Optimization of Bioethanol Production from Coffee Mucilage

Bianca Yadira Pérez-Sariñana,[a,*] Antonio De León-Rodriguez,[b] Sergio Saldaña-Trinidad,[c] and Sebastian Pathiyamattom Joseph [a,*]

A response surface methodology with 2^k full factorial design was applied to obtain optimum conditions for bioethanol production using coffee mucilage (CM) as the substrate and *Saccharomyces cerevisiae* NRRL Y-2034 as the inoculum. CM is an agro-industrial residue mainly composed of simple sugars; the product yield and productivity process were analyzed with respect to the fermentation, pH, temperature, and the initial sugar concentration. Employing the following predicted optimum operational conditions attained the highest bioethanol production: pH 5.1, temperature 32 °C, and initial sugar concentration 61.8 g/L. The estimated bioethanol production was 15.02 g/L, and the experimental production was 16.29 g/L ± 0.39 g/L, with a bioethanol yield of 0.27 g/L and a productivity process of 0.34 g/Lh. Glycerol was the predominant byproduct of the fermentative metabolism of *S. cerevisiae*. The response surface methodology was successfully employed to optimize CM fermentation. In the fermentative processes with yeast, optimizing the conditions of the culture medium is needed to fully exploit the potential of the strains and maximize the production of bioethanol.

Keywords: Fermentation; Bioethanol production; Optimization

Contact information: a: Instituto de Energías Renovables-UNAM, Temixco, Morelos, 62580, México;
b: Instituto Potosino de Investiación Cientifica y Tecnológica, San Luis Potosí S.L.P, 78216, México;
c: Universidad Politécnica de Chiapas, Tuxtla Gutiérrez, Chiapas, 29010, México;
** Corresponding authors: bipes@ier.unam.mx, sjp@ier.unam.mx*

INTRODUCTION

Carbon dioxide emissions affect the environment by contributing to global warming; because of this, it is necessary to find alternative sustainable energy sources (Kapdan and Kargi 2006). Bioethanol production by alcoholic fermentation has received a great deal of attention in recent years due to the high demand for fuel and need to supplement gasoline (Balat and Balat 2009; Yan and Lin 2009). One of the main benefits of this change is that biomass fuel is renewable and can potentially provide a sustainable fuel supply in the long term (Mabee and Saddler 2010).

Coffee is one of the most consumed beverages worldwide and is the second most commercialized product, after petroleum products (Mussatto *et al*. 2011). Due to the high demand for this product, large amounts of waste are generated in the coffee industry, which are toxic and pose serious environmental problems (Mussatto *et al*. 2012). Chiapas, Mexico, is one of the largest coffee producing states. The extraction process of the coffee bean generates waste as pulp, mucilage, and husk. Recently, investigations have been made using these residues for bioenergy generation (Mussatto *et al*. 2010; Mussatto *et al.* 2011; Choi *et al.* 2012).

By employing the response surface methodology and a 2^k full factorial design, the influence of pH, temperature, and initial sugar concentration of coffee mucilage were determined in this study, with the main objective to optimize the production of bioethanol.

EXPERIMENTAL

Materials and Methods

Substrate

Coffee mucilage (CM) was extracted using a mechanical extractor located in the municipality of Las Rosas, Chiapas, México. The composition was 4 kg of coffee cherry per liter of water. This was supplemented with 0.5 g/L ammonium sulphate as a nitrogen source (De Leon-Rodríguez *et al.* 2008). The CM was centrifuged at 7,000 rpm for 10 min, pasteurized at 65 °C for 25 min, and chilled for 20 min on ice (Alvarado-Cuevas *et al*. 2013). The initial pH was 4.5. The chemical composition of coffee mucilage is shown in Table 1.

Microorganism cultivation and preparation

Saccharomyces cerevisiae NRRL Y-2034 was obtained from the strain collection center of the Universidad Politécnica de Chiapas. The strain was maintained in YPD agar (1% w/v yeast extract, 2% w/v peptone, 2% w/v glucose, and 2% w/v agar) slants at 4 °C, and fresh cultures (24 to 48 h) in YPD were used as inocula. Strain was cultured in 250 mL shake serological bottles rotated at 200 rpm at 28 °C and using the growth medium and fermentation conditions as previously described. Growth proceeded overnight for 24 h to allow cell growth to reach the exponential phase, after which the broth was centrifuged at 10,000 rpm for 10 min, and the cells were re-suspended in the fermentation medium.

