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Production of ethanol from mesquite (*Prosopis juliflora* (SW) D.C.) pods mash by *Zymomonas mobilis* and *Saccharomyces cerevisiae*

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Abstract This study aimed to assess the use of mesquite pods hydrated mash as biomass for the growth of *Saccharomyces cerevisiae* UFEPEDA-1012 and *Zymomonas mobilis* UFEPEDA-205 and for ethanol production using a submerged fermentation. A 2^3 factorial design was used to analyze the effects of the type of microorganism, time of fermentation and condition of cultivation on the ethanol production in mesquite pods mash (30 g 100 mL^{-1}). From the obtained results the hydrated mesquite pods mash presented as a good substrate for the growth of *S. cerevisiae* and *Z. mobilis* in comparison to the standard media. The effect that most affected the ethanol production was the type of microorganism. The highest ethanol concentration (141.1 gL^{-1}) was found when *Z. mobilis* was cultivated in mesquite pods mash under static condition for 36 hrs. Ethanol production by *S. cerevisiae* was higher (44.32 gL^{-1}) after 18 hrs of fermentation under static condition. According to these results, the mesquite pods could be known as an alternative substrate to be used for biotechnological purposes, mainly for ethanol production.

Keywords: ethanol, mesquite, *Saccharomyces cerevisiae*, *Zymomonas mobilis*

INTRODUCTION

With the inevitable depletion of the world's petroleum supply, there has been an increasing worldwide interest in alternative, non-petroleum-based sources of energy. A growing, yet controversial, source of transportation fuel is fermentation-derived ethanol whose production cost still requires significant government subsidy to permit producers to remain in business (Montesinos and Navarro, 2000; Palmarola-Adrados et al. 2005). However, in the future, with the increased growth of energy crops and economies of scale, cost reduction may make biofuels competitive in their own right (Narendranath and Power, 2004; Gray et al. 2006).

Nearly all fuel ethanol is produced by fermentation of corn glucose in the United States or sugar cane sucrose in Brazil (Rosillo-Calle and Cortez, 1998), but any country with a significant agronomic-based economy can use current technology for fuel ethanol production (Mielenz, 2001; Atiyeh and Duvnjak, 2002; Bothast and Schleicher, 2005).

During the last two decades, technology for ethanol production from non-food-plant sources has been developed to the point at which large-scale production could be a reality in the next few years (Montesinos and Navarro, 2000). Moreover, agronomic residues such as corn stover (corn cobs and stalks), sugar cane waste, wheat or rice straw, forestry and paper mill discards, the paper potion of municipal waste, and mainly dedicated energy crops - collectively termed 'biomass'- can be converted to fuel ethanol (Datar et al. 2004).

Prosopis juliflora ((SW) D.C.), Leguminosae, popularly known as mesquite, is native to Central and South America and has spread to North America. Mesquite had been introduced to many arid zone countries, with rainfall of less than 200 mm/year, to combat desertification as it is an N-fixing legume and livestock consume its pods (Tabosa et al. 2000; Araújo et al. 2002; Mahgoub et al. 2005). Mesquite shows great potential for use as a multipurpose tree in different parts of the world, in comparison to several native and exotic species (Kailappan et al. 2000; Deans et al. 2003).

Table 1. Factorial design 2^3 for studies of the factors time of fermentation, type of microorganism and fermentation condition.

Experimental run	Codified variables			No codified variables		
	X_1	X_2	X_3	Time of fermentation (hrs)	Type of microorganism	Condition of fermentation
1	-1	-1	-1	18	*S. cerevisiae*	Static
2	1	-1	-1	36	*S. cerevisiae*	Static
3	-1	1	-1	18	*Z. mobilis*	Static
4	1	1	-1	36	*Z. mobilis*	Static
5	-1	-1	1	18	*S. cerevisiae*	Stirring
6	1	-1	1	36	*S. cerevisiae*	Stirring
7	-1	1	1	18	*Z. mobilis*	Stirring
8	1	1	1	36	*Z. mobilis*	Stirring

Mesquite pods are a significant feed source for livestock in many areas of the world (Batista et al. 2002). Pod production per tree can vary from a few kg to over 400 kg and is highly dependent on moisture availability to the plant. The mesquite pods production is of approximately 10 tons per hectare of planted tree. In the northeast region of Brazil, mesquite trees cover 150.000 hectares (Riveros, 1992).

