BIOETHANOL PRODUCTION IN BATCH MODE BY A NATIVE STRAIN OF *Zymomonas mobilis*

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Trabajo de Grado presentado como requisito para optar al título de Biólogo

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TITULO: BIOETHANOL PRODUCTION IN BATCH MODE BY A NATIVE STRAIN OF *Zymomonas mobilis**

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PALABRAS CLAVE: Bioetanol, *Zymomona mobilis,* biocombustibles

Se aislaron dos cepas nativas de *Zymomonas mobilis (ML1 y ML2*) a partir de melaza de caña obtenida en fincas de Santander, Colombia. Inicialmente, se realizó la selección de la mejor cepa productora de etanol, utilizando como valores de referencia los obtenidos con la cepa comercial de *Z. mobilis* DSM 3580. Esta cepa fue usada como patrón para la identificación morfológica, bioquímica y molecular (PCR) de las cepas nativas. Las tres cepas fueron cultivadas en un medio que contenía extracto de levadura, peptona, glucosa y sales, a pH 6 y agitación durante 62 horas. Los mejores resultados fueron los obtenidos con la cepa nativa *ML1* que alcanzó una concentración máxima de etanol de 79.78 g/L, rendimiento de etanol en biomasa ($Y_{P/X}$) 79,98 g/g, productividad volumétrica (Qp) 1,88 g/L*h, productividad específica qp 2,67 h^{-1} y velocidad específica de crecimiento μ 0,06 h^{-1}. Las cepas ML1 y ML2 fueron identificadas como *Z. mobilis*, de acuerdo con la morfología, caracterización bioquímica y molecular por PCR. Posteriormente, se evaluó el efecto de diferentes fuentes de nitrógeno sobre la producción de etanol. Los mejores resultados se obtuvieron utilizando urea en una concentración de 0,73 g/L. En este caso, la concentración máxima de etanol fue de 83,81 g/L, $Y_{P/X}$ 69,01 g/g, Qp_{max} 2,28 g/L*h, qp 3,54 h^{-1} y μ 0,12. Finalmente, se realizó un estudio del efecto de diferentes condiciones de cultivo mediante un diseño de experimentos de Placket-Burman, que indicó que las variables más significativas eran temperatura y agitación. Se logró un aumento notable en todas las variables respuesta, alcanzando una máxima concentración de etanol 93.55 g/L, $Y_{(P/X)}$ 195,81 g/g, $Qp_{max.}$ 3,89 g/L*h; qp 9,70 h^{-1}; y μ 0,11 h^{-1}. Estos resultados fueron obtenidos en las siguientes condiciones de cultivo: 35 °C de incubación, relación de carbono/nitrógeno 46, pH inicial 7 y sin agitación.

*Trabajo de Grado

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ABSTRACT

TITLE: BIOETHANOL PRODUCTION IN BATCH MODE BY A NATIVE STRAIN OF *Zymomonas mobilis* *

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Keywords: Bioethanol, *Zymomonas mobilis*, biofuels, batch fermentation

Two wild strains of *Zymomonas mobilis* were isolated (named as ML1 and ML2) from sugar cane molasses obtained from different farms of Santander, Colombia. Initially, selection of the best ethanol-producer strains was carried out using ethanol production parameters obtained with a commercial strain *Z. mobilis* DSM 3580. Three isolated strains were cultivated in a culture medium containing yeast extract, peptone, glucose and salts at pH 6 and 32 °C with a stirring rate of 65 rpm for 62 h. The best ethanol production results were obtained with the native strain ML1, reaching a maximum ethanol concentration of 79.78 g l^{-1}. ML1 and ML2 strains were identified as *Z. mobilis*, according to the morphology, biochemical tests and molecular characterization by PCR of specific DNA sequences from *Z. mobilis*. Subsequently, the effects of different nitrogen sources on production of ethanol were evaluated. The best results were obtained using urea at a 0.73 gl^{-1}. In this case, maximum concentration of ethanol was 83.81 g l^{-1}, with kinetic parameters of yield of ethanol on biomass ($Y_{P/X}$) = 69.01(g g^{-1}), maximum volumetric productivity of ethanol (Qp_{max}) = 2.28 (g l^{-1} h^{-1}), specific productivity of ethanol (q_p) = 3.54 (h^{-1}) and specific growth rate (μ) = 0.12 h^{-1}. Finally, we studied the effects of different culture conditions (pH, temperature, stirring, C/N ratio) with a Placket-Burman experimental design. This optimization indicated that the most significant variables were temperature and stirring rate. In the best culture conditions, a significant increase in all variables of response was achieved, reaching a maximum ethanol concentration of 93.55 g l^{-1}.

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*Degree Work

**Science, Biology, Director Rodrigo Torres. Codirector Claudia Ortíz

INTRODUCTION

Recently, the search and use of both new alternative and renewable energy sources has increased. Likewise, there is great interest in expanding the use of biofuels and biomass energy sources because it is possible to get energy supply in a long-term and sustainable manner. Simultaneously, cleaner technologies that contribute to reduce the environmental impact associated with the use of fossil fuels have been developed. Therefore, many authors have proposed the use of bioethanol as a greener alternative, economically viable and significant potential energy source (Tao et al. 2005; Wu et al. 2007).

