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EFFECT OF CONCENTRATION OF SALTS IN ETHANOL PRODUCTION FROM ACID HYDROLYSIS OF CLADODES OF *Opuntia ficus indica* var. Atlixco

EFECTO DE LA CONCENTRACIÓN DE SALES EN LA PRODUCCIÓN DE ETANOL A PARTIR DE LA HIDRÓLSIS ÁCIDA DE CLADODIOS DE *Opuntia ficus indica* var. Atlixco

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Abstract

Acid hydrolysis from cladodes of *Opuntia ficus indica* var Atlixco was performed. The total reducing sugars released showed a linear relation to the concentration of phenolic compounds released. Three wild microorganisms were isolated, which showed fermentative capacity on nopal hydrolysates. Molecular identification of isolated showed the presence of *Candida intermedia*, *Saccharomyces paradoxus* and *Zygosaccharomyces bailii*; microorganisms that proved capable of producing ethanol. The results showed that pH is the principal factor that impacted ethanol production, and that when associated with conditions of oxygen limitation generated yields ($Y_{p/s}$) of 48 % compared to the maximum theoretical value. Also, adding magnesium salt to the culture medium at a concentration of 0.5 g/L had the greatest effect on ethanol production for *S. paradoxus* and *Z. bailii*. In other results, a slight reduction in product formation was observed for *Z. bailii* when $(NH_4)_2SO_4$ was used as the nitrogen source, while for *S. paradoxus*, ethanol production increased slightly, from 1.64 to 2.11 g/L when the hydrolized is used with 5 g/L of nitrogen salts. These results show that, in general, adding the nitrogen source did not promote product formation.

Keywords: *Opuntia*, carbohydrates, ethanol production, hydrolysate, wild yeasts.

Resumen

Se llevó a cabo la hidrólisis ácida de cladodios de *Opuntia ficus indica* Var. Atlixco. Se observó una relación lineal de los azúcares reductores totales y la concentración de compuestos fenólicos liberados. Se aislaron tres cepas de levaduras con capacidad fermentativa sobre los hidrolizados del nopal. La identificación molecular de las tres cepas indicó la presencia de *Candida intermedia*, *Saccharomyces paradoxus* y *Zygosaccharomyces bailii* con capacidad de producir etanol. El pH fue el principal factor que incidió en la producción de etanol asociado a condiciones de limitación de oxígeno originando rendimientos ($Y_{p/s}$) del 48 % con respecto al valor teórico máximo. La adición de sales de Magnesio al medio a concentraciones de 0.5 g/L fue la que tuvo mayor efecto en la produccion de etanol en *S. paradoxus* y *Z. bailii*. Por otra parte, para *Z. bailii* se observó una ligera disminución en la formación de producto al emplear $(NH_4)_2SO_4$ como fuente de nitrógeno, mientras que para *S. paradoxus* la cantidad de etanol aumentó de 1.64 a 2.11 g/L utilizando 5 g/L de esta sal. Estos resultados mostraron que en general, la adición de la fuente de nitrógeno no promueve la formación de producto.

Palabras clave: *Opuntia*, carbohidratos, producción de etanol, hidrolizado, levaduras silvestres.

1 Introduction

Nopal is the common name of cacti of the genus *Opuntia* in Mexico, which contains 377 recognized

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species, 48 of them utilized by man (Reyes-Agüero *et al*. 2005; Stintzing & Carle 2005; El-Samahy *et al*. 2006). The most common of these recognized species is *Opuntia ficus indica*, which is cultivated in several areas of the world. *Opuntia ficus indica* produces an edible stem called a cladode (*penca* in Spanish), a synonym of nopal (Guevara *et al*. 2010). Nopal is a viable energy source for extracting solid, liquid and gaseous biofuels, thanks to its high productive efficiency, quick adaptation and growth, and low demand for inputs. Ishurd *et al*. (2010) and Ginestra *et al*. (2009) have reported that fresh nopal cladodes have high water (95 % w/w), fiber (1-2 %), carbohydrate (3-7 %), protein (0.5-1 %), vitamin, and mineral content. These cladodes consist largely of a pulp whose structure is more complex than that of any other part of the plant (Majdoub *et al*. 2001). The main component of this pulp is mucilage which has been widely used for the production of biofilms (López-García *et al*., 2017), its concentration in dry cladodes is in the range of 9 to 20.8 % by weight (Sepúlveda *et al*. 2007). Mucilage is a branched polysaccharide (Matsuhito *et al*. 2006) made up of L-arabinose (in the form of pyranose and furanose) with D-galactose, L-rhamnose, and D-xylose as the principal sugars, and galacturonic acid (Cárdenas *et al*. 1997). Another important component of cladodes are thorns, which account for 8.4% of their dry mass, and are composed of a 96 % polysaccharide compound that includes mainly cellulose and arabinan, at 49.7 and 50.3 %, respectively (Malainine *et al*. 2003). The arabinan portion contains L-arabinose (94.3 %) and traces of rhamnose (1.6 %), galacturonic acid (1.4 %), glucose (0.7 %) and galactose (0.6 %) (Vignon *et al*. 2004). However, the precise composition and concentration sugars in different varieties of nopal cladodes depend on edaphic factors, the cultivation site, the season of the year, and the plant age (Ribeiro *et al*. 2010). The carbohydrate content of nopal cladodes makes it potentially susceptible for use as a substrate in the design of biotechnological processes; hence, it is necessary to define two important processes: 1) the pre-treatment applied to disarticulate the lignocellulose matrix and recover the fermentable sugars (Lipnizki 2010); and, 2) the hydrolysis of the lignocellulose using physical, chemical or enzymatic treatments, or a combination of these (Balat & Balat 2008). The composition of the hydrolysates is particularly important since they are rich in pentoses. But this requires the use of microorganisms like *Candida*, *Kluiveromyces*, *Pachysolen* and *Pichia* that can ferment these sugars under diverse conditions (Gong *et al*. 1993; Mussatto *et al*. 2112).

The fermentation process required to obtain bioethanol depends on the metabolic properties of the microorganism and the type of lignocellulosic residue used. In addition to nutritional requirements, aeration, pH and temperature all directly affect the conversion of substrate to ethanol (Balat & Balat 2008; Dias *et al*., 2009). Sreenath and Jeffreis (2000) indicated that the optimal temperature for ethanol production is 26 °C, and that the ideal pH range is 4-7. Aeration plays an important role in fermentation, since limiting the oxygen concentration induces fermentation in microorganisms like *Pichia stipitis* and *Candida shehatae*, although these microorganisms require oxygen to achieve maximum biomass growth and so optimize ethanol production. Another important factor in fermentation is the composition of the medium, which can be improved by adding nutrients like metals and a source of nitrogen, which can impact the process of sugar conversion and are required as co-factors in various metabolic pathways (Tomás *et al*. 2009; Tomás *et al*. 2012).

The objective of the present study was to elucidate the influence of micronutrients and processing conditions on ethanol production using wild yeasts isolated from the cladodes and fruits of *Opuntia* and hydrolysates from the cladodes under soft acid conditions.

