to 40 mL with distilled water. Was stirred and let to stand for 24 hours. It was to the presence of a precipitate in the test tube bottom in the range from 0.6 to 3.0 mL which indicates that the honey is pure (IAL, 2005).

For the verification of the overheating or added sugar syrups in honey, was made the reaction with resorcine in acid medium. Was weighed 5 g of honey and was added 5 mL of ether. The ether layer was transferred to a test-tube. Was added 0.5 mL of hydrochloric solution of resorcine and let to stand for 10 minutes. In the presence of glucose commercial or overheated honey, the liquid will have an intense red color, indicating fraud. The result is expressed in positive or negative (IAL, 2005).

To search for the presence of starch and dextrans in honey, was weighed 10 g of sample and was added 20 mL of distilled water with stirring. Was allowed to in the boiling water bath for 1 hour. After cooled at room temperature, was added 0.5 ml of Lugol's solution. In the presence of commercial glucose syrups or sugar, the solution is colored reddish brown to blue. The result is expressed in positive or negative (IAL, 2005).

Dry matter (moisture) contents were measured by conventional drying method. The total protein content was measured using the Kjeldahl method for proteins, based on the conversion of the organic nitrogen present in the sample to $(\text{NH}_4)_2\text{SO}_4$ (AOAC, 1990). The pH value was measured in 10% aqueous solution, with a pH meter (Quimis).

Preparation of honey must and verifying the influence of the addition of pollen and brewer's yeast in the fermentation

For obtaining the must use in the fermentation experiments, the honey was diluted in mineral water (chemical composition: 49.74 mg.L⁻¹ of carbonates, 50.60 mg.L⁻¹ of sodium, 0.87 mg.L⁻¹ of calcium, 0.02 mg.L⁻¹ of magnesium, 0.10 mg.L⁻¹ of potassium, 1.70 mg.L⁻¹ of sulphates, 0.65 mg.L⁻¹ of chlorates, 0.21 mg.L⁻¹ of fluoride and 0.07 mg.L⁻¹ of vanadium) until 17-18 °Brix. The acidity was corrected to an

approximate pH of 3.6 with tartaric acid (Roldán *et al.*, 2011). The must was divided into equal volumes (2.7 L), added in two fermenters with a capacity of 5.0 L and made by the addition of 2.5 g.L⁻¹ of lyophilized *Saccharomyces cerevisiae* (Saf-Instant) in each fermenter.

After preparing the must, for verification the influence of the concentration of pollen and brewer's yeast in alcoholic fermentation, were carried out the following experiments: Control-C (without pollen or brewer's yeast), experiments with addition of 1 g.L⁻¹ (P1), 10 g.L⁻¹ (P10), 20 g.L⁻¹ (P20) of the pollen and 1 g.L⁻¹ (B1), 10 g.L⁻¹ (B10), 20 g.L⁻¹ (B20) of the brewer's yeast. The concentrations of pollen and brewer's yeast were defined according to previous tests and research of Roldán *et al.* (2011). The pollen or brewer's yeast were added in fermenters after the addition of yeast and immediately homogenized.

The fermentation occurred in the BOD chamber (Solab) at temperature of 25°C for a maximum time of 108 hours. It was considered the end of fermentation when there was no production of bubbles of CO₂ by the *Saccharomyces cerevisiae*. Every 12 hours, samples of the fermented were collected for determination of physicochemical parameters.

Physicochemical parameters determined over the fermentation

To obtain the physicochemical characteristics of the must during fermentation were determined for each sample drawn, the following parameters: reducing sugars (glucose), alcohol (ethanol) and growth of the biomass. All analyzes were performed in duplicate. For the determination of reducing sugars, was used the Lane-Eynon method employing standardized Fehling's solution with 0.5% glucose (IAL, 2005). The alcohol content in must (in terms of ethanol) was obtained using the methodology of relative density at 20°C/20°C, using a pycnometer, which was calibrated relative to the mass of the must at the beginning of fermentation (time zero) and throughout the fermentation at 20°C. The relation between the mass and volume results in the relative density (IAL, 2005). The concentration of alcohol was converted to g.L⁻¹ for the calculation of the yield and kinetic parameters. To obtain the biomass, was used the methodology by Mendes-Ferreira *et al.* (2010). An aliquot of 14 mL of the fermented was centrifuged at 4,000 rpm for 15 minutes. The supernatant was discarded and the cell mass was resuspended in distilled water and centrifuged under the same conditions and the supernatant is again discarded. The biomass was determined by drying in an oven (Cienlab) at 90°C to constant weight.

