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Optimization of mead production using Response Surface Methodology

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ABSTRACT

The main aim of the present work was to optimize mead production using Response Surface Methodology. The effects of temperature (x_1: 20–30 °C) and nutrients concentration (x_2: 60–120 g/hL) on mead quality, concerning the final concentrations of glucose (Y_1), fructose (Y_2), ethanol (Y_3), glycerol (Y_4) and acetic acid (Y_5), were studied. Twelve operational conditions were tested. No delays and moods were observed during fermentations. The second order polynomial models determined produced satisfactory fittings of the experimental data with regard to glucose ($R^2 = 0.646$, $p = 0.001$), ethanol ($R^2 = 0.741$, $p = 0.049$), glycerol ($R^2 = 0.899$, $p = 0.002$), fructose ($R^2 = 0.902$, $p = 0.033$) and acetic acid ($R^2 = 0.913$, $p = 0.001$). The optimum extraction conditions determined in order to maximize the combined responses were 24 °C and a nutrients concentration of 0.88 g/L. The mead produced under these conditions had the following characteristics: ethanol concentration of 10.2%, acetic acid 0.54 g/L, glycerol 7.8 g/L, glucose 1.8 g/L and fructose 2.5 g/L. These values were in agreement with the predicted and were within the safe limit established for acetic acid and the recommended range for glycerol. Furthermore, the residual sugars concentration was also low, decreasing the possibility of occurring undesirable refermentations.

1. Introduction

Beekeeping is a very important economic activity in the European Countries. However, it is sometimes difficult for beekeepers to sell all the honey, being it marketed at prices below the production cost. In this context, it is very important to find out alternatives, such as mead production.

Mead is an alcoholic drink (8–18% (v/v)) produced by fermentation of a diluted solution of honey. According to Gupta and Sharma (2009) this beverage contains many nutritional elements required by the organism and has favorable effects on digestion, metabolism and has advantages in the treatment of anemia and chronic diseases of the gastrointestinal tract. Mead can be classified as dry, sweet and frothy, according to its manufacturing technology. Mead production depends on honey variety, yeast strain, yeast nutrition and pH used, among other factors (Navrátil et al., 2001). Due to high sugar content, low pH and low mineral content of honey, mead production is a time-consuming process, often taking several months. Delays and moods during fermentation and the production of unwanted flavors are some of the problems encountered in mead production, often associated with yeast response to stressful conditions unfavorable to their growth (Pereira et al., 2013), also causing negative effects on mead quality and its subsequent marketing. These undesirable conditions may result in volatile acidity and in production of abnormal esters, decreasing organoleptic quality of the final product (O'Connor-Cox and Ingledew, 1991). Furthermore, in many situations honey mixed with brandy is sold under the label of "mead". However, these drinks do not result of any type of fermentation and are only honey liqueurs.

In order to solve some of the problems mentioned above, optimization of fermentation conditions may be advantageous. The traditional method of optimization that takes into consideration one factor at a time is laborious and time consuming. Moreover, the interaction between various factors is ignored. As such, the chances of determining the true optimum conditions are low.

Several statistical methods of experimental design (ED) have been used in development, improvement and optimization of bioprocesses. ED combines technical and statistical knowledge to plan an experiment or series of experiments, involving variables that affect the characteristics of a particular product with the objective to determine the magnitude of change, detect interactions between factors and predict their optimal combinations.

Response Surface Methodology (RSM), originally described by Box and Wilson (1951), is appropriate to identify the effect of individual variables and their interactions and also to find out the best conditions for a multivariable system. RSM is important in the de-

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sign, formulation and development of new products. In addition, it is also effective in improving existing ones. This method has been applied in modeling and optimization of several biochemical and biotechnological processes (Senanayake and Shahidi, 1999, 2002; Castro et al., 2000; Levigne et al., 2002; Vohra and Satyanarayana, 2002; Telez-Luis et al., 2003; Cacace and Mazza, 2003; Li and Fu, 2005; Tanyildizi et al., 2005; Ozer et al., 2006). The main advantage of RSM is to reduce the number of experiments required even when a large number of variables are involved, being less laborious and time consuming than other methods (Wu, 2002). With RSM it is also possible to determine the operating conditions that favor high yields and productivities of certain products (Costa et al., 2001).