2 Materials and methods

2.1 Isolation and selection of fermenting microorganisms for nopal hydrolysates

Samples of cladodes from *Opuntia sp* were collected from various fields in the municipality of Zempoala, Hidalgo, Mexico; then 1 g of each sample was mixed with 9 mL of a sterile saline solution to prepare serial dilutions. The isolation and counting of the yeasts was conducted in differential Wallerstein Laboratorio Nutrient (WLN) medium (Wu *et al*. 2014). The culture medium was complemented with gentamicin (75 μg/mL) to inhibit bacterial growth. Dishes were incubated for 48 h at 28 °C. Selection of levaduriform microorganisms was carried out by staining of colonies with bromocresol green reagent mixed in the WLN medium. Final isolation was performed in yeast potato dextrose agar (YPDA) medium to obtain pure cultures. Strains were stored

at 4 °C until use.

2.2 Preparing the inoculum

The isolated strains were cultivated in solid medium with 1 % yeast extract, 2 % bactopeptone, 2 % dextrose and/or agar at 2 % (YPD) and supplemented with gentamicin at a concentration of 75 μg/mL. This preparation was incubated for 24 h at 28 °C. The culture was then centrifuged at 6000xg for 5 min to separate the biomass. Finally, the pellet of biomass was re-suspended in the isotonic solution used as the inoculum.

2.3 DNA Extraction

Total genomic DNA extraction was performed with the cultures of the three strains of yeast isolated, following the methodology described by Hoffman & Winston (1987).

2.4 PCR amplification

The following primers were used: ITS1F: 5´ CTTGGTCATTTAGAGGAAGTA 3´ and ITS4: 5´ TCCTCCGCTTATTGATATGC 3´ (MWG-Biotech, Germany). The PCR reaction conditions described by Fernandez *et al*. (1999) were employed.

2.5 Analysis of PCR products

Analysis of the PCR products was conducted by manually correcting the chromatogram using CROMAS software. The search for similarity was conducted with sequences from the database at the National Center for Biotechnology Information (NCBI) using BLAST software from the website http://www.ncbi.nlm.nih.gov (Rao *et al*. 2008; Lee *et al*. 2011).

2.6 Hydrolysates and experimental design

Cladodes from 1-year-old nopals were cut into 2 cm^2 cubes and dried at 60 ºC for 72 h, next, they were ground up utilizing a commercial cereal mill. The flour obtained was stored in plastic bags and kept in a cool and dry place until use. The flour was used to prepare experimental units in 50 mL Erlenmeyer flasks with a working volume of 40 mL. Based on a 3^k factorial design, the effect on solid load was evaluated at three levels, 5, 7 and 10 % w/v at sulfuric acid concentrations of 1, 3 and 5 % v/v, it was determined the amounts of sugars and phenolic compounds released under each treatment. Each experimental unit was kept at 121 °C and 1 atm of pressure for 40 min after determining the hydrolysis conditions (data not shown), later experimental units were neutralized with NaOH, and centrifuged at 6000xg for 10 min (Thermo-Scientific Sorvall Legend XTR). Supernatants were separated and filtered through a 0.45 μm membrane to determine Total Reducing Sugars (TRS), phenolic compounds, carbohydrates and oligosaccharides in the medium.

In 40 mL of hydrolysates, using Erlenmeyer flasks, the strains *Candida intermedia*, *Saccharomyces paradoxus* and *Zygosaccharomyces bailii* were inoculated in triplicate. Each flask at an Optical Density (OD) of 0.25 was adjusted to three pH levels (4.5, 6, 7.5) under the following conditions: agitation (100, 150, 200), aeration (0.3 vessel volumes per minute (vvm) of air), anerobiosis (0.3 vvm of nitrogen for 5 min), oxygen limitation (0 vvm), and temperature (28 °C, 30 °C and 35 °C). The effect of the factors was evaluated following the design of the Box-Bhenken experiments (DBB). Additionally, the addition of nutrients $(NH_4)_2SO_4$ (0, 3, 5 g/L), K_2HPO_4 (0, 2, 4 g/L), and $MgSO_4$ (0, 0.5, 1 g/L)- was evaluated using a 3^k factorial design. The operative conditions of 150 rpm, 28 °C and pH 6 were used in both experimental designs. The response variables were ethanol production and biomass generation.

2.7 Statistical analysis

Results were evaluated using analysis of variance and comparing means at a level of significance of $p = 0.05$. Surface response methodology (RSM) was applied to study the influence of the factors. A second-order polynomial model was used to describe the responses of ethanol (g/L):

$$Y = b_0 + \sum b_i X_i + \sum b_{ii}^2 X_{ii}^2 + \sum b_{ij} X_i X_j \quad (1)$$

where: b_0 represents the intersection, b_i the linear term, b_{ii} the quadratic term, and b_{ij} the term of the effect of the interaction of factors. The factors were: X_1: temperature, X_2: agitation; X_3: aeration, and X_4: pH. All statistical analyses were performed with Statgraphics centurion XVI software.

2.7.1 Analytical determinations

The concentration of reducing sugars was determined using the dinitrosalicylic acid method described by Miller (1959) with glucose as the standard. Determination of total phenolic content (TPC) was

performed following the Folin-Ciocalteau method described by De Ascensao & Dubery (2003) with gallic acid as the standard. The amount of yeast was quantified by spectrophotometry after elaborating the calibration curve by measuring absorbance at 600 nm, in accordance with the methodology proposed by Xavier *et al*. (2010). Glucose, galactose, mannose, fructose and oligosaccharide concentrations were determined by HPLC (Thermo-Scientific Dionex) with automatic injection of 5 μL per sample, using a Rezex RCM Ca^{+} monosaccharide column and Rezex oligosaccharide column (Phenomenex, Torrance, CA, USA), equipped with a controlled-temperature oven at 80 °C. The mobile phase was HPLC-grade water at 0.6 mL/min and 0.3 mL/min, respectively. Quantification was carried out after elaborating the calibration curves for each sugar. Ethanol determination was quantified using a Trace 1310 gas chromatograph (Thermo-Scientific Ultimate 3000) equipped with a Phenomenex ZB column (Phenomenex, Torrance, CA, USA) and a flame ionization detector, with helium at 1.5 mL/min as the gas carrier. The oven was maintained at 40 °C for 1 min, then ramped up by 25 °C/min to 250 °C with an isothermal period of 1 min. Each injection contained 1 μL at a Split relation of 66:1. The temperature of the injector was 250 °C. All supernatants were filtered through a 0.2 μm membrane before chromatographic analysis. Supernatants were not analyzed immediately, but stored at -20 °C.

3 Results and discussion

3.1 *Release of sugars by acid hydrolysis of cladodes from Opuntia*

During the hydrolysis process of the flour from cladodes of *Opuntia ficus indica* var Atlixco, the operational variables considered were: solid load (5, 7, 10 % w/v) and the concentration of sulfuric acid (1, 3, 5 % v/v); while the response variables were the concentrations of TRS and the TPC. The concentration of TRS released during diluted acid hydrolysis of the nopal flour showed a linear relation ($p < 0.05$) with respect to TPC concentrations, since as the amount of solids (5 to 7 %) increased, the concentrations of both TRS and TPC increased proportionally, as shown in Fig 1. Observations showed that after 7 % of solids there was no significant difference ($p < 0.05$) between the TPC and TRS released compared to the treatment with 10 % solids.