Bioethanol is mostly obtained by fermentation of sugars, which are converted into ethanol and carbon dioxide by some microorganisms. *Saccharomyces cerevisiae* yeast are the most frequently used in this type of bioprocess (Dien et al. 2003; Baptista et al. 2006; Lin et al. 2006). Another promising microorganism for the production of bioethanol is *Zymomonas mobilis.* This is an anaerobic, facultative and Gram negative bacterium, which produces ethanol from glucose via the *Entner-Doudoroff* (ED) pathway (Bai et al. 2008). In this way, *Z. mobilis* generates a highly efficient glucose metabolic flux towards product with low bacterial growth, reaching productivities of ethanol 3 to 5 times higher than *S. cerevisiae,* and product yields on substrate about 97%, converting 1 mol of hexose to 1 mol of ethanol (and releasing 1 mol of ATP) (Bai et al. 2008; Cazetta et al. 2007). However, *Z. mobilis* presents a narrower range of useful substrates for fermentation than yeasts, because it only has the ability to utilize glucose, fructose and sucrose (Swings and De Ley 1977). Additionally, using sucrose as substrate, *Z. mobilis* can form other products such as sorbitol, levan polysaccharides and fructose oligosaccharides (FOS), causing a significant decrease in ethanol production (Skotnicki et al. 1981; Liu et al. 2010).

Furthermore, *Z. mobilis* tolerates high concentrations of ethanol (higher than 16% v/v) and has a high yield of product using glucose (Davis et al. 2006; Zhang et al. 1995). It has a wide temperature range (between 30 and 39 °C) for microbial growth (Swings and De Ley 1977). It also displays high tolerance for changes in pH (between 3.5 and 7.5 $\pm$ 0.2), the range between 5.0 and 7.0 $\pm$ 0.2 being the most suitable for ethanol production (Tao et al. 2005). In this way, *Z. mobilis* can ferment sugars to ethanol for a longer time than yeast before being inhibited by acidification of culture medium (Bai et al. 2008; Fu and Peiris 2007).

This study was aimed to isolate and select wild strains of *Z. mobilis* obtained from typical products of the region (Santander-Colombia), and to study the effect of the composition and culture conditions on production of ethanol in batches at laboratory scale.

1.METHODS

1.1 Isolation of native strains of *Z. mobilis* from plant sources

Z. mobilis were isolated by taking samples from sugar cane, apple juice, pear juice, sugarcane juice and molasses available in Rio Negro farms (Santander, Colombia). The samples were collected and transported under aseptic conditions. Subsequently, each sample was suspended in saline solution with 1:10 dilution. Finally, these were seeded in solid agar Petri dishes containing (in g l^{-1}) the following composition: yeast extract: 10, agar: 15, peptone: 10 and glucose: 20, in aerobic conditions (Tao et al. 2005). The isolation of strains was made based on microscopic, macroscopic, biochemical features and the ethanol production performance in liquid medium. A collection strain of *Z. mobilis* DSM 3580 was used as a reference strain. Microscopic morphology was visualized by Gram stain methodology (Dworkin et al. 2006; Brenner et al. 2005). All bacteria were stored at 4 °C in the culture medium mentioned above for further use in fermentation and molecular characterization.

1.2 Biochemical identification

Bacteria identification was done by the following biochemical tests: catalase, citrate, D-Glucose, fructose, sucrose, indole, triple sugar iron (TSI), methyl red / Voges Proskauer (MR / VP), lysine iron agar (LIA) (Dworkin et al. 2006).

1.3 Molecular identification of native strains by PCR

- *DNA extraction*

Genomic DNA was obtained using the GenElute bacterial genomic DNA extraction kit (Sigma), according to manufacturer instructions.

- *PCR for the detection of Z. mobilis*

Specific *Z. mobilis* primers were selected in the 3´ region of the 16S rRNA gene Z16p3 (5´-CAAGCCTGCAAAGGTWAG- 3´) and in the 5´ region of the 23S rRNA gene Z23p5 (5´-ATGCTCTTACCTCACGCT-3´) [14]. PCR mix was made as follows: 20 pmoles of Z16p3 and Z23p5 in the presence of 2 mM $MgCl_2$ and 1U *Taq* polymerase (Fermentas). The following PCR amplification conditions were used: 95°C for 5 min, 30 cycles of 95°C for 45 s, 50°C for 45 s, 72°C for 1 min with a final extension of 72°C for 5 min. PCR samples (25 µl) were run on a 0.8% (w/v) agarose gel in 1X TBE and visualized by ethidium bromide staining (Bio-Rad) (Coton et al. 2005).

