INTEGRAL AND SUSTAINABLE USE OF AGAVE

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INTEGRAL AND SUSTAINABLE USE OF AGAVE

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2019

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Preface

Preface

Once again, CIATEJ (Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco, A.C.) brings a new edition of Integral and Sustainable use of the Agave that consists of the most recent advances in scientific and technological research around the agave genus. The research reported in this book is the result of the effort and the scientific interests of researchers working in México and other latitudes around the world. In these lines, the sponsorship of CONACyT-México, CIATEJ, ADESUR, and AGARED is highly appreciated.

This work is divided into five major topics: 1. Scientific trends on Agave (nine chapters); 2. Science and technology of Agave beverages and other derivatives (six chapters); 3. Biological effects of Agave fructans and other by-products (six chapters); 4. Industrial processing of Agave wastes and subproducts (six chapters); and 5. Social and ethnobotanical aspects (four chapters).

Chapters in the first topic (pages 9 to 59) report advances and improved in vitro techniques for the propagation and conservation of agave germplasm, the finding of genes related to pathogen resistance, the control of agave weevil (*Syphophorus acupunctatus*), improved method for the *Agrobacterium*-mediated transformation of Agave, and the use of imaging spectroscopy in the study of the CAM physiology.

The second topic (pages 61 to 97) contains diverse chapters about yeasts and non-*Saccharomyces* microorganisms that participate in the mezcal artisanal and industrial production, the physicochemical quality of extra-aged, and novel crystalline tequilas, among others.

In the third topic (pages 99 to 133), six chapters report studies about the characterization of agave fructans from different species and their actions for the control of obesity, cancer, and other human diseases. Also, there is a report on the potential elicitor activity of fructans against the pathogen *Phytophthora capsici* in pepper plants and of their use for the microencapsulation of probiotics for human consumption.

Topic four (pages 135 to 171) tells about the research progress on the processing of agave of vinasses and bagasse and their use mainly for hydrogen production and as silage for ruminants.

Finally, the last topic in this book (pages 173 to 201) deals with the social, economic, and technical aspects of the Chiapas “Comiteco” drink. Also, this fifth topic deals with the challenges facing the mezcal industry concerning production costs, quality, and precise definitions regarding its origin terroir.

Benjamín Rodríguez-Garay

Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco, A.C.
A CONACyT Research Center.

Scientific trends on Agave

Application of two *in vitro* culture techniques for the conservation of agave germplasm.

Herrera-Isidrón, L.[1], Isidrón-Pérez, MF.[2] and Núñez-Palenius, HG.[3*]

ABSTRACT

The *Agave* genus is one of the most important and diverse in Mexico, however, natural and anthropogenic causes have reduced agave populations. Agave plants have been produced by tissue culture for conservation purposes, mainly using semisolid media. The use of liquid medium for *in vitro* micropropagation is often described as a way of reducing the cost of plantlets production. In order to develop an alternative method for agave conservation, the effects of two culture systems were studied. Axillary buds from *A. weberi, A. mitis, A. vilmoriniana, A. sisalana, and A. celsii* were transferred to a temporary immersion system (TIS) and to a semi-solid medium, both supplemented with 0.5 mg/L of Benzylaminopurine. Shoot regeneration was obtained in all the cultures after 12 months, but more than 90% was observed under the TIS.

Key words: Agave, tissue culture, temporary immersion, conservation, axillary buds.

INTRODUCTION

The genus *Agave* is endemic to the American continent and encompasses approximately 200 species, from which 75% are found in Mexico (Gentry, 2004). This genus contains a good number of economically important species. However, the rate of loss of these species is very high because their slow growth and relative low reproductive rates. Also, human activity has put several agave species at risk.

Nowdays, one of the critical challenges is the conservation of the agave diversity, being necessary the developing of strategies for their conservation and sustainable use (Ashmore, 1997).

Typically, the tissue cultures are sustained by a semisolid medium using agar as gelling agent. The development of temporary immersion systems (TIS) for *in vitro* culture is also an efficient means of micropropagation. TIS are based on the use of liquid culture medium, which involves the flooding of plant tissue at regular time intervals. TIS is considered to be more effective than a semisolid culture system due to that it provides close contact and uniform access to nutrients by the plants (Watt, 2012). TIS protocols for Agave species are still limited. The aim of this study was to evaluate the growth of five agave species in TIS with respect to semisolid medium, providing a new technology for the preservation of agaves.

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METHODOLOGY

Agave plants from five species (*A. weberi, A. mitis, A. vilmoriniana, A. sisalana, and A. celsii*) were surface-sterilized using standard disinfection methodologies. The plant material was planted in culture flasks containing a volume of 30 ml of 50% MS medium (Murashige and Skoog, 1962). The media were autoclaved at 121 ºC for 20 min after pH adjustment.

Axillary buds from each species were transferred to temporary immersion system or semisolid medium (8 g/L of agar), both supplemented with 0.5 mg/L of Benzylaminopurine. The flasks were maintained in a growth chamber at 26 ± 2 ºC and at 16:8 h photoperiod (37.5 µmol m-2s-1).

In the case of the semisolid system, plantlets were subcultured for 4 times on the same medium. The immersion frequency in the temporary immersion system was 3 min every 24 h. Several growth parameters and survival were determined after 12 months.

RESULTS AND DISCUSSION

Measuring explant length revealed the effectiveness of slow-growth in both culture systems, although it was slightly higher in TIS. Shoot regeneration was obtained in all the cultures. Irrespective of the media, rooting also occurred (Table 1).

Table 1. Effects of the culture type on agave growth.

Agave specie	Length increment (cm)		Shoot production (%)		Root production (%)	
	MSS*	TIS*	MSS	TIS	MSS	TIS
A. weberi	1.85	1.74	100	100	100	100
A. mitis	1.47	0.83	87	64	100	97
A. vilmoriniana	2.31	1.75	96	83	100	100
A. sisalana	2.49	2.44	82	73	100	99
A. celsii	2.57	2.28	99	94	100	100

*TIS: temporary immersion system, MSS: gelled media

In 12-month-old cultures, a higher survival (91–100%) was observed on TIS where no subculture procedure was necessary, than in the semisolid culture system. With the increase in age of the cultures, survival rates marginally declined in MSS (77-88%) (Figure 1). Additionally, plants cultivated in TIS developed more leaves compared to semisolid media (Figure 2).

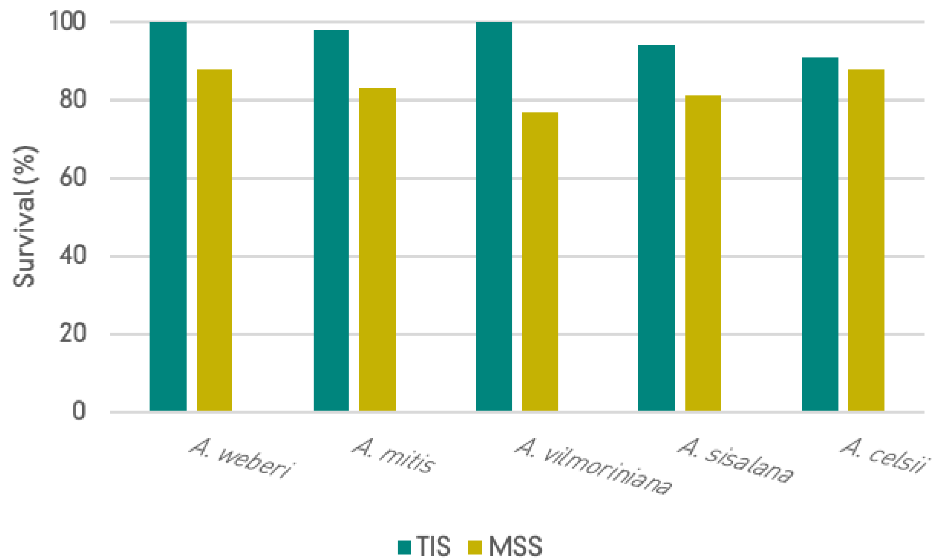

Figure 1. Survival (%) of cultures of agave species on two culture systems.

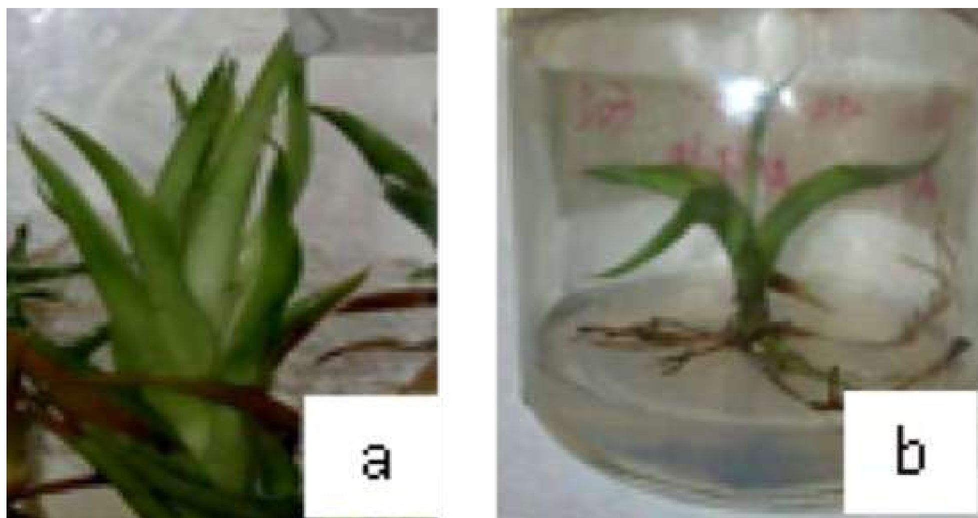

Figure 2. Cultures of *Agave vilmoriniana* on temporary immersion system (a) and gelled media (b).

According to the Operation Manual of the In Vitro National Collection of Agaves, Camote de Cerro and Achiote UG-SAGARPA (Nuñez-Palenius et al. 2015), the current semisolid method of *in vitro* culture of agave germplasm is laborious due to the high number of subcultures (four subcultures a year using semisolid medium).

The advantages of liquid media for enhancing shoot propagation have been reported for several species of plants (Georgiev et al. 2014). Such a system (TIS) allows cultures having only temporary contact with a liquid nutrient medium to ensure normal growth of cultures, and thus avoiding the hyperhydricity problem. In the present study, the agave plants were maintained for up to twelve months on TIS, without any sub-culture.

CONCLUSION

Conservation of germplasm in temporary immersion systems was established for the first time for five agave species. Using TIS was possible to conserve the agave germplasm in the mid-term, avoiding the periodic subcultures and the use of expensive reagents such as agar.

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Applied Cryobiotechnology for the Long-Term Conservation of *Agave sobria* spp *frailensis*.

Tin, J. and Folgado, R[1*]

ABSTRACT

The agaves are emblematic plants, and they are strongly bounded to the economy and history of Mexico. However, many of them are threatened with extinction. Botanical gardens, such as The Huntington can play a crucial role in their *ex-situ* conservation. In addition to the traditional methods such as field collections and seed banks, these gardens can maintain tissue culture collections thus reducing costs and space. Besides the tissue culture techniques, cryobiotechnological methods can help with long-term conservation of agave genetic resources. Tissues can be stored in small spaces at ultra-low temperatures (i.e., liquid nitrogen LN, -196 °C) and they can be regenerated into a plant when needed. A protocol for cryopreservation of shoot tips from *Agave sobria* spp *frailensis* was developed. This agave species occurs only in a few localities along the Gulf of California. Shoot tips from *in vitro* donor plantlets were dissected and cryopreserved using a droplet-vitrification technique. Before the procedure, donor plants were pretreated in two different media: control medium and sucrose enriched medium. After rewarming, there was a significant difference in the plant recovery; shoot tips from sucrose treated donor plants recovered double (87%) than those from control plants (40%). These results showed that the donor plants acclimated to osmotic stress and that this treatment improves the tolerance to the cryoprocedure in the studied agave. The protocol developed for this agave will serve as a reference to cryopreserve other agave accessions from different taxa.

Key words: Cryobiotechnology, *ex-situ* conservation, agave, droplet-vitrification, sucrose.

INTRODUCTION

The agaves have an enormous impact on the culture and economy of Mexico. They have been used for centuries in textiles, construction, food, medicine and alcoholic beverages (Colunga-García Marín et al. 1993) and they have also been appreciated as a productive bioenergy crop (Escamilla-Treviño, 2012). However, many of them are threatened with extinction, and there is an increase of the industries that use agave, which makes the conservation of the germplasm a priority (Colunga-García Marín et al. 2007). *Agave sobria* spp *frailensis* Gentry is restricted to few localities along the Gulf of California between Cabo Frailes and Punta Los Mangles on small granite hill slopes and no other agaves grow with it in Baja California Sur (Webb and Starr, 2015).

Botanical gardens, such as The Huntington, can play an essential role in *ex-situ* conservation by maintaining field collections of agaves and by creating repositories for seeds and tissue culture collections (Folgado et al. 2018). Agave seeds can be used for the conservation of the germplasm since many species are highly efficient. Unfortunately, some species have very long lifespans, and they may flower once before dying (Pavliscak et al, 2015). Others are asexually sterile clones and unable to produce seeds (Gentry

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1982). For these species, plant tissue culture is an effective method for conservation, especially those that are threatened or endangered (Liao et al. 2006).

Moreover, cryobiotechnological methods can help with the long-term conservation of agave genetic resources. Tissues can be stored in small spaces at ultra-low temperatures (i.e., liquid nitrogen LN, -196 °C) and they can be regenerated into a plant when needed (Sakai and Engelmann, 2007). The droplet-vitrification method has been successfully applied to many crop species (Panis et al. 2011; Folgado 2014) and sucrose pretreatment is often used to increase the viability after the cryoprocedure (Sakai and Engelmann 2007). Various studies had been reported for the micropropagation of agave (Rosales et al. 2008; Salazar et al. 2009; Chen et al. 2014; Aureoles-Rodríguez et al. 2008). However, there is no method for the long-term conservation of agave. Thus, this study aimed to develop the cryopreservation of shoot tips of *Agave sobria* spp *frailensis* using a droplet-vitrification method.

METHODOLOGY

Plant material and growth conditions

In vitro shoots of *Agave sobria* spp *frailensis* (#100266, The Huntington Botanical Gardens San Marino, CA, USA) were used as donor plants for the cryopreservation experiments. The shoots were subcultured every 4-6 weeks in Magenta GA7 boxes (Magenta Corp., Chicago, IL, USA) containing basal medium consisting of MS salts and vitamins (Murashige and Skoog 1962), 30 g/L sucrose and 30 mg/L MES buffer (PhytoTechnology Laboratories, Shawnee Mission, KS, USA). The pH was adjusted to 5.7 and 8 g/L of agar (PhytoTechnology Laboratories, Shawnee Mission, KS, USA) was added before sterilization at 121 ºC for 20 minutes. The cultures were kept under 40 µmol m-2 s-1 with a 16h/8h day/night photoperiod and 25 ºC.

Water Content

Six-week old plantlets were transferred onto a sucrose enriched medium, basal medium with 102.7 g/L sucrose for the treatment times: 0, 1, 2, 3 and 4 weeks. Each week, 10-15 plants of relative sizes were selected, cleaned and weighed (fresh weight, FW). Fresh samples were dehydrated in an oven at 40 °C for two days and then 95 °C for one day, before the dry weight (DW) was recorded. Water content (WC) of the shoots was calculated (WC = ((FW – DW)/FW) x 100) as a percentage of the water present in the tissue.

Cryopreservation

Pre-treatment of donor plantlets and dissection of meristems

Donor plants were pretreated in two different media for three weeks: control medium consisting of basal medium with 30 g/L sucrose and sucrose enriched medium with 102.7 g/L sucrose. Then, the shoot tips were dissected under a binocular microscope, having the meristem covered by one leaf primordia and a 1 x 1 mm base, and placed in 25 mL plastic vials containing 10-15 mL of basal liquid medium with 20 mg/L of L-ascorbic acid (AA) until dissection is completed.

Droplet-vitrification

Shoot tips were cryopreserved using a modified droplet-vitrification technique (Panis et al. 2005). Liquid media was removed and replaced with 15 mL of loading solution (LS; filter sterilized liquid MS basal medium with 137 g/L sucrose, 164.4 g/L glycerol, 20 mg/L AA) for 20 minutes at room temperature (RT) in the dark. The LS was replaced with 15 mL of iced plant vitrification solution 2 (PVS2; filter sterilized liquid MS basal medium with 137 g/L sucrose, 267.5 g/L glycerol, 148.1 g/L ethylene glycol, 150.3 g/L DMSO) (Sakai et al. 1990) for 15 min on ice at 0 °C in the dark. About three minutes before the end of the treatment, shoot tips were transferred onto an aluminum strip (0.5 x 2 cm) with a dropper. Aluminum strips were cooled 5 minutes prior to the transfer by putting them in a Petri dish placed on top of a frozen container, to maintain the temperature at 0 °C. Excess solution was removed from the strips, and the aluminum foil strips were then plunged into liquid nitrogen for at least 30 minutes before rewarming (Figure1). Sterile plastic Pasteur

pipettes (dropper) were used to remove the solutions and transfer the shoot tips.

Regeneration after rewarming

For rewarming, aluminum foil containing the shoot tips were taken out of liquid nitrogen and immediately rinsed in a small petri dish filled with unloading solution (US; filter sterilized liquid MS basal medium with 410.6 g/L sucrose, 20 mg/L AA) at RT in the dark for 20 minutes. After 10 minutes, half of the US was removed and replaced with fresh US. After the rewarming, the shoot tips were picked up using a dropper and placed on a sterile filter paper (42.5 mm) in small Petri dishes with recovery medium 0 (RM0, sucrose enriched medium supplemented with 20 mg/L AA) for one day in dark conditions. Then, the shoot tips were transferred to RM 1 (basal medium supplemented with 0.1 mg/L BA). One week after the cryoprocedure, the plates were taken out of the dark and placed in low light (4 µmol m-2 s-1) for two weeks and afterwards, they were moved to regular light. The shoot tips were transferred to basal medium for three days and then to the second recovery medium (RM2, basal media supplemented with 0.2 mg/L BA and 1 g/L charcoal) for two months. Finally, the regenerated shoots were placed onto the basal medium.

Shoot tips were observed on a weekly basis for at least four weeks, and data were recorded: survival (shoot tip was alive or dead) and growth (growth from the meristem, parts of the primordial leaf only, or callus only). Full plant recovery (shoot tips fully regenerated into a complete *in vitro* plant with normal shoot and root growth) was recorded six months after the cryoprocedure.

Statistics

Results are presented as mean percentages with standard deviation. Ten shoot tips x 3 replicates were used for each experimental condition. Data were arcsine transformed and analyzed using the one way-ANOVA with the least significant difference (LSD) and pairwise multiple comparison procedures using Tukey's test at p→0.05, using the SYSTAT version 13 (Systat Software, San Jose, CA).

RESULTS AND DISCUSSION

A preliminary trial was performed with different exposure times of the cryoprotectant solution PVS2, and a 15-min PVS2 treatment was selected for further experiments (results not shown).

The relative water content of donor plants was reduced during the first three weeks after the osmotic stress was applied and this time was chosen to acclimate the plants before the cryopreservation (Figure 1).

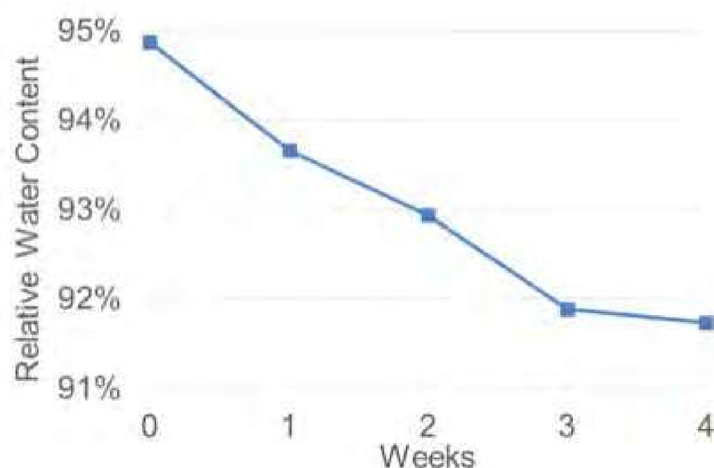

Figure 1. Reduction of the relative water content in shoots of *Agave sobria* spp *frailensis* cultured onto the sucrose enriched medium from 0 to 4 weeks.

The survival percentages were recorded two weeks after the rewarming of the shoot tips, and regrowth could be observed after four weeks onto the regeneration medium (Figure 2). The exposure to liquid nitrogen did not have a significant effect on the viability and regeneration of the shoot tips, regardless of the pretreatment (results not shown).

When comparing the pretreatment of donor plants, the survival percentages showed no significant influence between control and sucrose (73 % and 93 % respectively) (Figure 3a). The final regeneration percentages (rooted plants recovered) were significantly higher when the rewarmed shoot tips were dissected from donor plants pretreated with sucrose enriched medium (87%) than when plants were cultured in control conditions (40%) (Figure 3b).

Moreover the shoot tips from sucrose treated regenerated faster (Figure 2) The use of antioxidants during the cryoprocedure or the pre-culture of explants prior to the cryoprocedure may reduce the accumulative oxidative damage to the plant tissues, which prevent them from recovering into a healthy plant (Uchendu et al. 2010; Fki et al. 2013; Folgado et al. 2015). Our results indicate that a sucrose pretreatment improves their tolerance to the cryoprocedure in the studied agave.

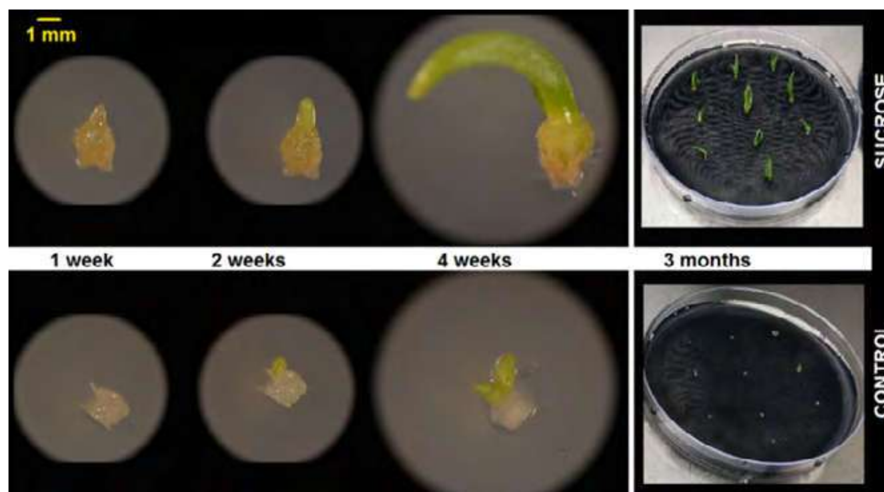

Figure 2. Cryopreserved shoot tips from *Agave sobria* spp *frailensis* one, two and four weeks after rewarming. Donor plants were pretreated in control (below images) and sucrose enriched (above images) media for three weeks before the shoot tips were excised for the cryoprocedure.

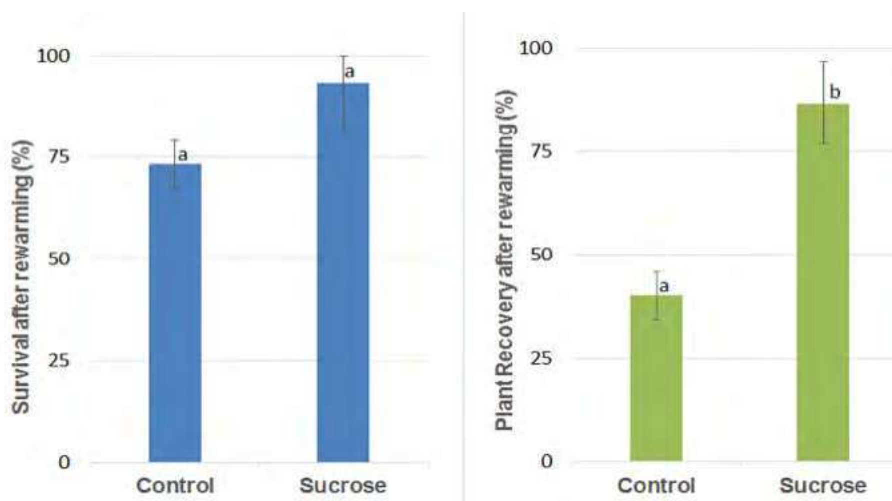

Figure 3. Response of meristems (Survival, left; plant recovery, right) from *Agave sobria* spp *frailensis* after rewarming. Donor plants were pretreated in control and sucrose enriched media for three weeks before the shoot tips were excised for the cryoprocedure; different letters indicate significant difference at p≤0.05 (ANOVA test). Error bars indicate standard deviation.

CONCLUSION

The treatment of donor plants with sucrose enriched medium enhanced the plant recovery after cryopreservation of *Agave sobria* spp *frailensis*. The protocol developed in this study will be used as a reference to apply the cryobiotechnology to other threatened and rare agave species, as well as hybrids with commercial use.

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Cellulose-degrading bacteria isolated from *Agave angustifolia* Haw.