Physical and chemical characterization of honey spirits with and without supplementation with pollen and brewer's yeast in the fermentation step

For the physicochemical characterization of the honey spirits, the following determinations were performed: real alcohol content, total acidity, pH, methyl alcohol, total aldehydes, esters and total furfural. The real alcohol content was quantified according with the relative density of distillate at 20°C (IAL, 2005). To determine the total acidity, it was transferred 25 mL of the sample to an Erlenmeyer flask containing 200 mL of distilled water. This mixture been titrated with a solution of 0.1 N sodium hydroxide until pH 8.2. The total acidity was expressed in grams of acetic acid per 100 mL of sample ($\text{g}\cdot 100\text{mL}^{-1}$) (Brazil, 2005). For obtaining the values of aldehydes, esters, furfural and methanol was used chromatographic method according to the normative instruction number 24 of 09/08/05 of the Ministry of Agriculture, Livestock and Supply (Ministério de Agricultura, Pecuária e Abastecimento) (Brazil, 2005).

Statistical analysis

Data were analyzed using the Statistica 10.0® (StatSoft, Inc.) Significant differences ($p < 0.05$) between means were identified using Tukey procedures. All the experiments were carried out in duplicate.

Results

Characterization of honey and nitrogen content of pollen and brewer's yeast

The values obtained in the physicochemical characterization of honey were $48.96\% \pm 1.04$ for glucose (reducing sugars), $16.53\% \pm 0.04$ to moisture, $0.67\% \pm 0.18$ for protein and 3.67 ± 0.05 for pH. Regarding the determinations indicate that the purity of honey, was obtained negative results for Fiehe reaction (honey overheating or adding sugar syrups) and the reaction with Lugol (presence of starch and dextrans) and to the reaction of Lund (presence of albuminoid) the amount of the precipitate obtained was $1.90 \text{ mL} \pm 0.10$ results that indicate the purity of honey for use in fermentation experiments. It is found that in relation to the nitrogen content (results of elemental analysis) the pollen corresponded to 16.31% of protein and the brewer's yeast corresponded to 46.56% of protein. As for the remaining of the elements, the values found were presented near.

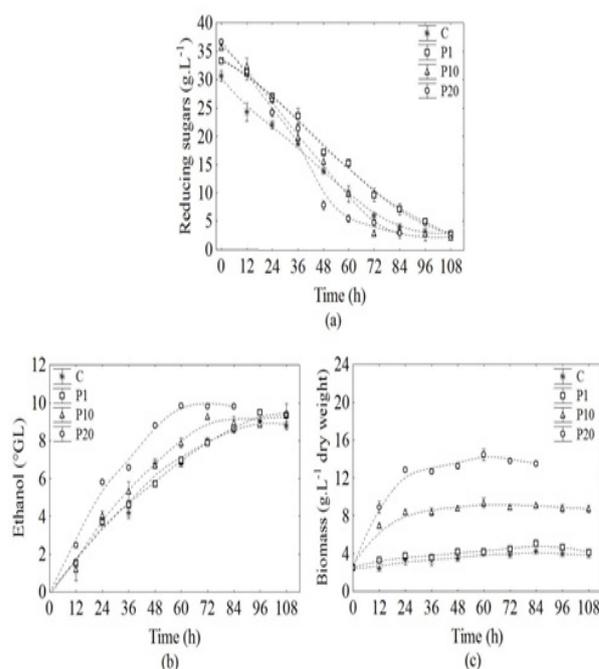


Figure 1. Profiles of the consumption of reducing sugars (a) production of ethanol (b) and cell growth - biomass (c) for experiments with and without supplementation using pollen

Profiles of the substrate, product and biomass production during fermentation of the honey must using pollen as supplement

The Figure 1 shows the profiles of the substrate consumption (reducing sugars), production of product (alcohol) and production of cells (biomass) over time of fermentation of honey must with and without supplementation of pollen. For all experiments, the profiles are characterized by a decrease in the concentration of reducing sugars (Figure 1a), an increase in alcohol concentration (Figure 1b) and increase in biomass concentration (Figure 1c) along the fermentation time, indicating that the microorganisms have adapted to the environment and consumed reducing sugars to produce alcohol and/or biomass. However, there are differences from experiments with and without supplementation, which can be visualized by the individual characteristics of the curves and the end time of the complementary fermentation.