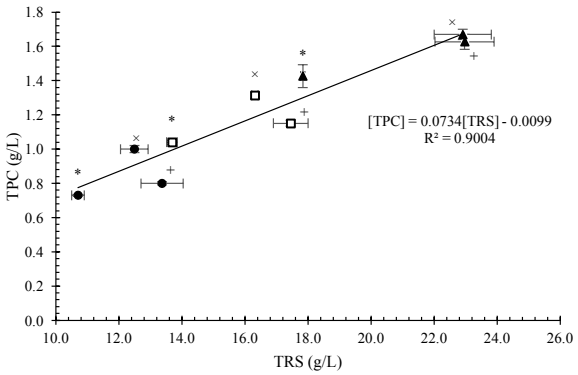

Fig. 1. Relation between the TRS production and TPC in the (·) 1 %; (□) 7 %; and (▲) 10 % of solids (w/v) and (*) 1, (x) 3, (+) and 5 % of H_2SO_4 (v/v).

In addition, prior of the hydrolysis, a rehydration process of the biomass was observed, which caused a limitation to the mixing of the system. This effect could be due mainly to the fact that the mucilage present in the cladode meal on rehydration show viscoelastic properties, an effect that is dependent on the mucilage concentration in each sample (Medina-Torres 2000; León-Martínez *et al*. 2011), which causes it to become a non-Newtonian fluid of pseudoplastic type with thixotropic behavior (El-Samahy *et al*. 2006), this has an effect on the homogenization of the mixture at the beginning of the hydrolysis because it favors the formation of molecular aggregates (Cárdenas *et al*. 1997). According to Ginestra *et al*. (2009), the mucilage is present in both fruits and cladodes in about 14 % in dry weight, whose main physiological function is to regulate the water content in the cell and the flux of calcium in the plant (Nobel *et al*. 1992).

This minimal difference between TRS and TPC was detected at concentrations greater than 7 % solids. This minimal difference could be due to two factors: first, the degradation of sugars caused by hydrolysis of the oligosaccharides into compounds with low molecular weight and the subsequent degradation of the products obtained into furfural derivatives (Pajaró *et al*. 2004; Behera *et al*. 2014); and, second, the solid load in the acid pre-treatment (Adebote *et al*. 2014; Qin el al. 2016). Binod *et al*. (2011) affirm that increasing the amounts of solids generates an increase in sugar concentrations, but this also produces a high concentration of toxic compounds such as furfural that can cause inhibition during fermentation processes. However, this amount may decrease with the neutralization process, generating detoxification at concentrations that can be tolerated and metabolized

by yeast (Palmqvist & Hahn-Hägerdal 2000; Millati *et al*. 2002; Purwadi *et al*. 2004).

A study by Guevara-Figueroa *et al*. (2010) designed to determine the amounts of phenolic compounds in lyophilizates from ten varieties of nopal found a range of 2-20 mg/g in the samples. Santos-Zea *et al*. (2011) observed concentrations of phenolic compounds of 0.3-0.9 g/g under drying conditions similar to those used in the present study. Their concentrations are higher than the ones obtained in our work (0.0178 g/g), even for the treatments with 10 % solids. Phenolic compounds such as ferulic acid, p-coumaric acid, 4-hydroxybenzoic acid, caffeic acid, salicylic acid and gallic acid have been identified in nopal samples (Guevara-Figueroa *et al*. 2010) as well as flavonoids (Stintzing & Carle 2005). It is known that these types of low molecular weight phenolic compounds are more toxic and cause a loss of membrane integrity, decreasing their selective capacity transport affecting the fermentation processes of lignocelullosic hidrolysates in addition; Compounds such as 4-hydroxybenzoic acid and vanillin at concentrations of 1 g/L can decrease fermentation yield by up to 30 % (Palmqvist & Hahn-Hägerdal 2000).

For the treatments performed in our study, observations revealed that the concentration of monosaccharides increased with greater solid loads and acid during hydrolysis; such that for the highest solid load (10 % of nopal flour), glucose concentrations of 2.5, 3.9 and 4.5 g/L were obtained, respectively, for the treatments with 1, 3 and 5 % of acid. Similar results were reported by Kuloyo *et al*. (2014), who obtained 7.4 g/L of sugars using a treatment with 1.5 % (w/w) of H_2SO_4 and 30 % (w/v) of *O. ficus-indica*. This demonstrated that the amount of sugars that can be obtained is proportional to the amount of solids used. Therefore, treatment conditions play an important role in the concentration of the compounds released during hydrolysis (Akanni *et al*. 2015).

Chromatographic analysis of the samples revealed the presence of such oligosaccharides as xylopentose, xylotetrose, xylotriose, xylobiose and maltose at concentrations that were a function of the treatment applied. Xylotriose was obtained in the treatments with 1 % of H_2SO_4 under the conditions evaluated (> 1 g/L), but this oligosaccharide was not detected when the acid concentration was increased (Table 1). The presence of oligosaccharides may be due to a larger amount of acid make it a more aggressive treatment that causes the elimination of most of the insoluble hemicellulose from the surface of the cellulose microfibrils, which then degrade into various soluble oligosaccharides (Qing *et al*. 2013). This effect was observed by Akpinar *et al*. (2009), who found that the amount of reducing sugars increased with time and acid concentration. In general, was observed that the amount of TRS can be increased if the chemical hydrolysis process is coupled to enzymatic treatments to increasing the amount of monosaccharides (Dagnino *et al*., 2013; Sun *et al*., 2016). However, due to the diversity of sugars and oligosaccharides such as glucose, xylose, fructose, arabinose and xylose in the hydrolysates, it is necessary that the microorganisms to fermentation have the metabolic capacity to transform the hydrolysates to ethanol.

3.2 Identifying the strains of wild yeast

Three strains of wild yeast, called CN-25, AT-51 and AT-52, were isolated from samples of nopal cladodes. Each yeast was identified using ITS as a molecular marker.

Table 1. Composition (g/L) of the hydrolysates obtained by factorial design for the acid-solid treatments.

Solids (% p/v)	**Acid (% v/v)**	**Glc**	**Gal/Xil**	**Ara/Frc**	**Xylopentose**	**Xylotetrose**	**Xylotriose**	**Maltose**	**Xylobiose**
5	1	2.4	1.4	3.1	0.05	0.82	1.84	0.43	1.05
5	3	1.9	2.5	1.9	ND	0.04	1.44	0.35	0.12
5	5	1.8	2.2	1.5	0.22	0.01	1.47	1.26	0.67
7	1	1.8	1.1	3.7	0.32	0.78	2.21	0.48	1.75
7	3	2.6	3.2	2.7	0.17	ND	ND	2.36	0.39
7	5	2.9	3.3	1.8	0.07	0.62	ND	2.55	0.87
10	1	2.5	0.9	5	0.28	0.33	1.35	1	1.94
10	3	3.9	4.4	4	0.29	0.19	ND	2.4	0.9
10	5	4.5	4.9	3.3	1.3	0.08	ND	1.74	0.79

Table 2. Identification of the sequences of the PCR amplification of the yeasts isolated: CN-25, AT-51 and AT-52.

Key	Name	% similarity	GenBank access no.
CN-25	*Candida intermedia*	98	MF278340
AT-52	*Zygosaccharomyces bailii*	95	MF189725
AT-51	*Saccharomyces paradoxus*	99	MF278339

The fragments obtained were sequenced and the sequence obtained was compared using the GenBank database. Results of this identification are shown in Table 2. These species of yeast have been previously isolated from decomposition processes on fruit surfaces (Lee *et al*. 2011) and fermentation of wines and mezcal (Fernández *et al*. 1999; Sheela *et al*. 2010; González-Hernández *et al*., 2012). The yeast isolated from nopal cladodes and prickly pears (tunas), were identified as *Candida intermedia*, *Saccharomyces paradoxus* and *Zygosaccharomyces bailii*, reports mention the isolation and identification of such microbial genera as *Kluyveromyces* (Sheela *et al*. 2010), *Wickerhamomyces anomalus*, and *Pichia anomala* (Lee *et al*. 2011).

