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Comparative study of native microorganisms isolated from watermelon (*Citrullus lanatus*) waste and commercial microorganism (*Clostridium thermocellum*) used for bioethanol production

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This study was to examine the efficiency of native microorganisms, in relation to the commercial bacterium *Clostridium thermocellum* ATCC 27405 for bioethanol production from waste watermelon on a pilot scale. The raw material was subjected to a grinding process for the later stage of hydrolysis under different temperatures using *Aspergillus fumigatus* and *Leuconostoc dextranicum* native microorganisms. This was followed by fermentation using control strains of *Zymomonas mobilis* (native microorganism) and *C. thermocellum* ATCC 27405. After hydrolysis, the fermentation product was evaluated by physico chemical analysis, pH determination and reducing sugars concentration using refractometry. Finally, the product obtained was subjected to vacuum distillation to increase the grade of purity. It was there after analyzed using pycnometry and gas chromatography (GC). GC results revealed that the yield of ethanol from hydrolysis and fermentation by native strains was improved by 27.62%, compared to yields of ethanol obtained from *Z. mobilis* (11.62%) and *C. thermocellum* (3.10%). It was therefore concluded that the native strains were more efficient in hydrolysing and fermenting watermelon compared to the commercial *C. thermocellum* and *Z. mobilis*.

Key words: *Aspergillus niger*, *Clostridium thermocellum* ATCC 27405, *Zymomonas mobilis*, hydrolysis, fermentation processes.

INTRODUCTION

Global warming and environmental pollution daily increases with the use of fossil fuels; to mitigate this problem, it is necessary to investigate new alternative energy sources, especially those derived from plant biomass that do not affect ecosystems and plants production for agricultural use. Currently, there is ongoing search for new and better ecological strategies to produce bioethanol, using agricultural waste with high

fermentable sugars content, such as watermelon waste fruits. Global warming is a phenomenon of significant increase in average temperature caused by the use of fossil fuels, oil industrialization, burning forests, industry and carbon monoxide fumes caused by the automotive park. These activities have caused an imbalance between land, ocean and atmosphere, which has changed global air circulation and water patterns. In recent years, there has been a gradual increase in temperature and it is estimated between 0.19 and 0.38°C (Melendo, 2014). According to databases, 1998 was the warmest year. As a result of global warming, snow-covered areas have been considerably reduced (Melendo, 2014).

Dependence of world society on fossil resources is high, reaching 80% of primary energy consumed in 2004 and 2006 in Europe and Spain; consumption was 79 and 83%, respectively (Eritja, 2015). The use of fossil fuels and global energy engine is cost effective and causes depletion; its effects on global warming and urban pollution attributed to combustion have forced research on the use of alternative energy sources, especially those derived from plant biomass. In this context, bioethanol obtained from sugars, starches or cellulosic material is an alternative to be used as a supplement or substitute for gasoline, engine power, thus reducing energy dependence in addition to the social benefits derived from their production (IRENA, 2015). The main sources of bioethanol production worldwide are: sugar cane, beets, wheat, corn, barley, cassava, sweet sorghum, etc. Also, agricultural residues such as olive residues, rice bran, fruit peel, etc. are included. Among the most commonly used for ethanol production energy crops, sugarcane is the raw material most commonly used in tropical countries such as Brazil and India. In North America and Europe, ethanol fuel is derived from corn starch and cereals (Colina, 2000).

Fermentation musts' sugar production is faster and adequate when further bacterial and fungal microorganisms are used, which by their rapid consumption of glucose help better develop the fermentation processes of plant materials (Mariscal, 2011). Ecuador began exporting oil from the Amazon rainforest since 1972, the date from which has been extracted more than 4 billion oil barrels. As a result of the activities of oil exploration, continued deforestation which affects ecosystems, causing loss of water in the Ecuadorian Sierra and the rest of the country is produced; so that Amazonian clouds are becoming less compact and this leads to a reduction in rain water flow (Baquero and Mieles, 2015). In this regard, the Department of Life Sciences and Agriculture at the Universidad de las Fuerzas Armadas (ESPE) seeks new and better ecological strategies to produce ethanol by hydrolysis and fermentation processes, using agricultural waste with high fermentable sugars content; this is the case of waste watermelon, Glory Jumbo variety. Watermelons can be grown in open fields and in greenhouses, so it is easy to dispose of waste fruits during the whole year. For this reason, fuel development from waste watermelon would not only have positive environmental effects, but would give an additional economic output to watermelon farmer (Salazar, 2010).