Bautista-Cruz, A.* **and Martínez-Gallegos, V.**[1]

ABSTRACT

Microorganisms carry out most of the cellulose degradation in nature. For many years, cellulose-degrading bacteria have been isolated from a variety of sources such as soil, decayed plant materials, organic matter, feces of ruminants and composts, and characterized to obtain more useful cellulases. Nonetheless, attempts to isolate and characterize cellulose-degrading bacteria in soils cultivated with agave espadín (*Agave angustifolia* Haw.) are scarce. *A. angustifolia* provides the basic ingredient for the production of "mezcal", a traditional Mexican alcoholic beverage. This work aimed to isolate, characterize and identify cellulose-degrading bacteria in the rhizosphere of *A. angustifolia*. A total of 54 cellulose-degrading bacteria isolates were found to be positive on screening media (carboxymethyl cellulose) producing a clear zone during aerobic incubation. Out of the 54 isolates, 23 morphologically different isolates were selected based on the largest diameter of the clearing zone. The clearing zone and the hydrolysis capacity value of the isolates selected ranged from 4.0 to 13.0 mm and 1.6 to 13.0 mm, respectively. Morphologically, all the bacterial isolates were Gram-negative bacilli (26.0%), coccobacilli (44.0%) and cocci (30.0%). Based on the biochemical and morphological tests, cellulose-degrading bacteria were predominantly *Burkholderia cepacia*, and only two isolates were identified as *Sphingomonas paucimobilis* and *Pseudomonas putida*. Soils cultivated with *A. angustifolia* support a high cellulose-degrading bacterial population; nevertheless, biochemical and morphological tests have not indicated broad genetic diversity among the strains.

Key words: Cellulose, cellulose hydrolysis capacity, cellulolytic bacteria, agave espadín, soil fertility.

INTRODUCTION

Cellulose from major land plants is a linear polysaccharide constructed from a monomer of glucose bound together with β-1, 4-glycosidic linkages, which is produced in the living plant cell through photosynthesis (Gupta et al. 2012). Plants produce 4×10^9 tons of cellulose annually (Coughlan, 1990). Microorganisms carry out most of the cellulose degradation in nature (Balamurugan et al. 2010). This biological process is controlled and processed by the enzymes of the cellulase system, which comprises three classes of soluble extracellular enzymes: 1,4-β-endoglucanase, 1,4-β-exoglucanase, and β-glucosidase (β-D-glucoside glucohydrolase or cellobiase) (Gupta et al. 2012). For many years, cellulose-degrading bacteria have been isolated from a variety of sources such as soil, decayed plant materials, hot springs, organic matter, feces of ruminants and composts and characterized in the search for more useful cellulases (Doi, 2008). Nonetheless, attempts to isolate and characterize cellulose-degrading bacteria in soils cultivated with agave espadín (*Agave angustifolia* Haw.) are scarce because of the difficult access to production

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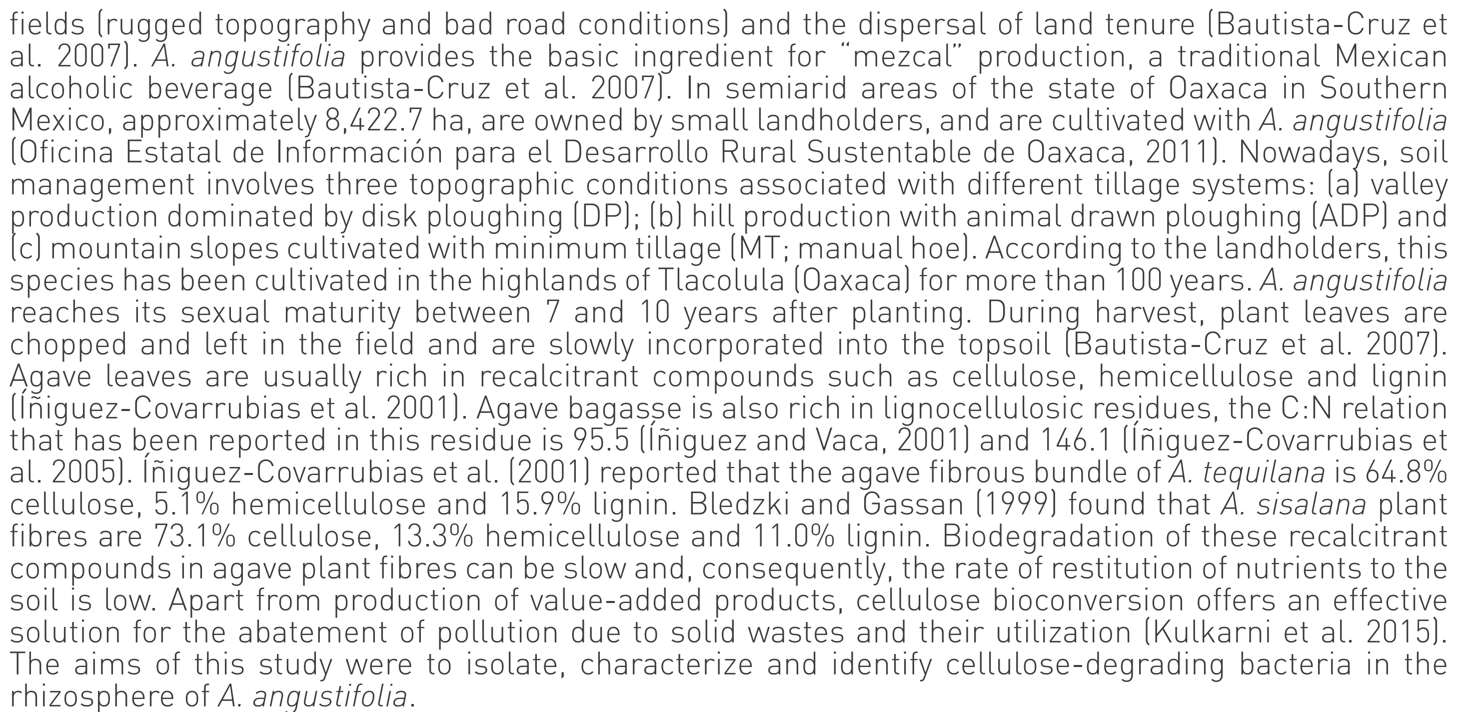

fields (rugged topography and bad road conditions) and the dispersal of land tenure (Bautista-Cruz et al. 2007). *A. angustifolia* provides the basic ingredient for "mezcal" production, a traditional Mexican alcoholic beverage (Bautista-Cruz et al. 2007). In semiarid areas of the state of Oaxaca in Southern Mexico, approximately 8,422.7 ha, are owned by small landholders, and are cultivated with *A. angustifolia* (Oficina Estatal de Información para el Desarrollo Rural Sustentable de Oaxaca, 2011). Nowadays, soil management involves three topographic conditions associated with different tillage systems: (a) valley production dominated by disk ploughing (DP); (b) hill production with animal drawn ploughing (ADP) and (c) mountain slopes cultivated with minimum tillage (MT; manual hoe). According to the landholders, this species has been cultivated in the highlands of Tlacolula (Oaxaca) for more than 100 years. *A. angustifolia* reaches its sexual maturity between 7 and 10 years after planting. During harvest, plant leaves are chopped and left in the field and are slowly incorporated into the topsoil (Bautista-Cruz et al. 2007). Agave leaves are usually rich in recalcitrant compounds such as cellulose, hemicellulose and lignin (Íñiguez-Covarrubias et al. 2001). Agave bagasse is also rich in lignocellulosic residues, the C:N relation that has been reported in this residue is 95.5 (Íñiguez and Vaca, 2001) and 146.1 (Íñiguez-Covarrubias et al. 2005). Íñiguez-Covarrubias et al. (2001) reported that the agave fibrous bundle of *A. tequilana* is 64.8% cellulose, 5.1% hemicellulose and 15.9% lignin. Bledzki and Gassan (1999) found that *A. sisalana* plant fibres are 73.1% cellulose, 13.3% hemicellulose and 11.0% lignin. Biodegradation of these recalcitrant compounds in agave plant fibres can be slow and, consequently, the rate of restitution of nutrients to the soil is low. Apart from production of value-added products, cellulose bioconversion offers an effective solution for the abatement of pollution due to solid wastes and their utilization (Kulkarni et al. 2015). The aims of this study were to isolate, characterize and identify cellulose-degrading bacteria in the rhizosphere of *A. angustifolia*.

METHODOLOGY

The study area was located in the District of Tlacolula, Oaxaca, Mexico, in the communities of Magdalena Teitipac (16° 54 N and 96° 33 W), San Baltazar Guelavila (19° 80 N and 96° 29 W) and San Juan del Río (16° 53 N and 96° 09 W). The primary soil types in the area include Regosols and Leptosols (Comisión Nacional de Biodiversidad, 2004). Altitude ranges from 1 060 to 1 700 m, average annual precipitation is 726 mm, and average annual temperature is between 28 and 32 ºC. The native vegetation is dry deciduous lowland forest (Lorence and García-Mendoza, 1989). Three tillage systems are used in *A. angustifolia* cultivation, and the method selected depends on the local topography. Minimum tillage is predominant in mountainous zones (San Juan del Río) with slopes of 35 to 45%; ADP in hilly zones (San Baltazar Guelavila) with slopes of 15 to 30% and DP in valleys (Magdalena Teitipac) with slopes of 3 to 10%. Three plots of land were delimited in each topographic area. The plots had a surface area of approximately 4000 m^2 with incipient agave plants of approximately 1.5 to 3.5 years old. Five agave plants were selected in each plot: one in the center and four at an approximate distance of 25 m, following the direction of the cardinal points with the central plant as a reference. These plants, including the roots and the attached soil to a depth of 20 cm, were extracted and maintained at a temperature of 4-5 °C until isolation of cellulose-degrading bacteria. The soil from the rhizosphere of each plant was mixed homogeneously to form one composite sample for each plot. Cellulolytic bacteria were isolated from soil by using serial dilutions and the spread plate technique. The medium used for isolation of cellulolytic bacteria was carboxymethyl cellulose (CMC) (Irfan et al. 2012). After incubation at 37°C for 48 h, the CMC agar plates were flooded with 1% Congo red and allowed to stand for 15 min at room temperature. A 1M NaCl was thoroughly used for counterstaining the excess of Congo red in plates. Cellulose-degrading potential of the positive isolates was qualitatively estimated by calculating hydrolysis capacity (HC), that is, the ratio between the diameter of the clearing zone and the colony. The selected isolates were identified morphologically and biochemically using the API 20 NE and API 20 E kits (bioMerieux, USA). The Gram stain test was carried out on the bacterial isolates as described in the Bergey Manual (Bergey, 1957). The selected isolates were identified through biochemical testing using API 20 NE and the API 20 E kits (bioMerieux, USA) and the identification was confirmed with the APIWEB™ software.

RESULTS AND DISCUSSION

The cellulose-degrading bacterial population density in the *A. angustifolia* rhizosphere soils ranged from 1.0×10^2 to 2.5×10^2 CFU ml^{-1} and was highest in valley soils. The variation in the population of cellulose-degrading bacteria under the different topographic conditions where *A. angustifolia* is cultivated may be attributed to many soil factors, such as nutrient status, pH, moisture content, organic matter, enzyme activities and soil management (Alia et al. 2013). A total of 54 cellulose-degrading bacteria isolates were found to be positive on screening media (carboxymethyl cellulose) producing clear zones during aerobic incubation. Out of the 54 isolates, 23 morphologically different isolates were selected based on the largest diameter of the clearing zone (Figure 1). The clearing zone and the hydrolysis capacity value of the isolates selected ranged from 4.0 to 13.0 mm and 1.6 to 13.0, respectively.

The use of Congo red as an indicator for cellulose degradation in an agar medium provides the basis for a rapid and sensitive screening test for cellulolytic bacteria (Hendricks et al. 1995). Teather and Wood (1982) proved that Congo red has a specific interaction with the cellulosic substrates. When cellulose is broken down by the action of cellulase, the Congo red no longer has affinity with the substrate and a clear zone will appear on the medium. The screening results revealed that each bacterium has diversified activity for the breakdown of

cellulosic substrates which indicates that the isolates have the ability to produce varying amounts of cellulase (Gunavathy and Boominathan, 2015).

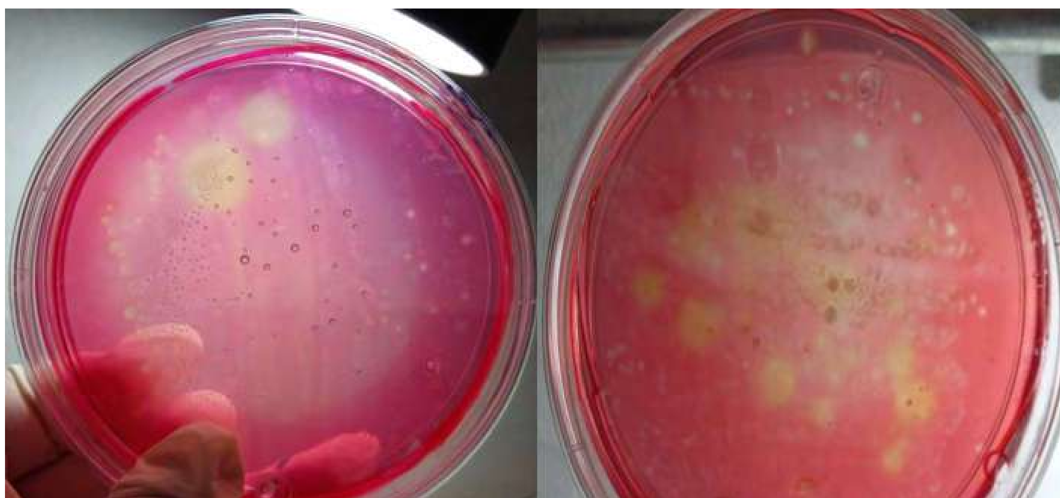

Figure 1. Zone of clearance on cellulose Congo red agar plates produced by the cellulose-degrading bacteria isolated from the rhizosphere soil of *Agave angustifolia* Haw. grown in the Tlacolula District, state of Oaxaca (Mexico). The formation of a clearing zone around the colonies confirms the secretion of extracellular cellulase.

Emmyrafedziawati (2013) reported higher values of clearing zones ranging from 2.2 to 3.18 cm in three strains of *Bacillus subtilis* isolated from oil palm empty fruit bunch compost. Hatami et al. (2013) found that the hydrolytic value ranged from 1.38 to 2.33 and 0.15 to 1.37 of cellulolytic bacterial isolates from farm and forest soils, respectively. Morphologically, all the bacterial isolates were Gram-negative bacilli (26.0%), coccobacilli (44.0%) and cocci (30.0%) (Figure 2). Based on the biochemical and morphological tests, the cellulose-degrading bacteria were predominantly *Burkholderia cepacia*, and only two isolates were identified as *Sphingomonas paucimobilis* and *Pseudomonas putida.*

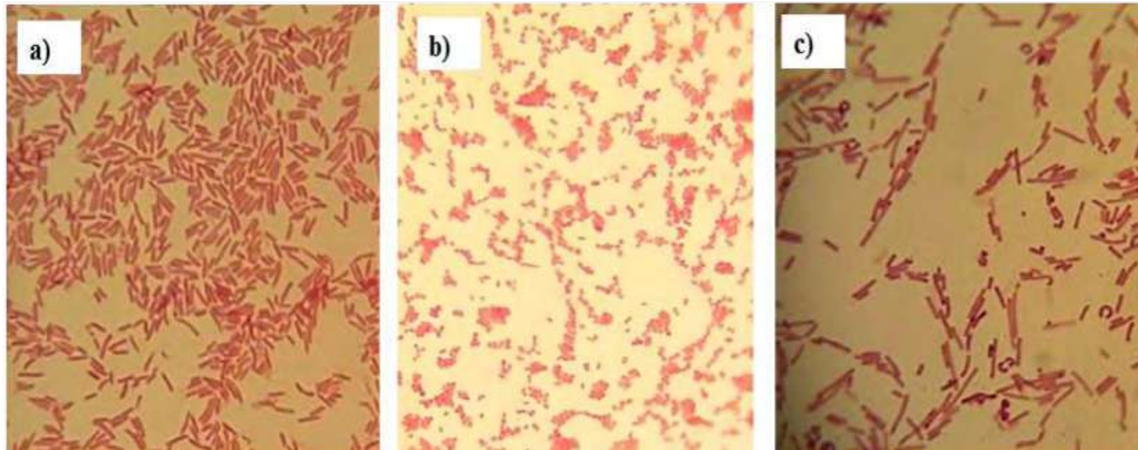

Figure 2. Micrograph of cellulose-degrading bacteria identified as a) *Pseudomonas putida*, b) *Burkholderia cepacia* and c) *Sphingomonas paucimobilis*.

CONCLUSION

Soils cultivated with *A. angustifolia* support a high cellulose-degrading bacterial populations; nevertheless, biochemical and morphological tests have not indicated high genetic diversity among the strains. *Burkholderia cepacia* was the dominant cellulose-degrading bacterium in the rhizosphere of *A. angustifolia*. Further studies are necessary to quantify the cellulase activity of these cellulose-degrading bacteria.

ACKNOWLEDGEMENTS

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Efficient method for agave seed germination.

Castañeda-Nava, JJ., Gutiérrez-Mora, A.* and Rodríguez-Domínguez, JM[1]

ABSTRACT

Currently in Mexico and around the world, interest in agave plants is increasing due to the demand as ornamental plants, and for the production of tequila, mezcal and syrups. The propagation of agaves is carried out by both sexual and asexual means. Germination pretreatments allow greater homogeneity at the moment of germination (breaking dormancy in the seeds), high percentage of germination and vigor, this is why it is employed in different species of economic importance. In the present study, different pregermination methods were evaluated, which permit germination of agaves to be more efficient. A first test was carried out where seeds were soaked in water at room temperature, hot water, hydrogen peroxide and cutting the seed coat. After the results of the first test, a second experiment was conducted by soaking the seeds in hydrogen peroxide, considering exposure periods (10, 15, 30 and 45 min) and different concentrations (1, 2 and 3%). In the first test it was observed that the hydrogen peroxide shows favorable effects in the germination, in the second experiment the treatment of 3% hydrogen peroxide and an exposure time of 10 min showed 100% of germination and was found to be within the statistical group with greater weight and length of the cotyledon. The hydrogen peroxide in the germination generates O_2, available for mitochondrial respiration and synthesis of proteins that stimulate plant growth.

Key words: *Agave salmiana*, Germination percentage, Hydrogen Peroxide, Pregerminative treatments, Vigor of germination.

INTRODUCTION

In Mexico and worldwide, the demand for agave drinks has increased, these being part of mexican traditions and culture. Commonly the agave species are propagated by means of shoots (suckers), however, this method limits the conservation of genetic variability, thus, an alternative for its propagation is by means of seeds (García-Mendoza, 2007). Given that the embryo and other structures that comprise seeds require specific conditions to initiate germination, it is necessary to know the factors and processes that are involved. Germination pretreatments allow greater homogeneity in germination periods (breaking dormancy of the seeds), high percentage of germination and increased vigor, which is why it is used in different species (Sánchez-Urdaneta et al. 2016). To allow the seed coat to be more permeable, scarification is carried out, which is any process that breaks, scratches, alters mechanically or softens the seed covers to make them permeable to water and gases. A large number of species do not germinate because the testa or seed coat is hard and prevents the entry of water and the seed does not germinate unless it is scarified (Insuasty et al. 2012). Another alternative is the treatment of the seed to chemical agents like acids, or others like hydrogen peroxide (hp). This last compound has effects on germination because it acts on the generation of O_2, available for mitochondrial respiration (Katzman et al. 2001); favors the appearance of cracks in hard seeds (Chien and Li 1994); contributes to oxidize

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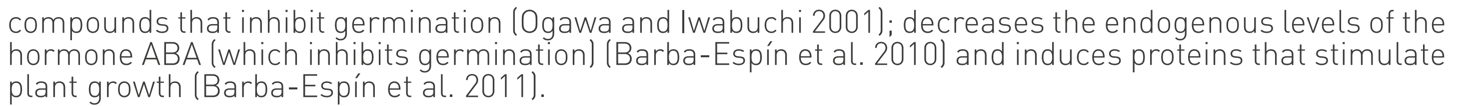

compounds that inhibit germination (Ogawa and Iwabuchi 2001); decreases the endogenous levels of the hormone ABA (which inhibits germination) (Barba-Espín et al. 2010) and induces proteins that stimulate plant growth (Barba-Espín et al. 2011).

Due to the demand that exists for agave plants, the objective of this work was to find an efficient seed germination method (greater germination and vigor).

METHODOLOGY

Plant material and culture conditions

Seeds of *Agave salmiana* were collected directly from the fruits in the field (location: San Felipe, Guanajuato, México). The seeds were placed on moist paper in Petri dishes under a photoperiod of 16 h light for 8 h of darkness at a temperature of 25 ± 2 ° C.

Evaluation of soaking, cutting and exposition to hydrogen peroxide (hp) of seeds

The following treatments were used for a first experiment; a) Control, seeds were put over moist paper. b) Seeds soaked in water 24 h, c) Soaked in hot water 45°C, d), Soaked in 3% hp for 30 min, e) Soaked in 3% hp for 24 h, f) Cutting on the seed coat and, g) Cutting on the seed coat + 3% hp for 24 h. Ten seeds per treatment were used.

Determination of time of exposition and concentration of Hydrogen Peroxide (hp)

A second experiment was conducted with the following treatments: a) 1% hp for 30 min, (b) 2% hp for 30 min, c) 3% hp for 30 min, d) 3% hp for 10 min, e) 3% hp 15 min; f) 3% hp for 45 min, and g), Control as above. The design was a completely randomized one with three replications per treatment, where each replication was ten seeds (one Petri dish).

Statistical analyzes

In the first experiment only the percentage of seeds that germinated was calculated. For the second experiment, an analysis of variance (ANOVA) and a multiple means comparison was performed by the Tukey test.

RESULTS AND DISCUSSION

In the first experiment, it was observed that hydrogen peroxide gave 100% germination followed by hot water at 45°C; and the cut of the seed coat was found good in a third place, These observations were made three days after the treatments were applied to all the seeds. For each species, pregerminative treatments have different effects influencing exogenous and endogenous factors of the seeds (Pérez et al. 2008). Our results showed that in agave seeds the treatment with hydrogen peroxide for 30 min favors 100% germination of the seed in 3 days. On the other hand, the extended periods, as in the case of the treatments e) and g), decrease the germination percentage (Table 1).

Table 1. Evaluation of different pregerminative treatments inseeds *Agave*.

Treatment	*Percentage of germinated seeds*
a. Control	60
b. Soaked in water 24 h	5
c. Hot water 45 ° C	80
d. Hydrogen peroxide (3%) for 30 min	100
e. Hydrogen peroxide (3%) for 24 h	35
f. Cut in the seed coat	70
g. Cut in the seed coat + Hydrogen peroxide (3%) per 24 h	20

In the ANOVA of the second germination experiment, significant differences were observed treatments ($p = 0.0003$). The best response with the highest germination percentage was observed with 3% hydrogen peroxide treatment and the total germination was reached after three days. For the response variable fresh weight of the cotyledon was significant ($p = 0.0110$), where the treatment with 3% hydrogen peroxide and 15 min exposure (treatment e) showed the highest germination average followed by the treatments d) and b). The analysis of variance showed that the response variable size of the cotyledon was significant ($p = 0.0000$). The highest average was achieved by treatment with 3% hydrogen peroxide and an exposure period of 10 min (treatment d). Treatments c) and e) were found in the same statistical group (Table 2 and Figure 1).

Table 2. Effect of Hydrogen Peroxide on germination and vigor of cotyledon of agave seeds.

Treatment	PSG	*CW (g)*	*SC (cm)*
a. 30 min + 1%	3.7 to *	0.085 ab	0.11 a
c. 30 min + 3%	5.0 ab	0.063 to	0.09 a
g. Control	9.0 bc	0.096 bc	1.68 c
b. 30 min + 2%	9.0 bc	0.100 bc	0.79 b
e. 15 min + 3%	9.0 bc	0.112 c	1.61 c
f. 45 min + 3%	9.3 c	0.097 bc	0.97 b
d. 10 min + 3%	11.0 c	0.106 bc	1.78 c

* In the columns the data with different letters are statistically different. PSG = germination average. CW = Cotyledon weight. SC = Size of the cotyledon.

Hydrogen peroxide oxidizes compounds that inhibit germination, but its effects can vary according to the dose (Ogawa and Iwabuchi, 2001) in this study, it was observed that not only the dose had influence, but also the exposure time is an important factor to yield a high percentage of germination and seedlings with a high vigor. García-Mendoza, (2007) mentioned that when germinating seeds, the seedlings are highly vulnerable environmental conditions, which is why they require a nurse plant. The use of Hydrogen Peroxide can benefit the seedlings to adapt to the environment that surrounds them, in addition to a greater number of germinated seeds.

Figure 1. Germination of agave seeds under de effect of Hydrogen peroxide.

CONCLUSION

In the case of agave seeds, hydrogen peroxide is observed improve germination at a suitable time and concentration. The use of hydrogen peroxide as pretreatment for germination allows a high percentage of germination, and seedlings with a high weight and size of the cotyledon. This practice of treatment for seed germination will allow having more homogeneous crops.

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Identification and characterization of new families of genes NBS-LRR in *Agave tequilana*.

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ABSTRACT

The Effectors Triggered Immunity (ETI), includes R genes, which code for proteins that are able to recognize pathogens and trigger the immune response (Meyers et al. 1998). The vast majority of R genes code for a group of genes called NBS-LRR (Jones and Dangl, 2006). It has been described that the presence of the NBS-LRR genes is related to the resistance of plants against diseases caused by different pathogens (van Ooijen et al. 2007). However, little is known in *Agave* species. This is the first work on the characterization of this type of sequences in *Agave tequilana*; it was possible isolate and identify gene sequences of response to pathogens of the NBS-LRR type *Agave tequilana*. In the analysis of the transcriptome were identified around of 96 sequences, which present the characteristic domain Coiled and Coil for this type of proteins. This information can help with the development of genetic markers for the search for pathogen resistant lines.

Key words: NBS-LRR, *Agave tequilana*, ETI, CNL, R genes.