Additionally, we show a sequencing of the amplified DNA of the best ethanol-producing strain (ML1), (Macrogen, Advancing through Genomics) whose sequence is available in Online Resource 1.

1.4 Selection of the best strain of *Z.mobilis* for ethanol production studies

For the selection of the best ethanol-producing strain, we increased glucose concentration in the culture medium to 150 g l^{-1}, according to previous studies (Tao et al.2005; Cazetta et al. 2007; Amutha and Gunasekaran 2001). Batch fermentations were performed in 250 ml Erlenmeyer flasks containing 160 ml of culture medium. Culture media were inoculated with a suspension of bacteria to get a standard concentration of 0.1 g l^{-1} in the culture medium. A pre-inoculum had previously been grown for 36 h at 32 ºC. Ethanol fermentations were kept at 32 °C and pH 6 ± 0.2 for 62 h with stirring at 65 rpm. Selection of the best strain was made based on growth kinetics, glucose uptake and ethanol production rates (Skotnicki et al. 1981).

1.5 Growth kinetics and ethanol production by *Z. mobilis*

Samples were periodically withdrawn and then centrifuged at 10,000 g for 15 min at 4 ºC. Biomass concentration was determined by the dry-weight technique and correlated with absorbance at 650 nm (Kesava et al. 1995). Cell density was

determined by measuring the absorbance at 650 nm of appropriately diluted sample using a UV-visible spectroscopy system (Shimadzu UV-1800, Maryland-USA). Cell dry weight (CDW) was determined by centrifuging 20 ml samples, and the pellets were washed twice with deionized water before drying at 95 °C to constant weight. One unit of OD650 was equivalent to 0.47 g l^{-1} of CDW. Glucose was quantified by the Di-Nitro-Salisylic acid (DNS) method, measuring absorbance at 540 nm (Miller 1959; Fu and Peiris 2007). Ethanol production was determined by gas chromatography (GC) using a flame ionization detector (FID) (Li et al. 2009). GC operating conditions were the following: DB-WAX column (dimensions of 60 m x 0.25 mm x 0.25 µm), with an initial temperature of 35 °C for 8 minutes and He as the carrier gas with a flow of 2 ml min^{-1}. Headspace operating conditions were the following: 10 min with average agitation for the sample balance at a temperature of 105 °C, and vial pressurization time of 0.03 min, samples loop fill time of 0.1 min and finally an equilibrium time loop of 0.1 min.

1.6 Study of the effect of medium composition and culture conditions on the ethanol production with a selected native *Z. mobilis* strain

Initially, the effect of the following nitrogen sources was studied: peptone, urea and ammonium sulfate (Ruanglek et al. 2006). The tests were carried out in 250 ml Erlenmeyer flasks containing 160 ml of culture medium. In this case, we used a reference medium (Cazetta et al. 2007), containing additionally 2 g l^{-1} of KH_2PO_4, 2 and 5 g l^{-1} of $MgSO_4$ 7 H_2O. We tested the following concentrations of nitrogen sources: 10 g l^{-1} peptone, 0.73 g l^{-1} of urea and 1.64 g l^{-1} ammonium sulfate. The experiments were performed at 32 ºC and initial pH 6, with a stirring rate of 65 rpm.

Subsequently, the effect of different growth conditions on the production of ethanol from *Z. mobilis* was studied by using a Plackett-Burman experimental design (Srinivas et al. 1994; Yu et al. 1997). The experimental design matrix for the growth conditions is presented in Table 1. In these experiments we used urea as the nitrogen source at different concentrations to obtain different C/N ratios. The other nutrients were added in the same concentrations as previously described. We

worked with different conditions of pH and temperature, and with and without stirring.

Table 1. Plackett-Burman design, to identify best growing conditions in a batch fermentation kinetics for thanol production using *Z. mobilis*

	pH	d1*	rpm	d2*	C:N**	d3*	T°
1	7	+	65	-	92	-	30
2	5	+	65	+	46	+	30
3	5	-	65	+	92	-	35
4	7	-	0	+	92	+	30
5	5	+	0	-	92	+	35
6	7	-	65	-	46	+	35
7	7	+	0	+	46	-	35
8	5	-	0	-	46	-	30

* Dummy variable

**Carbon/nitrogen ratio

1.7 Ethanol production in best growth and culture medium conditions using a native *Z.mobilis* strain

Best growth conditions were determined from the Plackett-Burman design. We performed additional tests to study the effect of the following C/N ratios: 9.2, 23, 46, 69 and 92. On the basis of obtained results, we selected the following culture conditions for ethanol production: temperature of 35 °C, ratio of C/N = 46, pH 7 and without agitation.

In all experiments, kinetic parameters were calculated at maximum values. We determined the following parameters: yield of ethanol ($Y_{P/X}$), defined as g of ethanol per g of biomass; volumetric productivity (Q_P), defined as g of ethanol per ml and

unit time; specific productivity (qp), defined as g of ethanol per g of biomass per unit of time, and specific growth rate (μ), expressed in h^{-1}. All experiments were performed in triplicate.

