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Effect of *Saccharomyces cerevisiae* cells immobilisation on mead production

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ABSTRACT

Mead is a traditional alcoholic beverage obtained by the fermentation of diluted honey performed by yeasts. In this work the potential of application of immobilised yeast cells on single-layer Ca-alginate or double-layer alginate–chitosan for mead production was assessed for the first time. The meads produced either with entrapped or free cells were evaluated in terms of quality and aroma profile. The immobilisation procedure had no adverse effect on cell viability, since minor differences were found in fermentation kinetics among the strains and immobilisation systems. The double-layer alginate-chitosan had no advantage compared with the single-layer Ca-alginate, as the number of free cells in the medium, resulting from cell leakage, was similar. Although meads obtained with entrapped yeast cells presented less ethanol and glycerol and more acetic acid, it exhibited larger amounts of volatile compounds. Immobilised cells produced meads with more compounds with fruity characteristics, such as ethyl octanoate and ethyl hexanoate; however the concentrations of undesirable compounds in such meads were also higher. The effect of immobilisation on the aroma profile was important, but the strain contribution was also of major importance. Thus, the sensory analysis of final product gives an important insight on the overall quality.

1. Introduction

Mead is a traditional honey-derived beverage containing 8–18% (v/v) ethanol. The beverage is produced by yeast alcoholic fermentation of diluted honey (Mendes-Ferreira et al., 2010; Ramalhosa, Gomes, Pereira, Dias, & Estevinho, 2011). Honey production is a significant economic activity in European countries, however to the development of honey-derived, such as mead, is of extreme importance to increase the profit of the beekeeping industry. Mead fermentation progress depends on several factors, such as yeast strain (Pereira, Mendes-Ferreira, Oliveira, Estevinho, & Mendes-Faia, 2013), honey type and composition (Navrátil, Šturdík, & Gemeiner, 2001), lack of essential nutrients such as a deficiency in available nitrogen (Mendes-Ferreira et al., 2010), low mineral concentration, low pH (Sroka & Tuszyński, 2007) and low buffer capacity (Maugenet, 1964). Several strategies have been introduced for the optimisation of mead fermentation through the use of an appropriate honey-must formulation to improve the alcoholic fermentation performance of yeast (Mendes-Ferreira et al., 2010), using starter yeast cultures isolated from honey/honey-wine (Pereira, Dias, Andrade, Ramalhosa, & Estevinho, 2009; Teramoto, Sato, & Ueda, 2005) or commercial yeast starter cultures (Koguchi, Saigusa, & Teramoto, 2009; Navrátil et al., 2001; Sroka & Tuszyński, 2007). It has been shown that supplementation of honey-must with ammonium significantly reduces fermentation length (Mendes-Ferreira et al., 2010). However, some residual sugars, other than glucose, still remain in meads after alcoholic fermentations despite the initial nitrogen concentration or the yeast strain used (Mendes-Ferreira et al., 2010). Recently, we have shown that increasing pitching rate impacts yeast fermentative activity, and significant time was saved in the fermentation process, with no detrimental impact on mead aroma composition (Pereira et al., 2013).

Microorganism immobilisation methods have gained attention in the last few decades and are being successfully applied in the

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production of alcohols (ethanol, butanol and isopropanol), organic acids (malic, citric, lactic and gluconic acids), enzymes (cellulose, amylase and lipase), the biotransformation of steroids for wastewater treatment and food applications (beer and wine) (Liouni, Drichoutis, & Nerantzis, 2008; Reddy, Reddy, Reddy, & Reddy, 2008), among others. Despite the great potential, the industrial use of immobilised cells is still limited because further application depends on the development of immobilisation procedures that can be readily scaled up (Kourkoutas, Bekatorou, Banat, Marchant, & Koutinas, 2004). The main techniques that enable biomass confinement are attachment or adsorption on solid carrier surfaces, entrapment within a porous matrix, self-aggregation of cells (flocculation) and cell containment behind a barrier (Pilkington, Margaritis, Mensour, & Russel, 1998). Entrapment involves imprisoning living cells within a rigid network that permits the diffusion of substrates and products, thereby making possible the growth and maintenance of active cells (Diviès & Cachon, 2005). The polymeric beads are usually spherical, with diameters ranging from 0.3 to 3 mm (Verbelen, De Schutter, Delvaux, Verstrepen, & Delvaux, 2006). Owing to the very gentle, simple and rapid procedure, the entrapment of cells in alginate hydrogels is still the most frequently used method for immobilisation (Pajic-Lijakovic, Plavsic, Nedovic, & Bugarski, 2010).