INTRODUCTION

Plants are continually subjected to stress (biotic or abiotic), and this can lead to losses in yield (Wang et al. 2017). Due to this, plants have developed defense mechanisms against pathogen attack (Glazebrook. 2005) like the physical barriers, such as the cell wall and wax and cuticle (García, 2004), however, beyond of these pre-existing physical barriers, and Pattern Triggered Immunity (PTI). Nevertheless, some pathogens are able to evade these defense mechanisms through the secretion of effectors against the PTI response, by which plants have developed another mechanism of defense called Effector Triggered Inmunyti (ETI) which are mediated by cytoplasmic receptors (Dodds et al. 2010). These receptor protein (R protein) recognize directly the effector coded by the Avirulence (Avr) gene, following the "gen by gen" model (Dangl and Jones, 2001) or indirectly, following the guard or decoy models (van der Hoorn and Kamoun, 2008).

These R proteins are encoded by a large number of gene families founded in large numbers within the genomes (Michelmore and Meyers et al. 1998). The majority of the R genes are encoding for a group of genes called NBS-LRR (Jones and Dangl, 2006) that are grouped in the genome (Marone et al. 2013) and are widely distributed among plant species. These genes can be classified into two groups based

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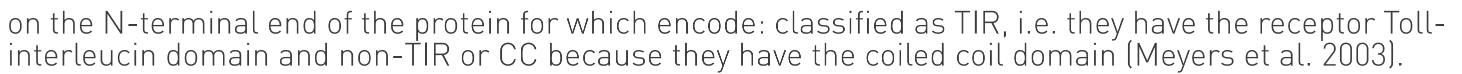

on the N-terminal end of the protein for which encode: classified as TIR, i.e. they have the receptor Toll-interleucin domain and non-TIR or CC because they have the coiled coil domain (Meyers et al. 2003).

These genes share domains as the binding nucleotides (NBS), which are able to hydrolyze ATP or GTP and work as a signal transduction for on/off switch after the recognition of the pathogen. It has been described that this region is formed by eight conserved motif (P-loop, RNBS-A, Kinase, RNBS-B, RNBS-C, GLPL, RNBS-D y MHDL. The highly conserved motif in this domain have been target to isolate and characterization of NBS-type sequences by PCR or genome analysis (Wang et al. 2017; Habachi-Houimli et al. 2018).

In the genus *Agave* it is little known about the NBS-LRR genes variety in the genome, including their structure and classification. Thus, goal of this work was to identify and characterize NBS-LRR genes of *A. tequilana*. The information obtained may permit the selection of NBS-LRR genes involved in the resistance against pathogens with the potential application of molecular marker.

METHODOLOGY

NBS sequence isolation

Two years old *Agave tequilana* Weber var. "Azul" plants were selected, and the amplification and identification of NBS sequences was previously conducted by using universal primers (Tamayo-Ordonez et al. 2012). The PCR reaction was carried out in 25 µl of final volume, the reaction contained 25 ng of genomic DNA, 130 µM of dNTPS, 10 mM of each primer, 2.5 units of Taq polymerase, PCR buffer 1 X and 1.5 mM $MgCl_2$. The reaction conditions included a home at 94° C for denaturation, followed by 35 cycles of 1 min at 94° C, 1.5 min at 60° C, 1 min at 72° C and finally 7 min at 72° C for the final extension. PCR products were separated by electrophoresis in 1.2% agarose gels stained with ethidium bromide. The bands were observed at 400 base pairs. The PCR product was cloned into the vector p-GEM T - easy, and the product of cloning was sequenced for confirmation of the identity of the insert. The obtained sequences were compared with sequences deposited in the genbank using the Blastn BlastP tool and the Mega software v. 5. They were alignments with sequences from different accessions of plant NBS-LRR genes for the identification, were grouped using the Neighbor-Joining method for amino acid and nucleotide sequences. With a boostrap of 1000 repetitions for confirmation of the identity.

Phylogenetic relationship of NBS from *A. tequilana*

Once confirmed the identity of these sequences, a search of these sequences in the transcriptome of *A. tequila* was conducted (Gross et al. 2013), using Blast with the whole transcriptome of *A. tequilana*. The sequences with a mayor identity were selected for the search of phylogenetic relationships. In order to group these base sequences on their similarities, we constructed a phylogenetic tree based on the nucleotide sequences using the Minima Evolution method with a bootstrap of 100 repetitions. For the characterization of sequences NBS-LRR candidates we used the MEME, CLC software viewer, and Mega v. 5. for the search of the motif and structure characteristic of this type of sequences.

RESULTS AND DISCUSSION

Identification of NBS sequences from *Agave tequilana*

The sequences obtained from the cloning were blasted with a subset of NBS-LRR sequences retrieved from Genbank. The analysis was performed by comparing these sequences from translated sequences, as reported by Rout et al. (2014). It is expected that even at the amino acid level the percentage of identity with other sequences are a must be around 70% or less (Rout et al. 2014).

In total, 11 sequences (NBS_A2,6,12,13,20, T4,7,8,9,11) were compared by the BlastP tool with known R gene sequences deposited in the GenBank. The level of identity of these sequences with other R proteins

of other plants varies among 37% for clone NBS_T11 and 41% for clone NBS_A13; being identified as NBS-LRR proteins with homology with putative proteins in *Oryza punctata* and RPM1-Like of *Phoenix dactylifera* respectively. Showing greater identity with R proteins of monocotyledonous plants mainly with *Oriza* species.

Likewise, the grouping of the NBS-type sequences isolated from *A. tequilana* with other sequences of R proteins known from other species including CNL, TNL from monocot and dicotyledonous plants (Figure 1), it was observed that our sequences are grouped with sequences of the non-TIR type, consistent with previous reports in other plant species (Wang et al. 2017; Alamery et al. 2018). It is probably that TIR-like proteins for monocotyledonous plants are rare or absent since they arise after the divergence between monocotyledons and dicotyledonous plant (Pan et al. 2000).

Within the clade of CNL, three subgroups were observed, one formed by the sequences isolated in this work, together with NBS type proteins obtained from the transcriptome of *Agave tequilana*, these are related to CNL-like proteins described for monocotyledonous plants such as *Triticum turgidum*, *Oriza punctata* and *Oriza brachyantha* (subgroup I). The other clade is formed only by TNL-like sequences of dicotyledonous species such as *Rosa rugosa*, *Arabidopsis thaliana* and *Linum usitatissimum*.

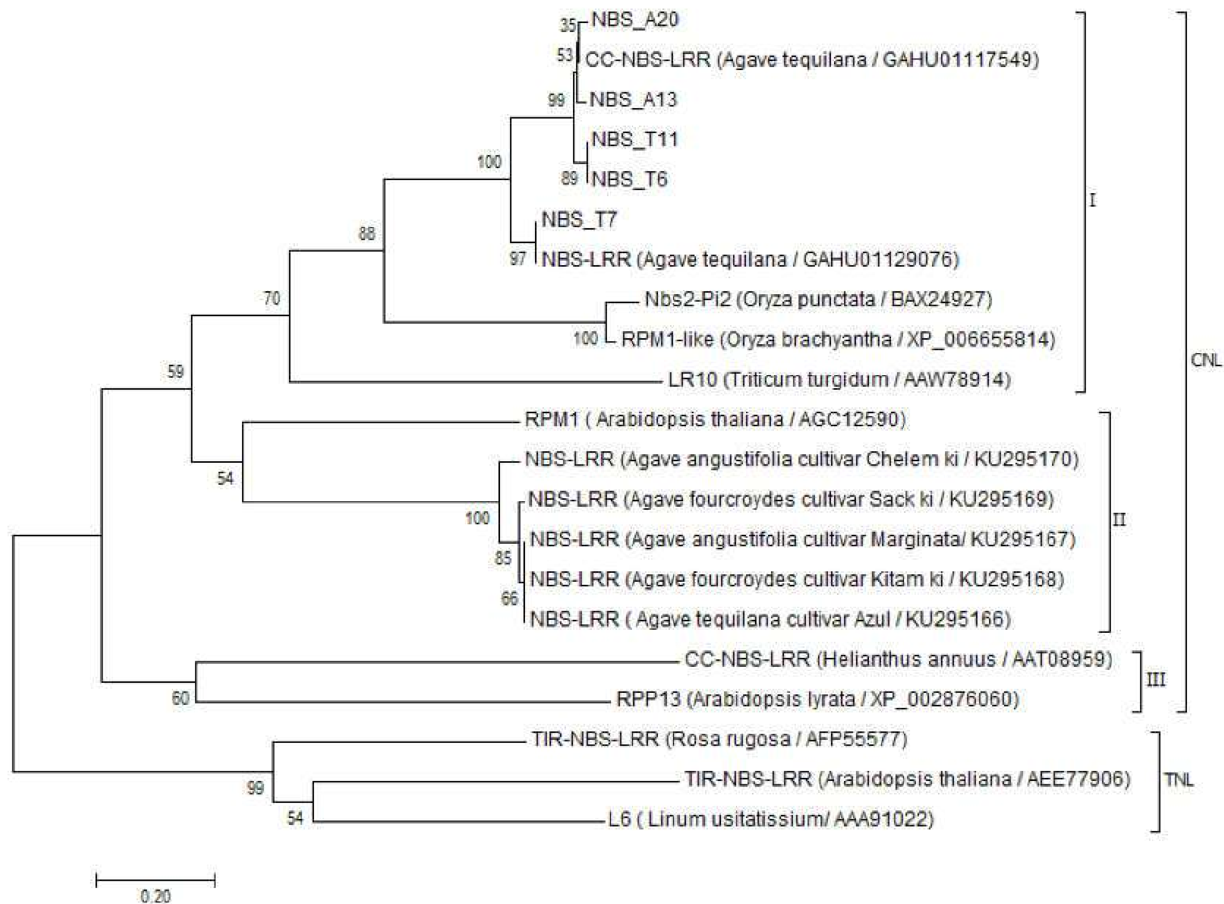

Figure 1. Phylogenetic tree based on the neighbor-joining method constructed with 21 sequences of R proteins (TNL and CNL). The sequences of the known R proteins were downloaded from the genbank with the following accession numbers: Pi2 (BAX24927), RPM1 (XP_00g55814), LR10 (AAW78914), RPM1 (AGC12590), CNL (AAT08959), RPP13 (XP_002876060), TNL (AFP55577), TNL (AEE77906), L6 (AAA91022). Transcribed sequences of genes encoding NBS type proteins were included for some Agave species found in the genbank with the accession numbers (GAU01117549, GAU1129076, KU295166, KU295167, KU295268, KU295169, KU295170).

Classification of NBS-LRR sequences in *Agave tequilana*

In order to carry out a broader analysis of the NBS type genes in *Agave tequilana*, the Blastn tool was used to find, the sequences with higher identity to the NBSS-type sequence previously isolated within the *Agave tequilana* transcriptome. The best hits of the transcriptome were used for the analysis of the structure and diversity of the sequences for this species. We selected a total of 96 (NBS_ATeq) sequences of which approximately 50% were discarded because they were too divergent for the analysis. The analysis was carried out searching the conserved domains and their position in the transcriptome sequences. The search of main domains in the transcriptome sequences of *A. tequilana* indicated that: out of the selected sequences, all belong to the non-TIR type, most of them having the characteristic domains CC, NBS and LRR. However, some of them do not present all the characteristic domains. This type of variation in terms of the domains found for this type of genes has been reported in other plant species, where different combinations between the characteristic domains are observed, including the absence of the characteristic NBS domain (Shimizu et al. 2014; Song et al. 2017). Finally, the selected NBS_Ateq sequences were translated, and were used for searching conserved motifs, the position they occupy and the logo of the motifs. The characteristic motifs for the NBS domain (P loop, RNBS-A, Kinase 2, Kinase 3, GLPL, MHD) were identified. We also identified some conserved regions in the CC and LRR domains.

CONCLUSION

It were identified NBS-LRR sequences of the *Agave tequilana* transcriptome through the use of universal primers. These sequences show a great diversity which may be indicating the neofunctionalization of some of them, besides that the conservation of the characteristic motifs as well as the conservation of the structure of the NBS domain may be indicating that they are sequences that code for functional proteins.

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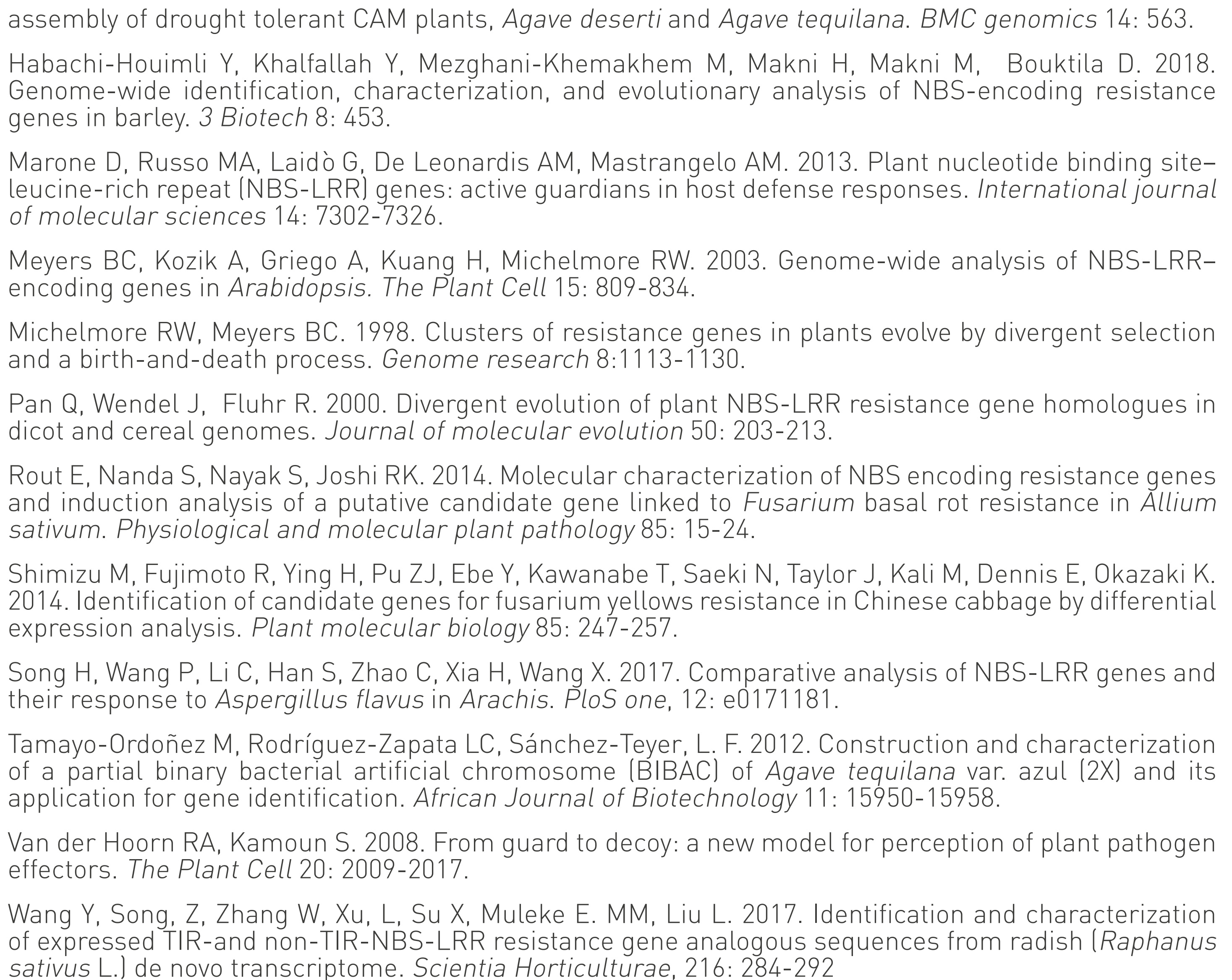

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Insights into the anatomy and physiology of water and carbohydrate storage in leaves of three *Agave species*.

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ABSTRACT

Agaves are succulent plants with constitutive CAM photosynthesis, among other adaptations that lead to water conservation. These plants produce a larger amount of water-soluble carbohydrates (WSC) and biomass under harsh conditions in comparison to other species, while reducing water loss. Therefore, succulence and carbohydrate metabolism, are important characteristics that should be studied in greater detail in different *Agave* species. In this study, anatomical traits, that are differentially expressed in young plants of *A. tequilana*, *A. striata* and *A. victoriae-reginae*, in relation to water and carbohydrate patterns in leaves, were determined and related to the CAM physiology of these species.

A combination of succulence parameters and Terahertz (THz) imaging spectroscopy allowed us to elucidate that *A. victoriae-reginae* is the most succulent, however, only a few traits, that have been characterized in other succulent species, were directly correlated with the succulence parameters in these three agave species. A 3D analysis of the succulence can help us to better understand how water is distributed and stored in agave tissues. Carbohydrates are more abundant in the TLC profiles of *A. tequilana*, while an *in situ* identification of total carbohydrates in tissue sections of leaves (PAS staining), shows an independent carbohydrate and water distribution that is species specific and does not correspond to conserved anatomical characters. Further studies will be focused on succulence physiology and how this contributes to CAM metabolism will be analyzed with this aim in more *Agave* species.

Key words: *Agave* **species, Crassulacean acid metabolism, Succulence, Water soluble carbohydrates, Anatomical traits.**

INTRODUCTION

All *Agave* species are considered to be succulent plants which carry out Crassulacean acid metabolism (CAM) based photosynthesis in addition to other adaptations that lead to water conservation (Lüttge, 2004). These species are considered as productive for exploitation of biomass and carbohydrates even under harsh, arid environmental conditions (Nobel, 2003, Benadom, 2010). Water-soluble carbohydrates (WSC) are the main photosynthates and contribute to the retention of water and stability of cellular structures under low water conditions (Males, 2017). The *Agave* genus is an example of the adaptive radiation of North America (Gentry, 2004) and is thought to have developed CAM independently of the *Yucca* and *Hesperaloe* genera that are also members of the *Asparagaceae* family (Heyduk et al. 2018).

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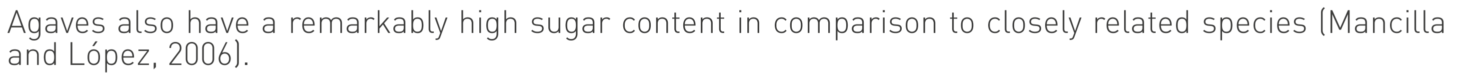


Agaves also have a remarkably high sugar content in comparison to closely related species (Mancilla and López, 2006).

Agaves are monocarpic perennials with a rosette of spirally developing leaves and are classified in two subgenera *Littaea* and *Agave*, based on the spicate or paniculate forms of their inflorescence, respectively. Species within each subgenus are distinguished by leaf morphology, contrasting in spines, teeth, shape, and size (Gentry, 2004) and molecular marker data support the taxonomic classification (García, 2007). Leaf traits and succulence seem to predate CAM metabolism (Heyduk et al. 2016) and are related to biogeography (Gross et al. 2013). However, the consensus definition of succulence is vague and is commonly only referred to as "storage of water in one or more living parts of the plant" (Males, 2017).

Agaves store a large amount of water-soluble carbohydrates (WSC) in the form of highly branched oligo fructans that are synthesized and stored in the vacuoles. The concentration and structure of the fructan molecules synthesized depend on the age of the plant and environmental conditions (Arrizon et al. 2010, Mellado and López, 2012). In leaves, these carbohydrates are the transitory products of photosynthesis found in vacuoles, vascular bundles and the apoplast (Wang and Nobel, 1998). In striking contrast to many other plant species, in agaves, carbohydrates are not stored in the form of starch which probably accumulates transiently in the shoot apical meristem and the primary thickening meristem where it seems to act as a carbohydrate reserve for these actively growing tissues (Zavala et al. 2018).

The relationship between succulence and carbohydrates in leaves was studied in three contrasting species with distinct leaf morphologies: *A. tequilana*, *A. victoriae-reginae* and *A. striata*. Knowledge of anatomical traits in combination with *in situ* localization of these resources will contribute greatly to the understanding of a unique CAM physiology of agaves.

METHODOLOGY

Biological material was obtained from ~3 year-old greenhouse grown plants of *A. tequilana* (Atq), *A. victoriae-reginae* (Avr) and *A. striata* (Ast). For each species, three plants were analyzed, and three leaves of each plant were excised and separated into basal leaf (the white and non-photosynthetic tissue adjoining the stem) and mid-leaf (the central section of the green, photosynthetically active part of the leaf). For THz imaging analysis, two plants per species and three leaves of each plant were hand sectioned with a razor blade as follow: four areas of the proximal and distal leaf regions were selected and two consecutive sections of 1 mm of thick of each area, were analyzed.

Analysis of thirteen traits, in addition to traditional measurements of succulence at the macro and microscale level (Table 1) were carried out (Mantovani, 1999, Heyduk, 2016, Males and Griffiths, 2018). For general carbohydrate identification, Periodic Acid-Schiff (PAS) staining was performed (Zavala et al. 2018), which was complemented with carbohydrate profiles obtained by Thin Layer Chromatography (TLC) (Avila de Dios et al. 2015). The cuticle was manually removed and stained with Safranin (Damián, 2013). Samples were visualized with an Olympus BX60 microscope and a Leica E24HD stereoscope (bright-field mode). Micrographs were obtained using ImagePro and LAS EZ software, then processed with the ImageJ software, while statistical analysis was performed in Excel and R computing.

Succulence was indirectly estimated based on absorbance by Terahertz (THz) spectroscopy for net water content determination (Castro et al. 2013). A terahertz time-domain imaging spectrometer (THz-TDI) with a Picometrix T-Ray 5000 device, was adapted to capture the transmitted absorbance in tissue sections. Data was recorded in the T-Ray Explorer 4.5.6 software, at an image resolution of 0.5 mm and a velocity of 15 mm sec^{-1}. Absorbance values plus the specific dielectric function of the leaf, were processed in Matlab and presented as images of water concentration and net % of water (Gente et al. 2018).

RESULTS AND DISCUSSION

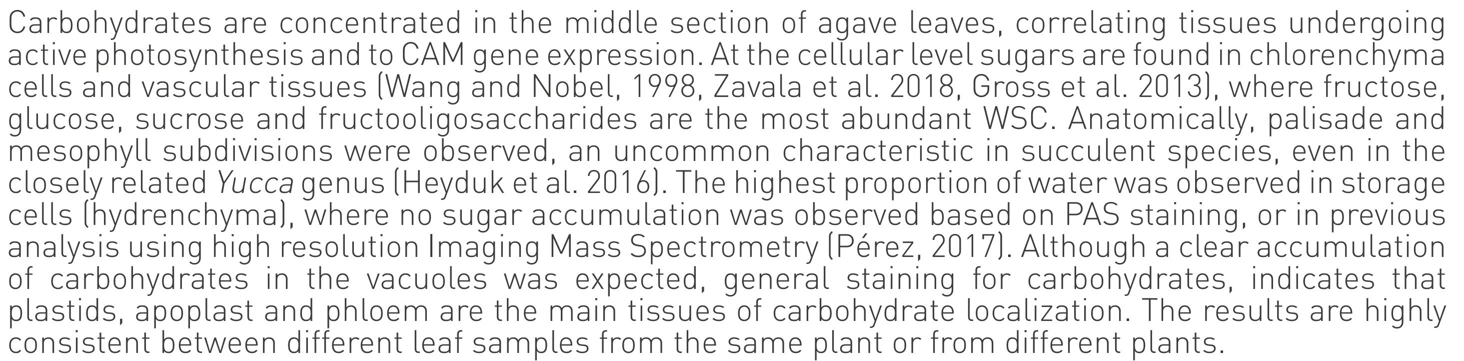
Differential patterns of carbohydrates in relation to water distribution were identified in leaf sections. Succulence parameters and 13 related traits (Table 1), were estimated and complemented with a net determination of water concentration *in situ* by employing THz-TDI spectroscopy. As was expected, Delf´s coefficient and leaf thickness were correlated to succulence, however anatomical traits such as stomatal size and cuticle thickness were also strongly correlated. Most of the traits that we measured that were previously reported as succulence indicators in other species (Males, 2017), did not show a direct correlation in this analysis, possibly due to the low number of species evaluated. With values of succulence ranging between 0.13 to 0.35 g cm^{-2}, agaves have the most succulent leaves within the *Asparagaceae* family (Heyduk et al. 2016). Linearity during the drying process was also strongly correlated with succulence (r^2=0.82) and was highest in the most succulent species. This may be an important trait in relation to the tolerance to arid conditions shown by most *Agave* species.

Succulence measurements in uniformly thin leaves, determine the amount of water in relation to leaf surface and normally correlate with leaf thickness (Mantovani, 1999). Nevertheless, in some species a gradient of succulence throughout the leaf from the base to the tip is observed, as in the case of *Agave* plants, where an average value is determined. Because of this, a 3D succulence estimation is needed. Here, based on absorbance determined by THz spectroscopy, the water content was indirectly estimated by the transmitted THz radiation. This method has been previously applied in other plant species, being this the first report of the application of the method to determine levels of succulence (Castro et al. 2013, Gente et al. 2018).

Table 1. Anatomical traits related to succulence analyzed for three Agave species.

