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Full Length Research Paper

Optimization of ethanol production from mango pulp using yeast strains isolated from "taberna": A Mexican fermented beverage

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Undamaged, uninfected and ripe mango fruits were collected from mango trees grown in Tuxtla Gutiérrez, Chiapas, México. In a first experiment, a Plackett-Burman statistical experimental design was used to study the effect of KH_2PO_4 (0.5 to 6.0 g/ll); $(NH_4)_2SO_4$ (0.5 to 4.0 g/l); $ZnSO_4$ (0.05 to 1.0 g/l); $MgSO_4$ (0.4 to 2 g/l); $CaCl_2$ (0.1 to 1.0 g/l); $CoSO_4$ (0.1 to 1.0 g/l) and the total sugar content of mango pulp (*Mangifera indica* var. criollo) (80 to 120 g/l) on ethanol production using two yeasts strains (denominated TL-ITTG-01 and TL-ITTG -06) isolated from a Mexican fermented native beverage. *Saccharomyces cerevisiae* NRRL-Y-2034 was used as reference strain. The variables studied had no significant effect on ethanol production by the yeast strain TL-ITTG-01 and *S. cerevisiae* NRRL-Y-2034. Highest ethanol productions were 24.89±0.17 for TL-ITTG- 01 and 51.24±3.40 g/l for *S. cerevisiae* NRRL-Y-2034. Substrate concentration, that is, sugars in the mango pulp, and $CoSO_4$, however, had a significant effect on the ethanol production by strain TL-ITTG-06 and the highest ethanol production for this strain was 47.92±0.82 g/l. Ethanol production by the strain TL-ITTG -06 was further optimized using a factorial design 2^2. The maximum ethanol concentration predicted by the model was of 48.56 g/l with 210.0 g/l sugar and 0.1 g/l of $CoSO_4$, but a fermentation with the aforementioned concentrations resulted in an ethanol production of 52.60±0.77 g/l.

Key words: Ethanol, Plackett-Burman design, mango pulp.

INTRODUCTION

New substrates are being tested constantly in fermentations to produce ethanol (Pimentel and Patzek, 2005; Teles et al., 2007; Ye et al., 2007; Hossain and Fazliny, 2010; Oyeleke and Jibrin, 2009). The mango (*Mangifera indica* L. var. criollo) is a fruit that has a total carbohydrate content ranging from 14 to 16% at maturity. It is rich in vitamins A and C, minerals, fibers and antioxidants (FAO, 2007) . Mango pulp is a suitable substrate for fermentation due to its high carbohydrate content and availability in Mexico. Since sugars are already available in a degradable form in mango pulp and yeast cells can metabolize sugars directly, these substrates are inexpensive to use (Lin and Tanaka, 2006).

Palm wine is a beverage obtained from different kind of palm trees, such as oil palm (*Elaeis guineensis Jacq.*), raffia (*Raphia hookeri Mann and Wendl.*) and others species and "taberna" is a Mexican fermented beverage obtained from the coyol palm tree (*Acrocomia aculeate*

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(Jacq.) Lodd. ex Mart.) (Alcántara-Hernández et al., 2010). Palm wine contains several bacteria and yeasts that have a potential to be used in fermentation processes. For instance, the yeast *Saccharomyces* can be found in palm wine and has a high alcohol and osmo-tolerance. As such, it can easily be used for alcohol production (Bechem et al., 2007).

Very high gravity (VHG) fermentation is a promising ethanol fermentation technology as it increases the ethanol concentration, reduces the energy cost and decreases the concentration of contaminating (yield–reducing) bacteria (Bafrncova et al., 1999; Bai et al., 2008).

Historically, *Saccharomyces cerevisiae* has most often been used for ethanol production. It can yield ethanol concentrations as high as 18% of the fermentation broth. This yeast can grow on both simple sugars, such as glucose, and on disaccharide sucrose. *Saccharomyces* is also recognized generally as a safe food additive for human consumption and is therefore ideal for producing alcoholic beverages and for leavening bread (Lin and Tanaka, 2006).

Concentrations of the major nutrients, that is, carbon, nitrogen and phosphorus, but also the amount of metal ions must be optimized to maximize ethanol production (Taillandier et al. 2006; Teles et al., 2007; Mary et al., 2008). Additionally, the effect of pH and incubation temperatures must be studied (Hossain and Fazliny, 2010).

Zhao et al. (2009) studied the impact of the zinc concentration on the tolerance and efficiency of ethanol production. They suggested that addition of zinc allows a reprogramming of the cellular metabolism, thereby increasing their tolerance towards ethanol. As such a larger ethanol production can be achieved. Birch et al. (2003) demonstrated that low magnesium: calcium ratios generally suppressed fermentation by yeasts in both synthetic and complex media. High concentrations of magnesium in the culture medium (0.8 g/l) stimulate yeast growth, ethanol production and sugar consumption. Potassium, cobalt and magnesium are considered to be cofactors for glycolysis (Crane, 1975).

