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Zymomonas isolated from different States of Mexico: limited producers of ethanol

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Abstract

The microbial synthesis of ethanol is conventionally by yeast, however the bacterial genus Zymomonas has an interesting capacity to synthesize ethanol, currently it is an ecological alternative, for the global demand for fuels other than oil. The objectives of this work were: i) to isolate Zymomonas that synthesize ethanol from natural sources ii) to select Zymomonas natural amounts for ethanol synthesis. The results show a wide distribution of Zymomonas in natural sources and limited natural ability to generate ethanol. It is reaffirmed that this genus is an alternative for the solution of the world energy crisis, based in to improve its natural ability for ethanol synthesized by biological means does not cause environmental pollution.

Keywords: Non-fossil fuel, natural selection, vegetable juice, bacterial fermentation.

INTRODUCTION

In tropical areas of America, Asia and Africa, alcoholic beverages from fruit juices are prepared and consumed by mixed fermentation with prokaryotes of the *Zymomonas* type and yeasts (1-3). The fermentation of sugars from these drinks is an ancient art. In Babylonia and Summaria 6000 years BC, yeasts were used to make beer (4). In the fourteenth century in China and the Middle East, alcoholic beverages were made from fermented grains (5). Cagniard de la Tour in France, Schwann and Kutsing in Germany, before Pasteur (1,3,4) reported that during fermentation there was: ethanol and carbon dioxide due to the activity of microscopic beings in the absence of oxygen (O_2). Pasteur in 1891 demonstrated that wine disease was caused by anaerobic bacteria that converted ethanol to acetic acid (4) In 1891 Barrer and Hillier isolated the genus *Zymomonas* as the agent responsible for low acidity in cider from the southwest of England (5). Between 1923-1924 Lindner in Mexico studied the fermentation of mead or sugary juice of the agave in the elaboration of pulque, an alcoholic drink with a content between 4 to 6% of ethanol, from that he isolated the genus and bacterial species called *Termobacterium mobile* (1-4). In 1931 Kluyver and Hoppenbrowers named it *Pseudomonas lindner*i due to its similar biochemical behavior with the *Pseudomonadaceae* family, but in 1936 Kluyver and Van Nielr identified it as *Zymomonas mobile* due to the differences with the *Pseudomonadaceae* family (5). In 1937 Shimwell isolated *Zymomonas* in the early fermentation stage of barley when brewingas well as on the brewery floor, on the wash brushes of the fermentation vats, and gave it the provisional name *Achromobacter anaerobium.* In

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1950 the name of *Saccharomonas* sp was proposed, as it was assumed to be a contaminant in brewing, it was called *Zymomonas anaerobia* (3,4). In 1956 he had already reported how: *A. anaerobium* and *T. mobile,* the latter ferments sucrose (6,7). In Bergey's Manual of Determinative Bacteriology 2005 edition (1,8), *Zymomonas mobilis* is classified as the main species, *Z. mobilis* exists in natural sources such as vegetable fruits, associated with yeasts, it is part of the microbiota of wine from palm, is cosmopolitan in: Indonesia, Far East, India, Africa, Brazil. Java, Mexico, etc. In palm wine it is the main genus and tolerant species of high glucose, fructose, and sucrose content and consequently alcoholic concentration, as in sugarcane juice in Brazil and in honey (9-111).