For reducing sugar, it is found that up to 36 hours a more accentuated decrease in the experiment C and after 48 hours the decrease is more pronounced for P20. For the alcohol, it appears that during the whole the time of fermentation, the highest production was observed for P20 experiment. The experiments C and P1 presented overlapping curves. Regarding the biomass, there a similar behavior, the biomass curve of the P20 showed higher difference compared to P10.

In relation to reducing sugars along fermentation

time, using the Tukey Test using with 95% of confidence, it is verified which for the experiment C, from 84 hours fermentation, no occurs statistical difference ($p>0.05$), therefore, not occur variation in the consumption of reducing sugars until 108 hours. For the P1, the same behavior occurs from 96 hours with values statistically equal to ($p>0.05$) until 108 hours. For the P10 and P20, there was no variation in the consumption of reducing sugars after 72 hours. The reducing sugars of the 72 hours to 108 hours showed no significant difference ($p>0.05$) for P10 and reducing sugars 72 hours 84 hours showed no significant difference ($p>0.05$) to P20. Therefore, in relation to the substrate consumption (reducing sugars) to C and P1, the end of the fermentation can occur in 84 hours and 96 hours, respectively, and to P10 and P20 in 72 hours of the fermentation.

For the production of alcohol for each experiment over time, is observed for C and P1, between 96 hours and 108 hours of fermentation, no occurs statistical difference ($p>0.05$). For P10, alcohol values obtained in 72 to 108 hours of fermentation are statistically equal ($p>0.05$) by Tukey test. For P20, alcohol values are statistically equal ($p>0.05$) for 60 minutes at 84 hours of fermentation. Therefore, in relation the production of alcohol, it is verified that the end of fermentation can occur in 96 hours for C and P1, 72 hours for P10 and 60 hours for P20.

For the production of biomass, is observed for C, that there was no statistically significant difference ($p>0.05$) between the samples from 60 hours of fermentation process up to 108 hours. For the P1, the maximum cell growth was in 84 hours of fermentation, however, the average is equal statistically ($p>0.05$) the average of the biomass obtained in 72 hours, 96 hours and 108 hours. For the P10, there is a rapid growth of microorganisms within 24 hours of process, and the averages for biomass for this time and the others (to 108 hours) showed no statistical difference ($p>0.05$). For the P20, the maximum value obtained for the biomass is in 60 hours of the process, but this value is equal statistically to the values 48 hours, 72 hours and 84 hours. In relation the biomass, the end of the fermentation can occur in 60 hours for C, 72 hours for the P1, 24 hours for the P10 and 48 hours for the P20.

The difference occurs between the substrate consumption, the alcohol production and the biomass for the experiments for the intermediate or end time of the fermentation process. For reducing sugars only in to 96 hours of fermentation occurs statistically equal ($p>0.05$) between values.

For alcohol production, it is verified that the end of each experiment, alcohol values were similar, it

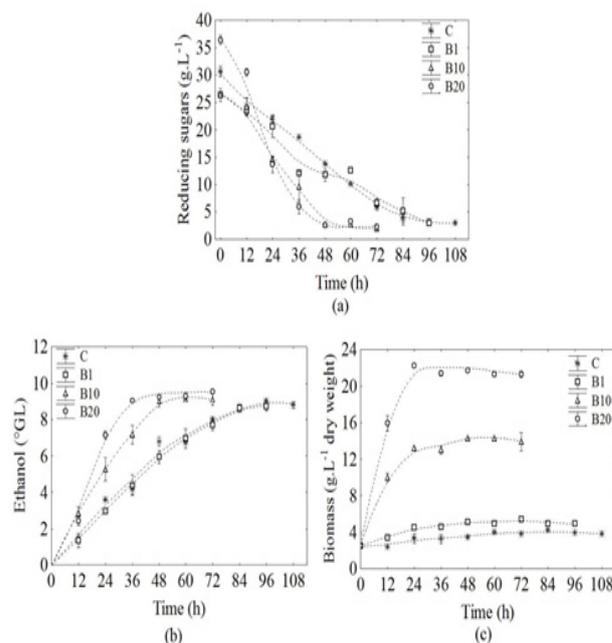


Figure 2. Profiles of the consumption of reducing sugars (a) production of ethanol (b) and cell growth - biomass (c) for experiments with and without supplementation using brewer's yeast

can be concluded that supplementation influences the rate of formation of alcohol, but at the end of each fermentation, alcohol concentration remained in a range of approximately 8 to 10 °GL.