Zymomonas mobilis has been considered a promising alternative to *Saccharomyces cerevisiae* in the synthesis of ethanol. Comparative with yeast, *Z. mobilis* has a higher tolerance to ethanol and better kinetic characteristics such as higher specific substrate uptake, ethanol synthesis rate and substrate yield to ethanol. Moreover, it has advantages for the fermentation of glucose to ethanol which include a high yield of ethanol from glucose consumed and a high specific rate of ethanol production (Joachimsthal et al. 1998; Tano and Buzato, 2003).

Table 2. Determination of glucose, total proteins and pH in liquefied mash prepared with different concentration of ground mesquite.

Mesquite concentration (g 100mL^{-1})	Total sugar* (g L^{-1})	Glucose (g L^{-1})	Total proteins (g L^{-1})
10	3.83 ± 0.04^{d}	1.29 ± 0.01^{d}	5.90 ± 0.28 ^{e}
15	5.75 ± 0.01^{c}	2.21 ± 0.03^{c}	10.40 ± 1.41^{d}
20	7.67 ± 0.07^{c}	2.29 ± 0.06^{c}	11.35 ± 1.48 ^{cd}
25	9.58 ± 0.02^{b}	3.46 ± 0.01^{b}	14.95 ± 1.34 ^{bc}
30	11.50 ± 0.06^{a}	3.99 ± 0.38^{a}	17.70 ± 1.13^{ab}
35	13.40 ± 0.08^{a}	3.98 ± 0.10^{a}	18.85 ± 0.92^{ab}
40	15.30 ± 0.05^{ab}	3.79 ± 0.07^{ab}	18.10 ± 2.97 ^{ab}
45	13.40 ± 0.01^{a}	3.88 ± 0.03^{a}	19.80 ± 2.45 ^{a}

^{a} average with different letters at the same column significantly differ ($p < 0.05$) according to the Duncan T test.
* sucrose.

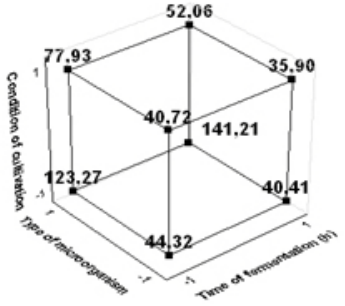
Regarding the high amount of carbohydrates present in mesquite pods (Bravo et al. 1994) and their high production in different countries, they could arise as a feasible alternative source for ethanol production. In this study the use of mesquite pods hydrated mash was assessed as biomass for the growth of *Saccharomyces cerevisiae* UFEPEDA-1012 and *Zymomonas mobilis* UFEPEDA-205 and also for production of ethanol by submerged fermentation. Moreover, an analysis of main effects was carried out in order to identify the best conditions for the ethanol production regarding the type of microorganism, condition of fermentation and the time of fermentation. To our knowledge there is a lack of studies about the ethanol production by *S. cerevisiae* and *Z. mobilis* using mesquite pods mash as fermentable substrate.