In Ecuador, ethanol can be produced on a large scale since there exist approximately 1513 ha. To obtain ethanol, watermelon cultivation in coast region which serve as raw materials were used, and this can provide individual, cheap and renewable energy source. Watermelon is grouped into the Cucurbitaceae botanical family, and comprises about 850 species of herbaceous plants that produce large fruits and protected by a hard crust. Watermelon plant has large and weighty fruits, reaching up to 30 cm in diameter and weighing between 15 to 20 kg. For domestic trade, watermelons usually weigh between 3 to 8 kg (Canales, 2003). *Citrullus lanatus* species is highly fermentative as per kg of fruit, 50% ethanol is obtained. The replacement of gasoline by ethanol is currently an economic problem rather than technical, to reverse this, it is necessary to find more efficient and better economic biotechnological alternatives (Alban et al., 2003).

Watermelon valued for its nutritional qualities, is very important in Ecuador, South America. However, about 20% of the harvest of this highly perishable fruit is lost due to post harvest decay. Decayed watermelons may be an important source of industrially-produced bioethanol. Industrial bioethanol production requires the use of efficient bacteria for fermentation. Therefore, the aim of this study was to produce bioethanol as clean energy alternative using bacteria, fungi, and native fruit with the purpose of fermenting watermelons waste as an energy material. The native microorganisms (bacteria and fungi) obtained from watermelon waste have equal or better efficiency in producing bioethanol compared with commercial microorganisms.

MATERIALS AND METHODS

Sample collection

Watermelon fruit waste was collected from the Membrillo locality; Manabi province, Tosagua Canton, Ecuador. Tosagua canton located northwest of Manabi province. Fruit that has no external damage was selected. Fruits collected in the field were used for the preparation of plant samples in laboratory. Experiments related to preparation of aqueous extracts, isolation of native microorganisms,

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biochemical tests, enzymatic hydrolysis, fermentation, distillation and quantification of bioethanol by gas chromatography were conducted under controlled conditions.

Sample preparation

Plant material was sterilized thoroughly with sterile water followed by removal of excess moisture. The sterilized material was immediately used. To obtain the microorganisms, triturated 100 g pulp and rinds was applied and this material was placed in Erlenmeyer flasks by adding 50 ml of distilled water and allowed to stir at 80 rpm and 50°C for bacterial and 25°C for fungal development through 8 days to obtain high levels of biomass with beneficial microorganisms (fungi and bacteria). Upon completion of the required time, serial dilutions were made to 1×10^{-4} and the last dilution was depth seeded in Potato dextrose agar (PDA) (Camacho et al., 2009).

Bacteria isolation and identification

For *Leuconostoc dextranicum* isolation, the Mayeux culture medium was used, containing (g): peptone 10, yeast extract 5, sucrose 100, sodium citrate 1, glucose 5, gelatin 2.5, agar 15 per liter of distilled water. Subsequently, the bacterial medium was autoclaved at 120°C during 20 min, dispensed and gelled in petri dishes at room temperature. For bacterial identification, Blast program was used (Bailón et al., 2003). *Zymomonas mobilis* isolation was performed in WL differential agar specific culture medium for 4 days and incubated at 25°C temperature. Bacteria obtained were massively multiplied in fermentation medium (g/L): glucose-100; yeast extract-10; KH_2PO_4- 2; (NH_4) $2SO_4$- 1; $MgSO_4.7H_2O$ - 0.5. *C. thermocellum* strain was purchased commercially (*C. thermocellum* ATCC 27405).

Fungi isolation and identification

Fungal colonies with different macroscopic characteristics were isolated, and then they were seeded in Petri dishes with PDA and antibiotic to inhibit bacterial growth. All fungal cultures were incubated at 25°C for 7 days. Once fungal colonies were fully developed, monosporic cultures were made in PDA to obtain axenic cultures. To identify fungal morphology, keys techniques were used. For the isolation of *Aspergillus niger* and *Aspergillus fumigatus*, the following culture media were used: agar Czapek, potato dextrose agar (PDA), agar broth nitrates and Bacto Peptone. To this, 30 mg of terramicina and cycloheximide antibiotics were individually added. 3,5-Dintro salicylic acid (DNS), sodium and potassium tartrate, with sodium hydroxide for the quantification of reducing sugars reagents were used. For DNS reagent, preparation of glucose stock was carried out, followed by washing of material with distilled water. To evaluate the effectiveness of *A. niger* relative to the native microorganisms (bacteria and fungi), the hydrolysis step was subjected to incubation for 8 days at temperatures (25, 30 and 35°C). Subsequently, *C. thermocellum* ATCC 27405 effectiveness and *Z. mobilis* with the native microorganism (bacteria) in the fermentation phase for 8 days at a temperature of (25, 30 and 35°C) were compared. Finally to obtain and determine ethanol concentration, distillation equipment and pump was used. Bioethanol production was quantified bygas chromatography.