2.RESULTS AND DISCUSSION

2.1 Identification of isolate strains of *Z. mobilis* from plant sources

First, we isolated different bacteria from vegetal sources available in our region. Two presumptive *Z.mobilis* strains were isolated from sugarcane molasses, which initially were called ML1 and ML2. It was not possible to isolate similar bacteria from the other substrates evaluated, even though these substrates have been recommended for growth of this type of bacteria (Swings J and De Ley J 1977). However, we obtained some ethanol producing yeast of *Candida* species, which may have inhibited the bacterial growth. These yeasts were used in other related studies. Isolates ML1 and ML2 presented macro and microscopic characteristics typical of *Z. mobilis.* The results of the characterization by biochemical tests matched with *Z. mobilis* (Table 2), and were according to those published by other authors (Zhang et al. 1995; Dworkin et al. 2006).

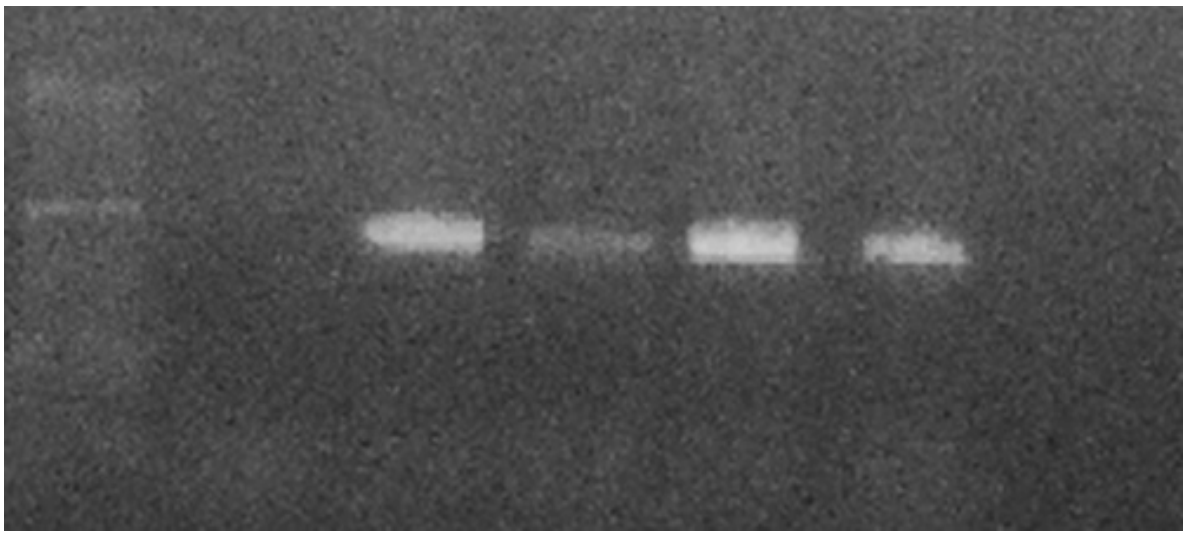
Table 2. Biochemical tests performed for the preliminary identification strains isolated from sugar cane molasses ML1, ML2 and *Z. mobilis* collection DSM 3580.

Pruebas	**ML2**	**ML1**	**DSM 3580**
Citrate	-	-	-
TSI	K/A	K/A	K/A
LIA	K/A	K/A	K/A
INDOL	-	-	-
RM/VP	-	-	-

Catalase	+	+	+
Glucose	+	+	+
Fructose	+	+	+
Sucrose	+	+	+

For the molecular identification of presumptive isolated bacteria, genomic DNA was isolated from strains ML1, ML2, and the strain DSM 3580. A fragment of 900 bp was amplified, using a pair of specific primers (16S rRNA gene Z16p3 y 23S rRNA gene Z23p5) for each of the strains, as presented in Figure 1.The results obtained confirmed that the bacteria isolated from sugarcane molasses belong to the species *Z. mobilis.* The results of amplification with the specific primer pair coincided with previous studies performed by other authors (Coton et al. 2005).

Fig 1. Amplification of genomic DNA of strain DSM 3580, ML1 and ML2. Lane 1, molecular marker HyperLadder II. Lane 2, negative control. Lane 3 and 4 amplified product of reference strain (DSM 3580) with dilutions 1 / 25 and 1 / 50 respectively. Lane 5, strain ML1. Lane 6, strain ML2.