MATERIALS AND METHODS

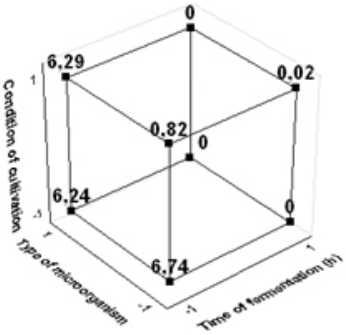
Microorganisms

Strains of *S. cerevisiae* UFEPEDA-1012 and *Z. mobilis* UFEPEDA-205 used in this study were gently supplied by the Microorganisms Collection, Department of Antibiotics, Federal University of Pernambuco, Recife, Brazil. Stock cultures of *S. cerevisiae* and *Z. mobilis* were kept in Standard Swings & De Ley - SSDL agar

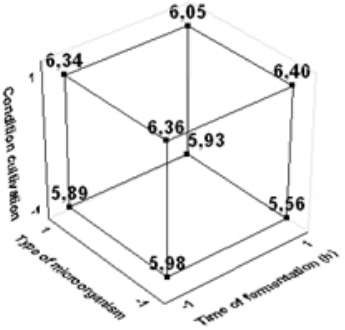
(glucose 20.0; yeast extract 5.0; agar 15 gL^{-1}) (Swings and De Ley, 1977) and Sabouraud agar (peptone 10.0; glucose 20; agar 17 gL^{-1}) slants, respectively, under refrigeration.



a. Ethanol (gL^{-1})



b. Glucose (gL^{-1})



c. Microbial count (Log cfu mL^{-1})

Fig. 1 Effects of the interaction of time of fermentation, type of microorganism and condition of cultivation on the ethanol production.
(a) Ethanol.
(b) Glucose.
(c) Microbial count.

For experimental assays, *S. cerevisiae* and *Z. mobilis* were grown in 50 mL of Sabouraud broth at 28ºC and SSDL broth at 37ºC, respectively. After the 48 hrs incubation, 5 mL of the culture was added to flasks containing 95 mL of the same growth media and allowed to grow at room temperature for 24 hrs under stirring (150 rpm).

Preparation of hydrated mesquite mash

Liquefied mash was prepared using healthy mesquite pods gently supplied by SUPRANOR (Suprimentos de Alimentos do Nordeste S/A, Rio Grande do Norte, Brazil). Pods were dried at 45ºC for 18 hrs, followed for grounding in hammer mill with a #4 screen to get the appropriate grind size. Mesquite pods ground presented moisture 5.8; total sugars 56.5; reducing sugars (glucose) 4.6; total fiber 7.2; total proteins 9.0; fat 2.1; and axes 0.2 g 100 g^{-1} (Silva et al. 2007). Hydrated mash was prepared using different concentrations of ground mesquite pods (10; 15; 20; 25; 30; 35; 40 and 45 g 100 g^{-1}) in order to find the best solubility. To prepare the mash, ground mesquite was slowly added to distilled water in a constant agitation. After the addition of the proper ground amount, the mash was heated to 50ºC, maintained at this temperature for 1 hr and submitted to centrifugation (3000 rpm for 15 min). The supernatant was vacuumed filtered using Whatman no. 1 and autoclaved at 121ºC for 15 min. The mash was cooled to room temperature and aliquots were aseptically dispensed in sterile Erlenmeyer flasks for fermentation.

Mashes at different concentrations (10-45 g 100 mL^{-1}) of mesquite pods ground were analyzed for total sugar (gL^{-1}) according to Instituto Adolfo Lutz (IAL, 2005), glucose (gL^{-1}) using the kit Glicose PAP - Liquiform (Labtest Diagnóstica, Minas Gerais, Brazil); total proteins (gL^{-1}) using the kit Total Proteins (Labtest Diagnóstica, Minas Gerais, Brazil); pH using a Micronal B474 pHmeter; and for total soluble solids using a refractometer (Shibuya Optical Co. Ltda, Japan). The mash at 30 g mL^{-1} was also analyzed for total sugar, reducing sugar (sucrose), proteins, total fiber, ashes, fat and tannin according to procedures described by Instituto Adolfo Lutz (IAL, 2005).