Experimental design

Completely randomized design (CRD) experimental design was used with 2 × 3 + 1 factorial arrangement to compare hydrolysis ability of each treatment. Where M has two factors: microorganism type and C temperature, with three levels each. M_1 (bacterium) - C_1=25; M_2 (fungus) - C_2=30; C_3=35 [Data were analyzed using the INFOSTAT package (INFOSTAT, 2000) version].

Table 1. Morphological characteristics of *Aspergillus* sp.

Aspergillus sp.	Conidiophore		Phialides (diameter in mm)
	Height (mm)	Width (mm)	
Aspergillus fumigatus	17.5	22.5	7.5
	17.5	20	7.5
	20	22.5	7.5
	12.5	20	7.5
	20	25	7.5
Average	17.5	22	7.5
Aspergillus niger	20	25.2	8.75
	21.2	27.5	8.75
	22.5	22.7	8.75
	23.5	26.7	8.75
	18.2	23.8	8.75
Average	21.1	25.2	8.75

Statistical analysis

Statistical analysis was determined by DMS Test 5% for microorganisms; Duncan test 5% overall treatment and temperature levels, regression and correlation of the temperature levels with each variable under study for each microorganism in the hydrolysis step. Statistical analysis for the fermentation phase was determined using tests by comparing average Duncan 5% for each treatment, normality and homoscedasticity test were used to verify the assumptions of ANOVA. For analysis of variance the quantity which must ferment in each experimental unit was used; that is regression and correlation between temperatures with each of the variables under study. To compare the ability of synthesis, fermentation and distillation amount of each treatment design was completely randomized. Where one factor is C levels and comprised: temperature and the microorganism (bacteria) which was more efficient than the control over a total of four treatments.

RESULTS AND DISCUSSION

After watermelon waste treatment, a high diversity of native beneficial microorganisms was obtained.

Fungi identification

Table 1 shows *A. niger* and *A. fumigatus* fungi macroscopic and microscopic identification. *A. niger* macroscopic characteristics were black or dark brown colonies, the reverse is colorless to yellow, dense colonies, flocosa granular, compact and white mycelium. Biseriate microscopically and radial conidial heads, stipes thick-walled, smooth, hyaline, yellowish or pale brown observed, almost spherical vesicles, brown globose

Table 2. Biochemical tests of *Leuconostoc dextranicum* and *Zymomonas mobilis*.

Zymomonas mobilis	*Leuconostoc dextranicum*
Gram-	Gram+
Catalase+	Fructose+
Oxidase-	Galactose+
Gelatinase-	Lactose+
Urease+	Maltose+
Tween 80-	Starch-
D-Glucose +	Sucrose+
Lactose+	Trehalose+
Citrate-	Dextran+
Mobility-	Arabinose-

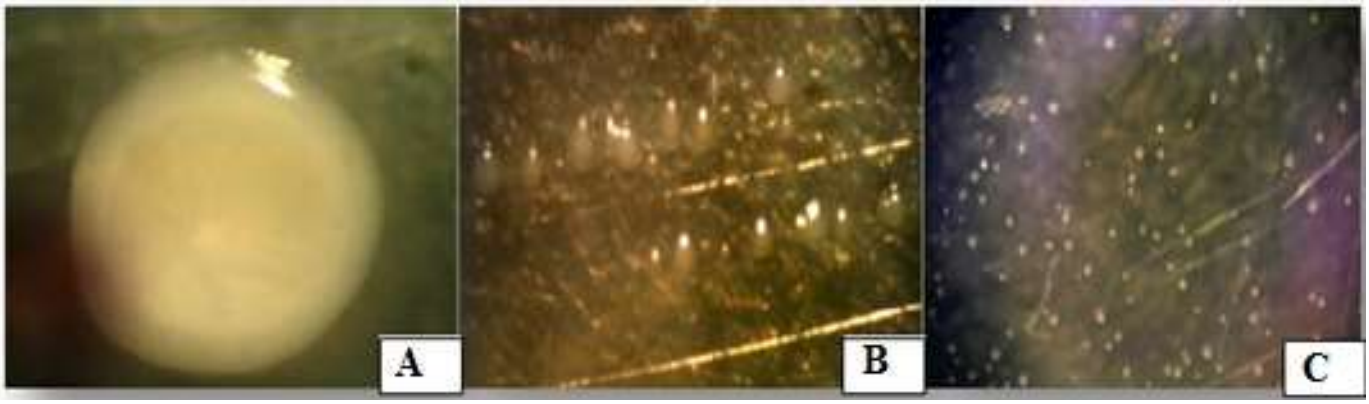

Figure 1. A. *Leuconostoc dextranicum*. **B.** *Zymomonas mobilis.* **C.** *Clostridium thermocellum* (ATCC 27405).