Fermentation

Batch fermentation experiments were carried out in serological bottles of 100 mL with constant shaking at 200 rpm for 48 h, using the initial culture conditions described in the experimental design (Table 2). Cell density was adjusted to an optical density (OD 600 nm) of 0.5. Culture samples of 1 mL were taken every 3 h and centrifuged at 10,000 rpm for 10 min. The supernatant was filtered through a 0.22 μm filter (Millipore, Bedford, MA, USA) after it was stored at -20 °C for later analysis. The validation of optimal conditions was assessed in triplicate.

In general *Saccharomyces cerevisiae* prefers acid pH (pH range of 3 to 7) (Pitt and Hocking 2009), the optimum pH is about 5 to 5.2 (Campbell 2003). *S cerevisiae* grow optimally between 25 and 30 ºC (Spencer and Spencer 1997) or 30 and 33 ºC (Campbell 2003). Its minimum growth temperature is reported as 4 ºC with a maximum growth temperature of 38 to 39 ºC (Pitt and Hocking 2009).

Based on the previous references, the ranges of pH of 4.5 to 5.5 and temperature of 28 to 38 ºC were selected for the present work. High and low sugar concentrations present in coffee mucilage were selected as 65 g/L and 35 g/L.

Bioethanol production (*BP*) is calculated as the amount of ethanol produced per liter of culture medium at the end of fermentation. The process yield (product/substrate) ($Y_{P/S}$, Eq. 1) is the amount of ethanol produced per sugar consumed, and the productivity process (*PP*, Eq. 2) is the amount of ethanol produced per liter and per hour. The process parameters were obtained as follows,

$$Y_{P/S} = \frac{P_f - P_i}{S_f - S_i} \quad (1)$$

$$PP = \frac{BP}{t} \quad (2)$$

where $Y_{P/S}$ is the process yield, P_f is the final concentration of bioethanol (g/L), P_i is the initial concentration of bioethanol (g/L), S_f is the final sugar concentration (g/L), S_i is the initial sugar concentration (g/L), *PP* is the productivity process (g/Lh), *BP* the bioethanol produced (g/L), and *t* is the time (h).

Experimental design

A 2^k full factorial design with three levels leading to 20 sets of experiments was realized to evaluate the effect of pH (factor X_1), temperature (factor X_2), and the initial sugar concentration (factor X_3) as independent variables of the fermentation. The following equation (Eq. 3) was used to build surface graphs for the model for each response variable and for predicting the optimal value,

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (3)$$

where *Y* is the predicted response corresponding to the bioethanol at the end of the fermentation process, X_1, X_2, and X_3 are independent variables, β_0 is an offset term, β_1, β_2, and β_3 are linear effects, β_{12}, β_{13}, and β_{23} are interaction terms, and β_{11}, β_{22}, and β_{33} are quadratic coefficients. The model was evaluated with significance, goodness of fit, and the R^2 values. The optimal values were obtained by solving the regression equation. The analysis of the response surface, the ANOVA, and the optimal conditions were obtained using the commercial software (Statgraphics Centurion XVI, Manugistics Inc., MD, USA). The adjusted models for *BP*, $Y_{P/S}$, and *PP* were evaluated by ANOVA analysis. The significant effects for the dependent variables were determined by a *t*-test with a probability value smaller than 0.05 (*P*-value <0.05).

Analysis

The protein concentration was determined by the Lowry method using bovine-serum-albumin (BioRad, CA, USA) as a standard (Lowry *et al.* 1951). The sugars, ethanol, glycerol, organic acids, and toxic compounds were determined by high-performance liquid chromatography (HPLC), using a column (Phenomenex, inc. USA) eluted at 60 °C with 0.0025 M H_2SO_4 at a flow rate of 0.5 mL/min and having a refractive-index detector. The redox potential was measured online with an autoclavable redox electrode (Applikon, Schiedam, Netherlands), and the data were registered in a PC interfaced with a potentiometer (B&C Electronics, Italy) using a RS232 port. Minerals were determined by ICP-OES (Inductively coupled plasma – Optic emission spectroscopy, Varian) device. Other compounds of CM were analyzed in a gas chromatograph 7820A, coupled with a mass spectrometer 5977E (Agilent Technologies, United States) using a 5% phenyl-methyl silicon capillary column 30 m long, 250 µm in inner diameter, and 0.25µm-film thickness. The MS detector was operated under electron impact ionization at 70 eV using the Scan mode at 45 to 450 aum. The compounds were identified by comparing their mass spectra to those obtained in the NIST 11 library of the MS database.