The production of ethanol in a fermentation process thus depends on several factors. In a process in which several factors affect the final product, a fractional factorial experimental design can be used as a first step to optimize the process.

Screening experiments are usually done in the early stages when it is likely that many of the factors considered initially have little or no effect on the response (Montgomery, 1997).

The Plackett-Burman design is an orthogonal array that allows testing the largest number of factors with the least number of observations (Montgomery, 1997; Vanaja and Shobha, 2007). The aim of this work was to optimize the ethanol production by native yeasts strains isolated from “taberna” using a culture medium from mango pulp and an experimental statistical design.

MATERIALS AND METHODS

Raw materials

Undamaged, uninfected and ripe mango fruits were collected from mango trees grown in Tuxtla Gutiérrez, Chiapas, México in April 2009. Fruits were washed with tap water. The pulp was extracted manually, characterized for total (Dubois et al., 1956) and reducing sugars (Miller, 1959), and stored at -18ëC until used. The pulp of mangoes had a maturity degree of 16.0±0.2ëBrix.

Microorganisms used

Two strains of yeasts, isolated from “taberna”, and *S. cerevisiae* NRRL-Y-2034, used as a reference strain, were maintained at 4ëC on agar slants in a medium containing: glucose (10.0 g/l), yeast extract (3.0 g/l), malt extract (3.0 g/l), casein peptone (5.0 g/l) and agar-agar (20.0 g/l). The initial pH of the medium was maintained between 6.4 and 6.8 with 0.1 N HCl. In preparing the inoculation cultures, the cells from agar slants were transferred to glass tubes containing 10 ml of the medium mentioned above, but without agar-agar and incubated at 30ëC for 48 h (Ratnam et al., 2007).

The strains were then transferred to 250 ml Erlenmeyer flasks with 50 ml of the same culture broth and incubated at 30ëC and 150 rpm for 10 h.

Fermentation conditions

Mango pulp was de- frozen and was filtered to obtain nectar. The nectar was diluted to obtain the different sugar concentration used in the treatments. Mineral salts were added as given in Table 1. The fermentation was done in 250 ml Erlenmeyer flasks containing 100 ml culture medium sterilized at 15 lb/in^2 for 10 min. All flasks were inoculated with 6×10^6 cfu/ml yeast cells and incubated at 30ëC and 100 rpm (Nielsen and Arneborg, 2007). After 36 h fermentation, the culture broth was filtrated and the filtrate was stored at -20ëC until analyzed.

Analytical methods

The concentration of ethanol in the culture broth filtrates was determined using the spectrophotometric method described by Magri et al. (1997). Total and reduced sugars were determined by the phenol -sulfuric and DNS method, respectively. The total biomass in the broth culture was determined using a Neubauer camera (Taillandier et al., 2007). Glucose, glycerol, acetic acid and ethanol in the culture broth filtrates were determined by HPLC (Waters 2695) using a Shodex 1011 column. The column was eluated at 30ëC with a degassed mobile phase containing 0.05 M H_2SO_4 at a flow rate of 0.5 ml/min. All the compounds were determined with a RI detector (Waters 2414).

Experimental design and statistical analysis

In a first experiment, a Plackett-Burman experimental design was used to test the effect of total sugar in mango pulp, and concentrations of KH_2PO_4, $(NH_4)_2SO_4$, $ZnSO_4$, $MgSO_4$, $CaCl_2$ and $CoSO_4$ on ethanol production of three strains, that is, TL-ITTG-01; TL-ITTG-06 and *S. cerevisiae* NRRL-Y-2034 (Table 1). Each strain was used in 15 different treatments. The central point treatment was done twice and all treatments were done in duplicate.

In a second experiment, based on the results of the Plackett-Burman experimental design, the effect of sugar concentrations in the mango pulp (120 and 210 g/l) and concentrations of $CoSO_4$

Table 1. Plackett-Burman experimental design.

Treatment	$(NH_4)_2SO_4$ (g/l)	KH_2PO_4 (g/l)	$ZnSO_4$ (g/l)	$MgSO_4$ (g/l)	$CaCl_2$(g/l)	$CoSO_4$ (g/l)	Total Sugar(g/l)
1	2.25	3.25	0.525	1.2	0.55	0.55	100
2	0.5	0.5	0.05	0.4	0.1	0.1	80
3	0.5	0.5	1	2	1	0.1	120
4	0.5	6	1	2	0.1	1	120
5	2.25	3.25	0.525	1.2	0.55	0.55	100
6	4	0.5	0.05	0.4	1	1	120
7	4	6	1	0.4	1	1	80
8	0.5	6	1	0.4	1	0.1	80
9	4	6	0.05	2	0.1	0.1	80
10	0.5	6	0.05	0.4	0.1	1	120
11	0.5	0.5	0.05	2	1	1	80
12	4	0.5	1	2	0.1	1	80
13	4	0.5	1	0.4	0.1	0.1	120
14	2.25	3.25	0.525	1.2	0.55	0.55	100
15	4	6	0.05	2	1	0.1	120

Table 2. Ethanol production for three strains tested using Plackett-Burman experimental design.