conidia, irregular roughness with ridges and bumps. *A. fumigates* macroscopic characteristics were blue-green to gray-greencolonies, whitemiocelio, colorless back, yellowish, reddish brown or green, velvety flocosa, flat or radial grooves texture. Uniseriate microscopic and predominantly columnar conidial heads, hyaline and smooth stipes, vesicular pear-shaped or spoon-shaped is observed, phialides occupying half or two thirds of the gallbladder, globose to oval, smooth or slightly rough conidia. These results are similar to those obtained by Abarca (2000), which corroborates the views expressed by Loustau (1964) and Tangarife (2011).

Bacteria identification

The two different bacterial isolates such as *Leuconostoc dextranicum* and *Z. mobilis* were characterized by their morphological and biochemical properties as shown in Table 2, where the results are consistent with studies reported by Matiz (2002), with *Zymomonas* sp. gender classification, however, *L. dextranicum* microscopic identification Gram positive forming short chains was observed unlike described by García (2007), who reported that *L. dextranicum* is a gram-positive coccobacillus and form short chains. In catalase and oxidase tests, positive results was obtained unlike the results obtained by API test identification where the results of oxidase and catalase tests were negative, with positive anaerobiosis and positive citrate; these characteristics are consistent with the reports described by Gonzáles (2013). The morphological difference microscopically observed in *L. dextranicum* is because growth ions are present in the culture medium; according to Fernandez (2010) cells grown on glucose or on solid media can have an elongation at bar, morphology formed, which occurred in the present study since the medium of solid and liquid culture had a source of sugar dextrose. *C. thermocellum* ATCC 27405 is a commercial strain classified as Biosafety classification on U.S. Public Health Service Guidelines; it is a gram positive bacilli with spore forming, which is consistent with the results reported by Demain et al. (2005). Cervantes (2007) states that the positive bacterial growth is due to the addition of carbohydrate, proteins, amino acids, vitamins and essential cofactors. Figure 1 shows the three types of bacterial colonies obtained.

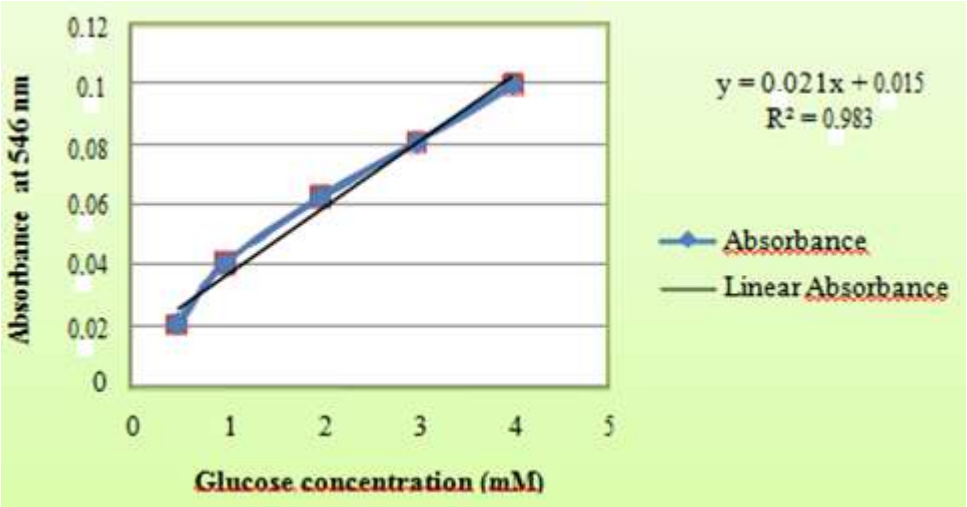

Figure 2. Reducing sugars concentration in waste watermelon fermentation - hydrolysis.