Even though some studies on mead production have already been performed (Vidrih and Hribar, 2007; Pereira et al., 2009; Gupta and Sharma, 2009; Mendes-Ferreira et al., 2010; Roldán et al., 2011), the main objective of the present study was to optimize the mead production process by applying RSM. As far as we know, no studies of this type have been undertaken for mead production. Nutrients concentration and temperature were the factors studied, being the aim of the work to develop fermentations without delays and moods, and to find out the best operational conditions to produce mead with high quality that means within the safe limit established for acetic acid and the recommended range for glycerol.

2. Materials and methods

2.1. Microorganism, standards and reagents

The *Saccharomyces cerevisiae* strain used in the present work was the *S. cerevisiae* ph.r. *bayanus* PB2002 of Fermol® Reims Champagne, produced by Pascal Biotech and distributed by the AEB group (Brescia, Italy). The commercial nutrients, Enovit, were from the AEB group (Brescia, Italy). Tartaric acid, sacarose, glucose, fructose, ethanol, glycerol and acetic acid were obtained from Sigma Chemical Co. (St. Louis, USA). Phosphoric acid was purchased to Fisher Scientific (Loures, Portugal).

2.2. Must preparation

Yeast cells (30 g/hL) were hydrated in water with sacarose (50 g/L) and incubated at 35 °C for 20 min, according to the manufacturers' instructions. At the same time, the must was prepared by mixing honey with spring water purchased in a market (395 g/L), followed by the addition of the commercial nutrients at 60, 90 or 120 g/hL, as well as SO_2 (8 g/hL) at 6% (v/v). The mixture was mixed and pH corrected to 3.5 with tartaric acid. All reagents and standards were p.a.

2.3. Fermentation conditions and measured parameters

The fermentations were performed in Erlenmeyer flasks of 500 mL, port sealed with a rubber septum for anaerobic sampling, using a working volume of 250 mL. After inoculation, the fermentations progressed at 20, 25 and 30 °C with nutrients concentrations of 60, 90 and 120 g/hL, as indicated by the experimental design. The fermentations finished after around 216 h (15 days). Temperature was constantly monitored, throughout the fermentations.

Biomass was determined periodically by optical density at 640 nm (Jenway Genova®). Glucose, fructose, ethanol, glycerol, and acetic acid were analyzed individually, following Pereira et al. (2013) method. A Varian HPLC system, equipped with a Rheodyne injector with a 20 μL loop, a Supelco Gel C-610H column (300 × 7.8 mm) at 35 °C and a refractive index detector RI-4 (Varian) were used. Isocratic elution was employed with a mobile phase consisting of 0.1% (v/v) phosphoric acid at a flow rate of 0.5 mL/min. Data was recorded and integrated using the Star Chromatography Workstation software (Varian). Glucose, fructose, ethanol, glycerol and acetic acid were quantified by external standard calibration.

2.4. Experimental design (ED)

In order to study the effect of temperature and salt concentration in mead production, a factorial design 3^2 was used with temperature (x_1) and nutrients concentration (x_2) as the independent variables. Table 1 presents the range and central point values of these variables. The choice of these temperature levels took into account that *S. cerevisiae* present higher fermentation rates at temperatures between 20 °C and 30 °C. On contrary, the fermentation rate decreases significantly for temperatures lower than 15 °C. In this context, high fermentation times would be observed if lower temperatures were applied. Additionally, it is known that the fermentation rate also decreases whentemperatures above 30 °C are used.