Anatomical traits		Agave species			Correlation [r^2] with succulence
		A. tequilana	*A. victoriae-reginae*	*A. striata*	
Succulence [g cm^{-2}]		0.29 ± 0.03	0.35 ± 0.08	0.13 ± 0.06	1.00
Maximum leaf thickness		1.01 ± 0.19	1.05 ± 0.07	0.27 ± 0.04	0.98
Delf's coefficient		0.33 ± 0.04	0.46 ± 0.07	0.15 ± 0.06	0.99
Lineal drying		0.64 ± 0.17	0.80 ± 0.17	0.60 ± 0.28	0.82
Leaf density [g cm^{-2}]		0.99 ± 2.12	0.68 ± 13.06	0.41 ± 12.32	0.67
IAS [%]		4.79 ± 0.02	4.39 ± 0.03	4.04 ± 0.14	0.67
Mass [g]		24.04 ± 4.32	4.45 ± 1.05	1.90 ± 0.82	0.35
A/V ratio		6.71 ± 1.07	4.21 ± 0.61	6.01 ± 1.23	0.33
Leaf volumen [cm^3]		24.25 ± 4.16	6.51 ± 0.83	4.62 ± 0.88	-0.05
Humidity [%]		86.55 ± 1.24	79.47 ± 20.05	81.62 ± 4.16	-0.50
Stomatal density [mm^{-2}]	adaxial	152.63 ± 6.36	55.50 ± 21.22	63.83 ± 16.29	0.17
	abaxial	187.32 ± 2.12	90.19 ± 13.06	62.44 ± 12.32	0.45
Stomatal size [µm]	adaxial	36.56 ± 2.89	51.25 ± 11.01	37.46 ± 13.64	0.68
	abaxial	76.88 ± 7.11	92.73 ± 12.21	35.30 ± 20.87	0.99
Cuticle cell size [µm]	adaxial	71.15 ± 2.55	35.73 ± 3.51	22.29 ± 3.46	0.50
	abaxial	66.11 ± 3.50	42.35 ± 3.03	32.08 ± 1.98	0.52
Cuticle thickness [µm]	adaxial	42.20 ± 10.70	45.53 ± 20.81	47.04 ± 21.34	-0.53
	abaxial	35.23 ± 9.70	98.05 ± 22.96	49.33 ± 13.95	0.55
Leaf area [cm^2]	blade	75.79 ± 14.49	10.18 ± 1.54	13.58 ± 2.61	0.20
	back	84.71 ± 12.08	17.02 ± 2.58	13.78 ± 3.54	0.29
	total	160.49 ± 25.35	27.20 ± 3.94	27.36 ± 5.55	0.25

Note: All of the measurements were analyzed in triplicate of 3 independent plants per species, where ± values corresponde to SE



Carbohydrates are concentrated in the middle section of agave leaves, correlating tissues undergoing active photosynthesis and to CAM gene expression. At the cellular level sugars are found in chlorenchyma cells and vascular tissues (Wang and Nobel, 1998, Zavala et al. 2018, Gross et al. 2013), where fructose, glucose, sucrose and fructooligosaccharides are the most abundant WSC. Anatomically, palisade and mesophyll subdivisions were observed, an uncommon characteristic in succulent species, even in the closely related *Yucca* genus (Heyduk et al. 2016). The highest proportion of water was observed in storage cells (hydrenchyma), where no sugar accumulation was observed based on PAS staining, or in previous analysis using high resolution Imaging Mass Spectrometry (Pérez, 2017). Although a clear accumulation of carbohydrates in the vacuoles was expected, general staining for carbohydrates, indicates that plastids, apoplast and phloem are the main tissues of carbohydrate localization. The results are highly consistent between different leaf samples from the same plant or from different plants.

In terms of succulence and water storage *A. victoriae-reginae* > *A. tequilana* > *A. striata*. Water is mainly stored in the mesophyll tissue of the leaf base, also the thickest section of the leaf. The highest levels of carbohydrates were found in *A. tequilana* and variation in localization of sugars and stored water varied between the species depending on leaf shape and morphology (Gentry, 2004). *A. victoriae-reginae* the species with the highest level of succulence is indigenous to the Chihuahuan dessert, however, a much wider study based on the natural distribution of species and levels of succulence is necessary in order to determine a correlation between these factors.

CONCLUSION

Independent distribution of carbohydrates and water throughout agave leaves was observed. Succulence was determined fundamentally at the cellular level and is not directly correlated to morphology. Succulence in agaves, not only depends on the amount of stored water but also tissue thickness and retention capacity, where macro and microscale traits must be considered. Interestingly and perhaps surprisingly, agaves show specific patterns of water accumulation that are independent to carbohydrate localization. Examination of species with highly contrasting anatomy such as those reported here, allow us to develop a better understanding of physiological adaptations that could be exploited in non-commercial agave species and incorporated into important but drought/temperature sensitive crop species such as cereals or grasses.

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Integrated management and control of the agave weevil at the national agave collection of the University of Guanajuato-SAGARPA.

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ABSTRACT

Agaves are plants of great importance for Mexico, since they are part of our culture and tradition; therefore, these plants represent our country. Mexico is the origin center of the subfamily Agavoideae, which comprises eight genera, and *Agave* genus stands out for its economic importance. In this subfamily, there are about 200 species, such as *A. tequilana* Weber, *A. salmiana* Otto Ex Salm, and *A. durangensis* , among others, from which, we obtain tequila, mezcal, and pulque. These are ancestral and important drinks for Mexico, nonetheless, they are internationally known as well.

The conservation of genetic reserves (DNA and RNA) is necessary for the agave's subsistence, since it contains the information for: plant development, morphology, resistance and/or tolerance to diseases or pests, the life cycle and future adaptations to climate change. In addition, it is necessary to have a genetic variation so that they possess certain evolutionary capacity. For the conservation of agaves, one of the essential points is to generate information on its genome, as well as knowing its diseases and pests (Espinoza, 2015). The objective of this investigation was the integrated management and control of the agave weevil (*Scyphophorus acupunctatus* Gyll) at the National Agave Collection of UG-SAGARPA, which received the advice of the State Committee of Plant Health of Guanajuato A.C. (CESAVEG).

Key words: Aggregation pheromone, trap, management strategies, *Scyphophorus acupunctatus* Gyll, CESAVEG.

INTRODUCTION

Agaves are plants of a great importance for Mexico, since they are part of our culture and tradition, therefore, these plants represent our country. Within the *Agave* genus, there are very important species, such as *A. tequilana* Weber, from which tequila is made. This is one of the most important alcoholic beverages in Mexico, and worldwide, since is highly appreciated. According to Espinoza (2015), in Mexico there are approximately 165 agave species.

The conservation of genetic reserves (DNA and RNA) is necessary for the agave's subsistence, since it contains the information for: plant development, morphology, resistance and/or tolerance to diseases or pests, the plant life cycle and future adaptations to climate change. In addition, it is necessary to have a

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genetic variation so that agaves possess certain evolutionary capacity. For the conservation agaves, one of the essential points is to generate information on its genome, as well as knowing its diseases and pests (Espinoza, 2015).

The agave cultivation in the State of Guanajuato, Mexico, is affected by different diseases and pests that cause considerable plant damage. The main diseases are marginal spot (*Phoma* sp. and *Alternaria* sp.) and dry spots (*Fusarium* sp. and *Alternaria* sp.) These diseases damage the leaves and cause a significant delay in the crop growth and development. Likewise, in the State of Guanajuato there are also other diseases with lower incidence, but higher severity: p. ex. the leaf blight caused by the fungus *Cercospora agavicola* and the bud rot caused by the bacterium *Erwinia* spp. Among the main pests, the armed scale (*Acutaspis agave*), the mealybug (*Paracoccus* sp.) and the agave weevil (*Scyphophorus acupunctatus* Gyll) at low infestation levels, are found (CESAVEG).

Regarding the agave weevil, *Scyphophorus acupunctatus* Gyll, is a very important pest in Mexico, since it affects many wild and cultivated agave species (Barrios et al. 2006, Aquino et al. 2007, González et al. 2007).

Concerning the agave germplasm of the University of Guanajuato–SAGARPA collection, the agave weevil has recently been found as a pest. Thus, in order to control it and reduce its effects, the integrated management is relevant to maintain the weevil threshold at levels that do not cause any problem. For this reason, the State Committee for Plant Health of Guanajuato A.C. (CESAVEG), in coordination with the Ministry of Agriculture, Livestock, Rural Development, Fisheries and Food (SAGARPA) and the Secretariat of Agricultural Development (SDA-Gto), implement the Phytosanitary Management Campaign for Agave in the Guanajuato State, in order to support the competitiveness of the agave sector in the State, and specifically at the University of Guanajuato.

METHODOLOGY

Location

The National Agave Collection of the University of Guanajuato-SAGARPA is located at north latitude 20°44'22" and west longitude 101°19'45", at 1750 masl. The average annual temperature is 19.4 °C, and an average of 58% relative humidity and 680 mm of pluvial precipitation. The climate is considered semi-warm and sub-humid with rains in the summer [BS (hw) (h) (e)].

Plant Material

The National Agave Collection of the University of Guanajuato-SAGARPA has more than 30 species, with 511 accessions, among them we find the following: *A. angustifolia*, *A. atrovirens* white, *Agave mapizaga*, *A. salmiana*, *A. salmiana* green, *A. tequilana*, *A. vilmoriniana*, among others (Figure 1). In addition, this collection has agave plants of different ages and sizes, since their dates of planting were variable, and its establishment was in 2002.

Figure 1. Some examples of *Agave* species at the National Agave Collection UG-SAGARPA. A) *A. angustifolia,* B) *A. atrovirens* white, C) *A. mapizaga* D) *A. tequilana*.

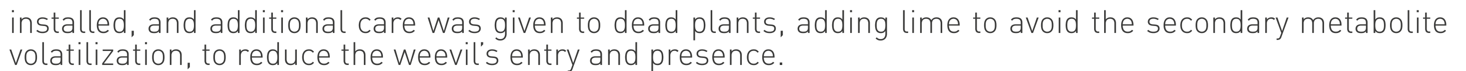
Integrated management of agave weevil

To carry out the agave weevil integrated management, poisoned baits, which consisted of leaf pieces (Figure 2A), were placed in perforated polyethylene bags and sprayed with Malation® (10 mL L^{-1}) (Figure 2B, C). In the cover lid of the trap, the aggregation pheromone (Agavenol®) was placed (Figure 2D, E). The traps were fixed within the agave rows, at ground level (Figure 2F), and the placement distance was every 100 linear meters, or one trap per hectare. In addition, the traps were changed every 2 months (Figure 2G, H), with fresh leaf pieces and chemical controls. Within the National Agave Collection UG-SAGARPA, a total of 10 traps with poisoned bait were placed. Every other week, the number of weevils per each trap were scored and the total average (10 traps) was obtained. After the evaluation of the weevil for each trap, if the insect number was above five specimens per trap (Figure 2I, J), a chemical insecticide was applied. CESAVEG´s suggestion was to apply 1 L of Lorsban® for every 1500 plants (Figure 2K). In addition, to avoid the inoculum source of agave weevil and its propagation, lime applications were made on agave dead plants (Figure 2 L).

Figure 2. Integrated management of agave weevil. A) Agave leaf pieces, B) Plastic bag with leaf pieces, C) Malation® spray, D) Aggregation pheromone (Agavenol®), E) Trap´s cover lid with aggregation pheromone (Agavenol®), F) Trap fixed within the agave rows, G) and H) Renewal of bait in the trap, I) Agave weevil counting inside the trap, J) Agave weevil in the leaf base, K) Chemical insecticide application, L) Covering agave residues and dead plants with lime.

RESULTS AND DISCUSSION

From February to December 2017, a log for different samplings was developed. It was observed that agave weevil´s population had different increases depending on the sampling date and the area within the National Agave Collection UG-SAGARPA (Table 1). The traps placed in the collection´s southern area had a greater number of adult weevils, in comparison to the other zones. Therefore, more traps were



installed, and additional care was given to dead plants, adding lime to avoid the secondary metabolite volatilization, to reduce the weevil's entry and presence.

CONCLUSION

According to the obtained data, in the National Agave Collection UG-SAGARPA, it was possible to know the different states of field infestation, as well as to apply the measures to reduce the incidence of the agave weevil pest. In the same way, with the integrated control of the agave weevil it will be possible to avoid the loss of plants, therefore, keeping the national agave germplasm.

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To the Life Sciences Division (DICIVA Spanish acronym), of the Irapuato-Salamanca Campus, University of Guanajuato, Irapuato, Guanajuato, for the support granted for this research. Likewise, to the CESAVEG´s personnel and authorities for all the support provided to carry out the present investigation.

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Installation of traps: Feb -16 - 2017 County: Irapuato Community: El Copal Farm: National Agave Collection UG-SAGARPA	Area: 11 ha Producer: Universidad de Guanajuato Planting year: 2002 Lead: M.Sc. Blanca Estela Orosco Alcalá

Trap	***Date of referral and trapping Number of agave weevil per trap***																	
	1x	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**
1	3	4	10	3	1	2	6	3	8	12	19	8	20	12	10	4	5	12
2	6	5	9	4	2	6	9	4	15	14	19	25	18	20	12	7	7	25
3	2	1	3	1	0	1	0	6	3	6	26	10	11	15	0	14	8	7
4	2	3	1	0	0	0	7	6	14	13	29	5	13	10	8	15	15	6
5	7	10	13	2	1	4	9	25	5	9	19	5	6	7	5	12	12	4
6	4	8	10	4	1	0	2	4	15	14	15	13	12	14	4	5	14	10
7	8	0	1	4	1	5	0	4	14	10	12	9	7	9	8	7	12	11
8	4	5	5	4	2	3	0	15	14	38	40	8	19	21	15	7	26	24
9	0	4	6	2	0	2	3	8	8	10	26	12	16	14	4	3	14	24
10	2	3	6	0	1	4	2	11	4	48	19	0	2	5	2	0	3	3
Total	38	43	64	24	9	27	38	86	100	174	224	95	124	127	68	74	116	126
Average	4.2	4.3	6.4	2.4	0.9	2.7	3.8	8.6	10	17.4	22.4	9.5	12.4	12.7	6.8	7.4	11.6	12.6

xSampling dates; 1=3/03/17; 2=03/17/ 17, 3=03/31/17, 4=04/12/17, 5= 04/28/17, 6= 05/12/17, 7= 05/26/17, 8= 06/09/17, 9= 07/11/17, 10=08/11/17, 11= 08/25/17, 12= 09/15/2017, 13= 09/29/17, 14= 10/13/17, 15= 10/27/17, 16= 11/10/17, 17= 11/24/17, 18=1

Micropropagation systems in *Agave* spp: common errors.

Delgado-Aceves L., Palacios H., Romo-Paz F. and Portillo L.[1]*

ABSTRACT

Micropropagation systems have been developed in *Agave* species, however regeneration systems are frequently confused. In this study, cellular structures from organogenesis and somatic embryogenesis are discussed with several protocols in literature, as reference for future works on *Agave*.

Axillary shoots, thin cell layer, and leaves as explants of *Agave tequilana* were stimulated and/or induced for micropropagation systems. Applied growth regulator doses were: for axillary shoot proliferation 5 mg/L benzylaminopurine (BA), for organogenesis 5 mg/L BA and 0.2 mg/L 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), and for indirect somatic embryogenesis 8 mg/L picloram (PIC) and 0.75 mg/L BA.

Samples for histological observations were embedded in polyethylene glycol (PEG) 1450, sectioned (10 µm) by the use of a rotation microtome, and stained with safranin and astral blue.

The results for axillary shoot proliferation, showed meristemoids (organogenic points) with dividing cells, close and similar to original meristems; then organogenesis occurs during it, obtaining axillary and organogenic shoots during the same process, leading to the confusing origin of both shoots.

The organogenic system showed unipolar structures with connection to the parent tissue, originated from meristemoids (multicellular origin). The somatic embryo system had autonomy from parent cells, and embryoids showed a bipolar structure from the very first asymmetrical division (unicellular origin), allowing germination at the same time of both, root and caulinar apexes. Therefore, morphological comparisons between somatic and sexual embryos should be used as another test to confirm the presence of somatic embryogenesis and not to confound with organogenesis from meristemoids.

Key words: Regeneration, biotechnology, maguey, organogenesis, somatic embryogenesis.

INTRODUCTION

Micropropagation strategies in *Agave* species have been widely studied. In 1977 Roenewald et al. reported plant regeneration in the *Agave* genus testing the effects of plant growth regulators on callus induction, since then, other protocols have been developed, including several growth regulators and additives in the culture medium (Powers and Backhaus, 1989; Santacruz-Ruvalcaba et al. 1999; Hazra et al. 2002; Nikam et al. 2003; Portillo et al. 2007; Chen et al. 2014). Seeds, stolon cuttings, bulbils from the inflorescence, leaves, and roots are biological materials commonly used as explant sources for micropropagation systems (axillary shoot proliferation, organogenesis, and somatic embryogenesis). The systems have been selected depending on the objective and the provision of biological material. Optimal protocols have

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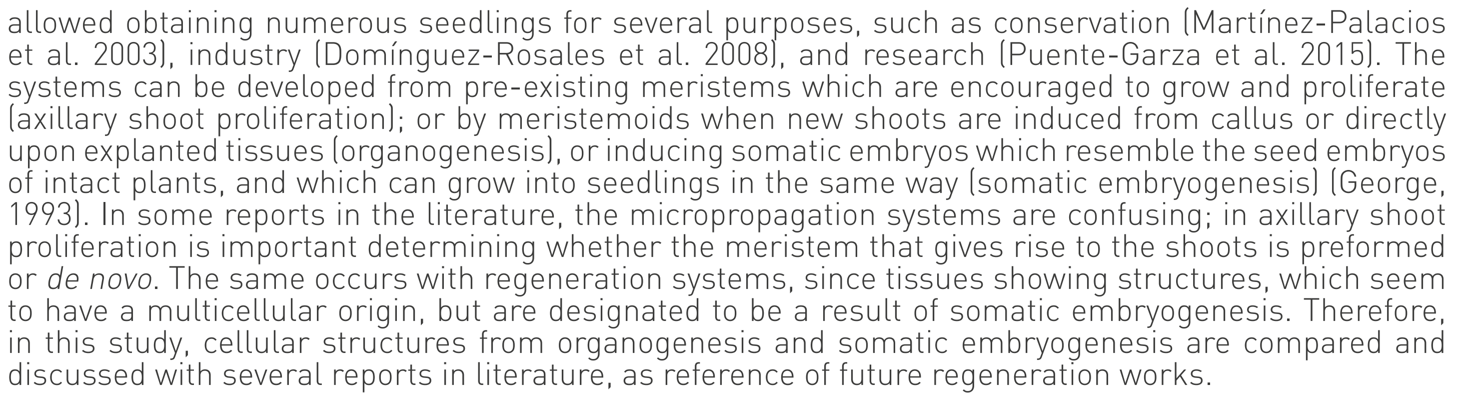

allowed obtaining numerous seedlings for several purposes, such as conservation (Martínez-Palacios et al. 2003), industry (Domínguez-Rosales et al. 2008), and research (Puente-Garza et al. 2015). The systems can be developed from pre-existing meristems which are encouraged to grow and proliferate (axillary shoot proliferation); or by meristemoids when new shoots are induced from callus or directly upon explanted tissues (organogenesis), or inducing somatic embryos which resemble the seed embryos of intact plants, and which can grow into seedlings in the same way (somatic embryogenesis) (George, 1993). In some reports in the literature, the micropropagation systems are confusing; in axillary shoot proliferation is important determining whether the meristem that gives rise to the shoots is preformed or *de novo*. The same occurs with regeneration systems, since tissues showing structures, which seem to have a multicellular origin, but are designated to be a result of somatic embryogenesis. Therefore, in this study, cellular structures from organogenesis and somatic embryogenesis are compared and discussed with several reports in literature, as reference of future regeneration works.

METHODOLOGY

The experiments were carried out in the Laboratory of Biotechnology of the University of Guadalajara. The genotype and embryogenic cell line (ES2) of *Agave tequilana* Weber cultivar azul was previously established in *in vitro* culture. Explants with meristems, thin cell layer cuts, and leaves were stimulated and/or induced for micropropagation systems. Shoot proliferation with 5 mg/L benzylaminopurine (BA), organogenesis with 5 mg/L BA and 0.2 mg/L 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), and indirect somatic embryogenesis with 8 mg/L picloram (PIC) and 0.75 mg/L (BA). All samples were boiled for 15 min and embedded in polyethylene glycol (PEG) 1450 in a 1:4 proportion (PEG:deionized water). Samples in PEG were sectioned by the use of a rotatory microtome (cuts of 10 µm) following a staining treatment with safranin and astral blue, both 1%, 1:2 proportion. Tissues analyzes were performed by light microscopy. Results were compared to those from other reports in literature.

RESULTS AND DISCUSSION

In the axillary shoot proliferation system (Figure 1A), meristemoids (organogenic points) could be observed with dividing cells, close, and similar to original meristems (Figure 1D) (Hernández et al. 2014). The organogenic system (Figure 1B) showed unipolar structures with connection to the parent tissue (1E), originated from meristemoids. On the other hand, the somatic embryo system (Figure 1C) had asymmetric divisions (Figure 1F), autonomy from parent cells, and embryoids showed a bipolar structure, allowing the germination of both, root and caulinar apexes at the same time; then their origin must be unicellular, not from meristemoids. Polarized embryogenic structures were observed with the first cell division with apical and basal cells, which was a pattern reported by Portillo et al. (2007). In some reports on the regeneration of *Agave*, frequently, the organogenic points are confounded with embryogenic clumps, and thus, the adventitious shoots are erroneously designated as embryoids (somatic embryos) (Nikam et al. 2003; Tejavathi et al. 2007; Monja-Mio et al. 2013). Organogenesis is a relatively easy process to achieve in *Agave* species, favored by the presence of meristemoids. However, for somatic embryogenesis, it should be necessary to confirm the unicellular origin, not only with asymmetrical division, since this process occurs in both regeneration processes (organogenesis and somatic embryogenesis). Then morphological comparisons between zygotic and somatic embryos should be used as another test to confirm the presence of somatic embryogenesis and preventing not to confuse with organogenesis from meristemoids. In the work of Portillo et al. (2007) there was a comparison between the morphology of zygotic and somatic embryos, confirming the presence of somatic embryogenesis with congruent morphology to the zygotic ones, due to auxin effect; however, those somatic embryos obtained with cytokinins emphasis, showed a different morphology, that now it is clear that those structures were just de novo shoots.

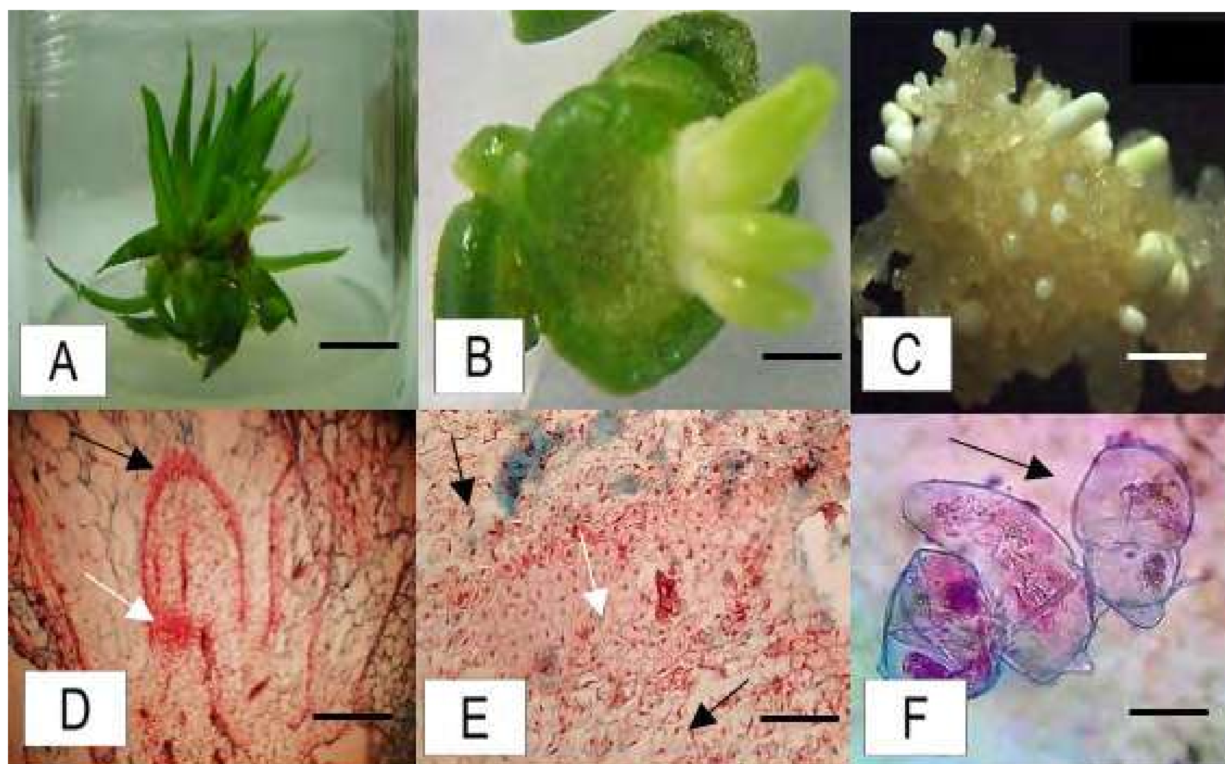

Figure 1. Micropropagation systems in *Agave tequilana* Weber cultivar azul. A) Shoot proliferation. *Bar* = 2 cm. B) Direct organogenesis by thin cell layer. *Bar* = 5 mm. C) Indirect somatic embryogenesis. *Bar* = 2 mm. D) Meristem (black arrow) and meristemoid (white arrow) with cellular division points. *Bar* = 500 µm. E) Organogenic process (white arrow) connected to the parent cells (black arrows). *Bar* = 300 µm. F) Asymmetric cell division (black arrow). *Bar* = 100 µm.

CONCLUSION

Histological sections showed different cellular structures in each micropropagation system. In shoot proliferation there are meristemoids generating adventitious shoots at the same time. All organogenic shoots always showed a connection to the parental cells and repeatedly are confound to somatic embryos. Morphological comparisons between somatic and sexual embryos should be used as another test to confirm the presence of somatic embryogenesis.

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Optimization of *Agrobacterium*-mediated transformation in *Agave tequilana* Weber Var. Azul.