Growth kinetics

The growth of *S. cerevisiae* UFEPEDA-1012 and *Z. mobilis* UFEPEDA-205 was evaluated in mesquite hydrated mash (added of $(NH_4)_2SO_4$ 0.1 g 100 g^{-1} and KH_2PO_4 0.2 g 100 g^{-1}) and in standard broth. Sabouraud broth was used as standard broth for *S. cerevisiae*, while for *Z. mobilis* it was SSDL. For this, a 5 mL of a 24 hrs old culture was added to 95 mL of the growth medium and incubated at room temperature under static and stirring (150 rpm) condition. At 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 and 24 hrs post incubation a 100 µL aliquot of the mixture was uniformly spread on sterile Sabouraud and SSDL agar Petri dishes at 28ºC and 37ºC for *S. cerevisiae* and *Z. mobilis*, respectively. Each experiment was made in triplicate and the results were expressed in Log of Colony Forming Units per mL (Log cfu mL^{-1}).

Table 3. Physico-chemical variables of mesquite hydrated mash at 30 g mL^{-1}.

Physico-chemical variables	Amount (gL^{-1})
Total sugars*	16.1 ± 0.4
Reducing sugar (glucose)	3.99 ± 0.3
Proteins	2.16 ± 0.5
Total fiber	3.99 ± 0.38
Ashes	1.80 ±0.1
Fat	0.63 ± 1.3
Tanin	0.09 ± 0.0
Brix	18 ± 0.01

* sucrose

Conditions of fermentation

Submerged fermentation of mesquite hydrated mash by *S. cerevisiae* UFEPEDA-1012 and *Z. mobilis* UFEPEDA-205 was analyzed. Mesquite mash (added of $(NH_4)_2SO_4$ 0.1 g 100 g^{-1} and KH_2PO_4 0.2 g 100 g^{-1}) was aseptically distributed in sterile 250 mL Erlenmeyer flasks, inoculated with a proper amount (5 mL 100 mL^{-1}) of a 24 hrs old culture and incubated at room temperature under static or stirring (150 rpm) condition. At 18 and 36 hrs of fermentation, samples were withdrawn and analyzed for ethanol concentration, pH value and microbial growth. pH and microbial growth analysis were carried out as before cited, and the ethanol concentration was determined using Gas Chromatography (GC).

GC analysis

Concentration of ethanol was determined using a gas chromatograph (HP 5890, Hewlett-Packard, Palo Alto, CA) fitted to a flame ionizer detector. A 2 µL-portion of the fermentation sample was injected onto a column (30 m; 0.25 mm inner diameter; 0.25 lm, J&W Scientific, Folsom, CA). The chromatographic conditions were as follow: sample (without dilution) injection volume 2 µL; hydrogen flow rate 5.0 mL min^{-1}; temperature program 120ºC (isotherm); injector temperature 100ºC; detector temperature 120ºC. The data were processed using the Millennium Computer Program (Waters Chromatograph Division, Milford, MA, USA).

Experimental design (2^3)

A 2 (times of fermentation: 18 and 36 hrs) x 2 (types of microorganism: *S. cerevisiae* and *Z. mobilis*) x 2 (conditions of fermentation: static and stirring) was carried out to optimize the fermentation process. This design provided eight assays (Table 1) which were repeated three times. The observed answers were ethanol concentration (gL^{-1}), glucose concentration (gL^{-1}) and microbial count (log cfu mL^{-1}) after 18 and 36 hrs of fermentation.

Table 4. Count (log cfu mL^{-1}) of *S. cerevisiae* in Sabouraud broth and mesquite liquefied mash during 24 hrs under static and stirring condition.