The choice of nutrients concentrations (60, 90 and 120 g/hL) was based on observations of previous studies in which some problems, such as fermentations' delays, were encountered when nutrients concentration less than 60 g/hL were used. Thus, it seemed adequate to start from this value and to increase nutrients concentration to 90 and 120 g/hL, in order to evaluate if this increase was needed or not.

In these work a Central Composite Design (CCD) was used with an α equal to 1. Outside points of the "safe" area were not tested. The dependent variables studied in this work were glucose, fructose, ethanol, glycerol and acetic acid contents, determined at the end of the fermentations, and usually regarded as the most important parameters in production of alcoholic drinks.

Analysis of the ED data and calculation of the predicted responses were carried out using the RSM of the Minitab® software. The relationship between dependent and operational variables was established by the model described by Eq. (1) that includes linear, quadratic and interaction terms:

$$y = B_0 + B_1x_1 + B_2x_2 + B_{12}x_1x_2 + B_{11}x_1^2 + B_{22}x_2^2 \quad (1)$$

where y is the dependent variable, B corresponds to the regression coefficients and x to the independent variables. Regarding B parameters: (i) B_0 is a constant; (ii) B_1 and B_2 are the linear coefficients; (iii) B_{11} and B_{22} are the quadratic coefficients; and (iv) B_{12} is the interaction coefficient between variables 1 and 2. Twelve fermentation conditions were tested as described in Table 2. The sequence was randomly established to limit the influence of systematic errors in the interpretation of results. It should be noted that Experiments 1–9 allowed the calculation of the regression coefficients, while Experiments 10–12 were replicas at the central point of the ED, in order to estimate the influence of experimental error. Two fermentations were carried out at each ED point and the mean values were reported as observed responses (Table 2).

3. Results and discussion

3.1. Fermentation kinetics

After performing the 12 experiments, meads with different compositions were obtained (Table 2). Due to the high number of fermentations performed in the present work, the authors decide to only show the fermentation processes for five situations (Fig. 1A–E), namely:

(A) $T = 20\,^{\circ}C$ (level-1) + salt concentration = 120 g/hL (level 1)
(B) $T = 20\,^{\circ}C$ (level-1) + salt concentration = 60 g/hL (level-1)
(C) $T = 30\,^{\circ}C$ (level 1) + salt concentration = 120 g/hL (level 1)
(D) $T = 30\,^{\circ}C$ (level 1) + salt concentration = 60 g/hL (level-1)
} Extreme conditions

(E) $T = 25\,^{\circ}C$ (level 0) + salt concentration = 90 g/hL (level 0)} Central condition

An exponential phase followed by a stationary phase was always stated. It was observed that the end of the exponential phase finished after approximately 70 h in most of the situations, except in Experiments B and D (both performed with 60 g/hL of nutrients) that finished earlier, after around 50 h.

Regarding biomass, the highest concentrations were obtained in Experiments 1A, 1C and 1E. These occurred at 20 °C (A), 25 °C (E) and 30 °C (C) with nutrients concentrations between 90 (E) and 120 g/hL (A and C). On contrary, Experiments B and D performed at 20 and 30 °C with a nutrient concentration of 60 g/hL, originated lower biomass concentrations, suggesting that low nutrients concentrations are undesirable.

In relation to ethanol, contents of 91.4 ± 0.06, 107 ± 0.02, 113 ± 0.03, 115 ± 0.22–119 ± 0.43 g/L were determined in Experiments A–E, respectively. Taking into account all the experiments performed (Table 2), the highest ethanol concentration was determined on the last experiment (12), corresponding to 124 ± 0.1 g/L, performed at 25 °C and 90 g/hL. On contrary, the lowest ethanol concentration was determined in Experiment 6, performed at 20 °C and a salt concentration of 120 g/hL.

Regarding sugars, it was observed in all the experiments that fructose and glucose were consumed during the fermentations in a similar way, as depicted in Fig. 1A–E. Generally, the consumption