For biomass, the increase in biomass concentration occurs in the experiments with higher concentrations of pollen. The supplementation affected the yeast growth more sharply than in the formation of alcohol. The final biomass concentration for the P20 is, approximately, 10 times higher than the concentration determined for the experiment without supplementation.

Profiles of the substrate, product and biomass production during fermentation of the honey must using brewer's yeast as supplement

Figure 2 shows the profiles of the substrate consumption (reducing sugars), production of product (alcohol) and production of cells (biomass) over time of fermentation of honey must with and without supplementation of brewer's yeast. The profiles obtained for experiments supplemented with brewer's yeast, shown in Figure 2a, also presented the different consumption reducing sugars along time, the same occurred with the supplemented experiments with pollen. It is verified that, both for B10 and B20, the decay profile ceases after 48 hours of fermentation. Already for the experiments C and B1, the consumption of the substrate by *S. cerevisiae* occurs up to 72 hours of fermentation. In relation the production of alcohol (Figure 2b), the experiments

Table 1. Productivities and yields for the total fermentation time and the time indicated by the statistical analysis of each experiment

	Fermentation total time						
	C	P1	P10	P20	B1	B10	B20
	(108 h)	(108 h)	(108 h)	(84 h)	(96 h)	(72 h)	(72 h)
P_p (g.L ⁻¹ .h ⁻¹)	0.081	0.086	0.087	0.117	0.091	0.126	0.132
P_x (g.L ⁻¹ .h ⁻¹)	0.012	0.015	0.058	0.131	0.025	0.159	0.261
$Y_{X/S}$ (gX.gS ⁻¹)	0.048	0.059	0.226	0.371	0.104	0.468	0.586
$Y_{P/S}$ (gP.gS ⁻¹)	0.322	0.342	0.340	0.331	0.371	0.372	0.297
$Y_{X/P}$ (gX.gP ⁻¹)	0.149	0.173	0.664	1.121	0.279	1.260	1.972
	Time of the fermentation indicated by statistical analysis						
	C	P1	P10	P20	B1	B10	B20
	(96 h)	(96 h)	(72 h)	(72 h)	(84 h)	(48 h)	(48 h)
P_p (g.L ⁻¹ .h ⁻¹)	0.094	0.099	0.129	0.136	0.103	0.188	0.193
P_x (g.L ⁻¹ .h ⁻¹)	0.015	0.022	0.089	0.157	0.029	0.246	0.400
$Y_{X/S}$ (gX.gS ⁻¹)	0.051	0.074	0.230	0.361	0.064	0.055	0.055
$Y_{P/S}$ (gP.gS ⁻¹)	0.327	0.331	0.332	0.313	0.406	0.379	0.291
$Y_{X/P}$ (gX.gP ⁻¹)	0.156	0.224	0.692	1.151	0.280	1.312	2.079

P_p is the productivity in product, P_x is the productivity in cell (biomass), $Y_{X/S}$ is the cell yield with respect to the substrate, $Y_{P/S}$ is the substrate yield with respect to the product, $Y_{X/P}$ is the cell yield with respect to the product. C is the control experiment, B1 is the experiment with 1g.L⁻¹ of brewer's yeast; B10 is the experiment with 10g.L⁻¹ of brewer's yeast; B20 is the experiment with 20g.L⁻¹ of brewer's yeast.

with 10 g.L⁻¹ (B10) and 20 g/L (B20) of brewer's yeast, the maximum production it was in 72 hours of fermentation, 24 hours unless that the maximum production of B1 and C. For cell growth (biomass), the profiles obtained were similar to those obtained for experiments supplemented with pollen, but the final cell concentration was superior to B10 and B20, demonstrating that supplementation with brewer's yeast further stimulated the multiplication of *S. cerevisiae*.

Analyzing along time for an experiment, for the experiment P1, the reducing sugars in do not present statistically significant difference ($p > 0.05$), using Tukey Test with 95% of confidence, from 72 hours of fermentation. For B10 and B20, the end of the fermentation, in relation of the substrate consumption, can occur within 48 hours, this time much lower when compared to C terminus time, which can occur in 84 hours. With regard to alcohol, it is verified that B1 has reached maximum production at 84 hours, and the value obtained is statistically equal ($p > 0.05$) to value of alcohol concentration obtained 96 hours of fermentation. For B10 and B20, the end of the fermentation, regarding the formation of alcohol can occur in 48 hours and 36 hours, respectively. These

times are relatively short compared to experiment C, which end can occur in 96 hours of fermentation. For biomass, to the experiment B1, the maximum production occurs in 72 hours and the value obtained for this time is statistically equal ($p > 0.05$) the values obtained for the time of 48 hours, 60 hours, 84 hours and 96 hours of fermentation. Thus, to B1, the process time compared to biomass can be fixed at 48 hours. To B10 and B20, the maximum production of biomass was obtained in 24 hours of process.