Bautista-Montes, E. and Simpson, J.[1]*

ABSTRACT

Blue agave (*Agave tequilana*) is mainly propagated through offsets due to its long-life cycle and low seed fertility. However, this generates some disadvantages such as the reduction of variability and increased vulnerability to certain diseases. Therefore, interest have been focused on the generation of a transformation protocol in agave that could help to modify characteristics of agronomic interest as well as to expand basic research. Currently, there is no report of an efficient protocol for transformation of *A. tequilana* by direct organogenesis, however in our group, we have been implementing a protocol by using *Agrobacterium tumefaciens* which shows good transformation potential and is relatively efficient (unpublished data).

Bulbils collected in Irapuato, Gto. were used in micropropagation assays to test the effect of different concentrations (100 mgL^{-1} - 500 mgL^{-1}) of timentin and cefotaxime during *in vitro* regeneration and an agar diffusion test was performed to assay the sensitivity of *A. tumefaciens* strain GV2260 to the different antibiotic concentrations mentioned above. Once the best antibiotic and concentration was selected, the transformation assays were set up by using a variety of strains of GV2260 carrying the *AtqKNOX1* and *AtqKNOX2* genes individually. Additionally, a histological analysis and immunolocalizations are being carried out in order to understand the organogenesis process at the anatomical level and to follow the expression of the *A. tequilana* KNOX genes in *in vitro* tissue during the regeneration process.

Tissue expressing the *AtqKNOX1* gene generates a greater number of regenerants, however most regenerated plantlets were not capable of developing correctly and eventually died. These preliminary results provide information about the differential responses obtained in the transformation protocol by expressing *AtqKNOX1* and *AtqKNOX2* genes during the development of shoots by organogenesis.

Key words: Agave, bulbils, co-cultivation, micropropagation, organogenesis.

INTRODUCTION

Blue agave (*Agave tequilana*) is principally known for being the source of raw material for tequila production, the most important alcoholic beverage produced in Mexico. Producers mainly propagate this species through offsets due to its long-life cycle and low seed fertility. However, this generates some disadvantages such as the reduction of variability and increased vulnerability to certain diseases that may negatively affect commercial plantations. According to the Tequila Regulatory Council (CRT, https://www.crt.org.mx/EstadisticasCRTweb/) the tequila production has been fluctuating over the last twenty years as a consequence of the scarcity of plants during some seasons (Robert et al. 2007). Therefore, by

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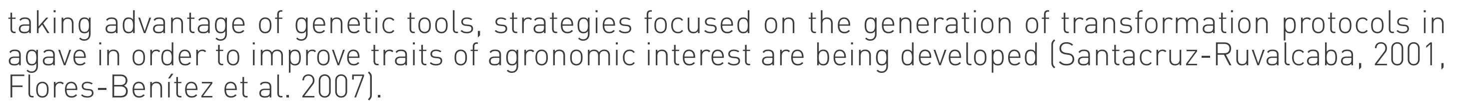

taking advantage of genetic tools, strategies focused on the generation of transformation protocols in agave in order to improve traits of agronomic interest are being developed (Santacruz-Ruvalcaba, 2001, Flores-Benítez et al. 2007).

There are two published reports of genetic transformation in *Agave* species based on embryogenic calli mediated by particle bombardment or *Agrobacterium tumefaciens* (Santacruz-Ruvalcaba, 2001 and Flores-Benítez et al. 2007). Although successful, these protocols are either cumbersome or time-consuming and transformation efficiency is not sufficient to contemplate routine, functional gene analysis. On the other hand, the development of a transformation protocol based on organogenesis has not been explored extensively and may represent an efficient alternative strategy, involving fewer tissue culture steps and investment of less time.

Previous reports (Srinivasan et al. 2007, Deng et al. 2009, Heidmann et al. 2011) have shown that expression of specific genes such as *BABY BOOM* during *in vitro* culture can lead to increased efficiency in regeneration protocols. Our group has shown that the meristem specific homeobox transcription factor genes *AtqKNOX1* and *AtqKNOX2* are expressed at the initial stages of new meristem development during bulbil formation in *A. tequilana* and that over expression of the *A. tequilana* KNOX genes in *A. thaliana* can lead to the lobed phenotype indicating which when severe can lead the formation of ectopic meristems (Abraham-Juárez et al. 2010). Based on these observations our group has been developing a protocol for transformation of *Agave* species by direct organogenesis using *Agrobacterium tumefaciens*. The aim of this work was to optimize the in vitro tissue culture conditions for this process and to explore the effects of expression of the transcription factors (TF) *AtqKNOX1* and *AtqKNOX2* on the induction of new meristems during the process of organogenesis with the aim of optimizing the transformation protocol.

METHODOLOGY

Biological material and antibiotic evaluation

Meristems from bulbils collected in Irapuato, Gto. were used in micropropagation assays to test the effect of cefotaxime and timentin during *in vitro* regeneration, using concentrations from 100 mgL^{-1} to 500 mgL^{-1}. *In vitro* conditions were based on Nava-Cedillo (1988) and Bernabé-Pérez (2017). In addition, an agar diffusion test was performed to assay the sensitivity of *A. tumefaciens* strain GV2260 carrying the expression vector pB7WG2D by exposing the bacteria to the selected antibiotic concentrations. Once the best option of antibiotic and concentration were selected, the transformation assays were set up by using a variety of individual expression vectors carrying cDNAs encoding the transcription factors *AtqKNOX1* and *AtqKNOX2*.

Transformation protocol

A. tumefaciens strains carrying the relevant vectors were grown on Minimum liquid media at 28 ºC until reaching 0.5 O.D., acetosyringone was added to increase the virulence of the strains. Co-cultivation media consisted of Erickson-Linsmaier and Skoog liquid media, supplemented with acetosyringone, and explants were cocultivated for 15 hours under low light conditions. Tissue was transferred to media supplemented with growth regulators for one week and then subcultivated to media supplemented with phosphinothricin at 0.75 mgL^{-1}. Two months later, surviving shoots were transplanted to Erickson-Linsmaier and Skoog solid media without growth regulators and an increased concentration of selective agent (0.85 mgL^{-1}). Presence of transgenes was confirmed by Polymerase Chain Reaction (PCR) and observation of the Green Fluorescent Protein (GFP) in roots.

Histological analysis and immunolocalizations

During *in vitro* regeneration, samples were taken at different time points, fixed for 7 days with Formalin, Acetic acid, Alcohol (FAA), dehydrated by using a series of ethanol solutions at different concentrations (50%, 60%, 70%, 80%, 90%, 95% and 100%) and prepared for the embedding process (Abraham-Juarez et al. 2010). Once the tissues were embedded in paraffin, sections of 8 μm thickness were prepared, and

the embedding process was reversed in order to carry out the staining procedure using safranin and methylene blue to localize nucleic acids and cytoplasm respectively.

The immunolocalization procedure was based on a protocol developed at the Hake laboratory at UC Berkeley using anti-KNOX antibodies from maize (Abraham-Juárez et al. 2015)

RESULTS AND DISCUSSION

During long-term culture, timentin did not produce necrosis in the tissue of explants in contrast to cefotaxime (Figure 1). However, no significant difference was observed between both antibiotics during the formation of new buds. According to these results, timentin at 500 mgL^{-1} was determined as the best option to be used during the transformation protocol.

The PCR analysis confirmed the regeneration of shoots carrying each of the *AtqKNOX* genes. Based on statistical analysis, tissue cocultivated with the *A. tumefaciens* strain carrying the *AtqKNOX1* expression vector showed a better response in relation to the number of regenerated shoots during the first two months of tissue culture when the major stimulus was received (Figures 2 and 3). However, a greater number of shoots survived following cocultivation with the *A. tumefaciens* carrying the *AtqKNOX2* expression vector.

This differential response reflects the results described in the heterologous system of *Arabidopsis thaliana*, where *AtqKNOX2* completely complemented the *A. thaliana KNAT1* mutant, whereas *AtqKNOX1* only partially rescued the *KNAT1* phenotype (Abraham-Juárez et al. 2010) indicating functional differences between the different *A. tequilana* KNOX genes. During bulbil formation in *A. tequilana*, *AtqKNOX2* is expressed in the initial stages of meristem development whereas *AtqKNOX1* is expressed in later stages when meristems are already visible. Additionally, *AtqKNOX2* is more widely expressed in other tissues whereas *AtqKNOX1* is almost exclusive to SAM tissue. This may be relevant for the results observed during the tissue culture process where although expression of *AtqKNOX1* can lead to the induction of an increased number of meristems/shoots, continuous, strong ectopic expression of *AtqKNOX1* may eventually lead to erroneous patterns of KNOX regulation and unviability of regenerating shoots. In contrast, *AtqKNOX2* expression may not lead to this deleterious effect. This hypothesis will be tested when regenerated plantlets expressing the *AtqKNOX* genes reach a size where they can be monitored extensively and compared in terms of phenotype and gene expression patterns.

Observation of roots for the presence of GFP, histological analysis and immunolocalizations are currently in progress.

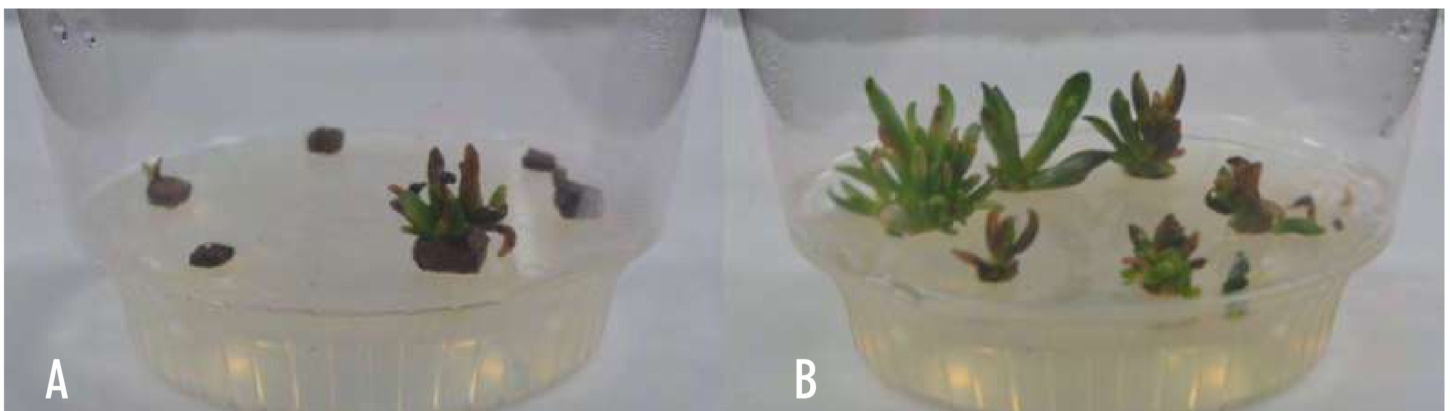

Figure 1. Regenerating shoots on Erickson-Linsmaier and Skoog solid media after 2 months of cultivation. Media supplemented with cefotaxime (a), media supplemented with timentin. (b).

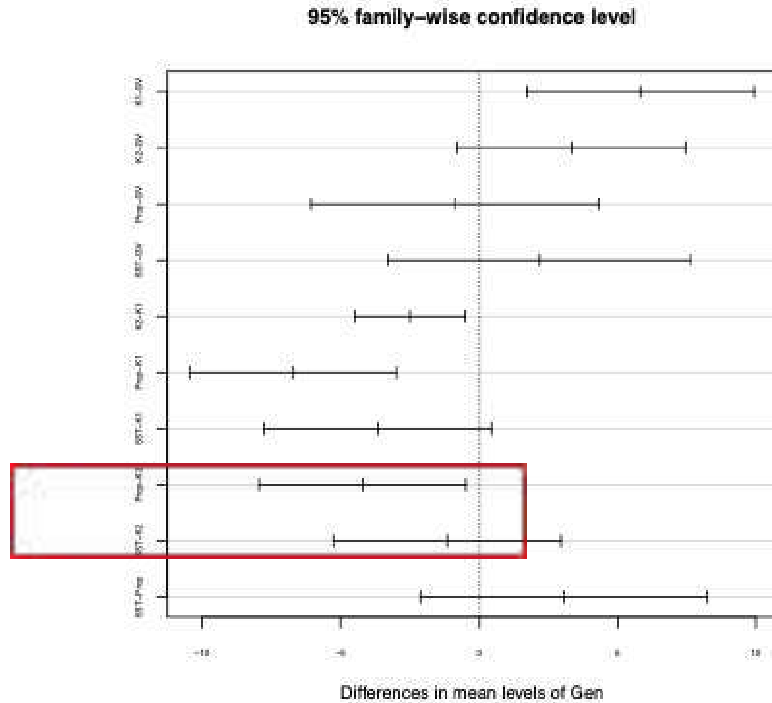

Figure 2. Box plot showing significant differences between the number of regenerants produced from cocultivation with different AtqKNOX genes (red square).

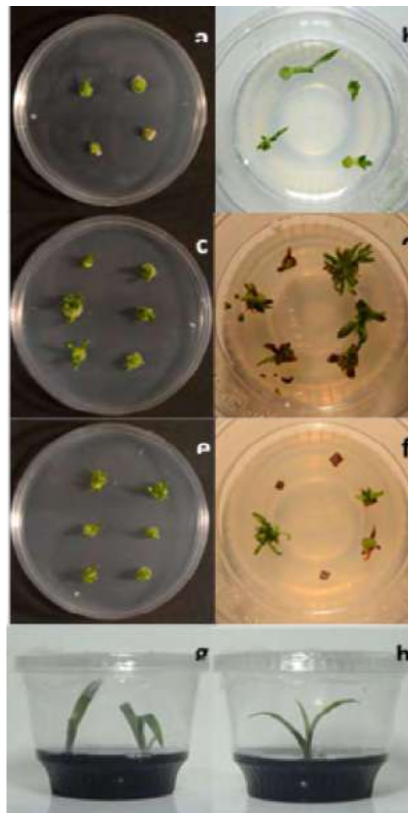

Figure 3. Transformation assays for *AtqKNOX* genes, negative control (a, b), samples cocultivated with *A. tumefaciens* strain GV2260 carrying *AtqKNOX1* gene (c, d) or *AtqKNOX2* (e, f). a, c, e after four weeks from cocultivation and b, d, f after eight weeks from cocultivation. g and h, eight months old plants from co-cultivation with *AtqKNOX1* or *AtqKNOX2*, respectively.

CONCLUSION

The use of timentin during the transformation protocol diminished contamination problems, although, an important part to prevent this, it is to have better practices during the handling of tissue and co-cultivation. Preliminary results gave us some information about the differential responses obtained in the transformation protocol between the *AtqKNOX1* and *AtqKNOX2* constructs.

ACKNOWLEDGEMENTS

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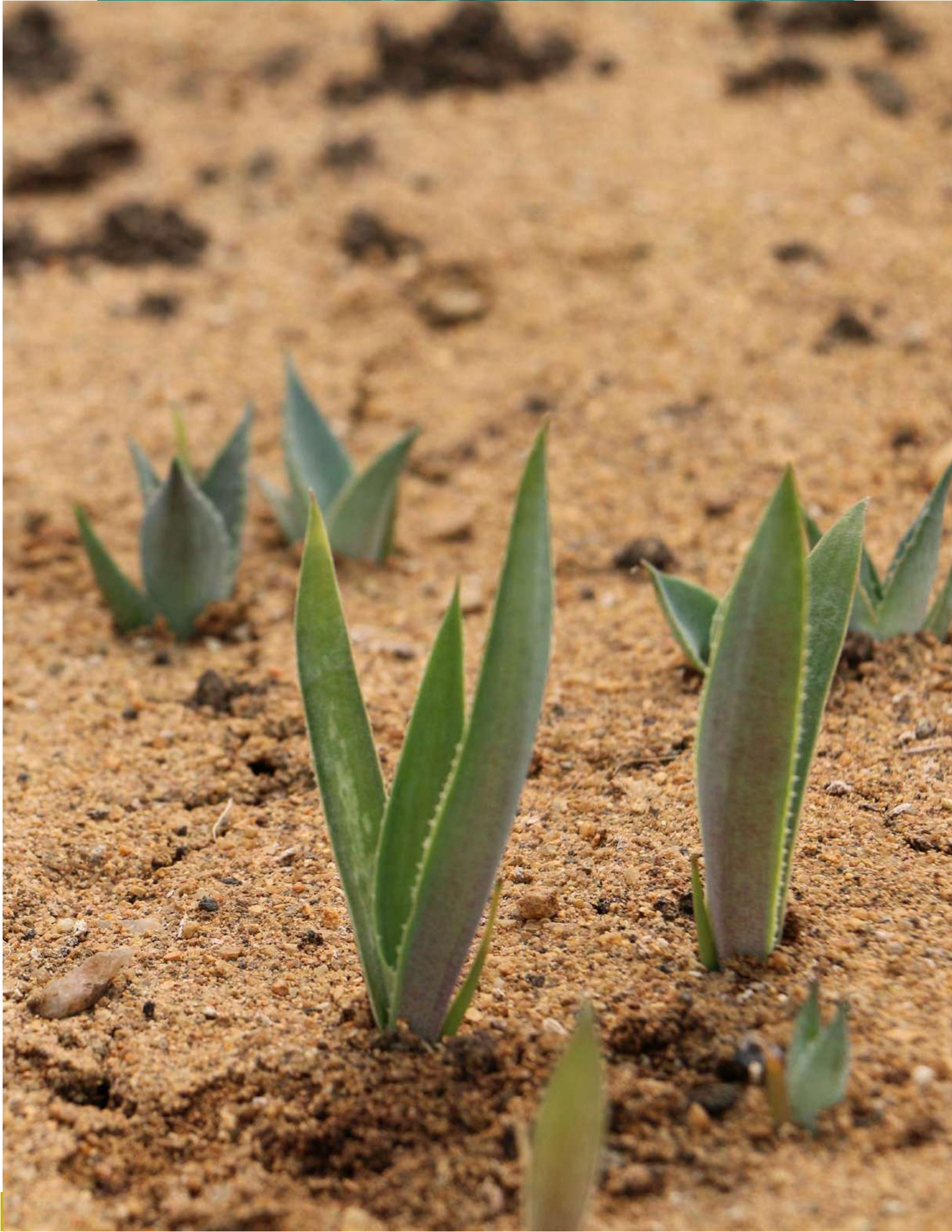
Science and technology of Agave
beverages and other derivatives



Analysis of the quality parameters of mezcal produced in the center region of the state of Guerrero.

Sierra-Martínez, P.*, Flores-Robles, D., Bello-Martínez, J. and Millán-Vega, A.[1]

ABSTRACT

In the center region of the state of Guerrero, the consumption of mezcal is a centuries-old tradition that has, little-by-little, been gaining ground, in part due to the fact that its production has become a significant socio-economic activity, one conducted on an artisanal basis by the vast majority of producers. The present study analyzed 59 mezcal samples obtained from six municipalities in the center region of the state of Guerrero, determining the alcohol, dry extract, higher alcohols, methanol and total acidity content, which, aside from this last characteristic, are considered parameters that indicate the physicochemical quality of a mezcal, according to Official Mexican Standard NOM-070-SCFI-2016: Bebidas Alcohólicas-Mezcal-Especificationes. The results obtained are evidence to be used by producers in their decision-making in order to improve the quality of their product, above all in the parameters of alcohol and methanol content, which, while not a major problem, were found to be above the value established in the NOM, corresponding to 25% and 42% of the total number of samples, respectively. Only the parameters for alcohol content and higher alcohols were found at the extreme lower end of the levels set by the NOM; however, none of the non-compliant samples surpassed the established standards by more than 20%. With regard to the dry extract, all samples analyzed presented values within the margin established in the NOM. In relation to total acidity, only 36% of the samples analyzed did not comply with the values set by NOM-070-SCFI-1994 for this parameter.

In general, the quality of the mezcal in the center region of the state of Guerrero was found to be acceptable, with only three parameters found to be above the upper limit in the samples, while, in terms of the lower limit, solely two parameters were observed, in the samples, to be below the established range. More than industrializing the production sector, extensive training is required in order that the 'master mezcal-makers' are able to match their ancestral knowledge with both modern technology and the legislation in order to establish more controlled processing conditions along the length of the agave-mezcal chain. This will ensure a product that complies with the requirements for consumption in both national and international markets and is in keeping with the customs and traditions of the communities involved in the production chain.

Key words: NOM-070-SCFI-2016, mezcal, total acidity, methanol, Guerrero.

INTRODUCTION

Colorless or slightly yellowish when rested, aged or smoothed, mezcal is a beverage with characterful and widely accepted *sui generis* aroma and taste. The Denomination of Origin Mezcal (DOM) has been in

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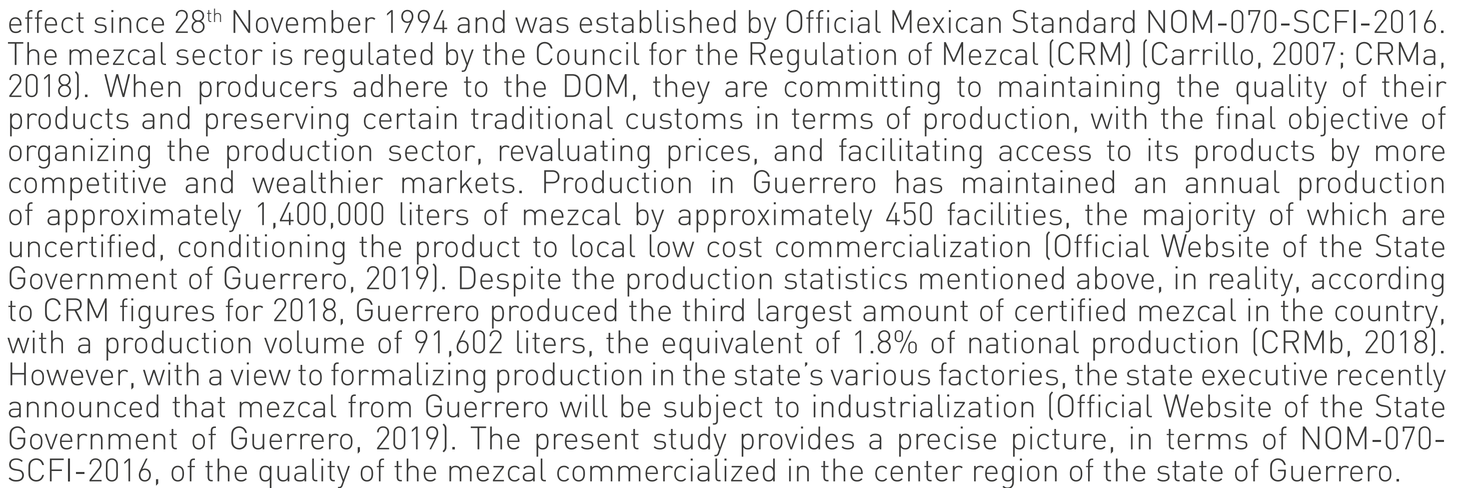


effect since 28th November 1994 and was established by Official Mexican Standard NOM-070-SCFI-2016. The mezcal sector is regulated by the Council for the Regulation of Mezcal (CRM) (Carrillo, 2007; CRMa, 2018). When producers adhere to the DOM, they are committing to maintaining the quality of their products and preserving certain traditional customs in terms of production, with the final objective of organizing the production sector, revaluating prices, and facilitating access to its products by more competitive and wealthier markets. Production in Guerrero has maintained an annual production of approximately 1,400,000 liters of mezcal by approximately 450 facilities, the majority of which are uncertified, conditioning the product to local low cost commercialization (Official Website of the State Government of Guerrero, 2019). Despite the production statistics mentioned above, in reality, according to CRM figures for 2018, Guerrero produced the third largest amount of certified mezcal in the country, with a production volume of 91,602 liters, the equivalent of 1.8% of national production (CRMb, 2018). However, with a view to formalizing production in the state's various factories, the state executive recently announced that mezcal from Guerrero will be subject to industrialization (Official Website of the State Government of Guerrero, 2019). The present study provides a precise picture, in terms of NOM-070-SCFI-2016, of the quality of the mezcal commercialized in the center region of the state of Guerrero.

METHODOLOGY

Origin of the samples and their processing

The 59 samples of young mezcal were obtained from six municipalities in the state of Guerrero corresponding to the economic area known as the center region. The study was conducted on mezcal samples procured at different randomly selected points of sale.

The municipalities included in the study were Chilpancingo de los Bravo, Eduardo Neri, Leonardo Bravo, Tixtla de Guerrero, Mochitlan and Martir de Cuilapan.

The measurements were carried out based on NOM-070-SCFI-2016 and NOM-070-SCFI-1994, for the determination of total acidity. The parameters applied in the present study were based on these standards.

A brief description of the methodology is given below, specifying only the particularities of the process.

Alcohol content

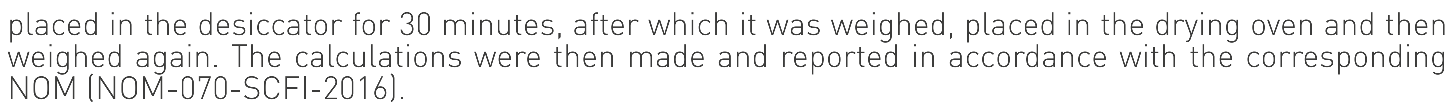
The equipment used was calibrated and verified, while the alcoholometers were certified and calibrated by means of a percentage scale of graduated volume by 0.1% Alc. Vol. in temperature conditions of 20°C. The thermometer used was calibrated to total immersion, on a scale of 0 to 100 °C and a minimal division of 1°C, while a Class A 250 mL volumetric flask was also used. Readings were taken by alcoholometer, according to their grade, from the previously distilled mezcal samples, which were placed in a 100 mL test tube. Said measurements carried out in a temperature controlled environment at 20°C, as indicated in the methodology (NOM-070-SCFI-2016).