Zymomonas spp in the synthesis of ethanol. *Z. mobilis* in batch cultures, ferments glucose, fructose and sucrose to ethanol, in continuous culture, enhanced by mutagenesis with ultraviolet light and nitrosoguanidine (10-12, 20). In 1977, Swings and De Ley , reported a yield of 1.5 moles of ethanol/mol of fermented glucose, that exceeds that of *Saccharomyces*, that synthesizes 49% ethanol from glucose, with the advantage that *Z. mobilis*, depending on the composition of the medium of culture is homo or heterofermenting (7,9,10) uses a minimum part of the sugar as a carbon source, and ferments 98%, while it only uses 2% in growth, but exclusively in anaerobiosis, in aerobiosis it generates one mole of ethanol/mole of glucose fermented, for this reason *Z. mobilis* is used in the manufacture of molasses drinks and milk enriched with fermentable sugars, especially in the industrialization of traditional drinks such as pulque (5,8,11). *Z. mobilis* has advantages in the synthesis of ethanol, compared to *Saccharomyces,* such as the following (13,14): a) it tolerates high concentrations of glucose and ferments to ethanol at a higher level than yeasts, especially in continuous culture in that it generates a high concentration of ethanol, b) that high concentration of ethanol, but low in biomass production compared to yeast genera used in ethanol synthesis (15,16). *Zymomona*s grows anaerobically, unlike yeasts, and it is not necessary to control the O_2 level to remain viable in continuous culture (17). *Zymomonas* spp has a high tolerance to ethanol of 70 to 80 g/L compared to *Saccharomyces cerevisiae* in continuous culture and more than 120g/L in batch (13,18). Therefore, wild isolates of *Zymomonas* from juices and naturally fermented beverages, such as pulque, are convenient to exploit this capacity to generate ethanol (19). In Mexico, pulque is consumed in the center and south of the republic, it is a source of isolation of *Zymomonas* (14), since it is reported that it synthesizes 1.8 moles of ethanol and 1.9 moles of carbon dioxide/mol of fermented glucose (16). Therefore, the objectives of this work were: i) isolate ethanol-producing *Z. mobilis* from natural sources of some States of Mexico ii) select *Z. mobilis* with a high capacity for ethanol synthesis.

MATERIAL AND METHODS

Origin of wild type *Zymomonas*.

Samples of natural vegetable fruit juices were collected in a 50 ml sterile bottle, transported on ice to the laboratory for isolation.

Detection of wild *Zymomonas*. It was carried out in tubes in yeast extract broth (BYE) g/L: 3g, malt extract 3, casein peptone 5, glucose 20; 100 ppm cycloheximide and 3% ethanol, pH 5.0 adjusted with lactic acid, w/inverted Durham tube. The tubes were incubated at 30°C/24-48 h, the presence of *Zymomonas* was detected by gas production and the typical morphology: short Gram-Negative diplobacillus. This step was repeated for the isolation in axenic culture. Isolation of *Zymomonas* spp. The positive tubes in the previous stage were seeded in Patri dishes with yeast extract glucose agar (YEGA) incubated at 30°C/24-48h. Those typical *Zymomonas* colonies were selected and by reseeding in YEGA without ethanol on the plate, thus axenic cultures were obtained (4). Identification of the isolates. The Bergey's Manual of Determinative Bacteriology, ninth edition of 2005, was used. Based on the tests for the identification of *Zymomonas* sp, the collection strain *Z. mobilis* ATCC 10988 was included as a reference and comparison standard (6-8). Conservation of isolates. The isolates, as well as the *Zymomonas* collection strain, were reseeded monthly in YEGA without ethanol with cycloheximide and kept refrigerated at 10°C (8,13,15).

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DNA extraction of wild isolates from different states of Mexico to complete its identification

Bacteria DNA was isolated using a physical breakdown method with glass beans. Bacterial cells were washed with distilled water, centrifuged and resuspended in 0.2ml of extraction mixture (2% triton, 1% SDS, 10mM NaCl, 10mM TRIS-HCl pH 8, USB, Cleveland, USA), EDTA 1mM, adding 0.2ml of 25:24:1 phenol: chloroform: isoamylic alcohol, 0.06g of 0.5-mm glass beads and 0.1ml sterile distilled water. Finally, the cells were homogenized by vortex for 1 min at high speed for 8 times. The tubes were chilled on ice for 1 min between runs. The upper layer was recovered and transferred to a clean tube and 1 volume of pure chloroform was added, the tube was mixed carefully and centrifuged at 13 000 *g* for 5 min. The aqueous phase was saved in a clean tube and 20 □**l** g ml^{-1} RNAase was added and incubated for 1 hour at 37°C. After 1 volume of 24:1 chloroform: isoamylic was added and centrifuged again. The aqueous phase was transferred to a clean tube with 10 µl of isopropanol and 4ml of ammonium acetate (4M) and carefully mixed and incubated for 15 min at room temperature. Supernant precipitate was centrifuged for 10 min and the DNA was washed with 70% v v^{-1} ethanol, vacuum-dried and dissolved in 30ml of TE prepared:10mM Tris-HCl, 1mM EDTA pH 7.5 (8,9,11).