Treatment	TL-ITTG-01(g/l)	TL-ITTG-06(g/l)	*S. cerevisiae* (g/l)
1	10.57±2.90	38.82±0.67	34.60±13.55
2	11.40±0.83	34.60±1.51	29.78±3.18
3	17.71±1.06	47.30±0.28	44.89±1.00
4	20.35±2.90	47.10±0.22	26.04±4.67
5	13.96±5.35	31.4±3.01	30.69±1.23
6	19.25±4.57	39.3±0.00	31.28±2.62
7	23.12±12.94	36.33±1.73	40.87±9.37
8	4.02±3.23	43.79±2.00	32.07±0.72
9	19.45±1.50	43.83±0.61	39.25±9.65
10	24.89±0.17	46.71±0.78	51.24±3.40
11	23.12±12.72	32.90±1.23	26.31±4.85
12	10.45±0.06	36.37±1.73	27.42±4.63
13	19.60±3.40	36.25±7.86	39.29±0.11
14	14.36±4.46	41.97±4.01	39.05±6.69
15	22.29±3.29	42.01±6.97	39.06±0.72

(0.01 and 0.1 g/l) were studied using a factorial 2^2 experimental design to optimize ethanol production of the TL-ITTG-06 strain. The culture medium in this experiment contained $MgSO_4$ (2.0 g/l), $ZnSO_4$ (0.05 g/l), $CaCl_2$ (0.1 g/l), KH_2PO_4 (6.0 g/l) and $(NH_4)_2SO_4$ (0.5 g/l). The central point treatment was done twice and all treatments were done in duplicate.

An analysis of variance (α=0.05) was used to test effect of the variables studied. All analyses were done with STATGRAPHICS plus 5.1 (Statgraphics, Statistical Graphics corp. USA.).

A first order equation was used to determine the optimal ethanol production.

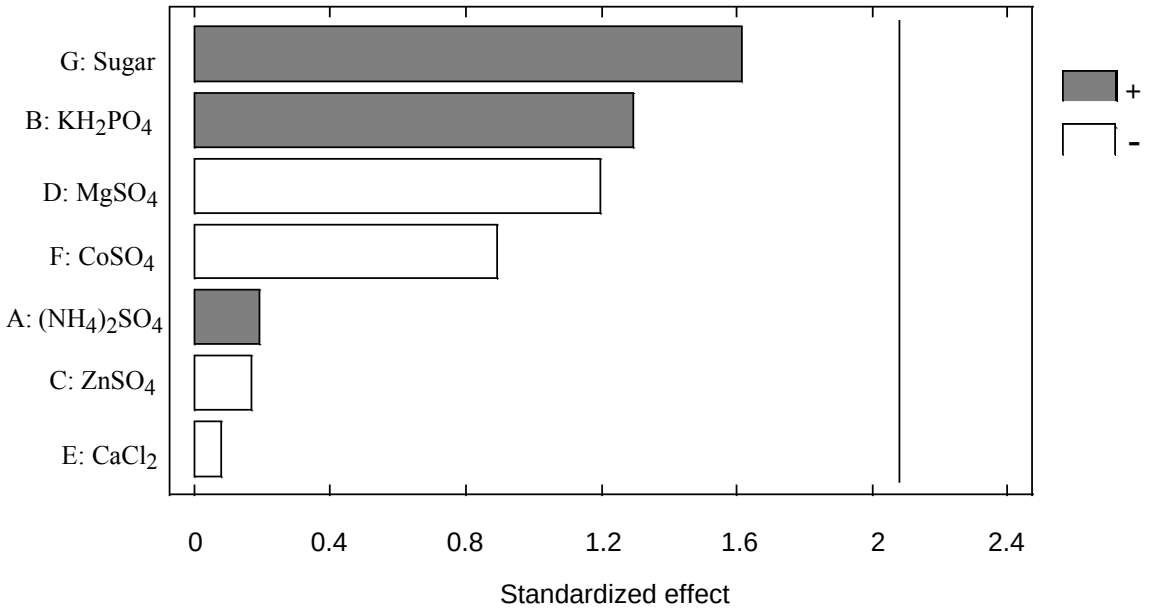
$$Ethanol\ \ (g/l) = a + bx_1 + cx_2 + dx_1 x_2 \quad (1)$$

with a, b, c and d the linear effects, and x_1 the total sugars (g/l) and x_2 the $CoSO_4$ (g/l) concentrations.