RESULTS AND DISCUSSION

Coffee Mucilage Composition

Carbohydrates are the most important constituents in coffee mucilage; for samples analyzed here, the sugar composition in CM was 37.67 g/L galactose, 35.65 g/L glucose, 1.06 g/L lactose, and 0.1193 g/L proteins (Table 1).

Table 1. Chemical Composition of Coffee Mucilage

Components	Concentration (g/L)
Glucose	35.65
Galactose	37.67
Lactose	1.06
Protein	0.119
Syringaldehyde	0.6100
Minerals	(mg/L)
Aluminum	nd
Arsenic	0.47
Sulfur	30.19
Boron	0.16
Barium	0.02
Beryllium	nd
Calcium	37.08
Cadmium	nd
Cobalt	nd
Chrome	nd
Copper	2.45
Iron	0.65
Potassium	239.8
Lithium	0.01
Magnesium	10.05
Manganese	0.07
Molybdenum	nd
Sodium	7.18
Nickel	0.01
Phosphorus	41.55
Lead	nd
Antimony	nd
Selenium	nd
Silicon	1.58
Tin	nd
Strontium	0.07
Thallium	nd
Vanadium	nd
Zinc	0.14

nd: not detected

Also, syringaldehyde, which is produced by lignin hydrolysis, was found in low concentrations. According to the ICP-OES analysis, CM contains several minerals. Potassium was the most abundant element, followed by phosphorus, calcium, sulfur, and magnesium (Table 1). Other compounds were also found, such as glycerine, caffeine, acetic acid, lactic acid, phenol, as well as 2,6 and 3,4-dimethoxyphenol.

Optimization of Fermentation Conditions

Table 2 shows a summary of the results for *BP*, $Y_{P/S}$, and *PP*. The *BP* achieved a maximum value 14.93 g/L and a minimum value of 8.28 g/L. The $Y_{P/S}$ values ranged between 0.19 to 0.32, while the *PP* achieved a maximum value of 0.31 g/Lh, and a minimum value of 0.17 g/Lh.

Table 2. Experimental Design and Summary of Results for Dependent Variables

	Independent variable			Dependent variable		
Treatment	Factor X_1	Factor X_2 (ºC)	Factor X_3 (g/L)	*BP* (g/L)	$Y_{P/S}$(-)	*PP* (g/Lh)
1	5.05	32.5	50	13.60	0.28	0.28
2	5.7	28	65	14.38	0.23	0.30
3	5.7	28	35	10.93	0.32	0.23
4	5.05	39.36	50	13.99	0.28	0.29
5	5.05	32.5	50	14.16	0.29	0.29
6	4.4	37	65	14.42	0.23	0.30
7	5.05	32.5	72.87	13.67	0.19	0.28
8	6.04	32.5	50	13.47	0.27	0.28
9	5.05	32.5	50	14.10	0.29	0.29
10	5.05	32.5	50	14.93	0.30	0.31
11	4.4	28	35	9.01	0.26	0.19
12	4.4	37	35	9.06	0.27	0.19
13	5.7	37	65	14.20	0.22	0.29
14	5.05	32.5	50	14.35	0.29	0.30
15	5.05	32.5	27.13	8.28	0.31	0.17
16	4.4	28	65	14.92	0.23	0.31
17	4.06	32.5	50	13.06	0.27	0.27
18	5.05	32.5	50	13.76	0.28	0.29
19	5.05	25.64	50	13.06	0.26	0.27
20	5.7	37	35	8.82	0.26	0.18

X_1: pH, X_2: temperature, X_3: sugar concentration. *BP*: ethanol production, $Y_{P/S}$: product yield, *PP*: productivity process

The statistical significance of the corresponding model equation was checked by F test analysis of variance (ANOVA, Tables 2, 3, and 4).