PCR amplification and cloning of 16S rDNA and ITS rDNA

The 16S rDNA regions of the bacteria were amplified using the forward primer 533F 5'-GTG CCA GCA GCC GCG GTA A -3' and the reverse primer 1492R 5'-GGT TAC CTT GTT ACG ACT T -3'. (Dojka *et al.* 1998). To amplify the ITS rDNA region from yeast the primers used were ITS1 5'- TCC GTA GGT GAA CCT GCG-3' and ITS4 5'- TCC TCCGCT TAT TGA TAT GC -3' which were described elsewhere by White *et al.* 1990. PCR amplification of ITS region was performed in 50ml volume on a thermocycler (Peltier PTC-200, M.J. Research, Wattham. MA, USA). Each reactions contained 2ml template, 8ml of dNTP's mixture (2.5mM, Roche Diagnostics, Germany), 5□l Extaq buffer 10X, 5ml $MgCl_2$ 25 mM, 0.25ml of Extaq polymerase 5U (Takara, Japan), and 1□l l of each primer (100pM, Invitrogen, Carlsbad, CA). Cycling conditions were 94°C for 4 min, followed by 30 cycles of (94°C for 1 min), annealing temperature of 55°C for 30 seconds), extension at 72°C for 2min and a final extension of 72°C for 10 min. PCR products were analyzed by electrophoresis with a 1.5% agarose gel in TAE buffer according the following: 40mM Tris-acetate, 1mM EDTA), 100 bp DNA ladder was used for the characterization of band size, following ethidium bromide staining and UV illumination. The 16S rDNA from each bacterial colony was amplified by PCR using the pair of primers 533F and 1492R. The PCR was performed in 50ml volume containing 0.25ml of Taq Polymerase (Invitrogen, Carlsbad, CA), 5ml of Taq Polymerase buffer 10X, 8ml of dNTP mixture, 1.0ml of each primer (100pM, Invitrogen, Carlsbad, CA) and 2□l of bacterial DNA extracted from each different colony as template in a final reaction volume on 50□l. For the amplification of 16S rDNA, the samples were incubated for 14 min at 94°C to denature the target DNA and then cycled 30 times at 94°C for 1 min, 50°C for 45s and 72°C for 2 min. The samples were then incubated for 12 min at 72°C for a final extension and were maintained at 4°C until tested. Each PCR product amplified (16SrDNA and ITS region respectively) were clonated into the PCR 4-TOPO vector kit sequencing (Invitrogen, Carlsbad, CA). Two microliters of ligation reaction were used to chemically competent *E. coli,* One-shot TOP10 cells including the TOPO TA cloning kit. Transformed cells were plated on LB-ampicillin plates. For bacteria, positive clones carrying approx. 1000 bp, 16S rDNA were identified by colony PCR (9,11).

Identification and phylogenetic analysis

In order to identify unique sequences of bacteria and yeast, we performed amplified ribosomal DNA restriction analysis (ARDRA) profiling for each positive clone. The unique ARDRA profiles belonging to partial sequences corresponding to *E. coli* 16S rDNA and ITS, were submitted to the non-redundant nucleotide database at GenBank using the BLAST program (www.ncbi.nlm.nih.gov) in order to determine the identity of the clone Inserts. A multiple alignment of 16S rDNA clones and reference 16S rRNA or rDNA sequences retrieved from GenBank database was performed using Clustal W program. A distance matrix calculation of nucleotide substitution rates and a phylogenetic tree was constructed

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with the Kimura 2-parameter and the neighbor-joining (NJ) method, respectively, using MEGA 3.1. In order to provide confidence estimated for the tree topology in the NJ method bootstrap methods were used 1000 replicates (9, 11).