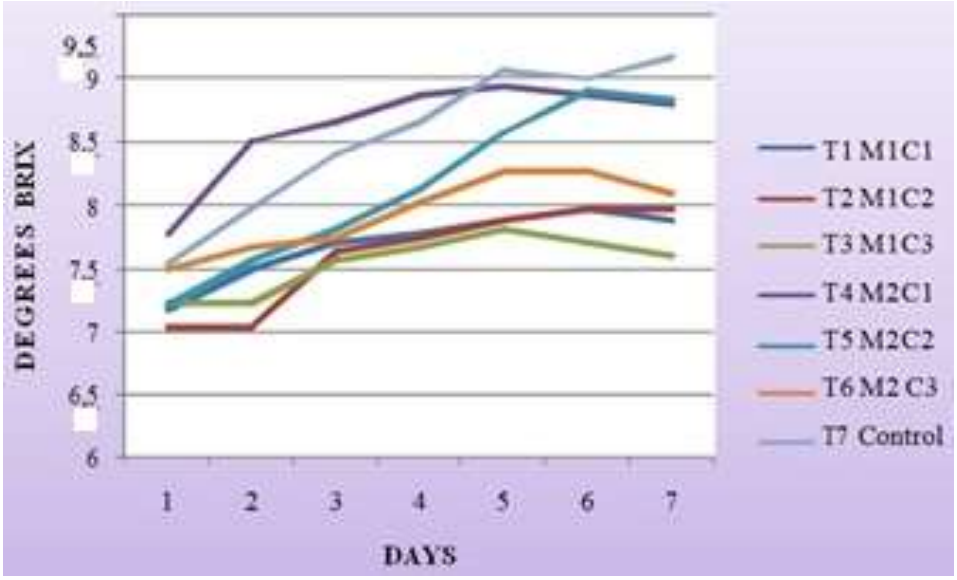

Figure 3. Microorganisms and temperature activity on watermelon degrees brix in the hydrolysis step.

Bacterial fermentation - hydrolysis

Figure 2 shows the amount of reducing sugars obtained with 3,5-dinitrosalicylic acid (DNS) (glucose) technique content in watermelon fermentation - hydrolysis by comparing these results with those obtained by Matiz et al. (2002), a 10 to 20% total sugars increase in relation to 3.878 g/L of initial amount was obtained. This demonstrates that the substrate used is nutritionally enriched and promotes native microbial populations growth. Figure 3 shows reducing sugars (Brix) production in enzymatic hydrolysis step using three native microorganisms: *L. dextranicum*, *A. fumigatus* and *A. niger* (control) at different temperatures (25, 30 and 35°C).

Compared to acid hydrolysis, *A. fumigatus* produced the greatest amount of reducing sugars as these fungi have ability to produce endoglucanase enzymes, which remove beta-1,4-glucosidic links chain cellulose (Landazábal, 2004; Cortínez, 2010). The enzyme activity was measured using refractometer. According to the results presented in Figure 3, in the hydrolysis process there were significant differences so that none of its values is discarded, however the sugars percentage was

found worthy (reducing sugars 8.38) and were higher than those obtained in the untreated raw material; these sugars were obtained using 3,5-dinitrosalicylic acid (DNS) technique, where the initial amount of reducing sugars is 3.88 g/L, demonstrating increased unfolded sugars and justifying the process carried out with any of the microorganisms *L. dextranicum* and *A. fumigatus*. Control was more functional because it presents greater degrees of brix number in relation to other treatments.

Fermentation

Hydrolysis yields enabled microorganisms used in fermentation step to convert the reducing sugar(s) to ethanol. According to Cervantes (2007), three types of bacteria occur in the must fermentation: acidic, alcoholic and viscous. The interaction of the three types determines an increase in microbial population counts, consumption of sugars, proteins and formation of ethanol gave a viscous appearance. *Z. mobilis* followed the metabolic pathway of Entner-Doudoroff pathway from which ethanol was obtained; *L. dextranicum* used heterolactate route for obtaining carbon and energy. Pyruvate as an end product of a biochemical pathway was converted to ethanol by the action of pyruvate decarboxylase and alcohol dehydrogenase; *C. thermocellum* ATCC 27405 uses acetone butanol route fermentation to complete energy metabolism. On the other hand, Cervantes (2007) reported that lactic acid bacteria such as *L. dextranicum* are responsible for viscose and alcoholic fermentation of fermented beverages. An interesting finding was the increase in gram-positive bacillus *L. dextranicum* being native bacteria that is more efficient at room temperature 25°C; which could correlate with increased viscosity, physical change in the mature leaven. Time and fermentation, pH 4.5, were within 8 days for all treatments since a decrease was observed in the bubbles produced during fermentation by CO_2 evolution.

Distillation

Distillation semi-micro equipment was used. By setting the variance analysis for the distillation volume obtained no significant differences was found. Never the less with T4 treatment (Control$_1$ *C. thermocellum* ATCC 27405), highest volume distillation was obtained (Table 3).