The adequacy of the models was expressed by the coefficient of correlation R^2, which was 0.97, 0.89, and 0.96. These values indicated that 97, 89, and 96% of the

variability of response in the bioethanol production, yield process, and productivity process, respectively, were explained by the model. Significant differences were indicated by a probability value less than 0.05 in ANOVA analysis.

The analysis ANOVA showed that *BP* was significantly affected by X_3 and X_3^2 (Table 3). The equation describing *BP* (Eq. 4), as a function of pH, temperature, and concentration of sugar, is as follows:

$$BP = -54.353 + 9.756X_1 + 1.058X_2 + 0.903X_3 - 0.555X_1^2 - 0.011X_2^2 + 0.006X_3^2 - 0.086X_1X_2 - 0.023X_1X_3 + 0.002X_2X_3 \quad (4)$$

Table 3. Analysis of Variance for the Adjusted Model for Bioethanol Production

Source	Polynomial coefficient	Sum of squares	d.f.	Mean square	F-Ratio	*P*-Value
Coefficient	-54.35					
X_1	9.76	0.18	1	0.18	0.53	0.48
X_2	1.06	0.22	1	0.22	0.66	0.43
X_3	0.90	73.82	1	73.82	218.72	0
X_1^2	-0.56	0.59	1	0.59	1.76	0.21
$X_1 X_2$	-0.09	0.51	1	0.51	1.52	0.25
$X_1 X_3$	-0.02	0.41	1	0.41	1.21	0.30
X_2^2	-0.01	0.59	1	0.60	1.76	0.21
$X_2 X_3$	0.002	0.148	1	0.15	0.44	0.52
X_3^2	-0.006	26.17	1	26.16	77.53	0
Total error		3.37	10	0.34		
Total (corr.)		106.02	19			

X_1: pH, X_2: temperature, X_3: initial sugar concentration, d.f.: degrees of freedom, F: Fisher test, *P*-value: probability distribution value. The correlation coefficient (R^2) was 0.97, adjusted correlation coefficient was 0.94 and the standard error was 0.58.

The maximum value of *BP* was 14.92 g/L, and it was obtained when the pH, temperature, and initial sugar concentration were 5.05, 32.5 °C, and 50 g/L (Fig. 1), respectively. A maximum point is observed in the sugar concentration, low pH there is a low bioethanol production. Because of this we can say that with increasing sugar concentration in the medium increases bioethanol production.

The analysis indicated that $Y_{P/S}$ was significantly injured by the X_3 and X_3^2 (Table 4). The equation 5 describing $Y_{P/S}$, as a function of pH, temperature, and concentration of sugar is as follows:

$$Y_{P/S} = -0.9236 + 0.2396X_1 + 0.0248X_2 + 0.0097X_3 - 0.9113X_1^2 - 0.00024X_2^2 - 0.0001X_3^2 - 0.0027X_1X_2 - 0.00062X_1X_3 + 0.00008X_2X_3 \quad (5)$$

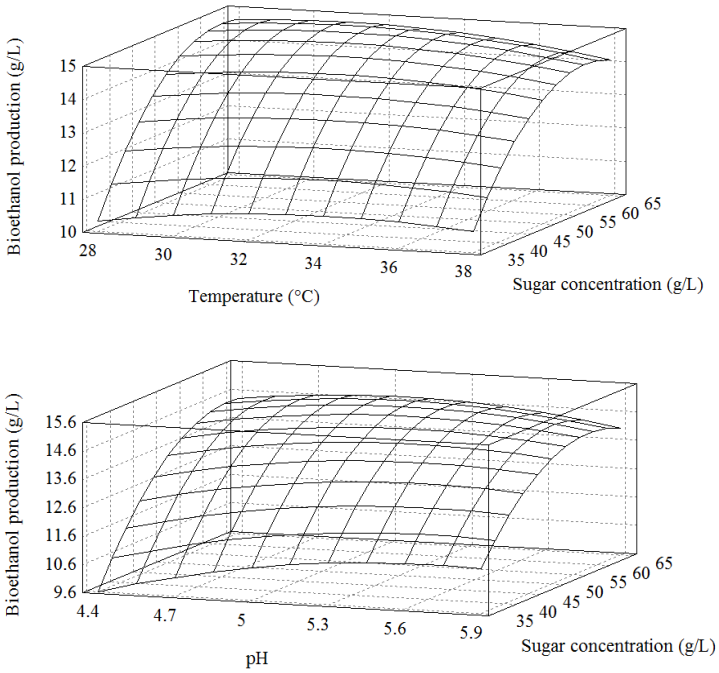

Fig. 1. Response surfaces described by the models representing the dependence of *BP* on the pH, temperature, and initial sugar concentration