Time (hrs)	Sabouraud broth		Mesquite hydrated mash	
	Stirring	Static	Stirring	Static
0	5.00 ± 0.11^{Aa}	4.98 ± 0.00^{Aa}	5.03 ± 0.07^{Aa}	4.97 ± 0.01^{Aa}
2	5.10 ± 0.03^{Aa}	4.72 ± 0.07^{Ab}	5.13 ± 0.03^{Aa}	4.87 ± 0.11^{Aa}
4	4.98 ± 0.23^{Aa}	4.79 ± 0.05^{Ba}	5.42 ± 0.06^{Aa}	5.29 ± 0.15^{Aa}
6	4.20 ± 0.30^{Ba}	4.85 ± 0.03^{Ba}	5.69 ± 0.12^{Aa}	5.06 ± 0.01^{Ab}
8	5.20 ± 0.02^{Ba}	4.83 ± 0.01^{Bb}	5.98 ± 0.06^{Aa}	5.31 ± 0.07^{Ab}
10	5.40 ± 0.03^{Ba}	4.87 ± 0.05^{Bb}	6.08 ± 0.03^{Aa}	5.66 ± 0.08^{Ab}
12	6.04 ± 0.05^{Ba}	5.55 ± 0.11^{Ab}	6.35 ± 0.03^{Aa}	5.68 ± 0.06^{Ab}
14	6.03 ± 0.06^{Ba}	5.02 ± 0.08^{Bb}	6.37 ± 0.02^{Aa}	5.70 ± 0.03^{Ab}
16	6.14 ± 0.03^{Ba}	5.45 ± 0.03^{Bb}	6.34 ± 0.01^{Aa}	5.71 ± 0.03^{Ab}
18	5.79 ± 0.01^{Ba}	5.22 ± 0.06^{Bb}	6.36 ± 0.01^{Aa}	5.92 ± 0.06^{Ab}
20	5.97 ± 0.01^{Ba}	5.22 ± 0.06^{Bb}	6.14 ± 0.05^{Aa}	5.89 ± 0.04^{Ab}
22	5.88 ±0.02^{Ba}	5.23 ± 0.04^{Bb}	6.28 ± 0.06^{Aa}	5.64 ± 0.03^{Ab}
24	6.17 ± 0.07^{Aa}	5.74 ± 0.06^{Ab}	6.11 ± 0.11^{Aa}	5.40 ± 0.01^{Bb}

[a] average with different caption letters at the same column significantly differ ($p < 0.05$) according to the Student T test.
[b] average with different small letters at the same line for each growth medium significantly differ ($p < 0.05$) according to the Student T test.

Statistical analysis

Data of microbial counts were evaluated for significant difference ($p < 0.05$) by Student t test. Data of total proteins, pH, sucrose and glucose were evaluated for significant difference ($p < 0.05$) by Duncan test. The answers obtained in the 2^3 factorial design were evaluated for main effects and their interaction. All statistical analyses were carried out using the software Statistica 6.0.

RESULTS AND DISCUSSION

Preparation of the fermentable substrate

Different concentrations (10-45 g 100 mL^{-1}) of mesquite pods ground were evaluated for best preparation of the fermentable substrate regarding the maximum solubility. For this, the concentration of glucose, sucrose, total proteins, total soluble solids in the mash prepared with different concentrations of ground mesquite were assessed (Table 2). The amount of sucrose, glucose and total proteins found for the mashes showed no significant difference ($p > 0.05$) as the amount of mesquite ground was 30 g 100 mL^{-1} or more. It suggests a possible saturation of these compounds (resulting in

a maximum limit of solubility) in mashes prepared with 30 g 100 mL^{-1} or more of ground.

No range was found for pH at the different mashes (data not showed). pH values (5.4-5.5) found for the mashes are considered as appropriate for fermentation (Mclellan et al. 1999).

Regarding these findings the mash prepared with 30 g 100 mL^{-1} of mesquite pods ground was chosen to be included in the assays for microbial growth and ethanol production. The physico-chemical variables of mesquite hydrate mash at 30 g mL^{-1} are shown in Table 3. Mean composition of the mash was 16.1 g 100 mL^{-1} of total sugars; 3.9 g 100 mL^{-1} of reducing sugars (glucose); 17.7 g 100 mL^{-1} of protein; 1.80 g 100 mL^{-1} of ashes; 0.6 g 100 mL^{-1} of fat; and 0.1 g 100 mL^{-1} of tanins. These results are in accordance with previous studies showing the high availability of fermentable sugars in mesquite mash besides a small amount of the phenolic tannin. It was found a total soluble solids value of 18ºBx. For sugar cane juice an amount of total soluble solids in a range of 12-18ºBx (g sucrose 100 g $juice^{-1}$) is regarded suitable for ethanol production (Tano and Buzato, 2003).