Concentration of higher alcohols

This measurement used the following: isobutyl alcohol and isoamyl alcohol, both from the brand Fermont; 100 and 1000 mL Class A volumetric flasks; 1, 2, 3, 4, 6 and 10 mL Class A volumetric pipettes; and, 15 mL test tubes with a ground-glass stopper. The volume of the samples was always measured at 20 °C (NOM-070-SCFI-2016).

Quantity of dry extract

The measurement of this parameter used an Ohaus analytical balance with a precision of 0.1 mg, a rack with a temperature regulator, a water bath with a temperature regulator, a drying oven with temperature control, thermometers, distilled water, a drying agent, porcelain capsules, a desiccator, capsule tongs, and 50 mL volumetric pipettes. The sample was evaporated in a water bath with distilled water and then



placed in the desiccator for 30 minutes, after which it was weighed, placed in the drying oven and then weighed again. The calculations were then made and reported in accordance with the corresponding NOM (NOM-070-SCFI-2016).

Methanol concentration

This measurement used an Ohaus analytical balance with a 0.0001 g resolution, an Agilent spectrophotometer, a burette graduated in tenths of 50 mL divisions, a water bath with a temperature regulator, an ice bath, methanol (*J. T. Baker*), sodium bisulfite (*Fermont*), ethyl alcohol (*Fermont*), 50 and 100 mL Class A volumetric flasks, 1, 2, 5, 10 and 15 mL Class A volumetric pipettes, and a 0 to 100 °C total immersion thermometer with 1°C subdivisions. The different solutions were added to the previously distilled samples, as indicated in the NOM, and were read by means of the spectrophotometer to an absorbance of 575 nm. The results were calculated with the formula stipulated by the NOM and reported as indicated (NOM-070-SCFI-2016).

Total acidity

The measurement of this parameter used a Hach potentiometer, a magnetic stirrer, a warming rack, a universal support, magnetic bars, an analytical balance, potassium hydroxide, potassium biphthalate, pH 4, 7 and 10 buffer solutions, distilled water, 250 mL beakers, a burette graduated in 25 mL divisions, and 50 and 100 mL volumetric pipettes. The previously distilled sample was titrated in duplicate with sodium hydroxide until a pH of 8.0-8.2 was obtained, with the calculations then made and reported as indicated in the NOM (NOM-070-SCFI-1994).

Concentration of higher alcohols

This measurement used the following: isobutyl alcohol and isoamyl alcohol, both from the brand Fermont; 100 and 1000 mL Class A volumetric flasks; 1, 2, 3, 4, 6 and 10 mL Class A volumetric pipettes; and, 15 mL test tubes with a ground-glass stopper. The volume of the samples was always measured at 20 °C (NOM-070-SCFI-2016).

RESULTS AND DISCUSSION

The results of the analysis conducted on the parameters of alcohol, dry extract, methanol, and higher alcohols content, as obtained from 59 mezcal samples collected in six municipalities in the center region of the state of Guerrero, are described and discussed below. Total acidity was incorporated into the present study as it is a significant parameter stipulated by NOM-070-SCFI-1994. In order to understand the results, it is necessary to ascertain the values established in both NOMs (NOM-070-SCFI-1994 and NOM-070-SCFI-2016), given that specifying parameters with well-defined values means that any measurement outside that range would be non-compliant with the NOM. Figure 1 shows the results obtained, indicating each parameter analyzed and their percentage distribution according to the values pre-established in the NOMs. It should be noted that 100% of the samples were compliant in terms of the dry extract content obtained, according to that stipulated by the NOM currently in force.

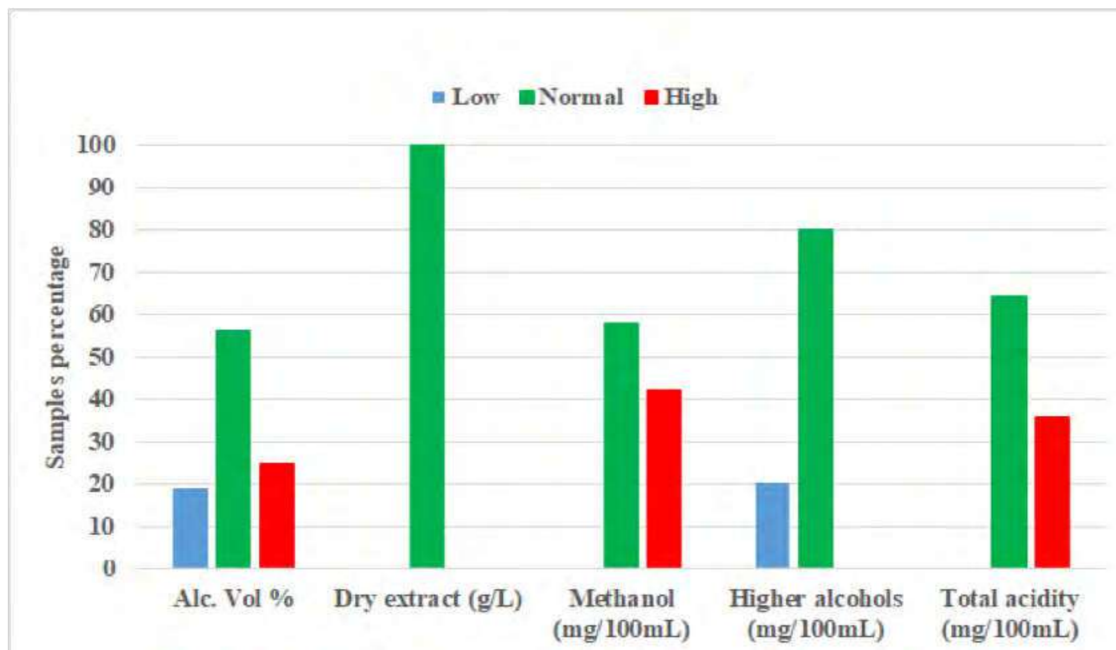
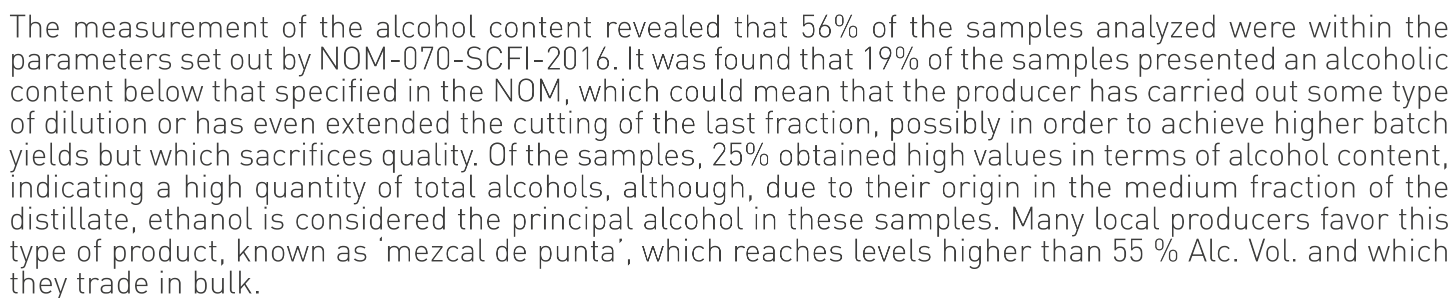


Figure 1. Percentage distribution of the samples analyzed for each physicochemical parameter. The green bars indicate the percentage of samples found to be in the range stipulated by the NOM currently in force. The blue and red bars indicate the percentage of samples that were found to be either below or above the parameters established in the NOM, respectively.

The dry extract parameter is found to indicate an absence of non-volatile compounds in the mezcal samples analyzed, with the values proposed in the NOM based on the carryover of salts and carbon compounds derived from the vat-making process, the fermentation process, the type of still used for distillation, the water used to lower the alcohol content, and the barrels used for ageing the product. Among the components identified in some distilled alcoholic beverages, the regulation of which is proposed for inclusion in the quality indices, are elements such as As, Zn, Cu, Pb, Cd and Fe, which mainly originate in the stills used during the distillation process, the water used for diluting the distillate, and, even, the ageing vats (Ávila, 2010).

The second highly controlled parameter in the process, as measured in the samples analyzed, was the higher alcohols, 80% of which were found to be within the range considered appropriate in the NOM. The remaining 20% contained a concentration of higher alcohols that was lower than that stipulated by the NOM, which reflects the fact that producers are mainly found to be correctly making the cut tips during the distillation process. In those cases in which the producers obtain low higher alcohol concentrations, it may be that they are cutting the distillate too late, namely that they are obtaining a higher number of cut tips and, with this, wasting part of the distillate that should constitute the body of the mezcal (Vera et al. 2009).

With regard to the methanol measurement, in which 42% of the samples surpassed the upper limit established by the NOM, it is observed that producers experience major problems with controlling the temperatures used for separating the compounds carried in the tail fraction. We concur with the finding that greater higher alcohol content is observed at the beginning of the distillation (the tips), decreasing until the process has been completed. This is the inverse of the behavior of methanol, which is more concentrated in the tails than in the tips, as reported in other studies (CIATEJ, 2014; Vera et al. 2009). Of the remaining samples, 58% indicate that the producers have mastered the mezcal-making process, given that the presence of methanol is attributable to the different stages comprising said process (Vera et al. 2009). The mixture of the tips and tails of the distillate with water also generates a composition of said characteristics, as does mixing the tails with the medium fraction (the body), which creates an abundant product in terms of volume, but one of poor quality.



The measurement of the alcohol content revealed that 56% of the samples analyzed were within the parameters set out by NOM-070-SCFI-2016. It was found that 19% of the samples presented an alcoholic content below that specified in the NOM, which could mean that the producer has carried out some type of dilution or has even extended the cutting of the last fraction, possibly in order to achieve higher batch yields but which sacrifices quality. Of the samples, 25% obtained high values in terms of alcohol content, indicating a high quantity of total alcohols, although, due to their origin in the medium fraction of the distillate, ethanol is considered the principal alcohol in these samples. Many local producers favor this type of product, known as 'mezcal de punta', which reaches levels higher than 55 % Alc. Vol. and which they trade in bulk.

Despite the fact the percentage of samples exceeding the upper limit for total acidity was not the highest for the parameters measured, a significant percentage of samples (36%) was considered in the present study, as this parameter shows the producer's capacity for preparing the wort and controlling the fermentation process. This is the level at which the largest quantity of organic acids is produced after the optimal sugar consumption time period has elapsed, at which point ethanol is converted into acetic acid in a process known as 'souring the wine'. This reinforces the notion that this parameter must be dealt with in a timely manner, given that, from the moment at which the cooked agave hearts are exposed to the elements, this conversion is magnified throughout the process until the acetic acid accumulates in the final product (Vera, 2009; CIATEJ, 2014). It should be noted that the behavior charted by the graphs for methanol and total acidity are very similar. This peculiarity is due to the fact that both compounds are found to migrate in the same fraction of the distillate, known by the producers as tails (Vera et al. 2009).

CONCLUSION

Analysis of the 59 mezcal samples obtained from six municipalities in the central region of the state of Guerrero revealed that the best-controlled parameters in the mescal-making process practiced by these producers were the dry extract (100% compliant with the NOM) and the higher alcohols (80% compliant). With regard to the parameters for alcohol and methanol content, more than 50% of the samples presented values within the ranges permitted by the NOM currently in force. With regard to total acidity, it was observed that more than 60% of the batches analyzed complied with that established in NOM-070-SCFI-1994.

In general, it can be concluded that the mezcal produced in the central region of the state of Guerrero is of good quality. However, producers lack a great deal of support in order that they are able to match their ancestral knowledge with both the legislation and modern technology, thus resolving the critical points in the mezcal-making process, specifically in the fermentation and distillation process. With their knowledge of these processes thus updated and working in concert, the mezcal of Guerrero would be more competitive at a national and international level, due to its rich sensory diversity and the quality standards imposed by the regulatory framework.

ACKNOWLEDGEMENTS

To the true mezcal masters, beyond the division of groups. To QBP. Manuel Joaquín Romero López for his advice and assistance in the laboratory work.

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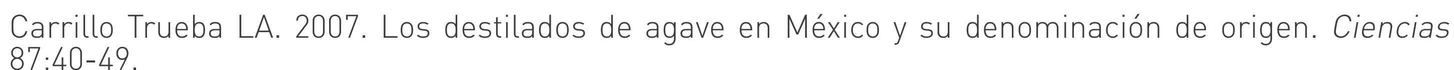

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Isolation and selection of yeasts with invertase activity during the artisanal processing of mezcal, from Oaxaca.

Granados-Alegría, I.[1], González-Flores, T.[1], Vázquez-Elorza, A.[2], [1*] and Sánchez-Contreras, A.[1*]

ABSTRACT

Various industries are interested in the enzyme that hydrolyzes sucrose into glucose and fructose. In different fermentative process, the presence of microorganism with the ability to produce invertase enzymes continues to be a research area in constant development. Invertase is important in the pharmaceutical and food industry, especially for the production of confectionery products. Because these enzymes are often obtained from yeasts, in this study, different artisanal distilleries ("palenques" in Spanish), of mezcal were visited in Matatlán, Oaxaca, Mexico, for the screening of wild yeasts as invertase producers. In this study, 49 wild yeasts were isolated, and only nine strains had the ability to grow on sucrose on BBL Crystal (ID) of Anaerobe (ANR) ™ test. To measure the extracellular invertase activity of the yeast, these strains were cultured in RPMI-1640 medium with 4% sucrose, and incubated at 37 °C for 24 h, separating the cell-free supernatant by centrifugation at 4 °C and using the Sigma MAK118 kit. In order to express specific invertase activity, was necessary to achieve the protein assay in the supernatant. The strain identified as 2F2, from the fermentation must of the artisanal distillery 2, presented the higher invertase activity (61.2 U invertase/mg). Cell growth in sucrose apparently was not related to invertase activity.

Key words: Invertase activity, wild yeast, mezcal, Oaxaca.

INTRODUCTION

The search is extensive for microorganism that are capable of synthesizing products of biotechnological interest. Invertase production of wild strains generates great interest due to its ability to catalyze a large number of specific reactions. The fermentation process of mezcal artisanal distillery has naturally yeast strains well adapted for sucrose degradation by the invertase enzyme. Alternative names for invertase include saccharase, glucosucrase, beta-fructosidase and the systematic name beta-fructofuranosidase, EC 3.2.1.26. (Bacon, 1995).

One unit of invertase is defined as the amount of enzyme required to release 1 µmol of glucose from sucrose per minute (Hernalsteens and Maugeri, 2008). Invertase is used in the pharmaceutical and food industry, especially in the production of confectionery products, such as chocolates, synthetic honey and jams in general, as well as in the production of artificial sweeteners and in the brewing industry (Veana et al. 2011).

The invertase activity is induced by high concentrations of sucrose and low glucose (Patel et al. 1994).,

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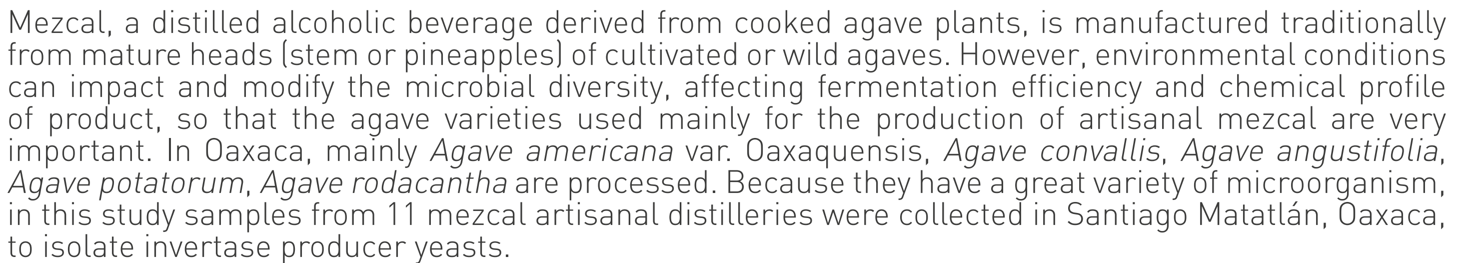


Mezcal, a distilled alcoholic beverage derived from cooked agave plants, is manufactured traditionally from mature heads (stem or pineapples) of cultivated or wild agaves. However, environmental conditions can impact and modify the microbial diversity, affecting fermentation efficiency and chemical profile of product, so that the agave varieties used mainly for the production of artisanal mezcal are very important. In Oaxaca, mainly *Agave americana* var. Oaxaquensis, *Agave convallis*, *Agave angustifolia*, *Agave potatorum*, *Agave rodacantha* are processed. Because they have a great variety of microorganism, in this study samples from 11 mezcal artisanal distilleries were collected in Santiago Matatlán, Oaxaca, to isolate invertase producer yeasts.

The production of artisanal mezcal has a disadvantage due to its non-standardized process (Ruiz Muñoz, 2016), otherwise yeast involved in mezcal fermentation remains unknown and is an important step the knowledge of native yeast that can be used in many applications.

METHODOLOGY

1. Sample collection

Sample collection of the Mezcal production processes was carried out in distilleries in the state of Oaxaca, Mexico at 11 artisanal distilleries, located in Matatlán, the municipality of Santiago Matatlán, and other nearby places like the highway between Macuilxóchitl de Artigas Carranza and Dainzu, San Dionisio, Villa Sola de Vega, Soledad Salinas, Minas, Chichicápam and San Pedro Taviche. The sample collection was completed in two visits: The first sampling of artisanal distilleries coded 1-6 was conducted in the month of March 2018, and the last coded 7-11 was conducted in May 2018 (Fig 1). Samples for the isolation of yeasts were identified according to the number of artisanal distillery from which they originated and stage of the mezcal´s elaboration process regarding the fermented agave must (F) and agave stems after cooking and grinding (PC). Agave must samples (15 mL) were collected at different times of fermentation, and "3M" transport swabs were used with Letheen medium (meat peptone, meat extract, soy lecithin and sodium chloride) to preserve the microorganisms.

2. Microbial enumeration and isolation

Fermentation samples were serially diluted in sterile water; 100 µL aliquots were spread plated in duplicate onto Potato Dextrose Agar (PDA, Fluka) supplemented with chloramphenicol (100 µg/mL, Sigma-Aldrich,) for yeast count. Plates were incubated at 29 °C for 3 days and were checked daily for population and morphology of colonies. Colonies were selected based on morphology characteristics and isolates were purified by Biochemical test. For each morphology, only one colony was isolated and preserved in glycerol stocks (40% at -20 °C) for subsequent tests.

3. Biochemical tests

Biochemical tests were performed with the help of the BBL Crystal (ID) of Anaerob (ANR). BBL Crystal ™ is a miniaturized identification method that uses modified conventional, fluorogenic and chromogenic substrates. These include tests for fermentation, oxidation, degradation and hydrolysis of various substrates. Test inoculum was prepared with the inoculum fluid and is used to fill all 30 wells in the base. When the lid is aligned with the base and snapped in place, the test inoculum rehydrates the dried substrates and initiates test reactions. Following an incubation period, the wells are examined for color changes or presence of fluorescence that result from metabolic activities of saccharides. Nine yeast strains were selected for their capacity for sucrose hydrolysis after 24 h.

4. Measurement of invertase-liberation activity

The selected strains yeast were cultured in RPMI-1640 medium (R1383 Sigma Powder pH 7.02) with of 4 % sucrose, for 24h (37 ° C, 150 rpm). Enumeration of yeasts in the culture was accomplished by Dilution Plating Technique and the fermentation broth was analyzed in order to determine if the yeast secretes any enzymes into the broth. The invertase-liberation activity assays were measured on the

supernatant using, invertase activity KIT (Sigma MAK118), the invertase cleaves sucrose to glucose and fructose, resulting in a colorimetric (570 nm) product, proportional to the invertase activity present. One unit of Invertase is the amount of enzyme that will catalyze the formation of 1.0 µmole of glucose per minute at pH 4.5 under the assay conditions. The specific activity it's expressed by concentration protein determinate to the cell free broth.

RESULTS AND DISCUSSION

1. Yeast strains

From the 11 artisanal distilleries sampled on the community of Santiago Matatlán of Oaxaca State, Mexico (Table 1), 49 yeast strains were isolated (Figure 1). Artisanal distilleries 1-3 presented yeasts with a greater growth after 24 h. Biochemical tests with BBL Crystal ™, identified the nine invertase producing yeast.

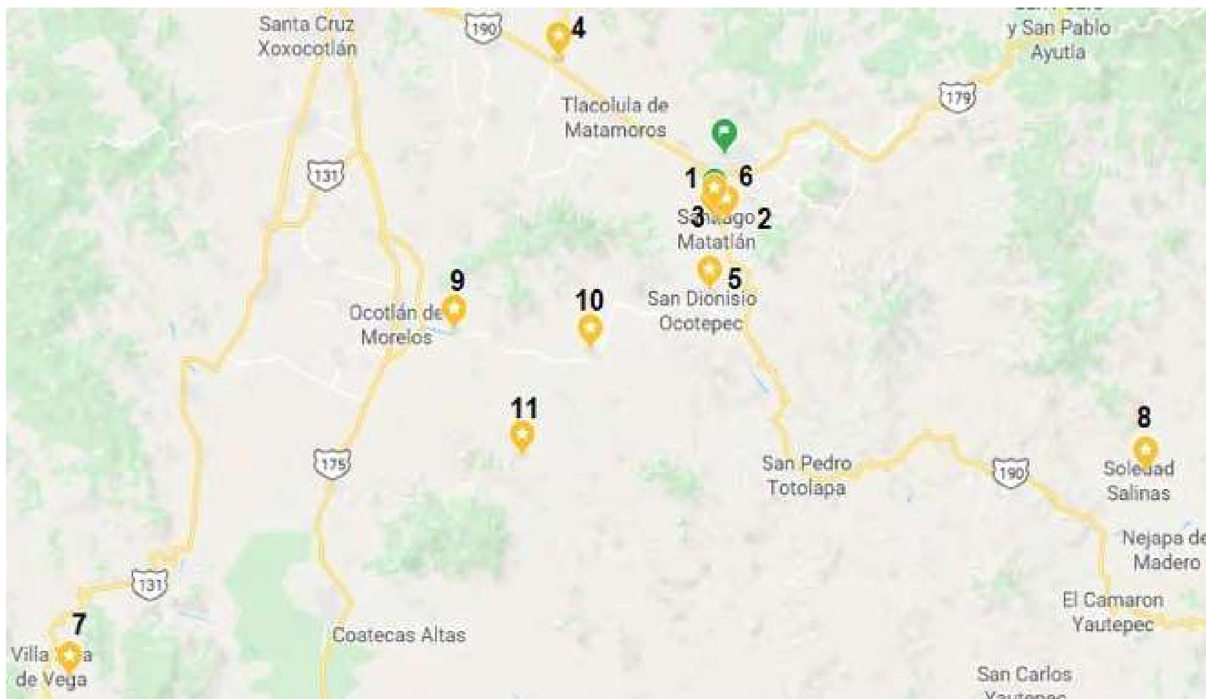


Figure 1. Location map for sample collection in the eleven Palenques of artisanal production of Mezcal, visited in the state of Oaxaca, México.

In the production of different alcoholic beverages, wild yeasts play an important role in the transformation of the agave must, producing an exceptional agave artisanal drink (Arrizon and Gschaedler 2002). In this study, nine wild strains were compared, isolated from different origin (fermented agave must and agave stems) for the technological interest in the production of invertase enzymes.

The yeast strain with the highest activity was isolated from the fermentation vats, sampled in the artisanal distillery 2in Santiago Matatlán, Oaxaca, Mexico, 2F2 strain reached 61.2±2.75 Uinvertase/mg, whereas other strains of yeast (like 2PC1, 2PC2 and 2PC3) that were isolated from cooked stems in the same artisanal distillery, only achieves between 8 and 10 Uinvertase/ mg, although their growth in sucrose was very similar in all strains from this artisanal distillery (between 3.54 and 2.01 x 10^7 CFU/ml). The characterization of the invertase activity of different yeasts strains, in one Palenque, emphasized the evidence of significant difference as a function of isolation origin.

Table 1. Origin of the nine strain isolated and selected and Distilleries location sampled.

Artisanal distilleries sampled from Santiago Matatlán Geographic location (latitude/longitude)	Yeast identification code	
	Fermented agave must (F)	Agave stems cooking (PC)
Artisanal distillery (16.872333 /-96.385851)	1F 1	1PC 1
Artisanal distillery (16.865134/ -96.375180)	2F 2 and 2F 3	2PC1, 2PC2 and 2PC3
Artisanal distillery (16.866179 /-96.386273)	3FC*, 3FE**	

Fermented agave must, center (C*) and Edge (E**) of the fermentation vats.

Further evidence that the cell growth of yeasts in sucrose, is apparently not related to the invertase activity comparing two strains isolated from the fermentation must in artisanal distilleries 2 and 3. The strain 2F2 had high growth and activity, another 3FC strain achieved better growth but had low activity. The invertase activity presented by yeast strain 2F2 was better than that reported by Veana et al. (2011) for *Aspergillus niger*, which achieved its highest activity in 6 days (293 U / mg), while 2F2 reached up to 61.2 ± 2.75 U / mg in just 1 day (Figure 2).