Table 4. Analysis of Variance for the Adjusted Model for Yield Process

Source	Polynomial coefficient	Sum of squares	d.f.	Mean square	F-Ratio	*P*-Value
Coefficient	-0.9236					
X_1	0.24	0.0002	1	0.0002	0.86	0.37
X_2	0.02	0.0001	1	0.0001	0.76	0.40
X_3	0.009	0.008	1	0.008	43.56	0.0001
X_1^2	-0.01	0.0002	1	0.0002	1.34	0.27
$X_1 X_2$	-0.002	0.0005	1	0.0005	2.71	0.13
$X_1 X_3$	-0.0006	0.0003	1	0.0003	1.62	0.23
X_2^2	-0.0002	0.0003	1	0.0003	1.46	0.25
$X_2 X_3$	0.0001	0.0003	1	0.0003	1.38	0.27
X_3^2	-0.0001	0.007	1	0.007	35.56	0.0001
Total error		0.001	10	0.0002		
Total (corr.)		0.02	19			

The correlation coefficient (R^2) was 0.89, adjusted correlation coefficient was 0.81 and the standard error was 0.01.

The maximum value of $Y_{P/S}$ was 0.32, and it was obtained when the pH, temperature, and initial sugar concentration were 5.7, 28 ºC, and 35 g/L (Fig. 2), respectively. It can be noted that as the sugar concentration increased, there were decreases in the process yield. When the temperature was maintained constant with a low pH, the process yield was low.

S. cerevisiae do not grow and do not produce ethanol at 5 g/L syringaldehyde. In the fermentations containing a variety of toxic compounds, there is no synergetic effect of multiple inhibitory compounds (Lee *et al*. 2010). In this work the yeast grew, but there was evidence of inhibition in bioethanol production. Therefore there was a low process yield. The inhibition was attribution to the main toxic compounds as syringaldehyde, phenol, acid acetic, and caffeine being present in coffee mucilage.

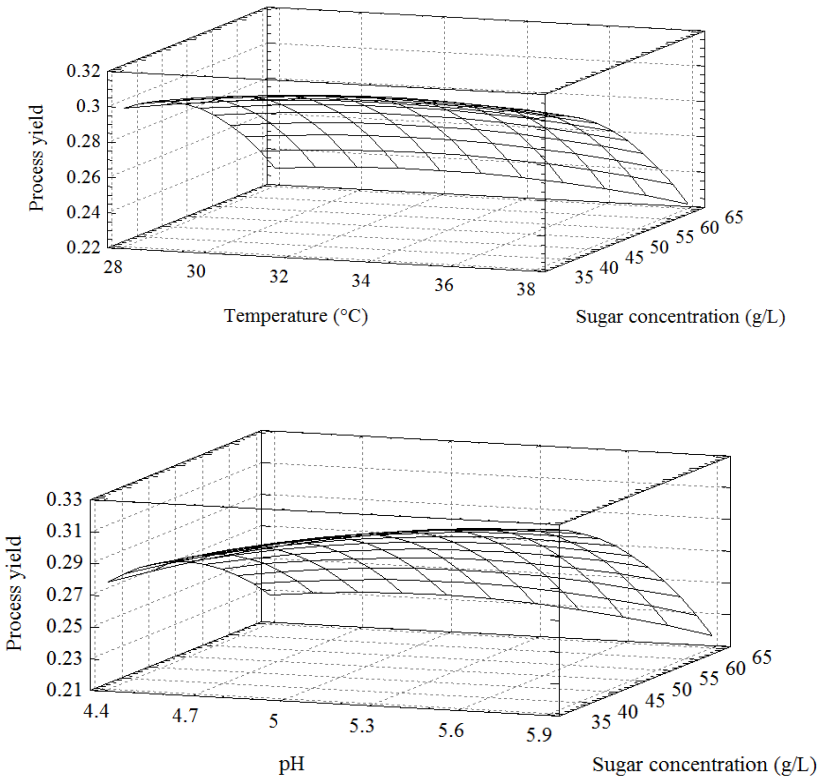

Fig. 2. Response surfaces described by the models representing the dependence of $Y_{P/S}$ on the pH, temperature, and initial sugar concentration

