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Evaluation of Alcoholic Fermentation During the Production of Mead Using Immobilized Cells in Kappa-Carrageenan

Andrés M. Martínez*[a], Giordan J. Vivas[b], Marta C. Quicazán[c]

[a]Agricultural Sciences Department. National University of Colombia (UNAL). Kra. 30 No. 45-03 Edifice 500. 111321. Bogotá. Colombia.
[b] Chemical and Environmental Engineering Department. National University of Colombia (UNAL). Kra. 30 No. 45-03 Edifice 453. 111321. Bogotá. Colombia.
[c] Institute of Science and Technology of Food (ICTA). National University of Colombia (UNAL). Kra. 30 No. 45-03 Edifice 500C. 111321. Bogotá. Colombia.
ammartinezh@unal.edu.co

Mead is an alcoholic beverage obtained by yeast fermentation of diluted honey. Crystallized honey is not appreciated in Colombian market because people think that this type of honey is false, so it is a cheap and excellent raw material for mead production, providing an important economic alternative for beekeeping chain in this country. The main objective of this work is to assess the fermentative process in mead production by using cells of *Saccharomyces cerevisiae* immobilized in carrageenan and to compare the process with free cells fermentation. Beads of carrageenan were made by the gellification method and the best conditions for it were stablished testing the beads formed in various KCl solutions. 2% (w/v) and 3% (w/v) carrageenan solutions were tested in 2%, 8%, 12% and 16% (v/v) KCl solutions for evaluation of beads formation. The beads were evaluated on its texture properties by a texture profile analysis. The spheres formed in solutions of 3% (w/v) carrageenan and 16% (w/v) potassium chloride showed the best texture results. Fermentation assays were carried out at 30 °C to compare free and immobilized cells, honey was diluted with water to 24 °Bx and pollen was used as nitrogen source. Glucose, fructose and ethanol concentrations were evaluated during the fermentation. Productivity and yield values showed significant differences; productivity with free cells is higher but efficiency with immobilized cells showed the best result. It was possible to reach a better yield, 0.40 g ethanol/g sugar, higher ethanol concentrations in the final product in a fermentation with immobilized cells (11.7% v/v in immobilized, 9.9% v/v in free cell), showing the advantages of the immobilization applied in mead production.

1. Introduction

Mead is an alcoholic beverage made from honey diluted in water or fruit juice. Since ancient times, it is known the empiric production process, similar in some aspects to fermentation in wine production; the consumption of mead is common for rituals and traditional celebrations. Currently, the production of mead is low and is still empiric (Ramalhosa et al., 2011). It is necessary to add a nitrogen source as pollen and the ethanol content in mead is between 8 % and 18 %v/v. Low uniformity in the final product, unpleasant odors, long fermentation times (Iglesias 2014) and some cultural reasons have limited the mead consumption.
Although in most of the countries, crystalized honey is recognized as a good product, in Colombia consumers do not accept it; it has low cost because they think that it has added sucrose for adulteration; frequently beekeepers heat this honey for melting the crystals to have liquid honey to sell. Crystalized honey is rich in reducing sugars (glucose and fructose) and has low water content (<17 %); because of this characteristics, this type of honey is very stable, has low acidity and it is not attacked by microorganisms. So, it is an excellent raw material for mead production; some Colombian beekeepers are interested in improving their artisanal process. The Instituto de Ciencia y Tecnología de Alimentos (ICTA) of Universidad Nacional de Colombia is working on mead production process to take advantage of crystalized honey and create economic value for the benefit of beekeepers (Hernández et al. 2015).

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Traditionally, free cells of *Saccharomyces cerevisiae* have been used carry out alcoholic fermentation. However, the interest in cell immobilization is growing in order to increase profitability and productivity in the process. The immobilized techniques need to be developed in order to allow scaling up for industrial process (Kourkoutas et al. 2004). There are advantages using immobilized cells in biotechnological process, comparing to traditional ones: easier handling, higher cell density, better control in continuous systems, higher cell stability, lower recovery of biomass costs and better environmental stress protection for the cells (Margaritis and Merchant, 1984).