Ethanol quantification by pycnometry

When comparing between T_1 and T_2 treatments (with *A. niger* hydrolyzed and fermented with *C. thermocellum* ATCC 27405 and *Z. mobilis*) it was determined that these treatments generated an average of 11.78 ± 0.059% lower alcohol which was produced by *A. fumigatus* (11.87 ± 0.059%) with T_1 and T_2 treatments (hydrolyzed with *A. fumigatus* and *C. thermocellum* fermented with ATCC 27405 and *Z. mobilis*). These values demonstrate that the activity of synergism between these microorganisms is developed to complete the procedure. These results agree with those obtained by Escudero (2015). Although in treatments not high percentage of alcohol is obtained, it is confirmed that the improvement in controlling process variables increases the efficiency in obtaining alcohol (Table 4). Ethanol percentages shown in Table 4 out-performed those obtained by Alban and Freire (2009), which means that the conditions for initial treatment and time directly influenced process quality. In fermentation process, using *L. dextranicum* native strain at 30°C, the highest percentage of 15.18 ± 0.059 was obtained and at 35°C, 13.26 ± 0.059 was achieved. With *Z. mobilis* ATCC 27405 and *C. thermocellum* control strains at 35°C, percentages of 13.09 ± 0.059% and 13.21 ± 0.059 were obtained, respectively.

Table 3. Distilled volume in watermelon fermentation.

Treatments	Distilled volume (mL)
T1 BC_1	15.25
T2 BC_2	15.50
T3 BC_3	14.75
T4 Control$_1$ *C. thermocellum*	16.25
T5 Control$_2$ *Z. mobilis*	15.00

Table 4. Ethanol percent determination by pycnometry method. Watermelon fermentation after 8 days of incubation under anaerobic conditions with controlled temperatures.

Treatments	% of ethanol by pycnometry		
	25°C	30°C	35°C
Ld+Ld	11.38	15.18	13.26
Ld+T_1	11.384	11.36	13.09
Ld+T_2	11.36	11.94	13.21
Af+Ld	11.98	11.95	12.48
Af+T_1	11.65	11.37	12.47
Af+T_2	11.87	11.372	12.53
An+Ld	11.38	12.48	12.51
An+T_1	11.37	11.64	11.94
An+T_2	11.35	12.47	11.92

Ld = *Leuconostoc dextranicum*; Af = *Aspergillus fumigatus*; An = *Aspergillus niger.*

Ethanol quantification by gas chromatography

When gas chromatography was performed, it was

Table 5. Ethanol percentage determination by Gas Chromatography. Watermelon fermentation after 8 days, under anaerobic conditions (25; 30; 35°C) with native and control strains.

Sample	Parameter	Method	Units	Results
Extract 1 NB+NBT° 25°C	Ethanol	Gas chromatography	g/100 ml	0.12
Extract NB+NBT° 30°C	Ethanol	Gas chromatography	g/100 ml	27.62
Extract 1 Af+NBT°25°C	Ethanol	Gas chromatography	g/100 ml	1.58
Extract An+LdT° 30°C	Ethanol	Gas chromatography	g/100 ml	0.42
Extract 1 NB+NBT° 25°C	Ethanol	Gas chromatography	g/100 ml	11.00
Extract Ld+LdT° 35°C	Ethanol	Gas chromatography	g/100 ml	7.89
Extract Af+NBT° 35°C	Ethanol	Gas chromatography	g/100 ml	3.14
Extract An+T2 T° 35°C	Ethanol	Gas chromatography	g/100 ml	3.10

NB = Native bacteria; Ld = *Leuconostoc dextranicum*; Af = *Aspergillus fumigatus*; An = *Aspergillus niger.*

determined that values decreased progressively, which coincides with Alban and Freire (2009) who reported that, synergism occurs when microorganisms occupy the same substrate. As reported by Bustamante et al. (2000), when the pH decreases, the *A. niger* growth increases, preventing other microorganisms growth, so the fermentative capacity of *Z. mobilis* also decreased. In the same way, increased pH caused decreased fermentation efficiency. So, optimum pH is best suitable for production of bioethanol by *Z. mobilis* (Khoja et al., 2015). Finally when comparing ethanol amount obtained by gas chromatography and pycnometry, different values were obtained (Table 5), however, the advantages of use of pycnometer is highlighted. When performing gas chromatography of all treatments after 8 days fermentation and at temperatures (25, 30, 35°C); highest concentration [27.62% (v/v)] was achieved with treatment NB+NB (*L. dextranicum*) at 30°C.