Comparing between experiments for the same time, for reducing sugars, from 48 hours to process occurs statistically significant difference ($p < 0.05$) between C and B1 when compared to B10 and B20. For B10 and B20, the consumption of reducing sugars is more pronounced until the end of fermentation, showing that the largest brewer's yeast concentrations tested stimulated the consumption of the substrate. Similarly, for the production of alcohol, it is found that for B20, to 24 hours fermentation there was statistical difference compared with the other experiments, the value being higher alcohol concentration in this experiment. From 48 hours, the same behavior occurs for the alcohol concentration occurred for the substrate, the statistically significant difference

($p < 0.05$) for C and P1 when compared to B10 and B20. For biomass, it is found that with increasing concentration of brewer's yeast, is increased cell growth. For B20, the values differ significantly from the others ($p < 0.05$) from 12 hours of process, and also is higher than the growth achieved for the experiments supplemented with pollen.

Productivities and kinetic parameters of fermentation of honey must with and without supplementation

In the previous step, by statistical analysis, the total fermentation time for each experiment can be set based on the results of substrate consumption and ethanol production. For the experiments C and P1, the total fermentation time was defined at 96 hours. For P10 and P20, the total fermentation time was defined at 72 hours. For B1, the total fermentation time may occur in 84 hours. B10 and B20 the time of the fermentation process may be in 48 hours.

Based on previous results, was calculated the productivities and kinetic parameters of the fermentation processes for the experiments with and without supplementation with pollen or brewer's yeast. The Table 1 shows the results of productivities and kinetic parameters calculated for the total fermentation time of each experiment and times of the process defined according to the statistical analysis.

It is verified in Table 1, for all the experiments, comparing the values obtained for the total fermentation time and the time defined according to the statistical analysis, there is an increase in the value of productivity in product (PP) and productivity in cells (PX). For YX/S, comparing between the two conditions of time of the fermentation, it is found that for C, P1, P10 and P20, the values remain close, demonstrating that the process time can be reduced without affecting the utilization of substrate for multiplication of the cells. However, to B1, B10 and B20, occur a reduction of 1.63 times, 10.65 times and 8.51 times, respectively, in the value YX/S, which demonstrates that on 96 hours of process to B1 and 72 hours to B10 and B20, occur a higher utilization of the substrate for the multiplication of the cells and non-alcohol production, which is not desired. For YP/S, which is the ratio of the metabolized substrate converted into product, it is found that for C, B1 and B10 occurs a slight increase in value, comparing the total process time and the time defined by statistical analysis, which demonstrates that the process time reduction can be used in these experiments as it does not affect this parameter. For P1, P10, P20 and B20, occurs a small decrease for the YP/S, this demonstrates that the reduction time is possible because the increase in 12 hours of process to P1 and

P20, 36 hours of process to P10 and 24 hour of process to B20 do not lead to product yields warranting the largest time in process.

The relation between the mass of cells and the product mass, represented by YX/P, demonstrates that there was an increase in this parameter for all experiments comparing the total process times and the times defined by the statistical analysis. Comparing the supplemented experiments with the experiment C, it is found that the values obtained for YX/P is larger both for the supplemented experiments with pollen (P1, P10 and P20) as for experiments supplemented with brewer's yeast (B1, B10 and B20), which may indicate that supplementation accelerated the cell multiplication.

Therefore it turns that supplementation induces the production of the cells and that these cells, during fermentation process, reached the maximum concentration of ethanol in less time compared to experiment C, which demonstrates an improvement in the process with the supplementation. However, the use of brewer's yeast accelerated the cell multiplication and this supplement may be used when the objective is the production of cells.

Physicochemical characteristics of the distillate fractions and the honey spirits produced with honey must with and without supplementation with pollen and brewer's yeast

After the definition the fermentation time and the kinetic and productivity parameters, was determined the alcohol content of the fractions of the distillate of spirits. The Table 2 shows the results of the alcohol content for the head, heart and the tail for the experiments without and with supplementation with pollen or brewer's yeast.