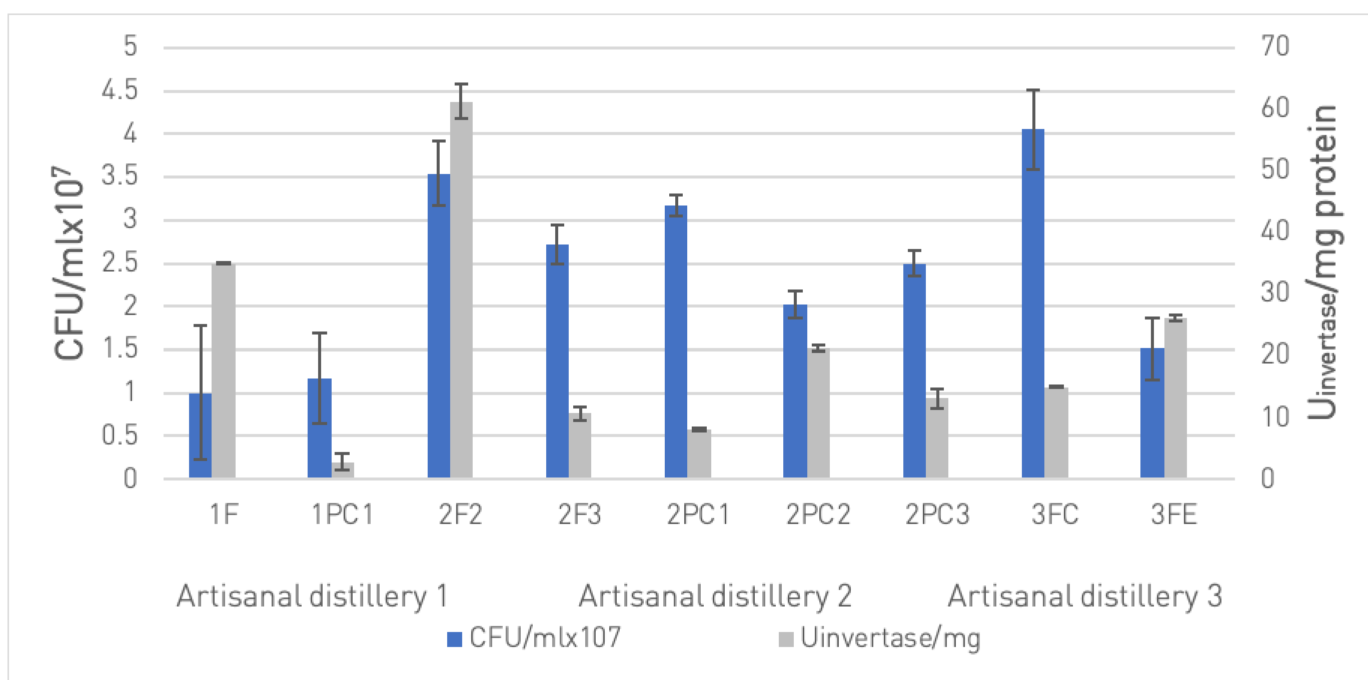
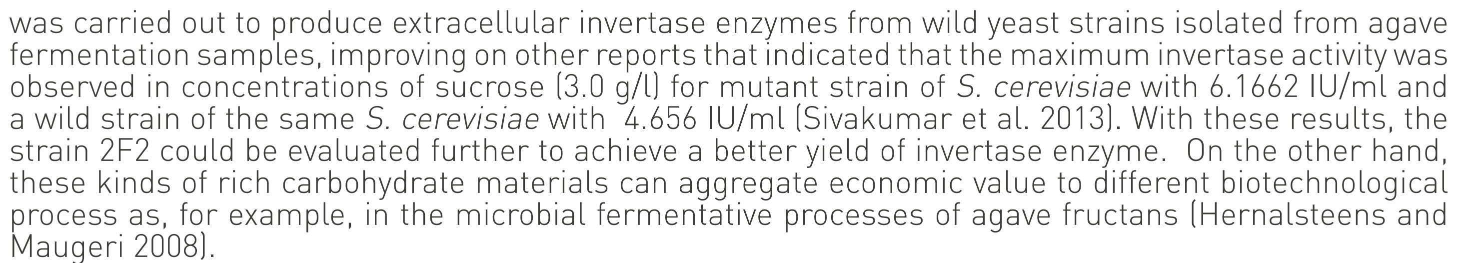


Figure 2. Measurement of invertase-liberation activity for nine yeast strains that were selected for their capacity of sucrose hydrolysis at 24 h. Artisanal distillery 1: Fermented agave must and Stem cooking (1F) and (1PC1), idem Artisanal distillery 2: (2F2, 2F3) and (2PC1, 2PC2, 2PC3). Artisanal distillery 3: Fermented agave must, center and Edge of the fermentation vats (3FC) and (3F3).

The yeast Saccharomyces cerevisiae is the main source of the invertase enzyme. It is mainly used in the food industry as a soluble or immobilized enzyme (Margetić and Vujčić 2016). The present study



was carried out to produce extracellular invertase enzymes from wild yeast strains isolated from agave fermentation samples, improving on other reports that indicated that the maximum invertase activity was observed in concentrations of sucrose (3.0 g/l) for mutant strain of *S. cerevisiae* with 6.1662 IU/ml and a wild strain of the same *S. cerevisiae* with 4.656 IU/ml (Sivakumar et al. 2013). With these results, the strain 2F2 could be evaluated further to achieve a better yield of invertase enzyme. On the other hand, these kinds of rich carbohydrate materials can aggregate economic value to different biotechnological process as, for example, in the microbial fermentative processes of agave fructans (Hernalsteens and Maugeri 2008).

CONCLUSION

The strain identified as 2F2 from the fermentation must of artisanal distillery 2, presented the highest invertase activity (61.2 $U_{invertase}$/mg), there was no quantitatively direct correlation between growth and invertase activity. Subsequently it is important to verify the invertase activity in the intracellular yeast and measure the transfructosilating activity for the synthesis of prebiotics like FOS.

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Native microbial diversity in hydrolysed and cooked agave juice at industrial level.

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ABSTRACT

At present day, the industrial manufacturing of tequila makes use of two procedures to hydrolyze fructans, one is achieved by cooking the agave stems and the other method consists in lixiviation. Through this method, juices are extracted and subsequently hydrolyzed using acids and/or temperature. However, the impact that each type of hydrolysis causes on the bacteria/yeast ratio and the variability of species and strains that grow in each type of juice is unknown.

This study was conducted to determine the microbial load of agave juices from different processes used by the industry to analyze how these impact on the bacteria/yeast ratio. This was done by both determining the species and their strain variability that grew in each juice obtained by one of the previously mentioned processes. Sixty-three yeasts and forty-six bacteria isolates from the musts belonging to nine genera of yeast and five bacterial genera, were considered.

In all juices the concentration of bacteria was greater than that of yeasts except in the agave juice obtained by diffuser coupled with thermal hydrolysis. It is possible that the juice contains a high amount of microbial growth inhibitors and this may affect mainly the bacteria.

On the other hand, it is noteworthy that some strains isolated from traditional processes such as T2 are very similar genetically, while in the case of hydrolyzed juices through a thermo-acid process they have the greatest genetic variability among them. This could be attributed to the fact that in juices that are not heated to high temperatures contain less inhibitors and different strains that are not adapted to these compounds are able to grow.

Key words: Hydrolysis, variability, genotype, agave juice, strains.

INTRODUCTION

Agave carbohydrates are compounds of great industrial interest and these are found mostly in the stems, commonly known as "piña", with a high content of fructans (20 to 30% W/W), which are composed of fructose polymers, and are synthesized as reserve carbohydrates (Mellado-Mojica and López 2012). For the elaboration of tequila, it is necessary that the fructans are hydrolyzed to produce must that will be taken to fermentation, that is, in the form of monosaccharides of fructose and glucose. Currently the industry uses two processes for hydrolysis, the first is the traditional process in which the agave stems are cooked and then the hydrolyzed sugars are extracted. The second process uses diffusers in which by

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means of leaching, the fructans are extracted directly from the steams of the previously crushed agave, later the fructans are hydrolyzed using acids, high temperature or a mixture of these (Gschaedler et al., 2015).

During cooking, not only are free sugars obtained, but they are also exposed to a complex series of reactions that transform them into colored compounds ranging from light yellow to dark brown. The products obtained contribute to the taste and aroma of tequila. Among the main reactions used are: the Maillard reactions, caramelization, oxidation-dehydration and the release of methanol. On the other hand, using an acid-thermal hydrolysis certain undesirable compounds are also generated, since by neutralizing the acidified hydrolysates, salts are generated which can also influence fermentation. Therefore, the process used to obtain hydrolyzed sugars from fructans directly impacts the characteristics of the must and generates important variants in the fermentation process (Gschaedler et al., 2015). This is because it has been reported that compounds such as furfural and hydroxymethylfurfural compounds are generated in the cooking stage and these work as inhibitors of microbial growth in low concentrations since they bind to biomolecules such as nucleic acids, proteins and lipids causing alterations to their biological function (Wierckx and Koopman 2011). The term "native microorganism" refers to the microbiota found in the fermentation process without the addition of any inoculant in order to promote the process, this microbiota has been described by different authors as being native in which they are found in most of the cases a great diversity of species and strains (Alcazar-Valle et al., 2019 and De Celis et al., 2019). Finally, due to these chemical changes in the juices, is it possible that according to the hydrolysis process of the fructans the bacteria/yeast ratio, as well as the species and variability of strains found, are different?

METHODOLOGY

Sampling was carried out in six tequila factories of which four perform a traditional cooking process (T1-T4) and two obtain the juices with a diffuser, one uses acid hydrolysis and low temperature (D1) and the second a thermal hydrolysis by autoclave (D2). The culture media used for yeast growth and bacteria were (WL) agar added with chloramphenicol, (YPD) agar and (MRS) agar respectively (Sigma Aldrich). It was started by making counts in Neubauer chamber to know the microbial load of the sample, once the microbial load is known, it is possible to carry out serial dilutions 1:10 to obtain one from 0.1 mL containing 30 to 300 colonies, the tests were carried out in triplicate. With the indicated dilution, 0.1 mL was added and plated by extension using a Drigalsky handle, to finally incubate for 24 h or until there was growth at 30 °C. Subsequently colony forming units (CFU) were calculated using the following formula: Viable cells = (count × 10) (dilution factor).

Identification of isolated yeasts and bacteria

The identification of isolates was achieved by desorption/ionization mass spectrometry with matrix-assisted laser (MALDI-TOF MS) on a MICROFLEX LT platform and the MALDI BIOTYPER 3.1 software from BRUKER DALTONICS (USA). Biomass from single colonies of a fresh culture was transferred with a sterile wooden stick onto a stainless steel MALDI target plate. Subsequently, 1 µL of alpha-cyano-4-hydroxycinnamic was added to each spot. The spectra obtained were compared against the reference database BDAL and a report of the genus and the species was generated.

DNA extraction and PCR amplification.

Biomass was obtained from a 24 h liquid cultures (media) of each strain in the corresponding culture media and centrifuged to obtain cell pellets. Genomic DNA was extracted following the protocol published by Tapia-Tussell *et al*. 2006. To determine the genotypic variability between species, the technique repetitive element palindromic PCR (rep-PCR) was used using the primer (5´GTG-GTG-GTG-GTG-GTG´3), described by Baleiras-Couto et al. 1996. For the mixture was used GoTaq® 2X (Promega) 12.5 µL, 9.5 µL of H_2O and 2 µL of primer 10 mM at a final reaction volume of 24 µL with 25 ng of each DNA template. The reaction conditions were: initial denaturation temperature of 95 ° C for 10 min, 30 cycles of 95 ° C for 30 s, 45 ° C for 1 min and 65 ° C for 8 minutes for finally a final polymerization temperature of 65 ° C for 16 ° C min. The same conditions were used for bacteria and yeast.

Electrophoresis conditions and analysis of the banding pattern.

The PCR products were separated by electrophoresis on a 2% agarose gel in 1X TAE buffer (40 mM Tris base, 20 mM acetic acid and 1 mM EDTA) stained with the GelRed® (Biotium) dye. The running conditions were 80 V for 4 h to finally be visualized in an ultraviolet light transilluminator. The banding patterns generated in the gels were analyzed using the program BioNumerics version 7.6.3 with the GelCompar II tool using the group method of unweighted pairs with arithmetic mean by the coefficient of Dice to determine the genotypic variability between the isolated strains of the different processes (Ishii and Sadowsky, 2009).

RESULTS AND DISCUSSION

In all processes, the concentration of bacteria was higher than yeasts, except in the juice that was hydrolyzed by leaching combined with autoclave (D2, Figure 1), which suggests that these juices could contain a high concentration of compounds that inhibit the growth of bacteria.

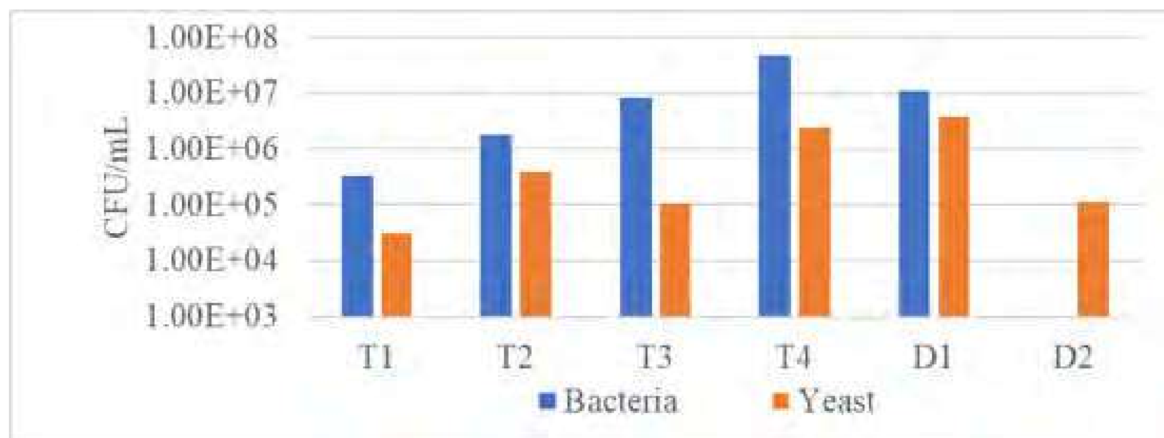

Figure 1. Concentration of microbial populations in different agave juices.

In the case of the different yeast genera found in the musts we can highlight *Saccharomyces, Clavispora, Hanseniaspora, Pichia, Kluyveromyces, Lachancea, Torulaspora* and *Zygosaccharomyces*; however, it should be noted that not all yeast genera were found in all processes and it is not certain if they grow in all the juices. The genera found in the present study agree with those reported by Lachance et al., 1996, a study focused on the characterization of yeasts in musts to produce tequila. At the end of the isolation 63 strains were obtained belonging to 8 genera with 11 yeast species.

In this study, a great similarity was observed in comparison with others carried out for the identification of bacteria in agave musts to produce mezcal, however, given the means used during the isolations in the study carried out by Kirchmayr et al., 2017, in the state of Oaxaca. in which it was possible to isolate *Lactobacillus, Leuconostoc, Weissella, Zymomonas*, as well as some acetic ones such as *Gluconobacter, Acetobacter* and other bacteria it allowed to find a greater number of environmental bacteria, this may be since in the study conducted in mezcal other culture media were used that favored the growth of another type of microorganisms. At the end of the bacterial isolation, 46 strains were obtained, of which 5 genera with 13 species were found.

Thirty strains of *Saccharomyces cerevisiae* were used for the study of genetic similarity applying a criterion of 90% similarity (Figure 2), the 90% criterion was established for the working group to finding the most significant differences between the strains. Twenty clusters were generated in which the strains with greater genetic similarity were those of the traditional process (T2), while the greater genetic variability

was observed in the juice obtained by diffuser with a thermoacid hydrolysis (D1). The global genetic variability of the strains was 45% using $(GTG)_5$ marker. The total of bands generated ranges from 9 to 16 depending on the strain and these bands were shown to be within the ranges of 590 to 3100 bp, among these the most predominant and that occurred in all strains showed bands of 590, 1700, 1900 and 2900 bp. These results agree with those reported for *S. cerevisiae* by Silva-Filho et al. 2005 whose project consisted in the screening of native strains found in different bio-ethanol distilleries.

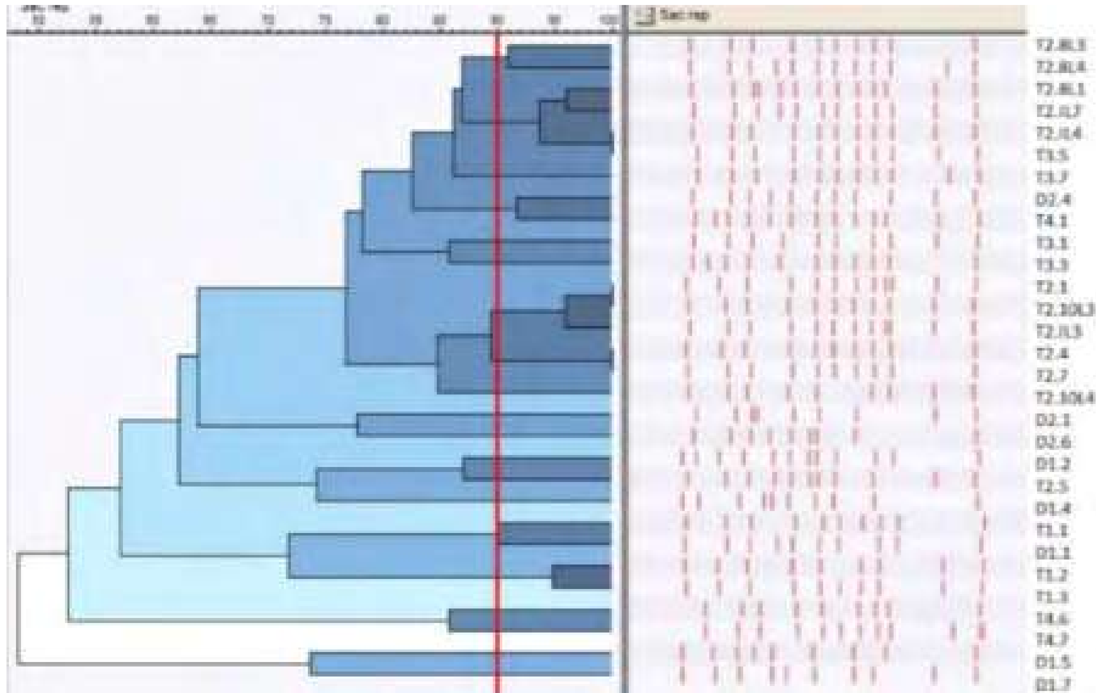

Figure 2. Genotypic variability of 30 native strains of *S. cerevisiae* isolates to agave juices of different processes based on rep-PCR using $(GTG)_5$ primers, red line indicates the 90% similarity between the strains, the more intense blue color indicates a greater genotypic similarity.

Of the 33 native non-*Saccharomyces* strains the same 90% similarity criterion was used to generate the clusters (Figure 3), resulting in a total of 22 clusters in which the genus with the greatest genetic similarity was *Hanseniaspora*, even though they were isolated from different tequila factories, showing a variability of less than 10% in some cases. On the other hand, the greatest genetic variability was observed in the *K. marxianus* strains in which some, despite being isolated from the same processes, present variability greater than 25%. The overall genetic variability between the genera was 75% in which the overall range of bands was observed between 200 to 12,000 bp. In a study conducted by Perez-Brito et al. in 2007, it was demonstrated that by using the primers $(GTG)_5$ and 6 more it was possible to differentiate between two groups of ascospores forms, so this technique proves to be effective for genotypically distinct strains. The number of bands observed was 5 to 18, in which the species that presented most of the bands was *K. marxianus* T1.4 whit 18 bands whereas *Pichia kluyveri* only 5. With this methodology it was possible to group the yeast species using this marker, which agrees with the results obtained by Capece et al. 2003 in non-*Saccharomyces* native wine strains.

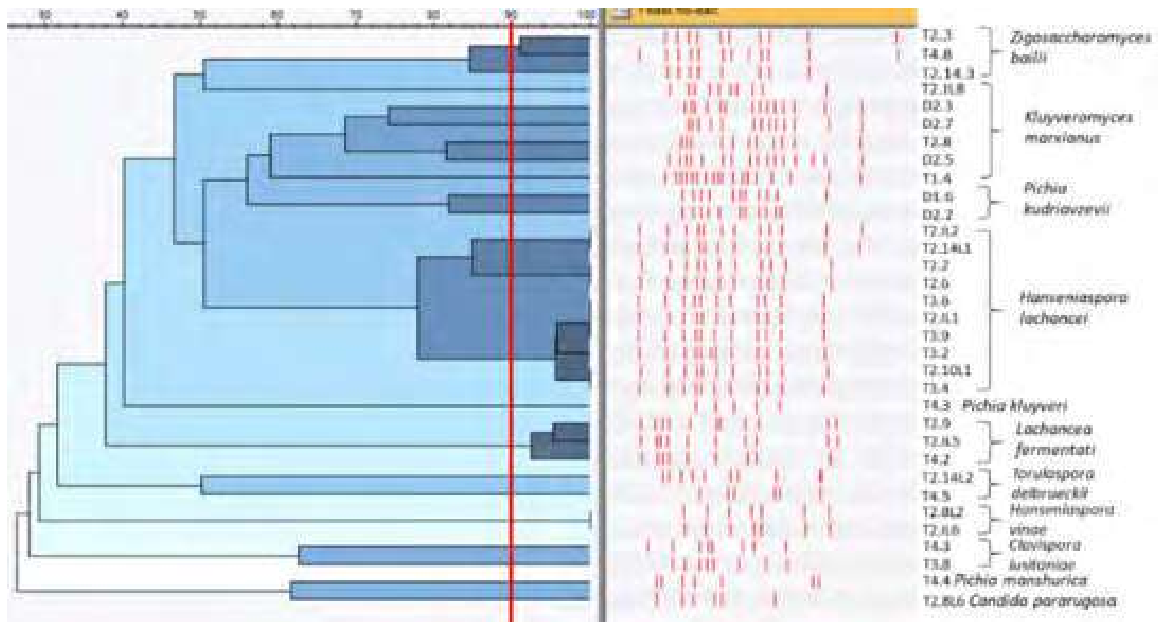

Figure 3. Genotypic variability of 33 native strains of different genera of non-*Saccharomyces* to agave juices of different processes based on rep-PCR with $(GTG)_5$ primers.

The genetic variability of bacteria was high (Figure 4), since is observed the formation of a cluster per strain; therefore, the overall bacterial genetic variability found is greater than 90%. In this case the range of bands between the bacterial genera was from 1 to 17 in which *Acetobacter cerevisiae* showed the highest number of bands while a strain of *Lactobacillus brevis* only amplified one band. The global range of bands was observed between 400 to 7000 bp. This coincides with some published work such as the case of Gevers et al., 2001 in which using strains from a collection of Lactobacilli and some isolates from different fermentations, were not grouped according to their banding pattern indicating that the bacteria show greater variability.

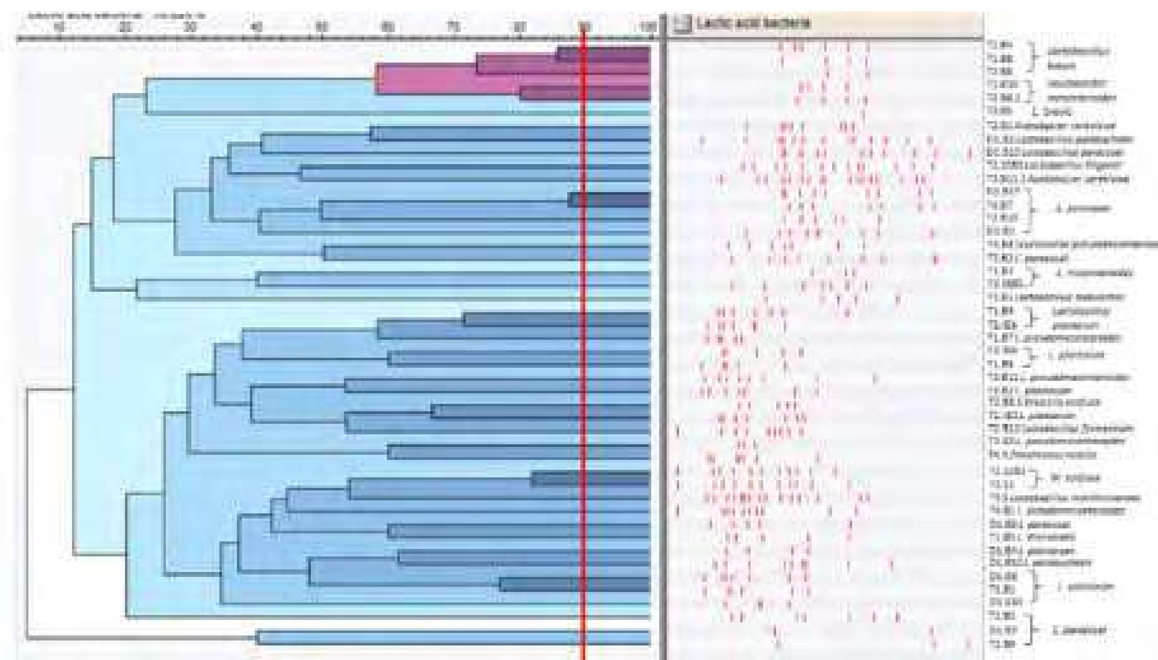

Figure 4. Genotypic variability of 46 native strains of different genera of bacteria isolates to agave juices of different processes based on rep-PCR with $(GTG)_5$ primer.

CONCLUSION

There are differences in the concentrations and in the relation yeast bacteria depending on the process of obtaining juice. There is a greater genetic variability in strains of *S. cerevisiae* isolated from hydrolyzed diffuser juices by an acid-thermal method in purchase with traditional juices or hydrolyzed diffuser juice by autoclaving. The molecular marker used allowed the non-*Saccharomyces* yeasts to be grouped according to their genus and species, except for the genus *Pichia*. In the case of isolated bacteria, the marker used did not allow to group neither species nor genera due to its wide genetic variability.

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Physicochemical quality of commercial Extra Aged and Crystalline tequilas.