Microbial growth

The results of the growth kinetic of *S. cerevisiae* UFPEDA-1012 in Sabouraud broth and mesquite hydrated mash along 24 hrs in static and stirring cultivation are shown in Table 4. Counts of *S. cerevisiae* in Sabouraud broth obtained for static and stirring cultivation from 8 hrs onwards significantly differed ($p < 0.05$). When growing in mesquite hydrated mash significant differences were found from 6 hrs onwards. Counts of *S. cerevisiae* were always higher in mesquite mash along the 24 hrs of incubation.

Highest counts of *S. cerevisiae* (> 6 log cfu mL^{-1}) in both assayed growth media were found in stirring cultivation. This phenomenon (named Pasteur Effect) is commonly found in *Saccharomyces* genus, where the yeast growth best in aerobic atmosphere resulting in a fast sugar consumption (Carvalho et al. 2006). The decrease in the counts of *S. cerevisiae* at 18 to 22 hrs of incubation could be possibly related to a metabolic repression (or enzymatic repression) resulting in a smaller microbial growth rate. Metabolic repression in some microorganisms occurs when high glucose concentration (> 3 g 100 g^{-1}) are found in the growth media causing a depressed synthesis of respiratory/oxidative enzymes and ultimately resulting an increasing fermentative metabolism even when oxygen is available (Atiyeh and Duvnjak, 2002; Mahgoub et al. 2005).

The results of the growth kinetic of *Z. mobilis* UFEPEDA-205 in SSDL broth and mesquite hydrated mash along 24 hrs under static and stirring condition are shown in Table 5. Counts of *Z. mobilis* in SSDL broth obtained for static and stirring cultivation were significantly different ($p < 0.05$) from 10 hrs onwards. Highest counts of *Z. mobilis* (> 9 log cfu mL^{-1}) in SSDL broth was noted in static cultivation. Previous studies found higher counts of *Z. mobilis* under anaerobic condition in comparison to aerobic one (Bringer et al. 1984; McLellan et al. 1999). It is reported that the extremely effective action of oxygen as electron acceptor could provide a disturbance in biosynthesis metabolic reactions of different microorganisms causing a decreased specific growth rate (O'Brien and Morris, 1971).

Z. mobilis presented higher counts when growing in SSDL in comparison to mesquite mash. Significant differences ($p < 0.05$) between the counts of *Z. mobilis* found in mesquite mash incubated under static and stirring condition were only noted from 18 hrs of cultivation on.

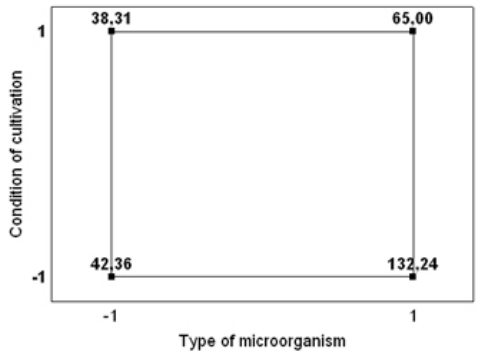


Fig. 2 Effects of the interaction of type of microorganism and condition of cultivation on the ethanol production (gL^{-1})in hydrated mesquite mash.

Along 24 hrs of fermentation the pH values of the growth media were in a range of 4.8-5.2 (data not showed). Bacterial contamination (mainly by *Lactobacillus*) in an industrial-scale ethanol production is the major cause for reduced ethanol yield. Sharply changes in pH of fermentable substrates could indicate the presence of contaminating bacteria in high amounts during the fermentation process (Mielenz, 2001).