3.3 Consumption of sugars in the hydrolyzate obtained from Opuntia

It was determined the consumption of the sugars present in the hydrolyzate of each of the yeasts with respect to the time, glucose was the main sugar consumed during the first 20 h, for both *C. intermedia* and *S. paradoxus*; Followed by fructose and galactose, with consumption of 74.3 and 66.3% respectively. Arabinose and xylose were the least assimilated sugars (Figure 2); This same effect was observed by Mussatto *et al*. (2012) in evaluating the fermentation capacity of *S. cerevisiae*, *Pichia stipitis* and *Kluyveromyces fragilis* in ground coffee hydrolysates; Fernandes & Murray (2010) mention that *S. cerevisiae* mainly ferments hexose and tolerates a broad spectrum of inhibitors and a high osmotic pressure. According to the analysis performed to evaluate the assimilation of xylose and arabinose, it was observed that these sugars were only used for the growth, being this limited, in this sense it has been mentioned that *S. cerevisiae* absorbs xylose using of glucose transporters although their affinity for this sugar is very low; in addition, competition with glucose restricts xylose assimilation (Jeffries 2006; Ortíz-Mendez *et al*., 2017). The main metabolic pathway of these pentoses is the conversion of these to D-xylulose-5-phosphate and its subsequent metabolism in the pentoses phosphate pathway (Fonseca *et al*., 2008; Fernandes & Murray 2010). In addition, it was observed that the assimilation of the sugar presents in the medium by *Z. bailli* began until after the 24 h of culture showing a period of adaptation to the medium, indicating that the environmental and nutritional conditions delayed the growth and metabolism of the Yeast, this effect could mainly be due to the presence of phenolic compounds which inhibited the assimilation of the sugars present in the medium as previously described.

3.4 Effect of operating conditions on ethanol production

The yeasts isolated were evaluated to determine their capacity to produce ethanol using hydrolysates obtained from the flour of nopal cladodes as the culture medium. Different conditions of agitation, aeration, temperature and pH were tested, as described above. The effects of these operating conditions were evaluated following the Box-Bhenken (DBB) experimental design without adding nutrients to the medium. The response variables were 1) generation of biomass; 2) ethanol production; and 3) yields of ethanol/sugars ($Y_{p/s}$) (Table 3). An ANOVA was used to analyze the DBB data obtained. For *C. intermedia*, this analysis showed that the principal factor that affected ethanol production was pH (Table 3). For this yeast, under the different operating conditions utilized, maximum ethanol production was detected at pH 6, ethanol production was minimal or even completely inhibited at pH 4.5. At 150 rpm, 30 °C, pH 4.5 and conditions of oxygen limitation (0 vvm), no ethanol production was detected under any conditions (Table 3). The operating conditions (pH, agitation and temperature) had an effect on ethanol production. According to Liu *et al*. (2015) , pH can alter the structure of the cell wall and modify the conformation of proteins in the plasmatic membrane, and so impact the organization of lipids and the function of the cell membrane by increasing its permeability to ions and other small metabolites. This, in turn, stimulates the passive diffusion of protons from the exterior towards the cytosol, which affects the growth rate and fermentation of yeast, while also influencing the constitution of fermentation products.

Table 3. Matrix of the Box-Behnken design for evaluating the environmental parameters that affect ethanol production in wild yeasts isolated from nopal cladodes.

					C. intermedia			*S. paradoxus*			*Z. bailii*		
No.	**T** (°C)	Agitation (rpm)	**Aeration**	**pH**	Biomass (g/L)	Ethanol (g/L)	$Y_{p/s}$ (gP/gS)	Biomass (g/L)	Ethanol (g/L)	Yp/s (gP/gS)	Biomass (g/L)	Ethanol (g/L)	$Y_{p/s}$ (gP/gS)
1	30	150	0	6	0.96^{ac}	6.08^{abe}	0.48^{acf}	0.80^{ac}	2.93^{ab}	0.15^{abc}	1.46	1.43^{ac}	0.16^{ac}
2	28	100	0	6	1.12^{ac}	7.51^{abe}	0.42^{acef}	0.62^{ac}	0.93^{ab}	0.1^{abc}	1.81	2.37^{ac}	0.34^{ac}
3	35	100	0	6	0.45^{ac}	4.52^{abe}	0.26^{acef}	0.46^{ac}	3.30^{ab}	0.18^{abc}	0.66	4.64^{ac}	0.62^{ac}
4	28	200	0	6	1.19^{ac}	7.11^{abe}	0.36^{ace}	0.96^{ac}	3.31^{ab}	0.21^{abc}	1.53	2.31^{ac}	0.16^{ac}
5	35	200	0	6	0.44^{ac}	4.99^{ab}	0.32^{ace}	0.91^{ac}	3.60^{ab}	0.22^{abc}	1.61	3.03^{ac}	0.36^{ac}
6	30	150	-1	4.5	0.21^{ab}	0.06^{acde}	0.01^{bdf}	0.49^{ac}	0.88^{ac}	0.08^{ab}	0.95	2.15^{ac}	0.55^{ac}
7	30	150	1	4.5	0.17^{ab}	0.00^{acd}	0.00^{bdf}	1.65^{ab}	0.01^{ac}	0.00^{ab}	2.31	0.05^{ab}	0.01^{ab}
8	30	150	-1	7.5	0.70^{ac}	4.90^{abde}	0.22^{adf}	0.84^{ac}	5.07^{ab}	0.37^{ac}	0.98	4.71^{ac}	0.50^{ac}
9	30	150	1	7.5	1.30^{ac}	3.28^{abd}	0.21^{adf}	1.11^{ab}	4.15^{ab}	0.36^{ac}	0.91	0.06^{ab}	0.01^{ab}
10	28	150	0	4.5	0.13^{ab}	0.00^{ace}	0.00^{bcf}	0.53^{ac}	0.45^{ac}	0.06^{ab}	1.81	0.81^{ac}	0.12^{ac}
11	35	150	0	4.5	0.23^{ab}	0.00^{ace}	0.00^{bcf}	0.46^{ac}	0.47^{ac}	0.08^{ab}	0.93	1.21^{ac}	0.18^{ac}
12	28	150	0	7.5	0.84^{ac}	6.38^{abe}	0.45^{acf}	1.34^{ac}	1.65^{ab}	0.09^{ac}	1.08	0.98^{ac}	0.11^{ac}
13	35	150	0	7.5	0.72^{ac}	7.04^{abe}	0.38^{acf}	0.70^{ac}	2.38^{ab}	0.14^{ac}	1.36	4.29^{ac}	0.51^{ac}
14	30	150	0	6	0.77^{ac}	6.42^{abe}	0.54^{acf}	0.75^{ac}	2.55^{ab}	0.13^{abc}	1.17	3.32^{ac}	0.34^{ac}
15	30	100	-1	6	0.46^{ac}	4.35^{abde}	0.39^{adef}	0.80^{ac}	3.55^{ab}	0.22^{abc}	0.72	2.59^{ac}	0.27^{ac}
16	30	200	-1	6	0.40^{ac}	4.03^{abde}	0.37^{ade}	0.87^{ac}	4.67^{ab}	0.28^{abc}	0.9	2.35^{ac}	0.22^{ac}
17	30	100	1	6	0.11^{ac}	0.15^{abd}	0.11^{adef}	0.91^{ab}	6.68^{ab}	0.3^{abc}	0.9	0.24^{ab}	0.02^{ab}
18	30	200	1	6	0.90^{ac}	0.01^{abd}	0.00^{ade}	1.77^{ab}	0.44^{ab}	0.07^{abc}	1.43	2.09^{ab}	0.19^{ab}
19	28	150	-1	6	0.52^{ac}	4.41^{abde}	0.25^{adf}	0.45^{ac}	2.61^{ab}	0.14^{abc}	0.42	4.10^{ac}	0.45^{ac}
20	35	150	-1	6	0.04^{ac}	3.92^{abde}	0.22^{adf}	0.63^{ac}	4.03^{ab}	0.25^{abc}	0.25	1.67^{ac}	0.23^{ac}
21	28	150	1	6	0.30^{ac}	4.41^{abd}	0.25^{adf}	1.81^{ab}	0.01^{ab}	0.00^{abc}	0.71	0.33^{ab}	0.04^{ab}
22	35	150	1	6	1.16^{ac}	5.38^{abd}	0.25^{adf}	1.09^{ab}	3.84^{ab}	0.26^{abc}	0.89	0.01^{ab}	0.05^{ab}
23	30	100	0	4.5	0.12^{ab}	0.08^{ace}	0.04^{bcef}	0.47^{ac}	0.49^{ac}	0.09^{ab}	0.64	0.69^{ac}	0.15^{ac}
24	30	200	0	4.5	0.13^{ab}	0.04^{ace}	0.00^{bce}	0.50^{ac}	0.30^{ac}	0.05^{ab}	1.01	1.07^{ac}	0.15^{ac}
25	30	100	0	7.5	0.59^{ac}	5.12^{abe}	0.33^{acef}	0.78^{ac}	3.01^{ab}	0.18^{ac}	0.75	3.08^{ac}	0.66^{ac}
26	30	200	0	7.5	0.69^{ac}	4.60^{abe}	0.24^{ace}	0.89^{ac}	2.40^{ab}	0.14^{ac}	1.89	1.81^{ac}	0.17^{ac}
27	30	150	0	6	0.84^{ac}	6.71^{abe}	0.44^{acf}	0.85^{ac}	2.78^{ab}	0.14^{abc}	1.32	2.26^{ac}	0.24^{ac}