The immobilised microbial cells in a hydrogel matrix are protected from harsh environmental conditions such as pH, temperature, organic solvent and inhibitors (Kocher, Kalra, & Phutela, 2006; Park & Chang, 2000). Cell growth in the porous matrix depends on diffusion limitations imposed by the porosity of the material and later by the impact of accumulating biomass (Kourkoutas et al., 2004). Cell immobilisation also allows easier handling of the cells and facilitates the clarification of the final product (Kocher et al., 2006; Kostov, Angelov, Mihaylov, & Poncelet, 2010; Kourkoutas et al., 2004; Park & Chang, 2000). Studies with immobilised cells in Ca-alginate (Qureshi & Tamhane, 1986) or pectate (Navrátil et al., 2001) in mead production have showed that fermentation length was reduced or fermentation rate increased, respectively. Therefore, the purpose of this study was to evaluate the effect of the yeast cell immobilisation of two yeast strains (QA23 and ICV D47) in a fed-batch system. The fermentation profile, cell viability, mead composition and mead aroma profile were evaluated in meads fermented with free or immobilised cells. Yeast cell immobilisation was accomplished using alginate high molecular hydrophilic polymeric gel at a concentration of 4%. In addition, single (Ca-alginate) or double layers (alginate-chitosan) were tested. The cells were entrapped in the gel using a drop-forming procedure.

2. Materials and methods

2.1. Yeast strains

Saccharomyces cerevisiae Lalvin QA23 (Lallemand, Montreal, Canada) and *S. cerevisiae* Lalvin ICV D47 (Lallemand, Montreal, Canada) were used in this study as active wine dry yeasts.

2.2. Honey

A dark multifloral honey was used that was derived primarily from the pollen of *Castanea* spp. and *Erica* spp. and was purchased from a local beekeeper in the northeastern region of Portugal. The characteristics and satisfactory quality of the honey were assured in accordance with the requirements established in Portuguese law (Decreto-Lei n° 214/2003, 18th September).

2.3. Preparation of honey-must for fermentation

The honey-must for fermentation with free or immobilised cells was prepared as described by Pereira et al. (2013). The honey was diluted in natural commercially obtained spring water purchased in the market (37% w/v) to achieve 23°Brix, corresponding to an alcoholic beverage with approximately 13.5% ethanol and mixed to homogeneity. Then, the insoluble materials were removed from the mixture by centrifugation (2682× *g* for 30 min; Eppendorf 5810 R centrifuge) to obtain a clarified honey-must. Titratable acidity was adjusted with 5 g/L of potassium tartrate (Sigma–Aldrich, St. Louis, USA), and pH was adjusted to 3.7 with malic acid (Merck, Darmstadt, Germany). The nitrogen content was adjusted to 267 mg/L with diammonium phosphate (DAP, BDH Prolabo, Leuven, Belgium). The parameters °Brix (Optic Ivymen System, ABBE Refractometer), pH (Five Easy FE20, Mettler-Toledo), titratable acidity and assimilable nitrogen concentration were determined prior to and after the adjustments. Titratable acidity was determined according to standard methods (Organisation Internationale de la Vigne et du Vin, 2006). Yeast assimilable nitrogen (YAN) was determined by the formaldehyde method as previously described (Aerny, 1996). After clarification, 10 mL of the sample was transferred into a 50-mL beaker and diluted with 15 mL of water. The pH was adjusted to 8.1 with 100 mM NaOH (Merck, Darmstadt, Germany) and 2.5 mL of formaldehyde (Merck, Darmstadt, Germany) at pH 8.1 was added. After 5 min, the pH was adjusted again to 8.1 by titration with 50 mM NaOH. Assimilable nitrogen was calculated using the following formula:

$$\text{YAN (mg/L)} = [(\text{vol. NaOH}) \times (\text{conc. NaOH}) \times 14 \times 1000]/(\text{sample volume})$$

The honey-must was pasteurised at 65 °C for 10 min and then immediately cooled. No sulphur dioxide was added to the honey-must.

2.4. Immobilisation of yeast cells

Starter cultures were prepared by the rehydration of 2 g of active dry yeast in 20 mL of sterilised water at 38 °C, according to the manufacturer's instructions, to obtain ca. 10^8 CFUs/mL. Sodium alginate (BDH Prolabo, Leuven, Belgium) was dissolved in distilled water at concentrations of 4% (w/v) and sterilised by autoclaving at 121 °C for 20 min.

To inoculate the honey-must with 10^6 CFU/mL, the appropriate amount of yeast suspension was added to 10 mL of sodium alginate solution. The polymer–cell mixture was added dropwise to a 180 mM $CaCl_2$ (Panreac, Barcelona, Spain) sterilised solution and left to harden in this solution for 30 min at 4 °C. Single-layer *S. cerevisiae* immobilised beads were rinsed three times with sterile distilled water. Then, the immobilised beads were transferred into the honey-must.

For double-layer immobilisation, after the cells were left to harden in $CaCl_2$ solution for 30 min at 4 °C, the beads were decanted and added to a chitosan (Sigma–Aldrich, St. Louis, USA) solution prepared according to Liouni et al. (2008) and maintained at 25 °C for 24 h at a rotational speed of 80 min^{-1}. Double-layer *S. cerevisiae* immobilised beads were decanted, rinsed three times with autoclaved distilled water and transferred into the honey-must.

2.5. Fermentation conditions and monitoring

The immobilised beads in the single and double layers were transferred into the honey-must for fed-batch fermentations. In