Few studies related to mead making with immobilized cells has been reported. Some authors have used immobilization matrix such as calcium pectate gels (Navrátil et al., 2001), calcium alginate (Qureshi and Tamhane, 1985) and alginate chitosan mixture (Pereira et al., 2014). Alginate gel is one of the most used matrix for immobilization process in fermentation; however, there are other substances suitable for its use as immobilization support such as: gluten pellets (Bardi et al. 1996), pectate (Rühlemann et al. 1990), and carrageenan (Tampion and Tampion 1987). Carrageenan is a sulfated polysaccharide obtained from certain red algae species; it has been classified according to its sulfation grade as gamma, kappa and iota. Kappa and iota carrageenan are more adequate to gel formation (Necas and Bartosikova, 2013). There are no studies related with cell immobilization in carrageenan applied to mead production; carrageenan has been used in production of other alcoholic beverages as wine (Gòdia et al. 1991) or beer (Kourkoutas et al. 2004).

The aim of this work was to find the best conditions for immobilizing yeast cells in carrageenan and compare their performance in the mead production with respect to the process with free cells. The study is based on previous results for fermentation conditions (water/honey ratio, fermentation temperature, nitrogen source, yeast type) obtained in the Instituto de Ciencia y Tecnología de Alimentos of the Universidad Nacional de Colombia.

2. Materials and Methods

2.1 Must preparation for fermentation

Honey from the department of Boyacá (Colombia) were diluted in water until reaching a sugar concentration equivalent to 24 °Bx, bee pollen was added in a proportion of 5 g/kg of total solution to act as nitrogen source, the final mixture is known as must. The must was pasteurized at 65 °C for 20 minutes.

2.2 Free cell fermentation

In the free cell fermentation, 0.5 g/L of *Saccharomyces cerevisiae* ex. Bayanus (Uvaferm BC) yeast were activated for 20 minutes at 37 °C in 10% of the total volume of the must. Then the inoculum was added to the remaining 90% of the initial must. The reactors used were glass flasks with one liter of capacity, with a metallic cap with adaptations of hoses for sampling and carbon dioxide vent. Fermentation was made at a temperature of 30 °C, stirring often the bio-reactor.

2.3 Immobilization conditions selection.

The matrix for immobilization was kappa-carrageenan, gellified over a solution prepared of potassium chloride salt of analytical grade distributed by MERK. For the carrageenan bead formation, 2% and 3% (w/v) carrageenan solution were made. The beads were formed through the drip method, which consists of loading the carrageenan solution in a syringe without piston and flowing of the solution through the nozzle by force of gravity, droplets of the solution enter in contact with a potassium chloride solution. The salt solution was tested in four levels (2%, 8%, 12% and 16% w/v) Hardness and other texture properties of the beads were measured using a TPA (*Texture Profile Analysis)* with a texturometer TA- XT2 (Stable Micro Systems). In the assay, 15 beads were placed in the sample holder and were pressed until half; a cylindrical probe of 31 mm diameter was used with a probe speed of 0.5 mm/s. This procedure was made for each carrageenan-KCl levels combination.

2.4 Immobilized cells fermentation

The yeasts (concentration: 0.5 g per liter of total must) were activated on 2.5% of the must volume in a temperature of 37 °C for 10 minutes. The must with the yeast were mixed with a 4% (w/v) carrageenan solution in a carrageenan: must ratio of 3:1 a 3 % (w/v) carrageenan solution was obtained. The mixture was located in a syringe without piston that it was flowed through the nozzle dripping in a 16% (w/v) potassium chloride solution at 4 °C. Once they were formed, the beads were stabilized at 4 °C for 24 hours. After that, they were washed with distilled water and added to the reactors with the remaining fraction of the must. Fermentation was made at 30 °C temperature, stirring often the bio-reactor.