2.2 Selection of the best strain of *Z.mobilis* for ethanol production studies

Strains of wild *Z. mobilis* were used (ML1 and ML2) in the selection of the best microorganisms for ethanol production, with *Z. mobilis* DSM 3580 as reference. We evaluated the growth kinetics, glucose uptake and ethanol production (See Fig. 2). Figure 2 shows that the wild strains (ML1 and ML2) have similar ethanol production up to 36 hours. However, at 48 hours we observed that ML1 shows a marked increase in ethanol production, achieving a maximum concentration of ethanol of 79.98 g l^{-1} at 62 h. On the other hand, ML2 presents a slight increase in ethanol production at 48 h, but obtains a maximum concentration of ethanol of 68.70 g l^{-1} at 62 h in the fermentation medium. Furthermore, *Z. mobilis* DSM 3580 achieved a lower ethanol production at 24 h of fermentation, ending in the stationary phase with a maximum concentration of 50.30 g l^{-1} of ethanol at 62 h. Selection of the best strain was carried out based on the kinetic parameters obtained for each fermentation (Table 3). According to these results, *Z. mobilis* ML1 was selected because this strain presented the greatest values of $Y_{P/X}$, Q_P, q_p and μ. These results are very close to those obtained in another study of selection of ethanol producing strains of *Z. mobilis* from fermented sugar cane juices (Cazetta et al. 2007).

Fig 2. Comparison of the kinetics of batch fermentation for ethanol production using wild strains and reference in the following culture conditions: pH 6, agitation 65 rpm and temperature 35 °C. A. Wild strain ML1, B. Wild strain ML2 and C. *Z. mobilis* DSM 3580.

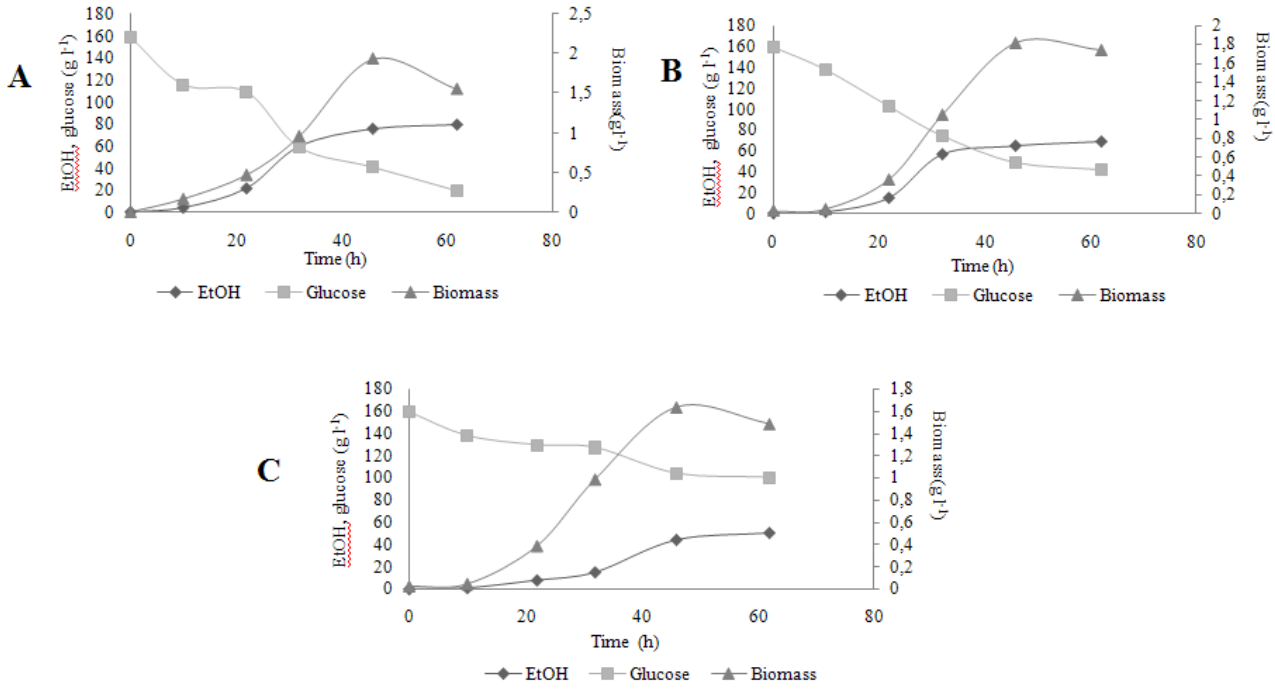


Table 3: Kinetic parameters of fermentation to choose the best wild strain producers ethanol under the following conditions: pH of 6, 65 rpm and 35 ° C. ($Y_{P/X}$) yield ethanol in biomass produced (Qp) volumetric productivity (qp) specific productivity (μ) specific growth rate.