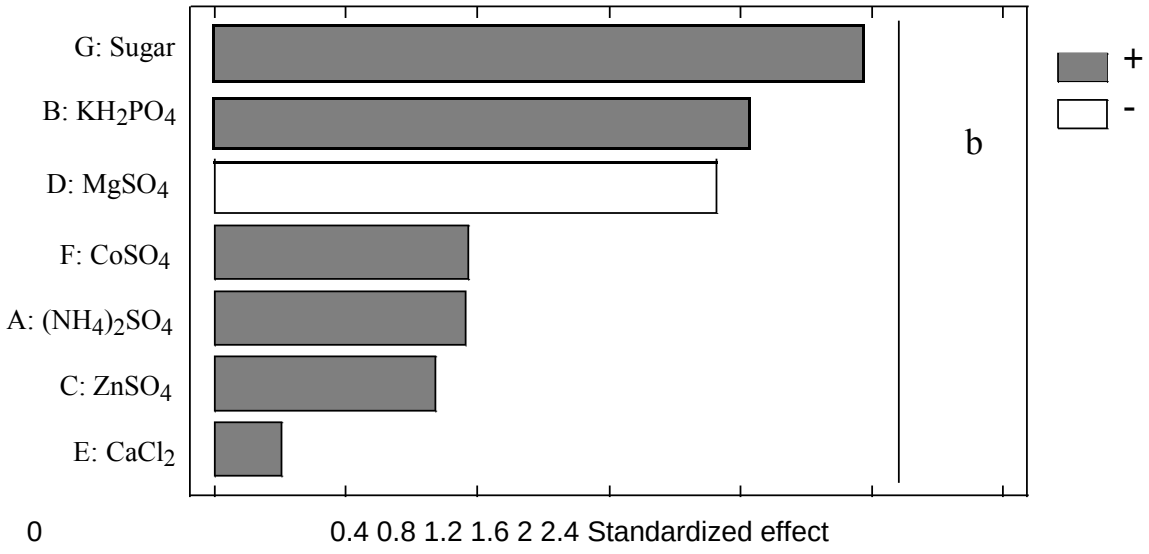
RESULTS AND DISCUSSION

Optimization with the Plackett-Burman experimental design

Average total and reduce sugars in the mango pulp were 18 and 4.8%, respectively. Results for ethanol production of all treatments with the three strains tested are shown in Table 2. The ethanol production was higher with the TL-ITTG-06 and *S. cerevisiae* NRRL- Y-2034 strain than with the TL-ITTG-06 strain. The highest ethanol production with TL-ITTG-01 and *S. cerevisiae* NRRL-Y-2034 strains was found in Treatment 10 and with TL-ITTG-06 strain in Treatment 3. The sugar concentration



(a)



(b)

Figure 1. Pareto chart of standardized effects for the ethanol production by *S. cerevisiae* NRRL-Y-2034 (a) and TL-ITTG-01 (b) isolated of "taberna" using a Plackett-Burman experimental design.

was 120.0 g/l and that of ammonium sulphate 0.5 (g/l) in Treatment 3 and 10.

Arrizon and Gschaedler (2002) reported that fermentation efficiency increases at high sugar concentrations with low nitrogen amounts. The N concentration (0.5 g/l) $(NH_4)_2SO_4$) appears to be the minimum required for protein synthesis of sugar transporting and glycolytics enzymes (Arrizon and Gschaedler, 2002).

The Pareto chart shows that the factors studied had no significant effect on ethanol production of the *S. cerevisiae* NRRL-Y-2034 and TL-ITTG-01 strains (Figure