2.5 Monitoring of the fermentation

Sugar consumption monitoring was made measuring the soluble solids content (°Bx) using a refractometer RSG-100/ATC (Sino Tech). Concentrations of fructose, glucose and ethanol were determined by HPLC. A

HPLC equipment with LC-2000 RI detector (JASCO), cation exchange column Sugar Pak I (300 x 6.5 mm x 10 µm, WATERS). Mobile phase was water type I in isocratic mode with flow of 0.47 mL/min; injected sample volume was 20 µL; oven temperature was 80 °C and detector temperature was 35 °C; run time was 20 minutes. The alcoholic grade was determined according to the Colombian standard NTC 5113, with a sample volume of 200 mL distilled until 140 mL. The distilled was completed with distilled water until 200 mL in a volumetric flask; the alcoholic grade of this mixture was measured with an alcoholmeter (ICONTEC, 2003).
Total acidity were determined by titration of a sample of the mead, NaOH 0.1 N was added until pH of 8.2, according to AOAC 962.19 (AOAC,2012). pH of the sample was measured in 10-15 mL of the product kept at 20 °C. Acetic acid, citric acid, malic acid and succinic acid determination was made through HPLC described formerly, equipped with a ion-exchange Rezex-ROA (300 x 7.8 mm x 8 µm) at 40 °C, sulfuric acid solution at 5 mM was used as mobile phase, at 0.5 mL/min and UV/VIS detector set at 205 nm.

2.6 Determination of yield, productivity and efficiency

Yield of ethanol (Yp/s) was calculated according to the ration between the produced ethanol and the consumed sugar. Productivity was calculated with the ratio between ethanol concentration per litter and the fermentation time. Efficiency was calculated with the ratio between the experimental ethanol produced per gram of glucose and its theoretical value (0.51 g ethanol/g of glucose).

2.7 Statistical analysis

The comparison between free cell and immobilized cell fermentation variables were done with analysis of variance (ANOVA) by using statistical software Statgraphics centurion ®.

3. Results

3.1 Beads characterization and immobilization conditions selection

The beads shape was irregular for 2% and 3% (w/v) carrageenan solution with 2% (w/v) potassium chloride solution, for the other concentrations of KCl (8%, 12%, and 16% w/v) the beads had sphere-like shape, however, none of the beads obtained were completely spherical. It was observed that in a higher concentration of carrageenan and potassium chloride solution, the beads had better characteristics of shape and deformation resistance. Nonetheless, some tests developed with a carrageenan solution of concentration above 3% (w/v), the bead formation was difficult producing gels without shape. Average size of the carrageenan beads was 3.9 ± 0.2 mm. The TPA () was not used on the beads which resulted of the potassium chloride solution of 2% (w/v) due to its fragility during the manipulation. The results obtained from the TPA of the 3% (w/v) carrageenan solution are shown in Table 1. Data analysis show that there are significant differences for the variables such as hardness, gumminess and chewiness ($p<0.05$), observing an increase in the values of the TPA parameters with the increase of the potassium chloride solution concentration. The potassium salt concentrations used in this study are greater than the used in other studies using carrageenan, 2.2% (w/v) (King and Zall, 1983) and 5.2% (w/v) (Sankalia et al. 2006).

Table 1: Texture analysis for carrageenan beads of 3% w/v with different potassium chloride solutions

KCl (%w/v)	Hardness (g)	Adhesiveness (g/s)	Springiness	Cohesiveness	Gumminess (g)
16	351±13.9^{a}	-11.4±1.1^{a}	0.89±0.02^{a}	0.62±0.005^{a}	218±6.7^{a}
12	310±1.3^{b}	-10.9±2.6^{a}	0.86±0.03^{a}	0.61±0.005^{ab}	190±1.0^{b}
8	265±6.1^{c}	-14.6±1.9^{a}	0.84±0.02^{a}	0.60±0.004^{b}	160±3.7^{c}

Different letters on the same column means significant differences. ($p<0.05$)

3.2 Immobilized cells fermentation

Carrageenan solution of 3% (w/v) and potassium chloride solution of 16% (w/v) were chosen to continue the fermentation process, due to that beads formed in those conditions had better shape and resistance characteristics. After first 24 hours of fermentation, carrageenan beads showed an increase in their diameter of about 30%; high CO_2 concentrations produced inside the spheres are able to cause changes in the strength of the gel structure (King and Zall, 1983). Other factors as low pH combined with temperature can cause gel instability, observing some of the spheres shredded or with non-spherical shape in later stages of the fermentation (Imeson, 1997).
In the Figure 1 are shown the behaviour of the sugars during the fermentation process. In free cell fermentation is observed that the value of °Bx stabilized in the eleventh day, whereas for the same day, in the immobilized cell fermentation still there is sugar consumption, stopping on the seventeenth day; fermentation times have significant differences ($p<0.05$). Final soluble solids concentration (°Bx) also had significant

differences between both experiments ($p<0.05$), immobilized cells had the lower value, evidencing a higher sugar consumption.