a, b, c, d, e, f indicate significant differences between treatments; Tukey α=0.05

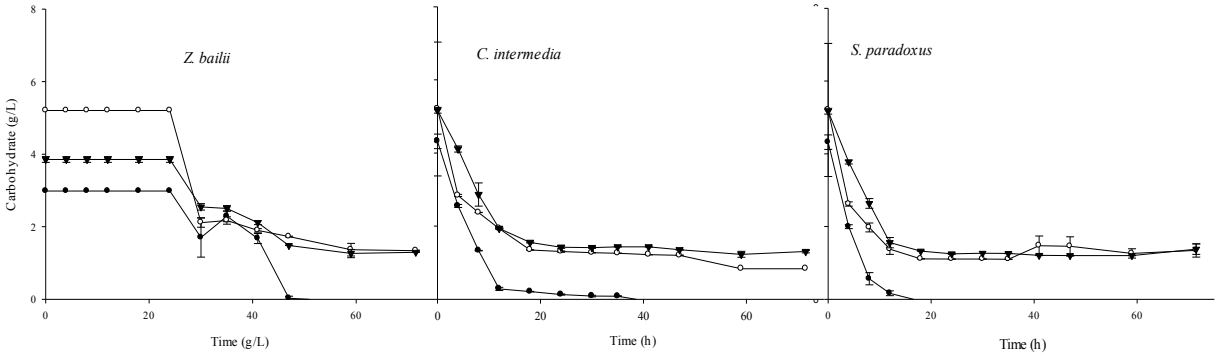

Fig. 2. Profile of sugar consumption of the hydrolysates by the three yeasts isolated; •glucose; o galactose-xylose; ▼ Fructose-Arabinose.

This effect was reflected in the amount of biomass produced, which was lower at 1 g/L with pH values of 4.5 than in the experiments conducted at pH 6, where results were higher at 1 g/L for all three strains evaluated. Singh & Bishnoi (2013) described a similar effect for S. cerevisiae in hydrolysates of wheat straw, observing maximum ethanol production at pH 5.5. Lopez-Rojo *et al*. (2017), described a similar behavior during the production of traditional Mexican fermented beverages (Tibico) where they observed that ethanol production decreased at low pH. Agitation also influenced ethanol production, since we detected the highest production of this compound between 100-150 rpm. The effect associated with the limited oxygen condition improvement yields ($Y_{p/s}$), as these reached values of 48 % of the maximum theoretical value (Fig 3).

Other observations revealed that limited oxygen conditions with no additional air supply (0 vvm; indicated as 0) promoted greater ethanol production by *C. intermedia* (treatment 2), which reached a maximum of 7.5 g/L; while in the cases of *S. paradoxus* and *Z. bailii*, ethanol production was linked to conditions of anaerobiosis (0.3 vvm of nitrogen during 5 min; treatment 8) (Fig 4). The other two microorganisms did produce ethanol under limited oxygen conditions. We were able to corroborate the effect of pH and the presence of oxygen on ethanol production by analyzing the results with surface response methodology and a non-linear multiple

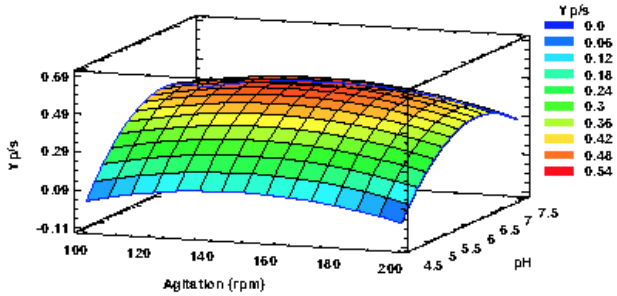

Fig. 3. Response surface of the effect of pH and agitation on yields of ethanol production for *C. intermedia*.