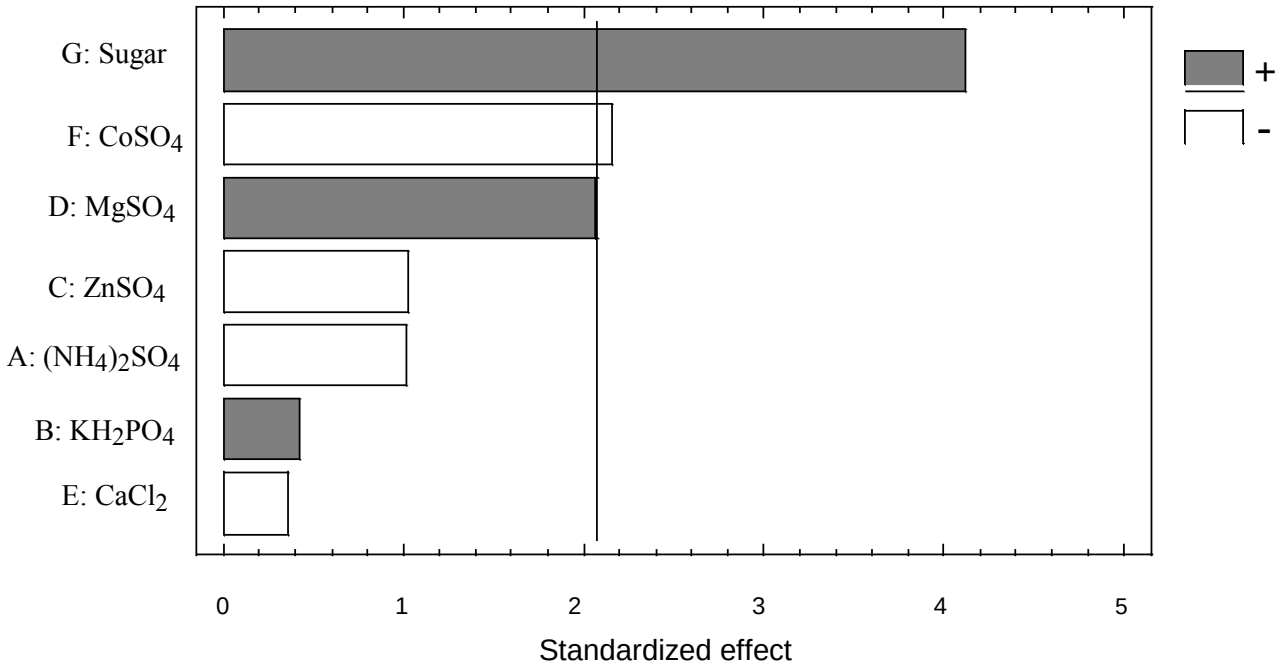


Figure 2. Pareto chart of standardized effects for the ethanol production by the strain TL-ITTG-06 isolated of "taberna" using a Plackett-Burman experimental design.

Table 3. Ethanol, glycerol and acetic acid production by the strain TL-ITTG-06 obtained using a factorial (2^2) experimental design.

Treatment		Ethanol (g/l)	Glycerol (g/l)	Acetic acid (g/l)
Total sugar (g/l)	$CoSO_4$ (g/l)			
210.0	0.01	46.74±0.56	5.86±0.23	0.33±0.01
210.0	0.1	47.92±0.82	5.09±1.23	0.38±0.09
165.0	0.055	44.05±1.81	5.80±0.40	0.35±0.01
120.0	0.01	28.51±1.37	4.56±1.41	0.47±0.04
120.0	0.1	46.6±1.67	3.78±0.58	0.40±0.04
165.0	0.055	46.62±0.26	5.09±0.84	0.42±0.10
165.0	0.055	41.17±1.60	2.94±0.20	0.39±0.04

1) This indicated that these microorganisms satisfied their metabolic needs with the micronutrients (cofactors) present in the mango pulp so it was not necessary to add more minerals to increase ethanol production. Mango pulp is thus a suitable substrate for yeast growth and ethanol production that contains sufficient micronutrients using the *S. cerevisiae* NRRL-Y-2034 and TL-ITTG-01 strains.

The total sugar and $CoSO_4$ concentrations, however, had a significant effect on ethanol production of the TL-ITTG-06 strain (Figure 2). The total sugar concentration had a positive and $CoSO_4$ a negative effect. The positive effect of an increased total sugar content suggests that the TL-ITTG-06 strain can grow at total sugar concentrations > 120 g/l with a possible increase in ethanol production (Shuler and Kargi, 2002).

Optimization of the ethanol production by the TL-ITTG-06 strain using a factorial experimental statistical design 2^2

Ethanol production by the TL-ITTG-06 strain was further optimized by changing the concentration of $CoSO_4$ and the amount of total sugar (Table 3). As such, the nutritional requirements of the native TL-ITTG-06 strain isolated from "taberna" were further characterized. The highest ethanol production by the TL-ITTG-06 strain was 47.92±0.82 g/l in Treatment 3 with 210 g/l substrate. This high substrate concentration (210 g/l) might increase the