Factorial design

Results of the analysis of main effects and their interaction in the 2^3 factorial design for ethanol production in mesquite hydrated mash by submerged fermentation are showed in Table 6. From the obtained results the effect that most affected (58.28) the ethanol production was the type of microorganism (X_2). The highest ethanol concentration (141.1 gL^{-1}) was found in the levels 1 (36 hrs), 1 (*Z. mobilis*) and -1 (static cultivation). Ethanol production by *S. cerevisiae* was higher (44.32 gL^{-1}) after 18 hrs of fermentation under static condition (Figure 1a).

The highest ethanol concentration (77.93 gL^{-1}) in stirring cultivation was noted for levels -1 (18 hrs) and 1 (*Z. mobilis*) in X_1 X_2 (Figure 1a). This ethanol amount is higher than that (23.34 gL^{-1}) found by Borsari et al. (2006) when *Z. mobilis* was inoculated in sugar cane juice for 20 hrs under stirring. Shene and Bravo (2001) studying the ethanol production by *Z. mobilis* in a mixture of glucose-fructose under static cultivation noted a highest concentration of ethanol (40 gL^{-1}) after 24 hrs of fermentation. According to our results, *Z. mobilis* when growing in mesquite mash under static condition produced higher amounts of ethanol in comparison to the findings of these researchers.

Time of fermentation (X_1) was the effect that most negatively affected (-5.02) the glucose concentration in the mash (Table 6 and Figure 1b). These results suggest

that up to 36 hrs of fermentation the glucose dispersed in the base media was totally metabolized for ethanol production. *Z. mobilis* presents a prominent capacity of hydrolyzing the sucrose dispersed in a growth media and rapidly metabolize fructose and glucose as carbon source for the ethanol production by the Entner-Doudoroff way (Swings and De Ley, 1977). According to Favela Torres and Baratti (1988) the availability of glucose, fructose or sucrose in the growth media increases the ethanol yield by *Z. mobilis*. On the other hand, small yields of ethanol are found in substrates with high amount of cellulose, inulin or starch since the bacteria is not able to hydrolyze these polymers (Swings and De Ley, 1977).

Table 5. Count (log cfu mL^{-1}) of *Z. mobilis* in SSDL broth and mesquite liquefied mash during 24 hrs under static and stirring condition.

Time (hrs)	SSDL broth		Mesquite hydrated mash	
	Stirring	Static	Stirring	Static
0	5.68 ± 0.05^{Aa}	5.70 ± 0.07^{Aa}	5.72 ± 0.03^{Aa}	5.71 ± 0.03^{Aa}
2	5.23 ± 0.07^{Bb}	5.97 ± 0.16^{Aa}	5.81 ± 0.02^{Aa}	5.81 ± 0.00^{Aa}
4	5.82 ± 0.10^{Aa}	5.89 ± 0.20^{Aa}	5.88 ± 0.00^{Aa}	5.95 ± 0.04^{Aa}
6	6.04 ± 0.07^{Aa}	5.64 ± 0.15^{Aa}	5.93 ± 0.01^{Aa}	5.85 ± 0.02^{Aa}
8	6.38 ± 0.00^{Aa}	6.26 ± 0.18^{Aa}	6.04 ± 0.07^{Ba}	5.96 ± 0.01^{Aa}
10	6.42 ± 0.08^{Ab}	8.01 ± 0.02^{Aa}	6.10 ± 0.03^{Ba}	6.11 ± 0.04^{Ba}
12	7.11 ± 0.16^{Ab}	8.98 ± 0.18^{Aa}	6.10 ± 0.03^{Ba}	6.15 ± 0.08^{Ba}
14	7.56 ± 0.08^{Ab}	9.53 ± 0.09^{Aa}	6.75 ± 0.02^{Ba}	6.94 ± 0.17^{Ba}
16	7.69 ± 0.00^{Ab}	9.66 ± 0.11^{Aa}	6.78 ± 0.03^{Ba}	6.82 ± 0.02^{Ba}
18	8.05 ± 0.09^{Ab}	9.81 ± 0.06^{Aa}	7.07 ± 0.01^{Ba}	6.71 ± 0.03^{Bb}
20	8.27 ± 0.09^{Ab}	9.82 ± 0.08^{Aa}	7.09 ± 0.01^{Ba}	6.79 ± 0.04^{Bb}
22	7.46 ± 0.08^{Ab}	9.83 ± 0.02^{Aa}	7.10 ± 0.06^{Ba}	6.65 ± 0.04^{Bb}
24	7.85 ± 0.12^{Ab}	9.85 ± 0.03^{Aa}	7.09 ± 0.03^{Ba}	6.62 ± 0.01^{Bb}