The ANOVA showed that *PP* was significantly injured by the X_3 and X_3^2 terms (Table 5). The equation describing *PP* (Eq. 6), as a function of pH, temperature, and concentration of sugar, is as follows:

$$PP = -1.1915 + 0.2148X_1 + 0.0236X_2 + 0.0190X_3 - 0.0127X_1^2 - 0.00026X_2^2 - 0.00014X_3^2 - 0.0018X_1X_2 - 0.00048X_1X_3 + 0.00004X_2X_3 \quad (6)$$

The maximum value of *PP* was 0.31 g/Lh, and it was obtained when the pH, temperature, and initial sugar concentration were 5.05, 32.5 ºC, and 50 g/L (fig. 3), respectively. The same condition for *BP*, since *PP* is a function of time, as defined in equation 6.

Table 5. Analysis of Variance for the Adjusted Model for Productivity Process

Source	Polynomial coefficient	Sum of squares	d.f.	Mean square	F-Ratio	*P*-Value
Coefficient	-1.19					
X_1	0.21	7.75E-05	1	7.74E-05	0.46	0.51
X_2	0.02	9.61E-05	1	9.61E-05	0.57	0.47
X_3	0.02	0.03	1	0.03	190.84	0
X_1^2	-0.01	0.0003	1	0.0003	1.85	0.20
$X_1 X_2$	-0.001	0.0002	1	0.0002	1.33	0.28
$X_1 X_3$	-0.0004	1.76E-05	1	0.0002	1.05	0.33
X_2^2	-0.0002	0.0003	1	0.0003	1.85	0.20
$X_2 X_3$	4.18E-05	6E-05	1	6.38E-05	0.38	0.55
X_3^2	-0.0002	0.01	1	0.01	69.73	0
Total error		0.002	10	0.0002		
Total (corr.)		0.05	19			

The correlation coefficient (R^2) was 0.96, adjusted correlation coefficient was 0.93, and the standard error was 0.01.

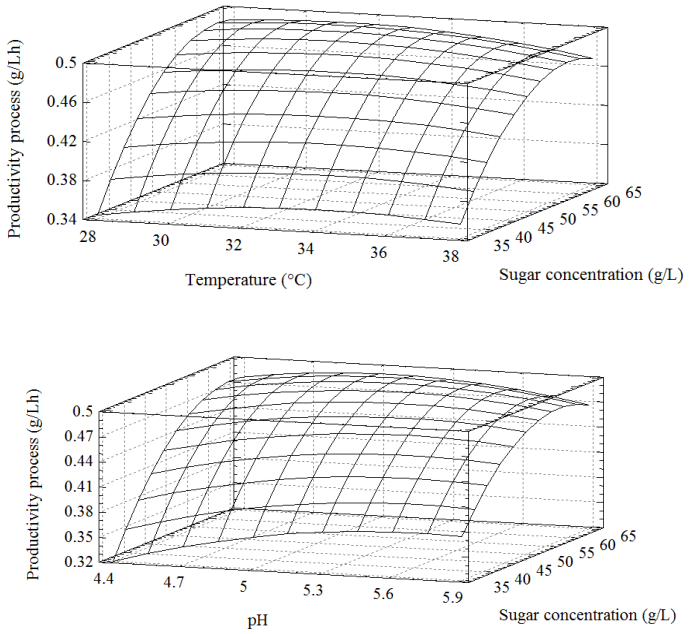

Fig. 3. Response surfaces described by the models representing the dependence of *PP* on the pH, temperature, and initial sugar concentration

Bioethanol is a biofuel; several investigations on the optimization of bioethanol production have been published (Chen 1981; Balusu *et al*. 2005; Bandaru *et al*. 2006, Pereira *et al*. 2010). Criteria such as yields, productivity process, and bioethanol production were used to evaluate the fermentations. For instance, a high bioethanol production and high process yield are not compatible, because the first one requires low substrate

concentrations, which cause substrate inhibition leading to a low ethanol productivity process (Balusu *et al.* 2005).