Strain	$Y_{P/X}$ (g/g)	Qp max (g l^{-1}*h^{-1})	qp (h^{-1})	μ (h^{-1})
DSM 3580	**31,21**	**0,81**	**0,49**	**0,11**

ML1	**56,79**	**1,28**	**0,66**	**0,12**
ML2	**38,17**	**1,10**	**0,60**	**0,09**

2.3 Study of the effect of medium composition and culture conditions on the ethanol production with a selected native *Z. mobilis* strain

Most media for culture of *Z. mobilis* are supplemented with ammonium salts as the nitrogen source (Ruanglek et al. 2006). This study evaluated the effect of different nitrogen sources including: peptone, urea and ammonium sulfate (Fig. 3). Fermentation results showed an increased production of ethanol and biomass using urea as the nitrogen source. Similarly, kinetic parameters obtained during fermentation of glucose by *Z. mobilis* confirmed that culture medium supplemented with urea was the best medium for ethanol production. In this case, maximum concentration of ethanol of 83.81 g l^{-1} was reached at 60 h. Table 4 shows the values of $Y_{P/X}$, Q_P, and μ achieved when urea was used as the nitrogen source. These values were higher than those obtained with peptone or ammonium sulfate. Thus, urea can be an interesting and alternative nitrogen source because it is very economical from an industrial point of view. Fermentation results showed that the culture medium supplemented with urea significantly favored ethanol production by 5%. These results are similar to those obtained in another studythat compared urea and ammonium sulfate as nitrogen sources (Ruanglek et al. 2006). However, volumetric productivity of ethanol did not change (1.77 g l^{-1} h^{-1} to 1.78 g l^{-1} h^{-1}), probably due to the presence of yeast extract and calcium pantothenate as culture medium supplements (Rakin et al. 2004).

Fig 3. Effect of different nitrogen sources on the kinetics of batch fermentation for ethanol production, using the wild strain ML1 previously selected and a relationC/N ratio of 92. Growing conditions were: pH 6, agitation 65 rpm and 35 °C. A. urea, B. peptone and C. ammonium sulfate

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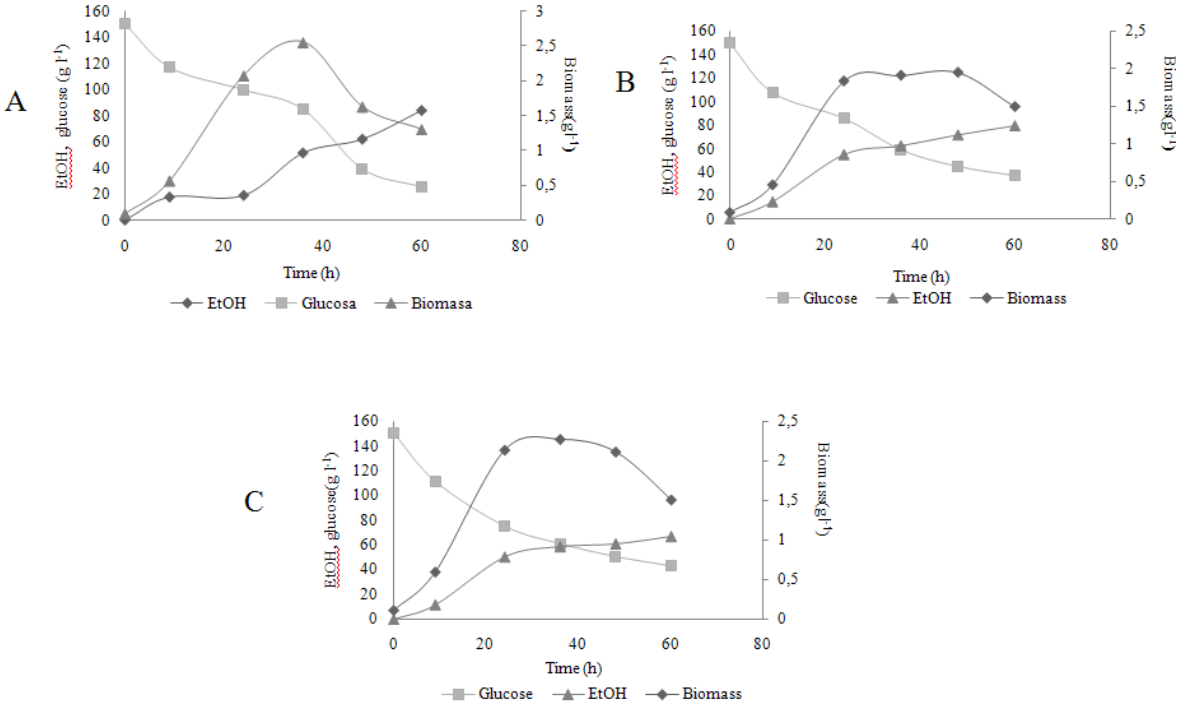


Table 4. Kinetic parameters of ethanol fermentation by *Z. mobilis* evaluating different sources of nitrogen to the following conditions: 65 rpm, pH 6, and a carbon-nitrogen ratio of 92. ($Y_{P/X}$) yield ethanol in biomass produced (Qp) volumetric productivity (qp) specific productivity (μ) specific growth rate.

Nitrogen source	**$Y_{P/X}$ (g/g)**	**Qp max ($g\ l^{-1}*h^{-1}$)**	**qp (h^{-1})**	**μ (h^{-1})**
Peptone	56,509	1,322	0,884	0,119
Ammonium	47,380	1,108	0,736	0,121
Urea	69,008	1,397	1,072	0,125

Results obtained in relation to the Plackett-Burman design are presented in Table 5. Significant increases in ethanol concentration and kinetic parameters are highlighted. The best results were obtained in run 7, which corresponds to conditions of pH 7, without agitation, C/N ratio of 46 and incubation temperature of 35 °C. Results achieved during ethanol fermentation by *Z. mobilis* were: ethanol concentration of 93.55 g l^{-1}, $Y_{P/X}$ 195.80 g g^{-1} and q_p 3.89 h^{-1}.