[a] average with different caption letters at the same column significantly differ ($p < 0.05$) according to the Student T test.
[b] average with different small letters at the same line for each growth medium significantly differ ($p < 0.05$) according to the Student T test.

Time of fermentation (X_1) and condition of fermentation (X_3) showed a slight influence on the microbial count in the mash (Table 6). There was no clear difference for the counts of *S. cerevisiae* and *Z. mobilis* after 18 (-1) and 36 (1) hrs of cultivation (Figure 1c).

The highest ethanol concentration (141.1 gL^{-1}) for the interaction $X_1.X_2.X_3$ was noted in the levels 1 (36 hrs), 1 (*Z. mobilis*) and -1 (static cultivation) (Figure 1a). $X_2.X_3$ (type of microorganism and cultivation condition) was the interaction that most affected the ethanol production (-31.60) (Table 6). Ethanol concentration was higher (132.24 gL^{-1}) in the mash inoculated with *Z. mobilis* under static cultivation (Figure 2). It is interesting to cite than even being found high counts of *Z. mobilis* in the mash kept under stirring condition, it did not mean higher ethanol production.

Tano and Buzzato (2003) noted a low ethanol production (29 gL^{-1}) by *Z. mobilis* in sugar cane juice during 24 hrs of fermentation in stirring condition. Anaerobic cultures of *Z. mobilis* have showed the highest ethanol yields (0.51 g $glucose^{-1}$) in different substrates, while small yields (0.13 g $glucose^{-1}$) have been noted in aerobic cultures (Kalnenieks et al. 2000; Prasad et al. 2007). Previous studies suggested a possible average yield of ethanol from mesquite pods of up to 260 L per ton, while for sugar cane it is purposed an average yield of up to 80 L per ton (Silva et al. 2003, Silva et al. 2007).

Table 6. Analysis of main effects and their interaction in the 2^3 factorial design for ethanol production in mesquite pods hydrated mash.

Variables	Answers		
	Ethanol (gL^{-1})	Glucose (gL^{-1})	Biomass (Log cfu mL^{-1})
Time of fermentation (X_1)	-4.17	-5.02	-0.16
Type of microorganism (X_2)	58.28	1.24	NS
Condition of fermentation (X_3)	-35.65	-1.46	0.45
$X_1 X_2$	NS	-1.25	NS
$X_1 X_3$	-11.18	1.47	NS
$X_2 X_3$	-31.60	1.49	-0.16
$X_1 X_2 X_3$	-10.72	-1.50	-0.20

NS: no significant for $p < 0.05$

However, these values of higher yield of ethanol from mesquite pods could be still analyzed cautiously in respect of the production of each culture. The average production of mesquite pods has been cited to be around 10 tons per hectare of planted tree, while for sugar cane the average production has been of approximately 90 tons per hectare (Rípoli et al. 2000; Maule et al. 2001; Silva et al. 2003). These previous findings suggest a possible average yield of ethanol from mesquite pods and sugar cane of up to 2600 and 7200 L per hectare of the culture, respectively.

Our results suggest that mesquite pods could be known as an alternative substrate for biotechnological purposes, mainly for ethanol production. Mesquite hydrated mash at 30 g 100 mL^{-1} presented as a suitable media for the growth of *S. cerevisiae* UFPEDA-1012 and *Z. mobilis* UFEPEDA-205 in submerged fermentation. Ethanol yield was found to be higher when *Z. mobilis* UFEPEDA-205 was cultivated in mesquite hydrated mash under static condition.

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