The optimum conditions for maximizing *BP* in serological bottles were calculated to be pH 5.1, temperature 32 °C, and a sugar concentration of 61.8 g/L. Under these conditions, the highest *BP* was estimated as 15.02 g/L.

In Fig. 4, the kinetic behavior of the batch culture for the optimal conditions is shown. In this work, a low concentration of glycerine was found. Most of the studies concerning glycerine formation have been carried out using yeast, *S. cerevisiae* (Mohammad *et al.* 2002).

Glycerine is a well-known metabolite formed by many microorganisms including bacteria, yeasts, molds, and algae (Spencer 1968; Vijaikishore and Karanth 1986; Rehm and Redd 2008); it is produced by anabolic reactions during anaerobic conditions by *S. cerevisiae* (Lagunas and Gancedo 1973; Oura 1977; Taherzadeh *et al.* 1996; Albers *et al.* 1998). It is also a predominant byproduct of the fermentative metabolism of *S. cerevisiae*; in this paper, about 3.1 g/L glycerol was obtained (Bisping and Rehm 1986; Bisping *et al.* 1989; Andre *et al.* 1991; Benito *et al.* 1994).

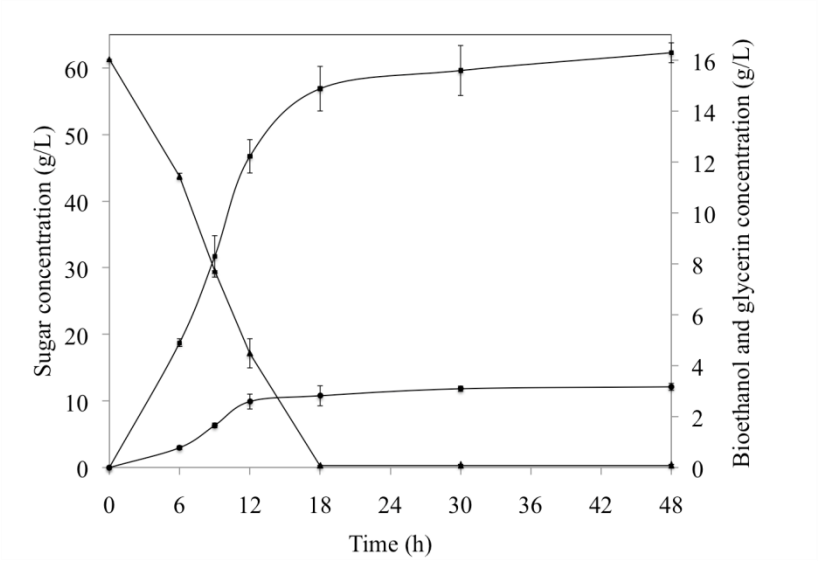

Fig. 4. (a) Bioethanol concentration (■), **(b)** glycerin concentration (●) and **(c)** sugar concentration (▲) over time

CONCLUSIONS

1. Coffee mucilage is an agro-industrial residue mostly composed of carbohydrates, galactose, and glucose that was used as substrate in fermentation process.
2. A response surface methodology was successfully employed to optimize CM fermentation and ammonium sulphate for the efficient production of ethanol by *Saccharomyces cerevisiae* NRRL Y-2034. Sugar concentration is a variable that significantly affect optimization of bioethanol production from coffee mucilage.

3. During the process there was inhibition of bioethanol production and low yield process, due to the presence of toxic compounds in low concentrations.
4. Optimal conditions for bioethanol production and productivity process were established as: pH 5.1, temperature 32 °C, initial sugar concentration of 61.8 g/L, *BP* of 15.02 g/L and *PP* 0.31 g/Lh. Under these conditions experimental bioethanol production was as 16.29 g/L ± 0.39 g/L. In this process, glycerol is a byproduct of the fermentative metabolism, producing 3.1 g/L. The optimizing the conditions of the culture medium is needed to fully exploit the potential of the strains and maximize the production of bioethanol.

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