Comparing between experiments, there is no significant statistical difference ($p > 0.05$) in relation to alcohol content in the "head". For the heart (honey spirit), the experiments C, P1 and B1 were statistically equal ($p > 0.05$), with the highest amounts of alcohol, being in accordance with to Brazilian law. The experiments P10, P20, B10 and B20, equals statistically ($p > 0.05$), have had the lowest alcohol content of honey spirit (heart) and these values are characterized as below the value established by the Brazilian legislation.

For the tail, it can be seen that with increasing concentration of pollen or brewer's yeast, also increased the alcohol content. The P1 and B1 experiments have the lowest alcohol content and are statistically equal ($p > 0.05$). However, comparing with the experiment C, it is found that all the contents of the alcohol in the supplemented experiments are

Table 2. Alcohol content and total acidity for distillate fractions for the experiments without and with supplementation with pollen or brewer's yeast

	Alcohol content (°GL)			Total acidity (mg acetic acid/ 100 mL of sample)		
	Head	Heart (spirit)	Tail	Head	Heart (spirit)	Tail
C	53.40 ^a ± 1.84	45.30 ^a ± 0.42	20.20 ^a ± 1.41	36.00 ^{ab} ± 5.66	40.40 ^{abc} ± 0.57	60.00 ^b ± 5.66
P1	53.85 ^a ± 1.91	39.05 ^a ± 1.34	6.40 ^c ± 0.99	32.00 ^{ab} ± 5.66	34.00 ^a ± 2.83	40.00 ^a ± 0.00
P10	55.45 ^a ± 3.46	33.35 ^b ± 1.77	10.45 ^b ± 2.90	40.00 ^{ab} ± 0.01	46.00 ^{abcd} ± 2.83	70.00 ^{bc} ± 2.83
P20	54.45 ^a ± 0.92	35.90 ^b ± 1.41	10.00 ^b ± 3.54	64.80 ^c ± 1.13	54.00 ^d ± 2.83	118.40 ^e ± 2.26
B1	50.80 ^a ± 1.13	40.15 ^a ± 0.35	7.75 ^c ± 0.49	28.00 ^a ± 5.66	36.00 ^{ab} ± 5.66	58.00 ^b ± 2.83
B10	52.00 ^a ± 1.84	33.35 ^b ± 2.47	12.60 ^b ± 0.14	40.00 ^{ab} ± 0.01	48.40 ^{bcd} ± 0.57	78.00 ^c ± 2.83
B20	53.70 ^a ± 0.71	34.50 ^b ± 0.97	11.30 ^b ± 1.56	46.00 ^b ± 2.83	51.20 ^{cd} ± 4.53	102.00 ^d ± 2.83

Letters equal, in column, are averages of alcohol content or total acidity equal to the same fraction of the distillate (head, heart or tail) in 95% level of confidence, using the Tukey Test. C is the control experiment, P1 and B1 are experiments with 1g.L⁻¹ of pollen and brewer's yeast, respectively; P10 and B10 are experiments with 10g.L⁻¹ of pollen and brewer's yeast, respectively; P20 and B20 are experiments with 20g.L⁻¹ of pollen and brewer's yeast, respectively.

lower and statistically different ($p < 0.05$).

For acidity, it is perceived that the fraction of honey spirit (heart) had values below 55% for all experiments. However, there is a tendency of the increasing of total acidity with increasing of the concentration of supplement. It is found that organic acids are in larger quantity on the "tail".

Therefore, based on the content of alcohol that is in accordance with Brazilian law, only C, P1 and B1 can be characterized as honey spirit. In this way, was performed the characterization in relation to the content of aldehydes, esters, furfural and methanol in these three experiments (C, P1 and B1), whose results are shown in Table 3. It is noted that the values of aldehydes and methanol were higher for P1. For esters, the value obtained for P1 was lower compared to C and B1. The presence of furfural was not detected for any of the experiments.

Discussion

The values obtained in the characterization of honey demonstrate a high concentration of sugars and the low availability of proteins. According with Roldán *et al.* (2011), the protein content of honey is 0.63%, value similar to that found in the present study (0.67%). Sugars (saccharides) represent the main components of honey. Besides the two main constituents, the monosaccharides glucose and fructose, there are the minor components consisting of about 25 oligosaccharides (disaccharides, trisaccharides, tetrasaccharides) (Anklam, 1998). Kirs *et al.* (2011), in characterization of different

honey types, found values of glucose of 28.84%-40.32%, moisture of 16.1-18.9% and pH of 3.48 to 5.12, being that values found in this study are within the range of values presented (48.96%, 16.53% and 3.67, respectively).