Table 5. Response variables of Placket Burman design used for the study of culture conditions on ethanol production using *Z. mobilis*

Run	EtOH (g/L)	Qp max (g l^{-1}*h^{-1})	qp (h^{-1})	Y p/x (g/g)	μ (h^{-1})
1	87,22	2,65	2,20	52,86	0,11
2	79,16	2,85	2,59	35,78	0,13
3	75,53	2,76	5,41	35,65	0,11
4	61,99	2,31	2,96	72,72	0,09
5	87,75	3,41	6,83	131,83	0,12
6	64,23	1,73	1,15	40,11	0,09
7	93,83	3,89	9,70	195,80	0,11
8	63,10	1,65	2,68	50,09	0,10

These results agree with the physiological characteristics of *Z. mobilis.* The use of appropriate temperatures for the metabolism of these bacteria improves performance of enzymes involved in the fermentation process [Philips 1996; Malvessi et al. 2010). On the other hand, absence of stirring allowed better production of ethanol in *Z. mobilis*. This trend coincides with previous studies carried out in static culture, where ethanol concentrations achieved in the fermentation broth were 2 folds higher than shaking cultures (Cazetta et al. 2007).

Furthermore, statistical analysis of the different variables analyzed from the Plackett-Burman design were carried out. We used regression coefficients and *t*-student values as criteria by which to analyze significance levels and select the most important variables for ethanol production based on the response fermentation parameters. For this reason, yield of ethanol on biomass ($Y_{P/X}$) and specific productivity of ethanol (q_p) were selected.

Table 6. Results of statistic analysis of Plackett Burman design on the effect of the conditions of the culture medium, taking into account the specific ethanol productivity (qp).

VARIABLE	T-STUDENT	SIGNIFICANCE (%)
pH	-0,186	No significant
rpm	-1,386	75
C:N	0,163	No significant
Temperature	1,609	80

Table 7. Results of statistic analysis of Plackett Burman design on the effect of the conditions of the culture medium, taking into account etanol yield in biomass($Y_{p/x}$).

VARIABLE	T-STUDENT	SIGNIFICANCE (%)
pH	0,802	No significant
rpm	-2,122	90
C:N	-0,213	No significant
Temperature	1,424	75

According to the values obtained (Tables 6 and 7), variables with greater effect on ethanol production were pH and C/N ratio. Nevertheless, low incidence of pH effects on ethanol production and microbial growth can be attributed to the wide

range of pH tolerates of bacteria from *Z. mobilis* species (Tao et al. 2005; Swings and De Ley 1977).

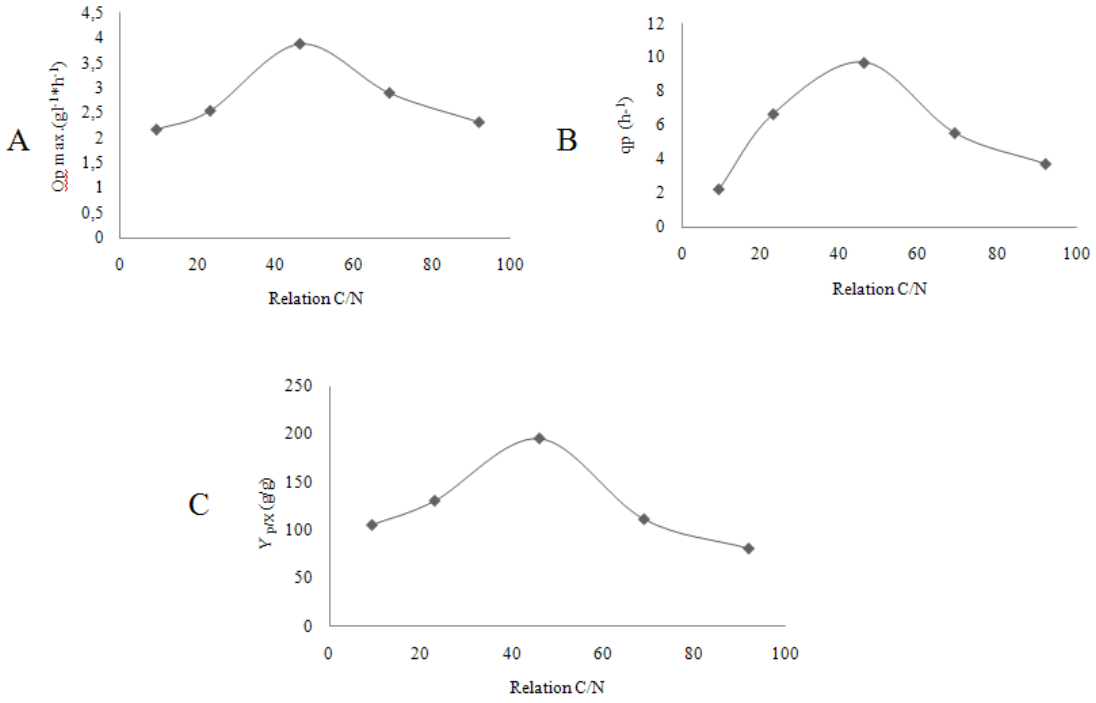
2.4 Ethanol production using best growth and culture medium conditions with a native *Z.mobilis* strain