Ethanol production. To check the ethanol synthesis capacity of the *Zymomonas* isolates, the following protocol was followed by the: fermentation. The isolates were activated in BYE without ethanol and cycloheximide, incubated at 30°C/24-48h, then one roast each was taken to sow a 500 ml flask with 250 ml of broth yeast extract glucose (BYEG), that was incubated at 30 °C, at 100 rpm in a rotary shaker/24-48h, from this to another flask was inoculated *Zymomonas* in BYEG for the synthesis of ethanol.

Ethanol production. For each isolate activated in YEGA, with glucose at 5% (v/v) was used for 250 ml flask with 150 ml/L for ethanol production medium with following chemical composition (g/L): KH_2PO_4 2, yeast extract 10 and 10, 30, 50, 100 and 150g of glucose in distilled water at pH 5.0 adjusted with lactic acid, salts added after base sterilization (8-9), each isolate was inoculated in triplicate, in a static condition and shaking at 100 rpm at 30°C for 120 h, taking 5.0 ml every 24 h in tubes with screw caps to measure. Ethanol quantification. The fermentation sample was frozen until the ethanol was measured, centrifuged at 3000 rpm/20 min at low temperature, the supernatant was transferred to vials, to later measure the ethanol concentration by Beckman Mod. GC 72-5 gas chromatography, with flame ionization detector, nitrogen as carrier gas and stainless-steel column 6 feet long and 1/8 inch in diameter packed with Porapack Q in chromatographic analysis, the following conditions were used (10,13). Nitrogen flow 40 c.c./min, Hydrogen flow 40 c.c./min, air flow 230 c.c./min, column temperature 180 °C, injector temperature 170°C, detector temperature 160°C, flame ionization detector, log attenuation 0.8, chart velocity 0.1 in/min., injected volume 1.0 microliter (6).

Growth measurement. Turbidity (O.D.) at 600nm (8,9) was measured for each isolate in a Coleman Junior II spectrophotometer, every 24 h during fermentation. Total sugars. The sample used for the quantification of ethanol was also used to determine total sugars in the culture medium, by the Anthrone method with a calibration curve with glucose standards (3). The experimental data were analyzed by the statistical test ANOVA/ Tukey HSD $P<0.01$ with Statgraphics Centurion XVI.I (10).

RESULTS AND DISCUSSION

Table 1 shows that 15 *isolates* from different states of Mexican republic obtained from maguey mead, sugar cane, pulque and grape juice from different states of México. This result demonstrates that this genus is indeed cosmopolitan in the sampled areas as it exists naturally associated with agaves in the various geographical regions of the country, as well as in traditional handcrafted beverages, that is why it was relatively easy to isolate according to reports in the literature (1-4, 8). Based sequences of these different clones were aligned with the database of GenBank isolates were identified as *Zymomonas mobilis.* According to the biochemical tests carried out, the wild isolates obtained in various areas of Mexico compared with the *Z. mobilis* ATCC 10988 collection strain based on Bergey in 2005 edition (5-8) had a cell morphology similar to the ATCC 10988 collection strain. This wild *Z. mobilis* were diplobacillus Gram negative, (Figures 1 and 2) mobile except for a single isolate coded as R4. However, *Z. mobilis* wild isolate registered the highest ethanol generation compared to 14 other isolates of *Z. mobilis* from several regions of México (data no showed based in ethanol production). Other wild-type *Zymomonas* isolates from different States of Mexico were negative for: oxidase, urease, gelatinase, indole reduction, and nitrates; but positive for catalase and glucose fermentation. Even that this pool of wild isolated are candidate for genetic engineering to improve its ethanol production (5, 9,10). The wild *Z. mobilis* coded as UI isolated from grape juice for wine was the only one selected from the 15 recovered in the Mexican Republic to compare to *Z. mobilis* ATCC 10988 for ethanol production: this wild *Z. mobilis* UI isolate was positive for hydrogen sulfide synthesis (H_2S), glucose gas production, fructose, sucrose fermentation and ethanol production (5-7)