Conclusions

*L. dextranicum*n, *A. fumigatus* and *A. niger*, were identified as *C. lanatus* native microorganisms and have hydrolytic and fermentative capacity. Enzymatic hydrolysis with native microorganisms was the best method for obtaining ethanol at a temperature of 25°C, with this temperature a complete conversation of reducing sugars of 9 g/L was obtained, which are transformed into alcohol in the fermentation step. In fermentation stage at different temperatures (25, 30 and 35°C), *L. dextranicum* was more efficient than *C. thermocellum* ATCC 27405 commercial strain and *Z. mobilis.* With *L. dextranicum,* 27.62% was achieved as ethanol maximum concentration. Watermelon wastes are suitable for production of clean bioethanol, abundant, inexpensive and help to reduce the negative effect in production of biofuels from sugarcane monocultures and corn.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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REFERENCES

Abarca L (2000). Taxonomía e identificación de especies implementadas en la aspergiliosis nosocomial, España, pp. 1-6, Extraído el 1 de Noviembre 2015. del sitio web:http://www.reviberoammicol.com/2000-17/S79S84.pdf.

Albán D, Freire D (2009). Tesis: Obtención de Bioetanol a partir de residuos de naranja "*Citrus sinensis*" provenientes del proceso Agroindustrial en la Provincia de Bolívar, Ecuador. [Revisión de tesis]. (pp.70-80). Quito. http://repositorio.espe.edu.ec/bitstream/21000/2597/1/T-ESPE-IASA%20I-004168.pdf.

Albán G, Arnao C, Mejía M (2003). Proyecto de producción de sandía para exportación en la Peninsula de Santa Elena, Ecuador. Pp. 1-175, Escuela superior politécnica del Litoral. Extraído el 3 de Septiembre, 2009, del sitio web: http://www.cib.espol.edu.ec/Digipath/D_Tesis_PDF/D-32674.pdf.

Bailón L, Cruz R, Cervantes A (2016). Atlas de pruebas bioquímicas para identificación de bacterias. Universidad Autónoma de México, 2003, pp. 1-175. México. Extraído el 15 de enero, 2016. , del sitio web:https://microbitos.files.wordpress.com/2010/06/atlasmicrobiologia1.pdf.

Baquero D, Mieles J (2015). Los booms petroleros, Qué cambió en los últimos 40 años. Pp. Revista de análisis y divulgación científica de economía y empresa. Extraído el 1 de diciembre 2015, disponible en el sitio web: http://foroeconomiaecuador.com/fee/los-booms-petroleros-cambios-40/.

Bustamante Z, Galindo E, Huanca M, Ballesteros F (2000). Obtención de bioproteina a partir de bagazo de naranja (*Citrus sinensis*) con *Aspergillus niger* Programa Fármacos, Alimentos y Cosméticos, (PROFAC), Facultad de Bioquímica y Farmacia, Universidad Mayor de San Simón. Bolivia. Extraído el 12 de Septiembre, 2009, del sitio web : http://www.umss.edu.bo/epubs/earts/downloads/49.pdf.

Camacho A, Giles M, Ortegón A, Palao M, Serrano B, Velázquez O (2009). Técnicas para el Análisis Microbiológico de Alimetos, 2da edición, Facultad de Química, UNAM, México. pp. 1-9.

Canales R(2003). Cadena Agroalimentaria de Sandía, pp. 1-96, INIFAP, Campeche. Extraído el 1 de Diciembre 2015, del sitio web:http://www.cofupro.org.mx/cofupro/Publicacion/Archivos/penit52.pdf.

Cervantes M (2007). El pulque: características microbiológicas y contenido alcohólico mediante espectroscopíaRaman, Ticoman México, pp. 101-112, Extraído el 25 de Diciembre 2015, del sitio web:http://www.unicolmayor.edu.co/invest_nova/NOVA/nova8_artorig3.pdf.

Colina A (2000). Obtención de Fuentes Alternativas de Energía. Universidad de Salamanca, España. Extraído el 3 de Septiembre, 2009, disponible en el sitio web: http://pdf.rincondelvago.com/bioetanol.html.

Cortínez V (2010). Comparación de pre tratamientos en residuos forestales para la producción de bioetanol de segunda generación, Universidad de Chile, Chile, pp. 1-133, Extraído el 8 de Octubre, 2014, del sitio web: http://repositorio.uchile.cl/tesis/uchile/2010/cf-cortinez_vv/pdfAmont/cf-cortinez_vv.pdf.

Del Valle Melendo J (2014). El cambio climático Cumbre de Varsovia, España, Extraído 6 de octubre, 2015, pp. 1-22, sitio web: http://www.ieee.es/Galerias/fichero/docs_opinion/2014/DIEEEO07-2014_CambioClimatico_JavierdelValle.

Demain AL, Newcomb M, Wu JD (2005). Cellulase, Clostridia, and Ethanol. Microbiol. Mol. Biol. Rev. 69(1):124-154.