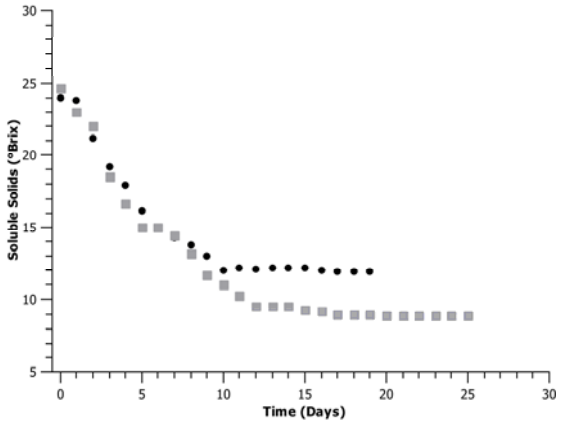

Figure 1: Behaviour of the soluble solids content during the alcoholic fermentation of mead with free cells (●) and immobilized cells (■)

3.3 Production of ethanol and sugar consumption

The results about the alcoholic grade shows that the product obtained using free cell has an ethanol concentration of 9.95% (v/v), while with immobilized cells was 11.77% (v/v) obtaining significant differences ($p<0.05$). In similar studies of mead, immobilized cell fermentation got a lower alcohol concentration compared to free cell (10.5% v/v vs 11.2% v/v), with yeast immobilized in alginate gel (Pereira et al. 2014). In wine fermentation were reported similar results as Pereira (Oliveira et al. 2011). Some studies report that carrageenan as immobilization matrix provides protection of yeast to environmental conditions such as inhibitory concentration of ethanol (Norton et al. 1995; Barros et al. 1987), this may explain the higher ethanol content achieved in immobilized fermentation.

The data obtained by HPLC (Table 2) shows that the main sugar concentrations in honey (glucose and fructose) decreases below 1% (w/v), with a higher speed at the beginning of the fermentation and a preferential consumption by the glucose. The values obtained for ethanol using free cells are similar to those reported by other authors (Mendes-Ferreira et al. 2010; Pereira et al. 2013).

Table 2: Monitoring of ethanol and reducing sugars in the fermentation with free and immobilized cells

Free Cell				Immobilized Cell			
Time (days)	Ethanol (% v/v)	Glucose (% w/v)	Fructose (% w/v)	Time (days)	Ethanol (% v/v)	Glucose (% w/v)	Fructose (% w/v)
0	0±0.00	11.18±0	12.18±0.12	0	0±0.00	11.5±0.71	12.4±0.60
3	4.26±0.04	8.45±0.25	8.12±0.00	1	0.32±0.03	8.6±4.56	9.01±5.42
5	6.27±0.12	4.35±0.02	5.97±0.02	4	2.37±0.89	3.9±0.38	6.48±0.69
7	7.7±0.3	1.16±0.66	4.35±0.03	7	6.35±1.68	3.08±2.05	5.8±2.23
9	9.92±0.38	0.98±0.69	3.3±0.18	11	NR	0.16±0.09	3.4±0.14
14	10.5±0.3	0.28±0.00	0.86±0.06	12	6.9±1.46	NR	NR
18	10.11±1.14	0.67±0.13	0.93±0.05	14	10.28±2.23	0.22±0,4	0.78±0.98
				18	12.31±0.27	0.22±0.02	0.73±0.01
NR: value not reported				26	12.52±0.62	0.22±0.02	0.37±0.04

3.5 Total acidity, volatile acidity and pH

Physicochemical quality parameters (Total acidity, volatile acidity, and pH) are presented in Table 3; the values of the parameters are compared to the Colombian technical standard for fruit wine (ICONTEC 2000), as in Colombia there are no specific regulations for mead.