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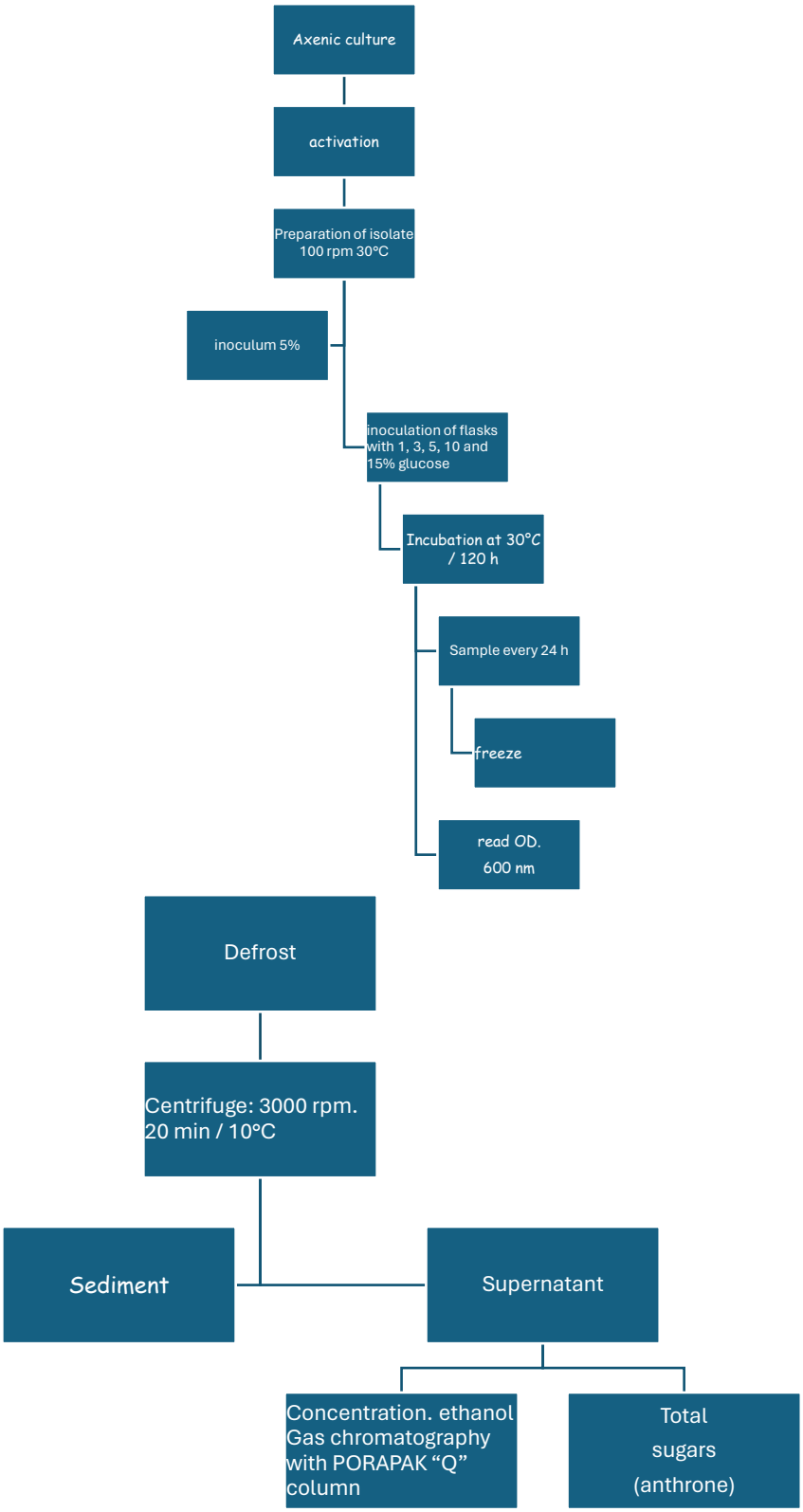

Figure ?