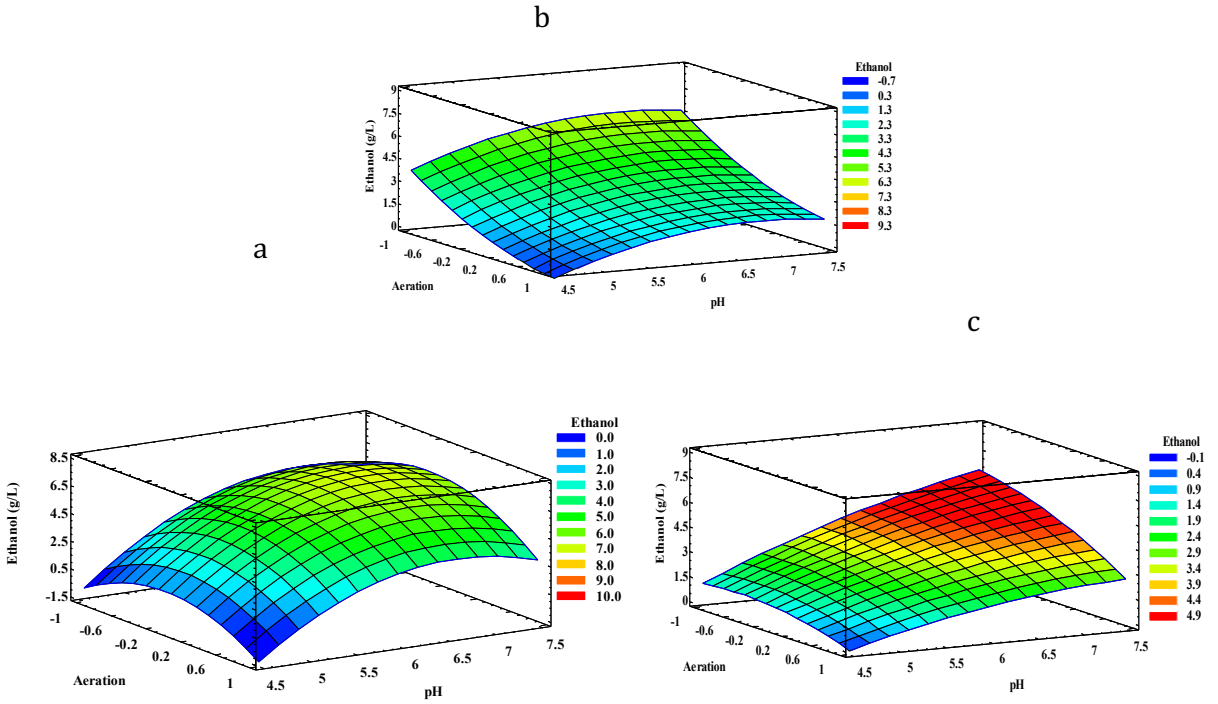

Fig. 4. Response surface of the effect produced by pH and aeration on ethanol production for the strains: a) *C. intermedia*; b) *S. paradoxus*; and, c) *Z. bailii*.

regression model between the level of aeration supplied and pH (Table 3; Fig 4). Khongsay *et al*. (2012) they mentioned that yeasts normally require oxygen in the medium to synthetize the lipids required to maintain membrane integrity. Rodmui *et al*. (2008) showed that at low concentrations of dissolved oxygen in combination with moderate agitation (0-50 rpm) high ethanol production could be generated (8 g/L), but that any increase in agitation caused the opposite effect. Hua & Shimizu (1999) observed similar effect on the growth and formation of ethanol, as they found that *Turulopsis glabatra* achieved a higher rate of growth at a level of dissolved oxygen (DO) >10 %; whereas levels of DO <1 % promoted ethanol formation. Morales *et al*. (2015) , meanwhile, observed a negative impact of aeration on ethanol production due to an increase in the amount of acetic acid in the medium compared to the cultures handled under anaerobic conditions.

In the range evaluated (28 to 35 °C) temperature was not a factor in ethanol production (p >0.05), though it was clear that the combined effect of pH (7.5) and temperature reduced ethanol production in the medium (Table 2).

Table 4. Effect between the environmental factors evaluated and the constants from the adjustment model with $\alpha = 0.05$.

	C. intermedia		*S. paradoxus*		*Z. bailii*	
Source	Value-P	model	Value-P	model	Value-P	model
Constant		-18.7308		-107.8850	-6.8862	
A: Temperature	0.4706	-2.1581	0.0597	5.3347	0.3230	-0.0550
B: Agitation	0.8615	0.1268	0.4491	-0.0126	0.5538	0.0934
C: Aeration	0.1392	-2.5633	0.3864	1.1649	0.0083	-3.9294
D: pH	0.0001	15.3422	0.0048	7.5364	0.0207	0.0645
AA	0.6310	0.0268	0.1705	-0.0789	0.9467	-0.0033
AB	0.7822	0.0012	0.8572	-0.0006	0.3814	-0.0027
AC	0.6436	0.1042	0.4421	0.1289	0.4344	0.1206
AD	0.8337	0.0314	0.9230	-0.0106	0.2213	0.1283
BB	0.0715	-0.0005	0.5979	0.0001	0.7257	0.0001
BC	0.9543	0.0009	0.0099	-0.0368	0.3635	0.0104
BD	0.8786	-0.0016	0.8643	-0.0014	0.4702	-0.0055
CC	0.0052	-2.2679	0.2101	0.6895	0.2587	-0.5679
CD	0.6213	-0.2600	0.9838	-0.0083	0.2716	-0.4250
DD	0.0016	-1.19685	0.0482	-0.5090	0.3191	-0.2212
Adjustment		71.9080		78.1534		84.6286

Table 5. Matrix of the 3^k factorial design for evaluating nutrients that affect ethanol production using wild yeasts isolated from nopal cladodes.