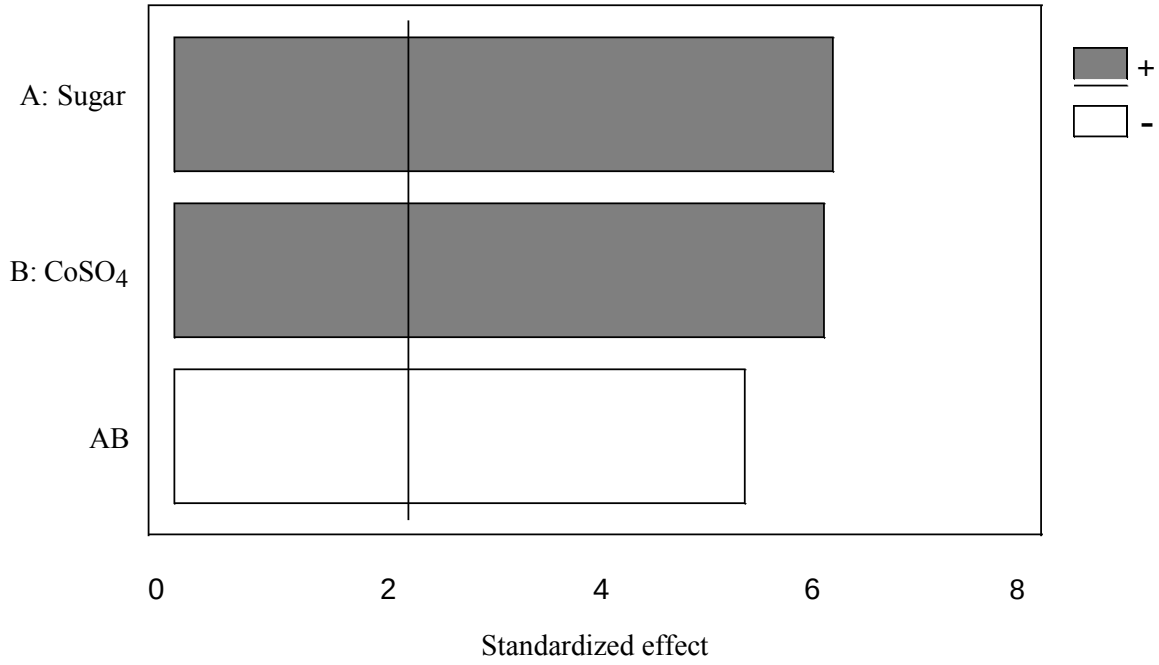


Figure 3. Pareto chart of standardized effects for the ethanol production by the strain TL-ITTG-06 isolated of "taberna" using a factorial 2^2 experimental design.

glycerol production. Glycerol is produced and accumulated in yeasts as a response to osmotic stress (Teles et al., 2007).

Therefore, the glycerol production was quantified (Table 3). Glycerol content varied between 2.94±0.20 and 5.86±0.23 g/l. Despite the high concentrations ob substrate, production of extracellular glycerol was low. Similar results were obtained by Teles et al. (2007) . They reported a production of 5.4 g/l of glycerol when using 87.7 g/l sugars of cashew juice and 5.8 g/l with 103.1 g/l.

Gibson et al. (2007) reported that in *S. cerevisiae* the polyhydric alcohol glycerol is a solute accumulated during osmotic stress confirming its capacity to withstand high extracellular osmorality. During ethanol production it is desirable that the extracellular glycerol production remains low event when high total sugar concentrations are used (Teles et al., 2007). Our results are interesting because low extracellular glycerol concentration obtained. This can be attributed to the zinc present in the culture medium, which accumulates in the yeast cells. Zinc is known to reduce the production of glycerol in the yeast cells, as it compensates for the osmotic stress induced by the sugars in the medium (Zhao et al., 2009).

The concentrations of acetic acid varied between 0.33±0.01 and 0.48±0.04 g/l (Table 3). Similar results were obtained by Delfini and Cervetty (1992). They reported values between 0.2 and 0.4 g/l during an alcoholic fermentation. Higher amounts can be found when bacterial contamination occurs. The Pareto chart shows that the sugar and $CoSO_4$ concentrations had a significant effect on the ethanol production (p > 95%) (Figure 3).

The polynomial regression first order model adjusted to the data was:

$$Ethanol(g/l) = 0.326+0.223x_1 +451.732x_2 -2.089x_1x_2 \quad (2)$$

with x_1 the total sugar (g/l) and x_2 the $CoSO_4$ (g/l) concentration.

The model contains three linear parameters (b, c and d) and one offset parameter (a). The R^2 value was 0.9139, which indicates that 91.39% of the variability in the response can be explained by the model (Equation 2).

Optimal values of the factors that maximize the ethanol production are given in Figure 4. The highest ethanol concentration predicted by the model was 48.56 g/l with 210. 0 g/l sugar and 0.1 g/l $CoSO_4$. Ethanol fermentations with TL-ITTG-06 strain were done in triplicate with 210.0 g/l sugar and 0.1 g/l $CoSO_4$ to verify the model prediction. The ethanol concentration obtained was 52.60±0.77 g/l or 8% bigger than predicted by the model.

Several reports and reviews have been published related to the production of ethanol by fermenting microorganisms (Oyeleke and Jibrin, 2010; Navarro et al., 2000; Caylak and Vardar, 1996; Vallet et al., 1996). Several bacteria, yeasts, and fungi have been used for the production of ethanol (Lin and Tanaka, 2006), with *S. cerevisiae* been most frequently used. Navarro et al. (2000), Caylak and Vardar (1996) and Vallet et al. (1996) reported ethanol concentrations of 70, 96.71 and until 91.8 g/l, respectively. These values are higher than those