Regarding the nitrogen content, the values obtained for the pollen (16.31%) and the brewer's yeast (46.56%) are similar to those found by other authors. The pollen contains, in addition to sugars, 7.4% of the moisture, approximately 20% of the protein (close to that found in the present study), 6% of lipids and 2.2% of ash, plus vitamins, minerals and carotenoids (Roldán *et al.*, 2011). Amino acid composition, however, may define the nutritional value of pollen more accurately than protein content, since the nutritional value is reduced when inadequate amounts of the essential amino acid. The predominant amino acids in pollen of 62 species, including 20 *Eucalyptus* spp., are glutamic acid, aspartic acid and proline (Roldán *et al.*, 2011; Human & Nicolson, 2006). Human and Nicolson (2006), in a study of the characterization of pollen of *Aloe greatheadii* var. *davyana*, they found 28.1% of protein, 7.6% of lipids and 60.7% of carbohydrate. Each 10 g.L⁻¹ of pollen added to a fermentation medium provides on average 70% of the amino acids that were already present in 1 L of honey-must (Roldán *et al.*, 2011).

Cells from yeast (brewer's yeast) are used as a supplement because it contains 45% to 60% of the protein. Yeast cells also contain lipids, vitamins and minerals (Chae *et al.*, 2001). Yeast extracts are obtained by biological methods from the protein-rich cell sap of brewer's yeast. This extract is rich

Table 3. Content of aldehydes, esters, furfural and methanol of the samples with and without supplementation with 1g/L of pollen or brewer's yeast

Experiment	Aldehydes (mg.100mL ⁻¹)	Esters (mg.100mL ⁻¹)	Furfural (mg.100mL ⁻¹)	Methanol (mg.100mL ⁻¹)
C	7.94	15.69	Not detected	8.20
P1	8.49	12.64	Not detected	9.92
B1	5.26	14.16	Not detected	8.10

in valuable amino acids and vitamin B-complex, nucleotides and other useful cell constituents (Andrews *et al.*, 2011), characterized as a good supplement.

The experiments using pollen and brewer's yeast in the fermentation showed similar profiles, however, the fermentation time used brewer's yeast was lower. Still, the supplemented experiments reached the final content of alcohol before the control experiment. The differences can be explained due to the other components in the pollen and brewer's yeast. Mendes-Ferreira *et al.* (2010) suggest that nitrogen is not the only growth-limiting factor of the *S. cerevisiae*, but, a more relevant increase in yeast cell biomass was only observed in the media with the highest initial nitrogen concentration, as occurred for B10 and B20, whose nitrogen concentrations were the highest in this study.

Excessive wort amino nitrogen tends to elevated higher alcohol concentrations by the stimulatory effect in cellular growth. On the contrary, very low amino acid nitrogen also yields an excessive higher alcohol concentration (Vidal *et al.*, 2013), this can be seen in experiment C. In this instance, the limited nitrogen content will promote enhanced endogenous synthesis of amino acids, stimulating the anabolic higher alcohol synthetic route (Vidal *et al.*, 2013).

In the study of Rondan *et al.* (2011), who verified the influence of pollen addition on mead elaboration, the alcohol values obtained for the control experiment, P10 and P20 were 9.4%, 11.74% and 11.80, respectively, by indicating the same behavior in this study, and comparing between the different concentrations of pollen, the fermentation rate also increased with pollen addition. Kawa-Rygielska and Pietrzak (2014), in study of ethanol fermentation of very high gravity (VHG) maize mashes by *Saccharomyces cerevisiae* with spent brewer's yeast supplementation, found that it after 48 h of fermentation, 95% of total ethanol was produced, this time equal to that obtained for the B10 and B20 experiments. Mussatto *et al.* (2008) studied the effects of medium supplementation and pH control on lactic acid production from brewer's spent grain, found that

addition of 5 g.L⁻¹ yeast extract enhanced the lactic acid volumetric productivity in 18% higher than that obtained from non-supplemented hydrolysate. Also, the bacterial growth in supplemented media was initially fast, but stopped after 12 h. On the other hand, in non-supplemented hydrolysate the cell growth was almost negligible, being not observed lag and log phases, as occurred in the present study for C, P1 and B1.