Because carbon/nitrogen ratios, and specially carbon or nitrogen substrate limitations, can dramatically influence productivity levels of ethanol (Godoy et al. 2006), we carried out different runs of fermentations using different C/N ratios under the best conditions obtained from the Plackett-Burman design (initial pH 7, without stirring and an incubation temperature of 35 °C). The results presented in Table 8 show that a C/N ratio = 46 is possible to achieve best yield and productivity values. These results are more evident in Figure 4, which presents the effect of different C/N ratios on the kinetic parameters Q_p q_p and $Y_{p/x}$.

Table 8. Fermentation kinetic parameters obtained from the validation of conditions obtained at the Plackett-Burman design, comparing different relation C-N ratio (35 ° C without agitation and initial pH 7).

	Ethanol [g/L]	**Qp max ($g^{-1}*h^{-1}$)**	**qp (h^{-1})**	**$Y_{p/x}$ (g/g)**	**μ (h^{-1})**
C-N=92	97,06	2,31	3,75	80,88	0,11
C-N=46	93,83	3,89	9,70	195,80	0,11
C-N=9,2	89,55	2,16	2,26	105,47	0,06
C-N=69	91,75	2,90	5,07	111,50	0,10
C-N=23	90,10	2,54	6,68	130,74	0,98

Fig 4. Effect of different relations C/N ratios on the kinetic parameters of fermentation using the ML1 strain previously selected. The culture conditions were determined from the Plackett-Burman design. Figure A. QpMax, Figure B. qp, Figure C. Y P / X



3. CONCLUSIONS

We achieved isolation of two native *Z. mobilis* strains from a plant source. One of these native strains (named ML1) was able to produce high ethanol concentrations compared to the other two strains evaluated and those published by other authors working with glucose as the main carbon source. Moreover, we used a Plackett-Burman statistical methodology to improve culture medium and conditions of ethanol production in a batch fermentation mode with *Z. mobilis* ML1, obtaining as best growing culture conditions for this strain: pH 7, without agitation, 35 °C and using as nitrogen source a carbon/nitrogen ratio of 46.

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5.ONLINE RESOURCE 1

>ML1 [Zymomonas mobilis]
NNNNNNNNNGNGCTAGCGGTATTGTTCTCTGCTACTCGAGAGCATAGAAGCGCGGAATCG
CTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCAGGCCTTGTACACACCGCC
CGTCACACCATGGGAGTTGGATTCACCCGAAGGCGCTGCGCTAACCCGCAAGGGAGGCAG
GCGACCACGGTGGGTTTAGCGACTGGGGTGAAGTCGTAACAAGGTAGCCGTAGGGGAACC
TGCGGCTGGATCACCTCCTTTCTAAGGATAGTCGAACATATCTTTGATAGTTTGACTGTA
CTAGAACATAAAAAATTCCGCCGTCCTCATGTCCCTTCATCTTGGAAAAAGCCTGTTGAG
CACTGTAGAGTGTTTGATGGTGGTAAAGTTAGCCTATGTGCTATTGCCAATGGGCCGGTA
GCTCAGGTGGTTAGAGCGCACGCCTGATAAGCGTGAGGTCGGAGGTTCAACTCCTCCCCG
GCCCACCATGGTTCAGGTAGGGAATTTCAAAGGGGCCTTAGCTCAGTTGGGAGAGCGCTA
GCTTTGCAAGCTTGAGGTCATCGGTTCGATCCCGATAGGCTCCACCAGAAATTATCTGGT
TGTTTTCCAAAGAAAGATGAAGAGGAAAAGAGATCCTTCTGATGATATCAGGAGGGTAGG
GGATTTATCCCTGAATGTTATTTGAAATTGTGAATGGAAATATTGAAAATCGATGCCGCG
ACTTCGATTTTTGAATGACTGCTTCGGTGGTTATTTGGAAAGCGAAAGTTGCATAAAGTA
AAGATATTCAATTGATGCTGAGATTTAACAAAAAGCATCATCGGTGGAAGAAGCAATTCT
GACATTGATGGTGGAAGTCTCAGNGNNGGGGANGGGCGNNNTNCTTTNAGGCGGGCTGAG
ATAAAAACAACATAAAGGGGGCCCCACAAAAATATATATTGTGGT----------
----------GGGTG