Escudero D (2015). Obtención de bioetanol a partir de inulina proveniente de biomasa vegetal mediante sacarificación y fermentación, Universidad Central del Ecuador, Ecuador, pp. 1-89, Extraído el 8 de enero, 2016, del sitio web: http://www.dspace.uce.edu.ec/bitstream/25000/4516/1/T-UCE-0017-111.pdf.

Fernández A (2010). Métodos de identificación bacteriana en el laboratorio de Microbiología, España, pp. 1-52, Seimc, Extraído el 20 de Diciembre 2015, del sitio web:https://www.seimc.org/contenidos/documentoscientificos/procedimientosmicrobiologia/seimc-procedimientomicrobiologia37.pdf.

García J (2007). Identificación de bacterias ácido lácticas mediante perfiles de fermentación y ribotipificación, pp. 1-96, Universidad autónoma del estado de Hidalgo, México, Extraído el 15 de Diciembre 2015, del sitio web:http://dgsa.uaeh.edu.mx:8080/bibliotecadigital/bitstream/handle/231104/382/Identificacion%20de%20bacterias.pdf?sequence=1.

Gonzáles A (2013). Caracterización microbiológica de productos lácteos tradicionales, Universidad de Oviedo, España, pp. 1-223. Extraído el 17 de Diciembre 2015, del sitio web:file:///C:/Users/IASA/Downloads/TDAlegriaGonzalez.pdf.

International RenewobleEnergy Agency (IRENA), (2015). Energías Renovables de América Latina. pp. 1-28. Informe de políticas IRENA. Extraído el 2 febrero 2016, del sitio web: http://www.irena.org/DocumentDownloads/Publications/IRENA_RE_Latin_America_Policies_2015_ES.pdf.

Khoja AH, Ali E, Zafar K, Ansari AA, Nawar A, Qayyum M (2015). Comparative study of bioethanol production from sugarcane molasses by using *Zymomonas mobilis* and *Saccharomy cescerevisiae.* Afr. J. Biotechnol.14(31):2455-2462.

Landazábal P (2004). Obtención de etanol a partir de almidón de yuca variedad chile en un proceso de 2 etapas: Hidrólisis (*Aspergillus niger*)-Fermentación (*Zymomonasmobilis*), Universidad industrial de Santander, Colombia, pp. 1-199, Extraído el 8 de Octubre, 2014, del sitio web: http://repositorio.uis.edu.co/jspui/bitstream/123456789/312/2/112241.pdf.

Loustau J (1964). Clave definitiva de especies del Género *Aspergillus*, pp. 1-63, Universidad de Murcia España, Extraído el 10 de Noviembre 2015, del sitio web:https://digitum.um.es/jspui/bitstream/10201/6483/1/N%203%20Clave%20determinativa%20de%20las%20especies%20del%20genero%20Aspergillus.pdf.

Mar CampinsEritja (2015). De Kioto a París: Evolución o involución de las negociaciones internacionales sobre el cambio climático. pp. 1-19, Instituto español de Estudios estratégicos IEEE.ES. Extraído el 1 marzo 2016, del sitio web: http://www.ieee.es/Galerias/fichero/docs_opinion/2015/DIEEEO61201 5_Kioto_paris_MarCampins.pdf.

Mariscal J (2011). Evaluation and selection of microorganismsfor etanol production at industrial level. pp. 1-99 Colombia, Extraído el 25 de noviembre, 2015, disponible en el sitio web:http://www.bdigital.unal.edu.co/4791/1/8108502.2011.pdf.

Matiz A (2002). Producción de etanol con células inmovilizadas de *Zymomonasmobilis*, pp. 216-223, Universidad de Córdoba Montería, Colombia. Extraído el 25 de Noviembre 2015, del sitio web:http://www.redalyc.org/pdf/693/69370204.pdf.

Matiz A, Torres C, Poutou R (2002). Producción de etanol con células inmovilizadas de *Zymomonasmobilis*spp. Universidad de Cordova. Colombia Extraído el 3 de Octubre, 2009, del sitio web: http://redalyc.uaemex.mx/redalyc/pdf/693/69370204.pdf.

Salazar A (2010). Análisis del mercado para un plan de exportación de sandía. pp. 1-81, UDLA, Ecuador. Extraído el 14 de Octubre 2015, del sitio web:http://dspace.udla.edu.ec/bitstream/33000/1731/1/UDLA-EC-TINI-2010-22.pdf.

Tangarife V (2011), *Aspergillus* spp. Universidad de Antioquia Colombia, pp. 1-20, Extraído el 15 de Noviembre 2015, del sitio web:http://aprendeenlinea.udea.edu.co/lms/moodle/mod/page/view.php?id=100812.