Table 2 shows the comparative analysis of the ethanol production capacity of the wild isolate UI of *Z. mobilis* at different glucose concentrations under static conditions and shaking with the *Z. mobilis* ATCC 10988 collection strain. Under static conditions, the wild-type isolate of *Z. mobilis* UI showed a

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limited ability to generate ethanol with no correlation between the glucose concentration used and the glucose concentration used. amount of ethanol produced. On the contrary, the strain of *Z. mobilis* ATCC 10988 generated ethanol in direct relation to the concentration of glucose, which was even equal to the amount of ethanol generated with 30 and 100 g/L of glucose, which confirms the osmophilic and tolerance capacity of the strain of *Z. mobilis* ATCC 10988 when using glucose via the Enter-Duduroff pathway (13,15). It was evident that the ethanol generation was directly proportional to the ethanol concentration under shaking condition especially in the ATCC 10988 strain reaching a maximum of 104 g/L of ethanol while static was 70 g/L both with 150 g of glucose/L in contrast to the relatively low ethanol generation with 3.0 g ethanol/L of the wild-type isolate of *Z. mobilis* UI under agitation (4,5). Exactly the opposite of the behavior of the ATCC 10988 strain, highly tolerant not only to glucose concentration but also to ethanol, a property that would have to be improved in the wild to be useful in ethanol generation (6,13,17). The dynamics of ethanol production under agitation of the ATCC10988 strain had no proportional relationship with the glucose concentration, except with 150 g/L of glucose (18,19). Different physiological behavior was observed with the wild isolate of *Z. mobilis* UI from grape juice, in that case the ethanol synthesis was directly proportional to glucose concentration except with 150g/L of glucose that did not favor the maximum ethanol production (16,19).

Table 3 shows the comparative analysis of the cellular yield coefficient (Y=w/s) of *Z. mobilis* ATCC 10988 compared to the wild isolate of *Z. mobilis* with different concentrations of glucose under static and shaking conditions. Regarding the *Z.mobilis* ATCC 10988 strain, the capacity to convert glucose in cells was from 0.17 to 0.009 for the U I strain at 10 and 50g/L. glucose, while for *Z. mobilis* ATCC 10988, it was 0.36 and 0.69 for 10 and 150g/L of glucose culture medium There, the maximum glucose conversion capacity as well as greater osmotolerance of *Z. mobilis* ATCC 10988 was evident (17,19,21), that, regardless of the glucose concentration, with the static condition as well as in shaking, surpassed the wild isolate of *Z. mobilis*, caused reduced cellular conversion by increasing glucose concentration especially in static condition. Since it was detected that mixing with yeasts such as *Pichia* and *Saccharomyces* species increased the synthesis of ethanol (22, 23) (data not shown) which belongs to a work in preparation.

CONCLUSIONS

It is concluded that the genus and species of *Z. mobilis* exist in different natural areas related to traditional artisanal beverages and that although the potential for ethanol production is limited, it is possible to carry out natural or induced selection to improve the capacity of these *Z. mobilis* in synthesis ethanol with the possibility of using yeast bacteria mixtures to achieve this aim.

Table 1. Origin of ethanol-producing *Zymomonas mobilis* isolates from maguey mead, sugar cane, pulque and grape juice from different States of Mexico.

Isolated	Source of isolation	Place
B 1[a]	Agave juice*	Bustamante, Nuevo León**.
B 2	Agave juice*	Bustamante, N. L.
B 3	Agave juice*	Bustamante N. L.
E 2	maguey mead	Iturbide, N. L.**
E 3	maguey mead	Iturbide, N. L.
R 3	Agave juice	Laguna de Sánchez, N. L**.
R 4	Agave juice	Laguna de Sánchez, N. L.
R 5	Agave juice	Laguna de Sánchez, N. L.
R 6	Agave juice	Laguna de Sánchez, N. L.
Z 4	24 h wort*	Tequila, Jal. ***
Z 5	24 h* wort.	Tequila, Jal.
P	Pulque	Matehuala, S.L. P+++
F	Pulque	Fortín, Veracruz +
T	Pulque	Yondije, state of México++.
U I	Grape juice (wine making)	Monterrey, N. L.**

*a=3 samples for each one *Juice or must in fermentation, **northeast, ***central west Jal=Jalisco, northeast+++ SLP=San Luis Potosi, +northeast coast, ++central Mexico.*

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Table 2. Comparative analysis of ethanol production by *Zymomonas mobilis* ATCC 10988 and *Z. mobilis* isolated from grape juice of one State of Mexico.