Based on the total fermentation time for each experiment and time determined by the statistical analysis, it was noted that reducing the fermentation time for experiments P10, P20, B1, B10 and B20 is possible without affecting the kinetic parameters. Therefore, the supplementation favored the fermentation. The same occurred in the study of Rondan *et al.* (2011). Was used pollen as supplement in honey must for the production of mead and without a fermentation activator like pollen, the fermentation lasted approximately 6 weeks, confirming that honey is low in nutrients for yeast fermentation. The control mead showed a significantly lower fermentation rate and a higher time to reach the maximum rate than the honey musts with added pollen. An increase in fermentation rate and reduction of the time to maximum with increasing concentrations of pollen were observed.

The experiments, C, P1 and B1 showed the alcohol content required by Brazilian law (38-54% by volume at 20°C) (Brazil, 2009). Miranda *et al.* (2007), to characterize the chemical quality of sugarcane spirits, determined in 94 samples, the alcohol content ranging from 34.24 to 50.29%, shown that even being marketed, there are products out of law.

The average values of volatile acidity found were below the limit of 150 mg of acetic acid per 100 mL of anhydrous ethanol, as established by Law. A quick increase in acidity is observed in the first hours of the process and sometimes combines with the low buffer capacity of mead wort to cause a rapid fall of pH causing the fermentation to stop. This phenomenon, caused mainly by the formation of succinic acid, is strongly dependent on the strain of

yeast and the presence of nitrogen compounds (Sroka & Tuszynski, 2007).

Ethanol is the main product of fermentation of sugar cane; however, the minority or secondary compounds (volatile acids, aldehydes, esters and other types of alcohol) are responsible for the characteristic aroma and flavor of the beverage. These compounds are produced by the degradation of some amino acids and once they have a considerable molecular weight, they are concentrated mainly in the "tail" (Parazzi *et al.*, 2008). Methanol is undesirable spirits (Miranda *et al.*, 2007). The values found in this work are in accordance with the law. Methanol is produced in small concentrations during fermentation because of the hydrolysis of pectins (Granato *et al.*, 2014).

Generally, aldehydes having up to 8 carbon atoms are pervasive and often nauseating smell, and are considered undesirable in distilled beverages. The main aldehyde associated with alcoholic fermentation is acetaldehyde which has penetrating aroma and generally cloying and are considered undesirable in spirits (Parazzi *et al.*, 2008). Higher alcohols and esters are of major industrial interest because of their high concentrations (with respect to other secondary metabolites) and aroma contribution. The other important flavours for defining the sensorial quality of spirit beverages are acetate and ethyl esters, since they are characterized by fruity and floral aromas. Acetate esters are formed by the condensation of acetyl-CoA with an alcohol, such as isoamyl acetate and isobutyl acetate, whereas acyl esters, such as ethyl hexanoate (ethyl caproate) and ethyl octanoate (ethyl caprylate), are produced by the condensation of CoA-activated medium-chain-fatty acids with ethanol. The concentrations of esters vary widely in alcoholic beverages (Vidal *et al.*, 2013).

In the context of spirit production, there is a certain difficulty to find out what concentration of nitrogen in the medium would be optimal for the cell population required. The nitrogen demand will depend of several parameters, as for example the type of cells, given by the strain of yeast used, the size of inoculum, and the type of fermentation conditions employed, such as aeration and sugar concentration (Vidal *et al.*, 2013).

Conclusion

This study showed that the supplementation of honey must with pollen and brewer's yeast can exert an influence on fermentation parameters. The process time are modified with the concentration of supplements, based on the time of 108 hours (total time for the control experiment - C). For the

experiments with addition of 1g.L⁻¹ of pollen (P1) and brewer's yeast (B1), the fermentation occurred in 96 hours and 84 hours, respectively. For other experiments, the time was 72 hours or less. The supplementation with concentrations of 10g.L⁻¹ and 20g.L⁻¹ of brewer's yeast (B10 and B20) induced the production of cells. The experiments C, P1 and B1 had alcohol content values in accordance with Brazilian law. The total acidity of the honey spirit and distillate fractions (head and tail) showed an increase in the production of organic acids with increase of supplemental nitrogen source. The supplementation with pollen and brewer's yeast was characterized as a good alternative to reduce the fermentation time without affecting the quality parameters of honey spirit, however, the nitrogen content can be variable as a function of other characteristics as composition honey, fermentation temperature, type of water used in the preparation of honey must, yeast strain, among others.

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