	C. intermedia			*S. paradoxus*			*Z. bailii*					
No.	**$(NH_4)_2SO_4$**	**KH_2PO_4**	**$MgSO_4$**	**Biomass (g/L)**	**Ethanol (g/L)**	**$Y_{p/s}$ (gP/gS)**	**Biomass (g/L)**	**Ethanol (g/L)**	**$Y_{p/s}$ (gP/gS)**	**Biomass (g/L)**	**Ethanol (g/L)**	**$Y_{p/s}$ (gP/gS)**
1	0	0	0	0.92	4.38	0.28	0.62^{a}	1.64^{a}	0.11^{ac}	1.35^{a}	1.77^{ab}	0.20^{a}
2	2.5	0	0	1	5.95	0.41	0.72^{a}	1.87^{a}	0.12^{ac}	1.33^{a}	1.30^{ab}	0.13^{a}
3	5	0	0	0.73	5.12	0.74	0.71^{a}	2.11^{a}	0.13^{ac}	1.42^{a}	1.08^{ab}	0.12^{a}
4	0	2	0	0.94	7.74	0.59	0.89^{ab}	2.02^{a}	0.13^{ac}	1.31^{a}	1.46^{ab}	0.15^{a}
5	2.5	2	0	0.83	4.39	0.34	1.01^{ab}	1.37^{a}	0.08^{ac}	0.98^{a}	1.40^{ab}	0.16^{a}
6	5	2	0	1.03	6.93	0.5	0.97^{ab}	1.46^{a}	0.09^{ac}	1.11^{a}	1.37^{ab}	0.15^{a}
7	0	4	0	0.88	6.26	0.47	0.99^{ab}	1.67^{a}	0.10^{ac}	1.59^{a}	0.92^{a}	0.12^{a}
8	2.5	4	0	0.83	6.41	0.54	0.95^{ab}	1.77^{a}	0.11^{ac}	1.29^{a}	1.09^{a}	0.13^{a}
9	5	4	0	0.81	6.63	0.58	1.01^{ab}	1.53^{a}	0.10^{ac}	1.54^{a}	1.23^{a}	0.18^{a}
10	0	0	0.5	0.84	5.18	0.34	0.98^{a}	2.79^{b}	0.16^{ab}	1.81^{ab}	3.01^{bb}	0.32^{ab}
11	2.5	0	0.5	0.8	6.62	0.35	1.02^{a}	3.68^{b}	0.21^{ab}	1.70^{ab}	3.40^{bb}	0.39^{ab}
12	5	0	0.5	0.82	6.55	0.45	0.89^{a}	4.61^{b}	0.23^{ab}	1.48^{ab}	2.54^{bb}	0.48^{ab}
13	0	2	0.5	0.97	7.3	0.41	1.00^{ab}	3.79^{b}	0.21^{ab}	1.66^{ab}	3.08^{bb}	0.43^{ab}
14	2.5	2	0.5	0.85	5.17	0.33	0.99^{ab}	3.50^{b}	0.19^{ab}	1.52^{ab}	3.47^{bb}	0.58^{b}
15	5	2	0.5	0.83	6.05	0.39	1.01^{ab}	3.39^{b}	0.18^{ab}	1.80^{ab}	2.59^{bb}	0.33^{ab}
16	0	4	0.5	0.72	6.62	0.39	0.97^{ab}	3.57^{b}	0.20^{ab}	1.71^{ab}	2.32^{b}	0.31^{ab}
17	2.5	4	0.5	0.88	6.55	0.35	0.88^{ab}	2.95^{b}	0.17^{ab}	1.57^{ab}	3.34^{b}	0.50^{ab}
18	5	4	0.5	0.78	5.56	0.31	0.99^{ab}	3.91^{b}	0.22^{ab}	1.43^{ab}	2.12^{b}	0.48^{ab}
19	0	0	1	0.84	5.12	0.36	1.23^{a}	2.96^{c}	0.16^{ab}	1.56^{a}	2.86^{cb}	0.36^{ab}
20	2.5	0	1	0.91	6.9	0.44	1.08^{a}	2.98^{c}	0.17^{ab}	1.35^{a}	2.89^{cb}	0.48^{ab}
21	5	0	1	0.97	6.3	0.38	1.04^{a}	3.26^{c}	0.21^{ab}	1.13^{a}	2.29^{cb}	0.41^{ab}
22	0	2	1	0.97	6.36	0.39	1.19^{ab}	2.86^{c}	0.16^{ab}	1.34^{a}	2.39^{cb}	0.31^{ab}
23	2.5	2	1	0.96	6.62	0.43	1.29^{ab}	2.27^{c}	0.12^{ab}	1.33^{a}	2.55^{cb}	0.41^{ab}
24	5	2	1	0.83	5.91	0.48	1.31^{ab}	3.12^{c}	0.18^{ab}	1.31^{a}	2.76^{cb}	0.26^{ab}
25	0	4	1	0.91	6.14	0.45	1.19^{ab}	4.04^{c}	0.25^{ab}	1.30^{a}	2.36^{c}	0.43^{ab}
26	2.5	4	1	0.87	6.74	0.56	1.15^{ab}	2.81^{c}	0.16^{ab}	1.39^{a}	2.31^{c}	0.48^{ab}
27	5	4	1	0.8	6.83	0.36	1.12^{ab}	2.84^{c}	0.18^{ab}	1.36^{a}	2.20^{c}	0.40^{ab}

a, b, c indicate significant differences between treatments; Tukey $\alpha = 0.05$.

This effect may have been due to a process of denaturalization of the cells caused by the combination of these factors (Boudjema *et al*. 2015). In a similar study, Wang *et al*. (2008) reported that ethanol production increased at higher temperature and pH, but that excessive temperature and pH increases reverted this tendency. The results obtained upon evaluating the hydrolysates of nopal cladodes as a medium culture showed that maximum ethanol production was achieved in the range of 28-30 °C. Pramanik (2003) found that *S. cerevisiae* achieved high ethanol production at temperatures of 35-38 °C; however, that author reported that the maximum ethanol concentration was reached at 30 °C.

3.5 Effect of nutritional factors on ethanol production

Finally, our study evaluated the effect of the inorganic salts Mg, P and N on ethanol production using the wild strains of yeast. The aim was to maximize ethanol production (Table 5). Earlier studies showed that adding these salts to the culture medium during fermentation can have positive effects, such as protecting against stress (Deesuth *et al*. 2012), or stimulating the growth and efficiency of ethanol production (Deesuth *et al*. 2012; Khongsay *et al*. 2012). Our results showed that, except for pH 4.5, ethanol production was generally similar to the effect of operating conditions on ethanol production (Table 2). With respect to the salts KH_2PO_4 and $(NH_4)_2SO_4$, statistical evidence for *C. intermedia* was insufficient to permit any determination of their effect on ethanol production, which meant that the results were similar to those observed for pH, temperature and agitation in the DBB, with values of 6.2 and 5.9 g/L of ethanol, respectively. However, it was clear that during the growth of *C. intermedia* the presence of $MgSO_4$ at a concentration of 0.5 g/L had a slightly negative effect, though biomass increased marginally at a concentration of 1 g/L, compared to the culture medium with no magnesium sulphate added (Table 5). In ethanol formation, in contrast, we observed an increase at 1 g/L compared to the medium without this salt (experiment 1), while concentrations of 0.5 and 1 g/L of $MgSO_4$ had no significant effect on product formation, as average ethanol concentration remained at 5.6 g/L. These results probably indicate that, for yeast, the culture medium prepared from hydrolysates of nopal cladodes has the required salt content, sufficient for good biomass development and ethanol formation.

Different amounts of minerals have been reported for cladodes of *Opuntia*. Salim *et al*. (2009), for example, determined the presence of calcium and magnesium in amounts of 12.4 and 18.8 mg/100 g of solid material, respectively; while Moβhammer *et al*. (2006) cite concentrations above 59 and 98.4 mg/100 g of these salts, respectively. Also, there are reports of the presence of salts of sodium, potassium and phosphorus in ranges typical of fruits (Kuar *et al*. 2012). Tomás-Pejó *et al*. (2012) , in turn, affirm that the hydrolysates obtained from lignocellulosic materials contain low amounts of nutrients and nitrogen, though wheat straw may have sufficient inorganic salts and trace elements to sustain growth.

Turning to *S. paradoxus*, the study found that the principal factor that controlled the generation of biomass was the source of magnesium in the medium ($p < 0.05$), while for *Zygosaccharomyces* a concentration of 0.5 g/L of magnesium salts yielded maximum biomass production at approximately 1.5 g/L (Table 6). However, the highest concentration of magnesium salts utilized inhibited growth, reducing ethanol formation. In this case, ethanol concentrations were lower than those obtained previously in the evaluation of the operating conditions for the three microorganisms. This effect is shown in the surface graphs in Fig 5, where it is clear that magnesium ions tend to promote ethanol production. Dombek and Ingram (1986) determined the importance of magnesium as the principal cation in such cell structures as ribosomes and the cell wall. Moreover, magnesium is an important regulator of metabolism during cell division, since many enzymes involved in this process require it as a co-factor. Udeh *et al*. (2013) also underscored the importance of magnesium as an element that promotes high ethanol and biomass yields, because its ions stabilize enzymes like the phosphorylases, enolases and alcohol dehydrogenases (Mahler & Nudel 2000). However, at high concentrations (> 0.7 mM) no effect on ethanol production was observed (Dombeck & Ingram 1986).