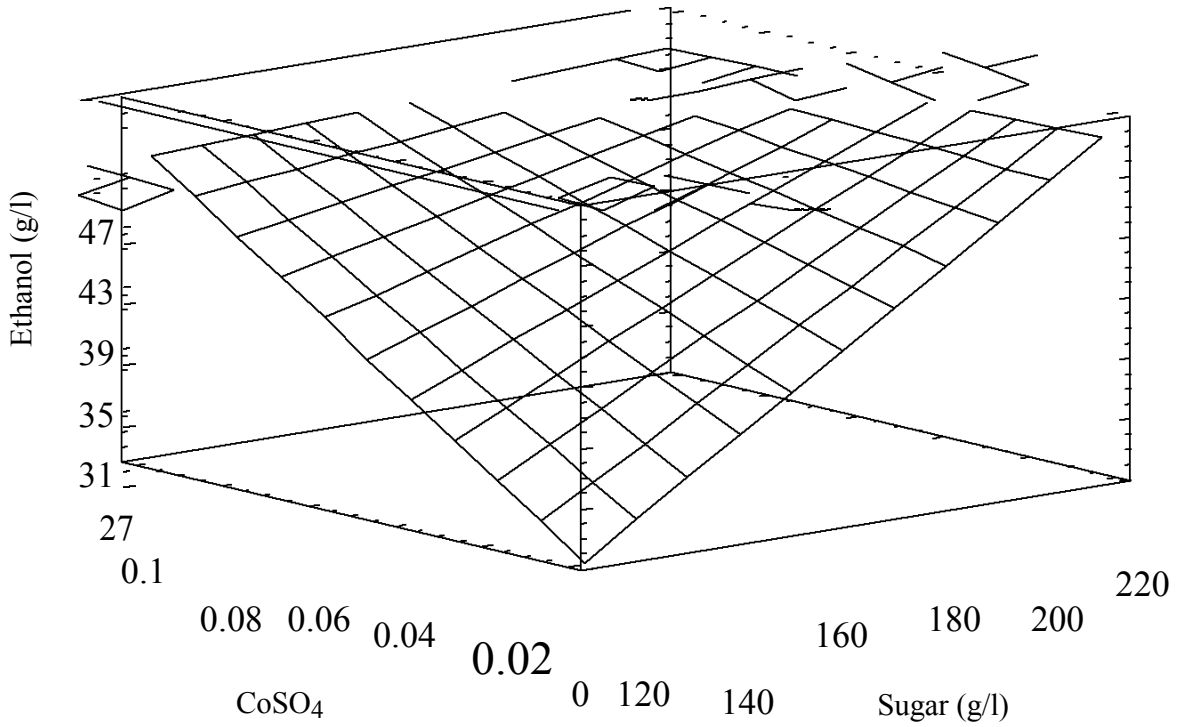


Figure 4. Response surface plot of the effect the sugar and $CoSO_4$ concentration on the ethanol production.

found in this study (52.6 g/l), but they used sugar, sucrose and glucose as C source, and other microorganisms for fermentation. Oyeleke and Jibrin (2010) reported a maximum ethanol concentration of 27.10 g/l from guinea corn and millet husk (18.24 g/l) using *Aspergillus niger* and *Zymomonas mobilis*. Differences in ethanol production are most likely due to the different organisms used, the degradability of the substrates and fermentation conditions.

Others authors have used fruit waste. For instance, Hossain and Fazliny (2010) reported a maximum ethanol yield production of 68.64 g/l using rotten pineapples wastes. Agulejika et al. (2005) using fresh fruit and waste fruits found ethanol concentrations of 64.01 and 21.14 g/l respectively using *Z. mobilis*. The higher ethanol yield was due to higher concentration of fructose and glucose in fresh fruit than in corn husk, millet husk and waste fruits.

It was found that the variables studied, that is, total sugars $CoSO_4$, $MgSO_4$, $ZnSO_4$, $CaCl_2$, KH_2PO_4 and $(NH_4)_2SO_4$, had no significant effect on ethanol production by TL-ITTG-01 and *S. cerevisiae* NRRL-Y-2034. Highest ethanol productions were 24.89±0.17 for TL-ITTG-01 and 51.24±3.40 g/l *S. cerevisiae* NRRL-Y-2034.

Substrate concentration, that is, sugars in the mango pulp, and $CoSO_4$, however, had a significant effect on the ethanol production by strain TL-ITTG- 06. The highest ethanol production for this strain was 47.92±0.82 g/l. Ethanol production by the strain TL- ITTG-06 was further optimized using a factorial design 2^2. The maximum ethanol concentration predicted by the model was of 48.56 g/l with 210.0 g/l sugar and 0.1 g/l of $CoSO_4$, but a fermentation with the aforementioned concentrations and $MgSO_4$ (2.0 g/l), $ZnSO_4$ (0.05 g/l), $CaCl_2$ (0.1 g/l), KH_2PO_4 (6.0 g/l) and $(NH_4)_2SO_4$ (0.5 g/l) resulted in an ethanol production of 52.60±0.77 g/l.

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REFERENCES

Agulejika EO, Olabode FI, Babatunde KA (2005). In: Oyeleke SB, Jibrin NM (2009). Production of bioethanol from guinea cornhusk and millet husk. Afr. J. Microbiol. Res., 3(4): 147-152.

Alcántara-Hernández RJ, Rodríguez-Álvarez JA, Valenzuela-Encinas C, Gutiérrez-Miceli FA, Castañón-González H, Marsch R, Ayora-Talavera T, Dendooven L (2010). The bacterial community in '*taberna*' a traditional beverage of Southern Mexico. Lett. Appl. Microbiol., 51(5): 558-563.

Arrizon J, Gschaedler A (2002). Increasing fermentation efficiency at high sugar concentrations by supplementing an additional source of nitrogen during the exponential phase of the tequila fermentation process. Can. J. Microbiol., 48: 965-970.