Table 3: Total acidity, volatile acidity and pH in the final product

	Total Acidity (g/L)	Volatile Acidity (g/L)	pH	Citric acid (g/L)	Malic acid (g/L)	Succinic acid (g/L)
Free cell	3.60 ± 0.07[a]	0.17 ± 0.04[a]	3.66 ± 0.01[a]	0.27 +0.05 [a]	3.63+0.96[a]	2.43+0.35[a]
Immobilized cell	3.27 ± 0.04[a]	0.22 ± 0.01[a]	4.00 ± 0.01[b]	0.29+0.01[a]	3.59+0.09a	2.48+0.08[a]
Reference value (NTC 708)	3.5-10	<1.2 g/L	2.8-4.0	N/A	N/A	N/A

Different letters on the same column means significant differences. ($p<0.05$)

Total acidity for free cell fermentation was in the range defined by Colombian regulations, nonetheless total acidity for immobilized cell fermentation is below the lower limit. The values for total acidity did not have significant differences. Total acidity in both fermentations are similar to that reported by some authors, 4.2 g/L (Acosta, 2012) 6.58 to 6.96 g/L (Pereira et al., 2014) 3.1 to 7.7 g/L (Mendes-Ferreira et al. 2010). Volatile acidity (acetic acid) was far below of the limit value set by Colombian regulations for fruit wines; the experiments did not have significant differences. Compared with the values reported by other authors, 0.5 g/L (Acosta, 2012), 0.34 to 0.43 g/L (Pereira et al. 2014), 0.51 to 0.84 g/L (Mendes-Ferreira et al. 2010) and 0.54 g/L (Gomes, 2013), the volatile acidity values of this study were lower. Significant differences were found on pH values, the immobilized cell fermentation had a higher value, which is in agreement with lower acidity values. Measured pH is alike with values reported by other authors, 3.2 (Acosta 2012), 3.60 to 3.67 (Pereira 2014) and 3.27 to 3.67 (Mendes-Ferreira et al. 2010). The values of citric acid, malic acid, and succinic acid did not have significant differences, showing that they are not affected whether the fermentations are with immobilized or free cells.

3.6 Yield, productivity and efficiency

According to the results shown in Table 4 fermentations with immobilized cells presented a higher increase in the performance and the efficiency in the process and lower productivity with respect to fermentation with free cells, this last one due to a longer time with immobilized cells.

Table 4: Performance, productivity and efficiency in fermentation with free cells and immobilized cells

	Ethanol (% w/v)	Initial Sugars (%w/v)	Final Sugars (% w/v)	Consumed Sugars (% w/v)	Yield (g. ethanol/ g. Sugar)	Time (h)	Productivity (g/L-h)	Efficiency (%)
Free	7.86±0.19 [a]	23.40±0.12	1.59±0.18	21.80±0.30	0.36±0.00[a]	288±0	0.27±0.01[a]	70.80±0.78[a]
Immobilized	9.30±0.09 [b]	23.90±0.08	0.59±0.11	23.30±0.19	0.40±0.01[b]	480±24	0.19±0.01[b]	78.14±1.42[b]

Different letters on the same column means significant differences. ($p<0.05$)

4. Conclusions

The immobilization of *Saccharomyces cerevisiae* in carrageenan beads is an alternative for mead production, as in its fermentation process is evidenced a higher concentration of ethanol compared with the concentration of ethanol in the fermentation with free cells,11.7 %v/v, 9.95 %v/v respectively. The fermentation process with immobilized cells had a higher yield compared to free cells (0.4 g ethanol/g sugar vs 0.36 g ethanol/g sugar) but their productivity was significantly lower. Regarding to the formation of beads of carrageenan a higher concentration in the solution of carrageenan and potassium salt improves the stability conditions of the beads; however very high concentrations of carrageenan make difficult the formation of them. For this investigation concentrations of carrageenan (3 %w/v) and KCl (16 %w/v) were chosen as the most appropriate.

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