Adding a source of phosphorus to the fermentation medium also had a significant effect on the biomass formation and production ethanol by *S. cereviseae*. At a concentration of 2 g/L of phosphate salts, ethanol concentrations as high as 1 g/L were achieved, compared to the medium with no added phosphate salts (Fig 5). However, higher concentrations of this salt decreased ethanol production in both *S. paradoxus* and *Z. bailii*, though not for *C. intermedia*. A similar effect to the one obtained in our study for *S. cerevisiae* was reported by Maruthai *et al*. (2012) for *S. diasticus*, where KH_2PO_4 was a nutrient that produced positive and negative effects on ethanol production at different concentrations, this effect ware observed by Yu *et al*. (2009) when were supplemented phosphorus and nitrogen on sorghum hydrolysates to produce ethanol, the bests values were 0,77 and 2.15 g/L of phosphorus and nitrogen respectively, while to 0.131 and 1.271 g/L of this salt the productivity was reduced. It is very likely that the efficiency of the alcoholic fermentation increases due to the presence of ammonium and phosphate ions (Gupta, *et al*. 2009). Anupama *et al*. (2010) also evaluated KH_2PO_4 as a source of phosphorus for *S. cerevisiae* at intervals of 1-7 g/L.

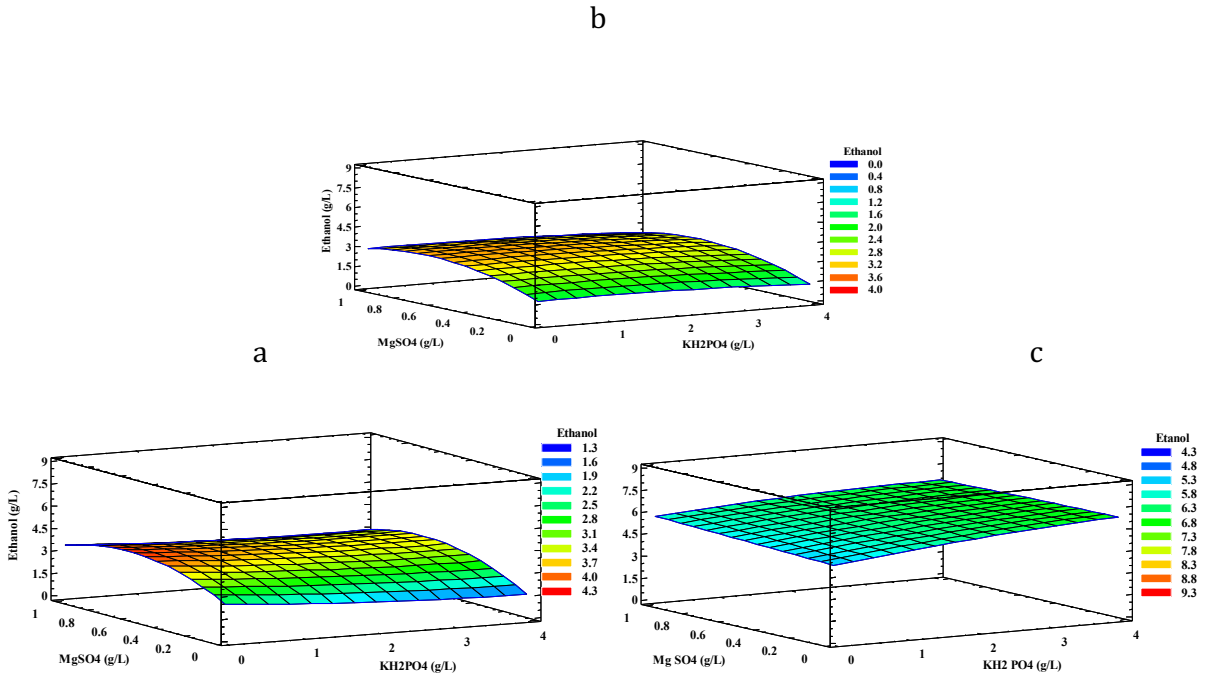

Fig. 5. Response surface of the effect on ethanol production of adding nutrients to the medium for the strains: a) *S. paradoxus*; b) *Z. bailii*; and, c) *C. intermedia*.

They obtained 5.3-4 g/L of ethanol. Serrat *et al*. (2011), meanwhile, found that adding (NH)4HPO$_4$ had no significant effect on ethanol production. In their study, Mukhtar *et al*. (2010) mention that phosphorus is one of the most important nutritional requirements for the growth and production of ethanol, and Rubio-Arroyo *et al*. (2011) observed that adding phosphate salts increases the efficiency of the fermentation process by increasing the formation of ATP. But they found the opposite effects upon adding phosphates to culture media formulated with hydrolysates of yucca and sorghum under similar operating conditions to those used herein. Thus, it is probable that consumption of phosphate salts is associated with the amount of fermentable sugars, such that more of the latter triggers higher consumption of the former.

Regarding *C. intermedia*, this study found that the presence of an additional source of nitrogen supplied as ammonium sulfate limited the amount of biomass generated during fermentation; thus promoting increased ethanol production at an average of 6.3 g/L, compared to the treatment without this source of nitrogen. However, upon increasing the concentration from 2.5 to 5 g/L, a decrease in the ethanol production was noted. The yeast *Z. bailii*, meanwhile, showed a slight decrease in product formation compared to the concentrations tested in the experiments without nitrogen, as the amounts obtained for the hydrolysate without nitrogen were 1.77, 1.3 and 1.42 g/L, and 2.5 and 5 g/L of $(NH_4)_2SO_4$ respectively; whereas in the case of *S. cerevisiae*, the amount of ethanol produced increased slightly, from 1.64 to 2.11 g/L, in the medium without this source of nitrogen, and with 5 g/L of this salt. These results show that, in general, adding the nitrogen source does not promote product formation and, hence, indicate that a process which seeks to optimize the hydrolysate of nopal cladodes does not require an additional source of nitrogen. This finding could reduce additional costs associated with the bioprocess for ethanol production.

Observations from a study with *Kluyveromyces marxiannus* by Serrat *et al*. (2011) showed that by increasing the amount of TRS supplemented to the medium to 2.48 g/L $(NH_4)_2SO_4$ and 2.73 g/L $(NH_4)_2HPO_4$, and adding such divalent cations as Ca^{2+} and Mg^{2+} (0.33 and 0.54 g/L, respectively) led to greater ethanol production. Adela & Loh (2015) evaluated the effect of diverse sources of nitrogen, including yeast extract, malt extract, peptone, and NH_4Cl, but they did not observe any effect on ethanol production after analyzing the lignocellulosic residue employed. Finally, Blomqvist *et al*. (2011) detected a

similar effect to those obtained in our study utilizing up to 2 g/L of $(NH_4)_2SO_4$ in agricultural residues.

Conclusions

We were able to isolate wild yeasts identified as *Candida intermedia*, *Saccharomyces paradoxus* and *Zygosaccharomyces bailii* from nopal cladodes, and these showed good adaptation and good fermentation capacity of hydrolysates obtained with 10 % w/v of flour solids from the nopal cladodes, and 3 % v/v of H_2SO_4. The culture conditions determined that a pH of 6 and the combined effects of limited dissolved oxygen and agitation in a range of 100-150 rpm, were factors that generated maximum ethanol production. Also, the amount of $MgSO_4$ at a concentration of 0.5 g/L was a determining factor in ethanol production in the medium for the strains *S. paradoxus* and *Z. bailii*; however, for *C. intermedia*, this factor did not show significant differences. Therefore, it is viable to utilize the hydrolysate obtained from nopal cladodes as a culture medium for obtaining ethanol. Furthermore, the native yeasts of this plant have the ability to ferment these hydrolysates and provide yields that attain 48 % of the theoretical value.

Acknowledgments

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