Glucosa (g/L)	***Z. mobilis* ATCC 10988**		***Z. mobilis* U I (wild isolate)**	
	Static	***shake***	***static***	***shake***
10*	5.30^{a**}	3.60^{b}	1.49^{c}	1.72^{c}
30	16.45^{a}	15.50^{a}	1.80c	3.28b
50	29.29b	33.60^{a}	1.60c	4.40b
100	54.50a	53.45a	1.27c	4.00b
150	70.00b	104.67a	1.34c	3.00d

*Fermentation conditions: 5% inoculum shaking 100 rpm 30°C, pH 5.0 * n=4 **Values with different letters had a statistical difference (P<0.05) according toANOVA-Tukey*

Table 3. Comparative analysis of cell production yield (Y=p/s) between *Zymomonas mobilis* ATCC 10988 and wild *Z. mobilis* UI from grape juice from one State of Mexico.

Glucosa (g/L)	***Z. mobilis* ATCC 10988**		***Z. mobilis* U I**	
	Static	***shake***	***static***	***shake***
10*	0.53^{a}**	0.36^{b}	0.15^{c}	0.17^{c}
30	0.54^{a}	0.51^{a}	0.06^{c}	0.11^{b}
50	0.58^{b}	0.67^{a}	0.032^{c}	0.08^{c}
100	0.54^{a}	0.53^{a}	0.013^{c}	0.04c
150	0.46^{b}	0.69^{a}	0.009^{d}	0.02^{c}

Fermentation conditions: 5% inoculum shaking 100 rpm 30°C, pH 5.0 n=3 **Values with different letters had a statistical difference (P<0.05) according toANOVA-Tukey*

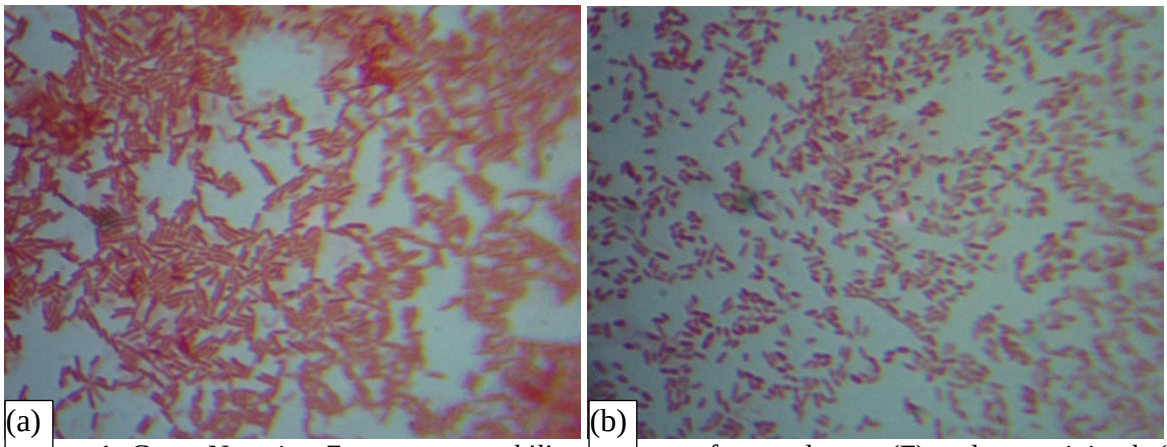

Figure 1. Gram Negative *Zymomonas mobilis* wild types from pulque a (F) and grape juice b (UI) morphology isolated from different States of Mexico.

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Author Contributions

Experimentation GGM, MMTM, and JMSY conceptualization GGM LJGW and JMSY methodology, GGM and MMTM software, GGM and JMSY; validation, GGM, LJGW and JMSY; results MMTM and JMSY.; investigation, GGM, MMTM, LJGW and JMSY; resources, GGM and JMSY writing—original draft preparation GGM and JMSY writing—review and editing, GGM and JMSY; visualization, GGM, LJGW and JMSY; supervision GGM, LJGW and JMSY. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

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Data Availability Statement

Data supporting these findings are available within the article.

Institutional review board statement

This study was conducted in accordance with the guidelines of the Declaration of Helsinki and was approved by the ethics committee of the university.

Informed consent statement

Not applicable.

Sample availability

Not applicable.

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