Bafrncova P, Smogrovicova D, Slavikova I, Patkova J, Domeny Z (1999). Improvement of very high gravity ethanol fermentation by media supplementation using *Saccharomyces cerevisiae*. Biotechnol. Lett., 21: 337-341.

Bai FW, Anderson WA, Moo-Young M (2008). Ethanol fermentation technologies from sugar and starch feedstocks. Biotechnol. Adv., 26: 89-105.

Bechem EET, Omoloko C, Nwaga D, Titanji VPK (2007). Characterization of palm wine yeasts using osmotic, ethanol tolerance and the isozyme polymorphism of alcohol dehydrogenase. Afr. J. Biotechnol., 6: 1715-1719.
Birch RM, Ciani M, Walker GM (2003). Magnesium, calcium and fermentative metabolism in wine yeast. J. Wine Res., 14: 3-15.
Caylak B, Vardar SF (1996). Comparison of different production processes for bioethanol. Turk. J. Chem., 22: 351-359.
Crane E (1975). Honey: Wines from the fermentation of honey. Heinemann, London, p. 400.
Delfini C, Cervetti F (1992). Study on metabolic and technological factors causing production of large amounts of acetic acid by yeasts during the alcoholic fermentation. Biol. Oggi., 35: 217-234.
Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956). Colorimetric method for determination of sugars and related substances. Anal. Chem., 28: 350-356.
Gibson BR, Lawrence SJ, Leclaire JPR, Powell CD, Smart KA (2007). Yeast responses to stresses associatedwith industrial brewery handling. FEMS Microbiol. Rev., 31: 535-569.
Hossain ABMS, Fazliny AR (2010). Creation of alternative energy by bio- ethanol production from pineapple waste and the usage of its properties for engine. Afr. J. Microbiol. Res., 4(9): 813-819.
Lin Y, Tanaka S (2006). Ethanol fermentation from biomass resources: current state and prospects. Appl. Microbiol. Biotechnol., 69: 627-642.
Magri AD, Magri AL, Balestrieri L, Sacchini A, Marini A (1997). Spectrophotometric micro-method for the determination of ethanol in commercial beverages. Fresenius J. Anal. Chem., 357: 985-988.
Mary AP, Naveen KT, Hema SRB, Shiva NM (2008). Screening and optimization of metal ions to enhance ethanol production using statistical experimental designs. Afr. J. Microbiol. Res., 2: 87-98.
Miller GL (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem., 31: 426-428.
Montgomery DC (1997). Design and analysis of experiments. 5th ed., pp 303. John Wiley and Sons, Inc, NY, USA.
Navarro AR, Sepulveda MC, Rubio MC (2000). Bio-concentration of vinasse from the alcoholic fermentation of sugar cane molasses. Waste Manag., 20: 581-585.
Nielsen MK, Arneborg N (2007). The effect of citric acid and pH on growth and metabolism of anaerobic *Saccharomyces cerevisiae* and *Zygosaccharomyces bailii* cultures. Food microbiol., 24: 101-105.
Oyeleke SB, Jibrin NM (2009). Production of bioethanol from guinea cornhusk and millet husk. Afr. J. Microbiol. Res., 3(4): 147-152.
Pimentel D, Patzek TW (2005). Ethanol production using corn, switchgrass, and wood; biodiesel production using soybean and sunflower. Nat. Resour. Res., 14: 65-76.
Ratnam BVV, Bandaru SR, Somalanka SR, Mendu DR, Imandi SB, Benjawada SR, Medicherla NR, Devarajan T, Karothi J, Chityala A (2007). Optimization of fermentation parameters to enhance the production of ethanol from palmyra jaggery using *Saccharomyces cerevisiae* in a batch fermentor. Appl. Biochem. Biotechnol., 143: 224-235.
Shuler ML, Kargi F (2008). Bioprocess Engineering Basic concepts. Ed. Prentice Hall International Series, Castleton, New York, Second Edition, p. 551.
Teles PAD, Ponte RMV, Macedo GR, Goncalves LRB (2007). Evaluation of cashew apple juice for the production of fuel ethanol. Appl. Biochem. Biotechnol., 148: 227-234.
Vallet C, Said R, Rabiller C, Martin ML (1996). Natural abundance isotopic fractionation in the fermentation reaction: influence of the nature of the yeast. Bioorg. Chem., 24: 319-330.
Vanaja K, Shobha RH (2007). Design of experiments: concept and applications of Plackett-Burman design. Clin. Res. Regul. Affairs., 24: 1-23.
Ye C, Ratna RSS, Chengci C (2007). Ensiling agricultural residues for bioethanol production. Appl. Biochem. Biotechnol., 143: 80-92.
Zhao XQ, Xue C, Ge XM, Yuan WJ, Wang JY, Bai FW (2009). Impact of zinc supplementation on the improvement of ethanol tolerance and yield of self -flocculating yeast in continuous ethanol fermentation. J. Biotechnol., 139: 55-60.