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ABSTRACT

Tequila has shown great growth in recent years due in large part to the consumption of its Premium products like Crystalline Tequila. Sales statistics for Crystalline tequila have grown by 40.4%, increasing the number of brands by up to 2000% due to its wide acceptance in the market. However, this type of tequila is not yet classified in the Official Standard (NOM-006-SFCI-2012) and therefore, its quality characteristics are not established. For this reason, in the present investigation, the parameters of physicochemical quality were determined to establish differences between Extra Aged and Crystalline tequilas. Six brands of tequila were used in this investigation. Two types of tequila from the same brand were acquired (Extra Aged and Crystalline) already existing in the market were chosen. The physicochemical determinations of alcoholic content and dry extract were made according to the Mexican Standard NOM-006-SFCI-2012. Besides, pH, color, and acidity analyses were also carried out. A greater amount of dry extract was determined in the samples of Crystalline tequilas than in Extra Aged tequilas; it should be mentioned that the value of the dry extract is outside to the limits established by the Mexican Standard NOM-006-SFCI-2012. The alcoholic content values are within what is established by the Mexican Standard NOM-006-SFCI-2012 for both types of tequilas. In the case of pH and acidity, similar results are observed, with greater acidity being observed in Crystalline tequila samples.

Key words: Distilled beverage, physicochemical parameters, quality, Extra-aged tequila, Crystalline tequila.

INTRODUCTION

Tequila is the Mexican alcoholic beverage with the greatest presence in the national and international markets. The tequila industry has constantly sought to innovate its products to remain in the market of alcoholic beverages, increasingly competitive achieving great growth in recent years due in large part to the consumption of its Premium products. In this subcategory, Crystalline tequila is classified. The first Crystalline tequila was launched on the market in 2011, the same year in which it reported a growth in total tequila production with 3.6 million liters more than the previous year. According to the Regulatory Council of Tequila (CRT), in 2018, there was growth with a total production of 290 million liters, attributing this increase to Crystalline tequila. A Crystalline tequila can be obtained from Aged, Extra-Aged or Ultra-Aged tequila, so it must maintain the characteristics of a product that was matured in barrels, but with the transparency of a Silver tequila. To obtaining Crystalline tequila, each distillery has established its.

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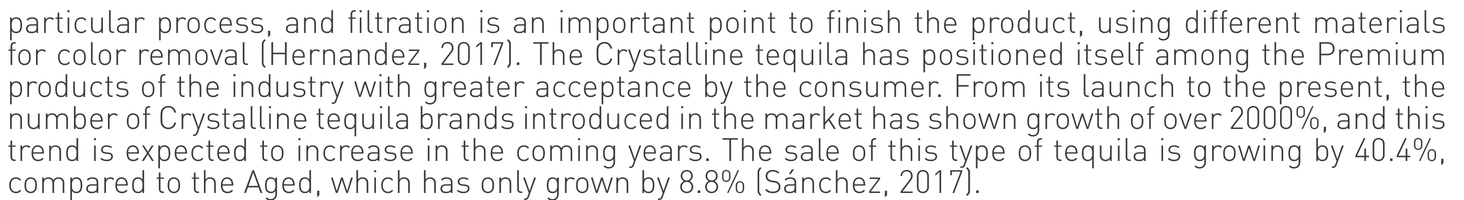

particular process, and filtration is an important point to finish the product, using different materials for color removal (Hernandez, 2017). The Crystalline tequila has positioned itself among the Premium products of the industry with greater acceptance by the consumer. From its launch to the present, the number of Crystalline tequila brands introduced in the market has shown growth of over 2000%, and this trend is expected to increase in the coming years. The sale of this type of tequila is growing by 40.4%, compared to the Aged, which has only grown by 8.8% (Sánchez, 2017).

However, despite commercial success, this type of tequila is not yet classified in the Official Standard of tequila (NOM-006-SFCI-2012), and therefore its quality characteristics are not established. Therefore, in the present investigation, the physicochemical parameters were determined with the purpose of establishing differences between Extra Aged and Crystalline tequilas. With the results obtained, composition limits were established for crystalline tequila that can be of great help in the tequila sector and so that in the future, these can serve as a reference for inclusion in the Official Standard appropriate.

METHODOLOGY

Tequila samples

Six commercial tequilas 100% agave were selected according to their positioning in the market, and later two types of tequila from the same brand were acquired (Extra Aged and Crystalline).

Physicochemical analysis

The analysis of the alcohol content was measured according to NMX-V-013-NORMEX-2013. Alcohol content expressed as % alcohol volume at 20ºC was measured with a densitometer DMA-48 (Anton Paar, Graz, Austria). The dry extract was quantified by evaporation to dryness according to NMX-V-017-NORMEX-2014, and the result was expressed as g/L. Total acidity was determined by titration according to NMX-V-015-NORMEX-2014. The results were expressed as mg of acetic acid/100 mL of anhydrous ethanol. pH was measured performed directly on a Thermo Scientific Orion Star A211 device. The color was measured on a Konica Minolta Spectrophotometer CM-5. 60 ml of sample were placed in a polystyrene cell culture flask Corning brand. The main volatile compounds in tequila: acetaldehyde, higher alcohols (s-butanol, n-propanol, i-butanol, n-butanol, i-amyl, and n-amyl), methanol, esters (ethyl acetate and ethyl lactate) and furfural were determined by gas chromatography-FID and were quantified as mg/100 ml of anhydrous ethanol according to NMX-V-005-NORMEX-2014, using 2-pentanol as internal standard.

Statistical analysis

The data obtained were analyzed by using Statgraphics Centurion XVI software (StatPoint Technologies, USA). Analysis of variance (ANOVA) and Fisher's multiple range test of minimal significant differences (LSD), allowed evidencing variables that showed significant differences (p-value = 0.05).

RESULTS AND DISCUSSION

The values that were obtained for the parameter % alcohol volume in both types of tequila were found within the range allowed by NOM-006-SFCI-2012 (35-55 % alcohol volume). For 100% of the analyzed samples of Crystalline tequila, minimum values of 35% alcohol volume were observed (Figure 1 A). These results coincide with those reported by Hernández (2017) and Diez (2015), in which they describe that Crystalline tequila has a mild profile, that is, a low alcohol content because this drink is strongly inclined to consumption by women (Micallef, 2017). On the other hand, only 8.3% of the total tequilas that were analyzed have an alcohol content higher than 40% of alcohol volume. The most frequent % alcohol volume value in the market was 38%.

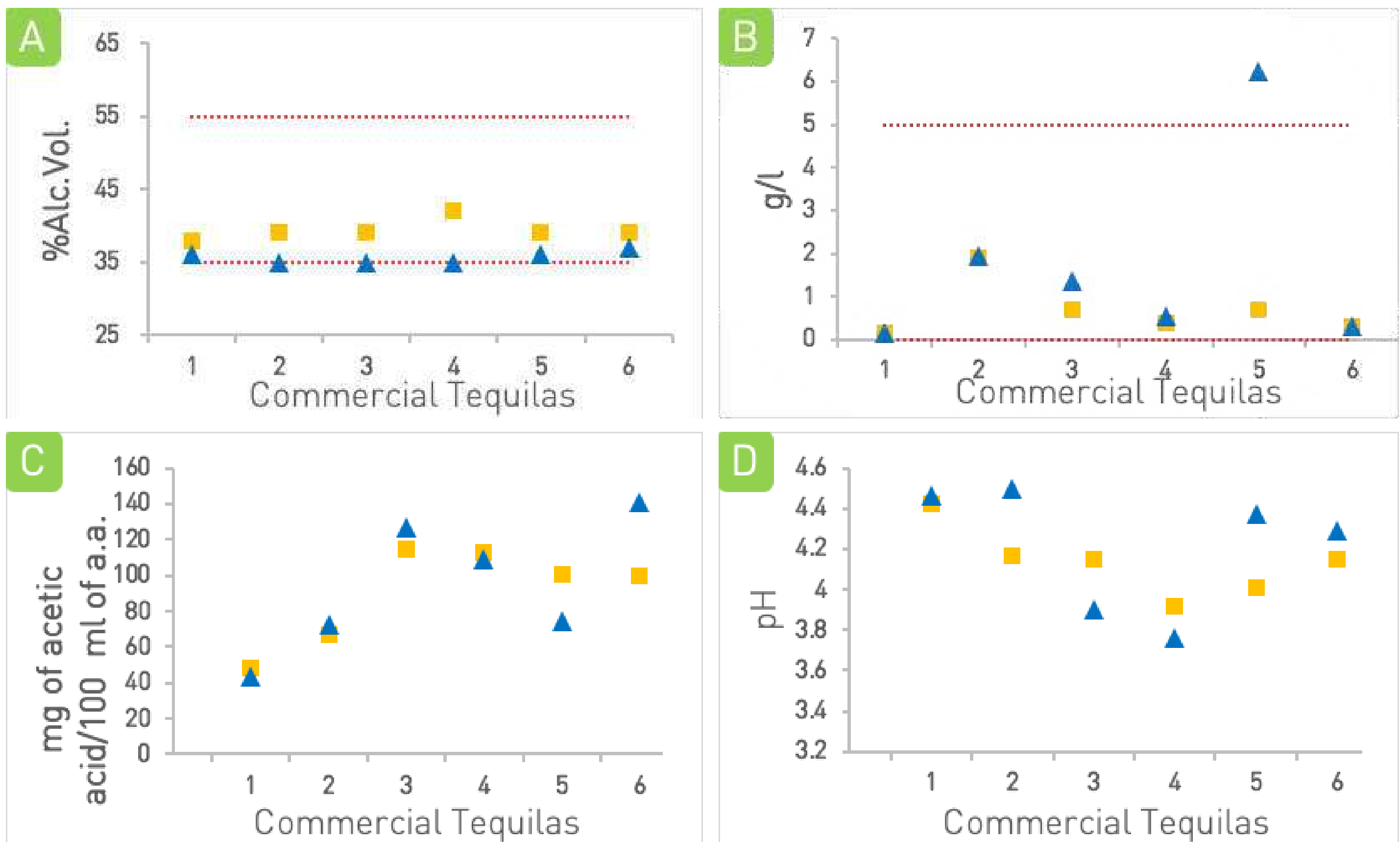

Figure 1. Results of physicochemical parameters: % Alcohol Volume (A), dry extract (B), total acidity (C), and pH (C). Dotted red lines indicate the maximum and minimum values established by the NOM-006-SFCI-2012.

Crystalline tequilas **Extra Aged tequilas.**

100% of the Extra Aged tequilas that were analyzed comply with the values established by NOM-006-SFCI-2012 for the dry extract (0-5 g/L). As previously mentioned, Crystalline tequilas are not yet officially recognized as a different type of tequila by CRT; these are identified in terms of their aging as Aged, Extra Aged, or Ultra Aged (Micallef, 2017). In other terms, the parameters with which Crystalline tequila must comply are those established for the Extra Aged that gave rise to it. In this way, 16.66% of the Crystalline Tequilas analyzed exceeded the amount of dry extract allowed by NOM-006-SFCI-2012 with 6.22 ± 0.5 corresponding to sample T5 (Figure 1 B). To obtain Crystalline tequila, the Extra Aged tequila passes through a filtering process by which, in addition to color, many other compounds are removed. Thus it is expected that the values for the dry extract in Crystalline tequilas are less or equal to the Extra Aged tequila that gave rise to it. Contrary to what was observed in this analysis, only 33% of the samples that were analyzed coincide with the values obtained for both types of tequila of the same brand, being the samples T6 and T1.

For total acidity, significant differences were found in the obtained data, comprising values of 43.94 ± 4.78 to 140.87 ± 4.63 mg of acetic acid/100ml of anhydrous alcohol (Figure 1 C). The highest values for total acidity belong to samples of Crystalline tequila being these of 127.20 ± 4.86 and 140.87 ± 4.63 mg of acetic acid/100ml of anhydrous alcohol corresponding to samples T3 and T6 respectively. The values of total acidity were like those reported by González-Manzano et al. (2009) for the brandy subject to resting in barrels. As previously mentioned, NOM-006-SFCI-2012 does not establish a range for the total acidity parameter in the case of tequila. However, for the case of other distilled beverages such as the Bacanora, a maximum allowed value of 170 mg of acetic acid/100 ml of anhydrous alcohol was established (NOM-168-SCFI-2004). Setting this limit as a reference, all the samples that were analyzed are below the established value.

pH and color parameters are not established in NOM-006-SFCI-2012; however, some tequila Companies measure it as part of the internal quality control of their products. The variation of pH can modify the flavor and stability of the drink (López-Ramírez et al. 2013). The pH values of the Extra Aged Tequilas (Figure 1 D) were like those reported by González-Manzano et al. (2009) for brandy resting in barrel. López-Ramírez et al. (2013) reports a pH range of 3.18 to 4.20, similar values to those obtained in this investigation for both types of tequila.

For color, the lower luminosity values, but higher in the transition from the yellow-blue chromaticity (b*), objectively showed the differences between the types of tequila evaluated. The aged tequilas acquire coloration during their resting in barrels while the crystallines have had their color removed. Table 1 indicates the values of ΔE for samples of commercial tequilas; the lower values indicate less coloration in the sample. In the case of Crystalline tequilas, no statistically significant differences were observed for the values of ΔE; this expresses that the color variations between the samples are imperceptible. In contrast to the Extra Aged tequilas, in which the value of ΔE showed significant differences between all the brands. In other words, there are notable variations of color between the different brands of Extra Aged tequilas. The change in color was previously attributed to the extraction of wood compounds during aging for Whiskey (Conner et al. 2003).

Table 1. Color evaluation of samples of commercial Extra Aged and Crystalline tequilas.

Sample	L*	a*	b*	ΔE	Sample	L*	a*	a*	ΔE
Extra Aged Tequila					Extra Aged Tequila				
T1A	93.43	-0.48	21.55	22.52	T1C	99.98	-0.18	0.97	0.98
T2A	75.10	12.88	67.72	73.28	T2C	99.91	-0.21	1.19	1.20
T3A	91.46	-1.80	32.77	33.90	T3C	99.75	-0.23	1.72	1.74
T4A	92.21	-1.46	34.21	35.11	T4C	99.92	-0.37	1.65	1.68
T5A	92.10	-1.76	37.70	38.55	T5C	97.30	-0.15	1.02	2.89
T6A	92.91	-0.98	26.48	27.42	T6C	99.68	-0.76	3.17	3.27

L*, a*, and b* are the chromatic coordinates. L* indicates luminosity. A positive value of a* indicates red, and a negative value indicates green. A positive value of b* indicates yellow, and a negative value indicates blue. A: Aged tequila, C: Crystalline tequila.

Among the different types of tequila, significant differences were observed for all the major compounds measured (Figure 2). However, all values were found within the specifications of NOM-006-SCFI-2012. In samples 2, 5, and 6 an increase in the concentration of higher alcohols in Crystalline tequila was observed concerning to Extra Aged. For methanol, an increase was observed in all samples of Crystalline tequila, except for sample 6. For the aldehyde, an increase was observed in Crystalline tequila in samples 4, 5 and 6. For the case of esters, an increase in Crystalline tequila for samples 2, 3 and 6. The furfural increase in samples of Crystalline tequila 3, 4, 5 and 6. For all the above mentioned, it is expected that the values obtained for the parameters measured between both tequilas of the same brand are similar or failing to observe a lower concentration in the lenses due to the filtration process to which they are subjected to obtain. In the opposite case to the one observed in this investigation where 100% of the Crystalline tequilas analyzed had a higher concentration for at least one parameter measured. Highlighting samples 5 and 6, these being the ones that increased the concentration in four parameters of the five measured.

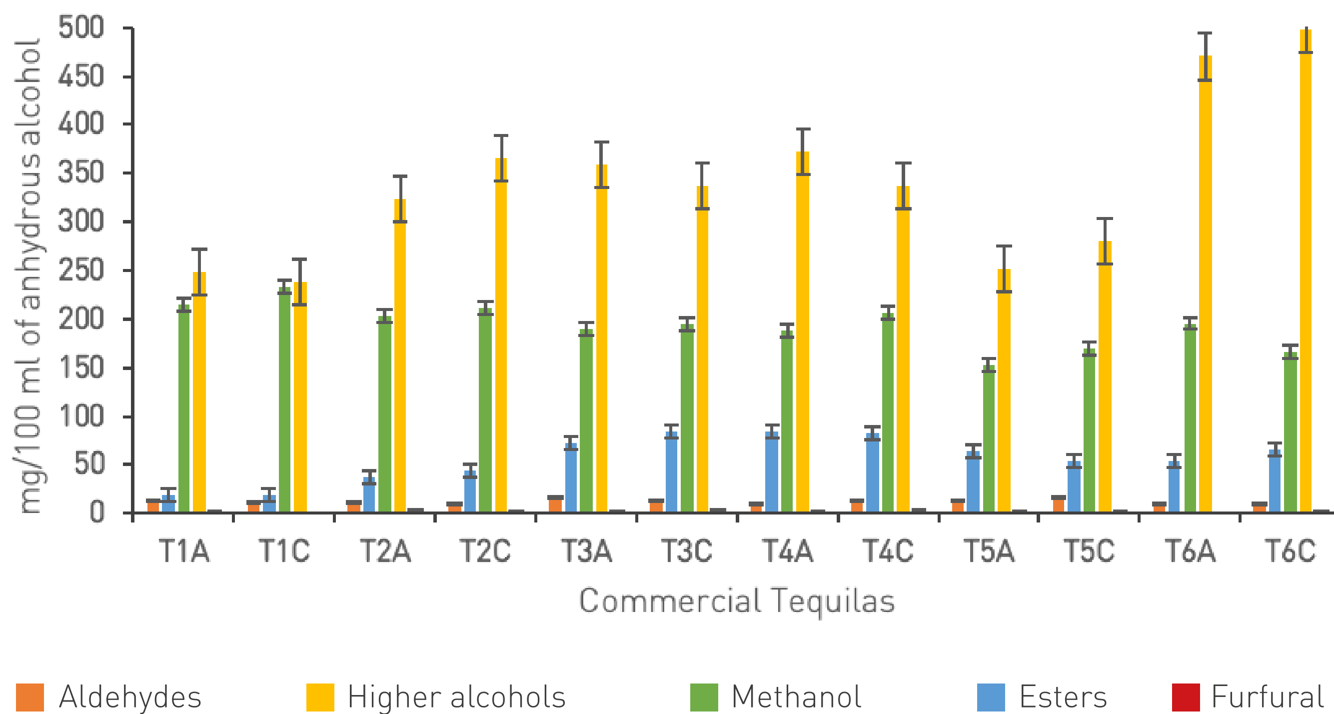

Figure 2. Results of main volatile compounds in commercial tequilas, Extra Aged and Crystalline.

CONCLUSION

The results obtained for physicochemical analysis showed significant differences between both types of tequila mainly for total acidity, dry extract, and color. Some physicochemical characteristics for crystalline tequila are consistent with the Official Mexican Standard NOM-006-SFCI-2012 for aged tequila. This finding demonstrates the need for broader studies to characterize crystalline tequila, and it is expected that the parameters established in this work can serve as a reference for inclusion in a future update of the corresponding Mexican Standard.

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Thermo-tolerance, osmo-tolerance and ethanol tolerance of *Pichia kluyveri* strains isolated from traditional fermentation processes.

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ABSTRACT

Non-*Saccharomyces* yeasts have been on the spotlight lately, due to their ability for producing several metabolites, such as, enzymes and aromatic compounds, that may be interesting in different industrial processes. It has been reported that *Pichia kluyveri* presents the ability to produce aromatic compounds like terpenes and esters, which may improve product quality in food and beverages like cocoa, coffee, wine, distillates, to mention some; Yet there is little information around this yeast. The aim of the present work is to achieve a physiological characterization, evaluating the thermo-tolerance, osmo-tolerance and the ethanol tolerance of 19 *Pichia kluyveri* strains that were isolated from three different traditional fermentation processes, Cocoa, Mezcal and Tejuino. This to have a better understanding of this yeast species. For the thermo-tolerance assay, the strains were grown in YPD agar and incubated at different temperatures: 20, 30, 40 and 50 °C. For osmo-tolerance assays, the strains were exposed to different concentrations of glucose and fructose, ranging from 200 g/L up to 800 g/L. Regarding to ethanol tolerance, the strains were exposed to different concentrations of ethanol, ranging from 4% to 11% (v/v), and making variations on the carbon source, glucose, fructose, equimolar mixture of glucose-fructose and no sugar added. The physiological characterization showed a wide range of behaviors even in strains that were closely related in terms of fermentation process and region where they were isolated. Interesting information was gathered regarding *Pichia kluyveri*, it was evident that despite of being strains of the same species, environmental differences have a significant impact on their behavior.

Key words: ***Pichia kluyveri*, non-Saccharomyces, Mezcal, Cocoa, Tejuino.**

INTRODUCTION

Yeasts are among the most important groups of microorganisms used in biotechnology. They play a main role in the production of several fermented foods all around the world, such as, bread, dairy and charcuterie (Bhalla and Savitri, 2017). They are fundamental in the production of alcoholic beverages like, beer, wine, cider, sake, distillates, to mention some; The intervention of yeasts in industrial processes is becoming a common practice, some of the different application areas are, bio-fuels, cellular proteins, fodder, enzymes and low molecular weight metabolites (Johnson, 2013). The presence of *Saccharomyces* in some of the most important traditional fermented products may be the reason why there is a large amount of information regarding to this genera; A result of this was that non-*Saccharomyces* species were left out, being even considered contaminants (Jolly et al. 2006;Padilla et al. 2016). Nowadays, non-

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Saccharomyces yeasts have gained importance due to their capability of producing several interesting metabolites, mainly enzymes and aromatic compounds (Maturano et al. 2015), making them a tool to give added value to alcoholic beverages such as beer, wine and distillates (Padilla et al. 2016). The tendency in alcoholic beverages seems to be that monoculture will be eventually be displaced by mixed cultures using *Saccharomyces* and non-*Saccharomyces* yeasts, looking to achieve more complex products and consequently products of higher quality (Beckner Whitener et al. 2015).

METHODOLOGY

Strains

On Table 1 all the *Pichia kluyveri* strains used in the present study are listed, along with interesting information: the process where they were isolated from, the region and sub-region they are native from and the year in which they were isolated.

Table 1. *Pichia kluyveri* strains used in the present study.

ID Strain	Species	Process	Region	Sub-region	Year
MG1	*P. kluyveri*	Mezcal	Guerrero	Chilpancingo	2002
MG2	*P. kluyveri*	Mezcal	Guerrero	Eduardo Neri	2014
ME1d	*P. kluyveri*	Mezcal	Mexico state	Malinalco	2016
ME2b	*P. kluyveri*	Mezcal	Mexico state	Malinalco	2016
ME3b	*P. kluyveri*	Mezcal	Mexico state	Malinalco	2016
ME1c	*P. kluyveri*	Mezcal	Mexico state	Malinalco	2016
ME4a	*P. kluyveri*	Mezcal	Mexico state	Malinalco	2016
TZB	*P. kluyveri*	Tejuino	Jalisco	Zapopan	2016
KH1b	*P. kluyveri*	Cacao	Chiapas	Tapachula farm 1	2016
KH1a	*P. kluyveri*	Cacao	Chiapas	Tapachula farm 1	2016
KB1h	*P. kluyveri*	Cacao	Chiapas	Tapachula farm 2	2016
KB1c	*P. kluyveri*	Cacao	Chiapas	Tapachula farm 2	2016
KB1f	*P. kluyveri*	Cacao	Chiapas	Tapachula farm 2	2016
KN23	*P. kluyveri*	Cacao	Tabasco	Cunduacan	2009
KB1e	*P. kluyveri*	Cacao	Chiapas	Tapachula farm 2	2016
KB3g	*P. kluyveri*	Cacao	Chiapas	Tapachula farm 2	2016
KB1b	*P. kluyveri*	Cacao	Chiapas	Tapachula farm 2	2016
KB2a	*P. kluyveri*	Cacao	Chiapas	Tapachula farm 2	2016
KB2d	*P. kluyveri*	Cacao	Chiapas	Tapachula farm 2	2016

Media

All the media were composed by a base mixture consisting of, yeast extract 10 g/L, bacteriological peptone 20 g/L and bacteriological agar 15 g/L, and it was labeled as YP, the addition of a carbon source was what made different each of the other media. YPD had the YP base with 20 g/L of glucose. YPF had the YP base with 20 g/L of fructose. YPDF had the YP base with 10 g/L of glucose and 10 g/L of fructose.

Physiological characterization

Thermo-tolerance, osmo-tolerance and ethanol tolerances assays were performed in solid media based on experiments presented by De la Torre-González et al. (2016). For Thermo-tolerance assays, YPD agar was used as culture media, four different temperatures were assayed, 20º, 30ºC, 40ºC and 50ºC. Osmo-tolerance assays were performed with fructose and glucose, the media composition was, yeast extract (10 g/L), peptone (20 g/L), bacteriological agar (15 g/L) and increasing amounts of fructose (200, 400, 600, 650, 700, 800 g/L) and glucose (200, 400, 500, 600, 700, 800 g/L). Ethanol tolerance assays were performed with glucose (20 g/L), fructose (20 g/L), an equimolar mix of glucose and fructose (10-10 g/L) and no sugar added, the media composition was, yeast extract (10 g/L), peptone (20 g/L), bacteriological agar (15 g/L), sugar (20 g/L) and increasing amounts of ethanol (4% - 11%). The strains were activated in YPD broth, culture conditions were, 30ºC, 200 rpm, 18 hours. O.D. was adjusted to 0.5 (Sample A), cells were counted on neubauer chamber, then 4 dilutions were obtained, 10, 10^2, 10^3 and 10^4. 10μL of each dilution were used for each treatment.

RESULTS AND DISCUSSION

Figure 1 left shows the results of the thermo-tolerance assay applied to the 19 Pichia kluyveri strains, it can be observed that all the strains grew at 20ºC and 30ºC, however, at 40ºC only three strains presented growth, ME1c, ME3b and ME4a, it should be noted that these three strains belong to the ones isolated from Mezcal process. No strains presented growth at 50ºC.

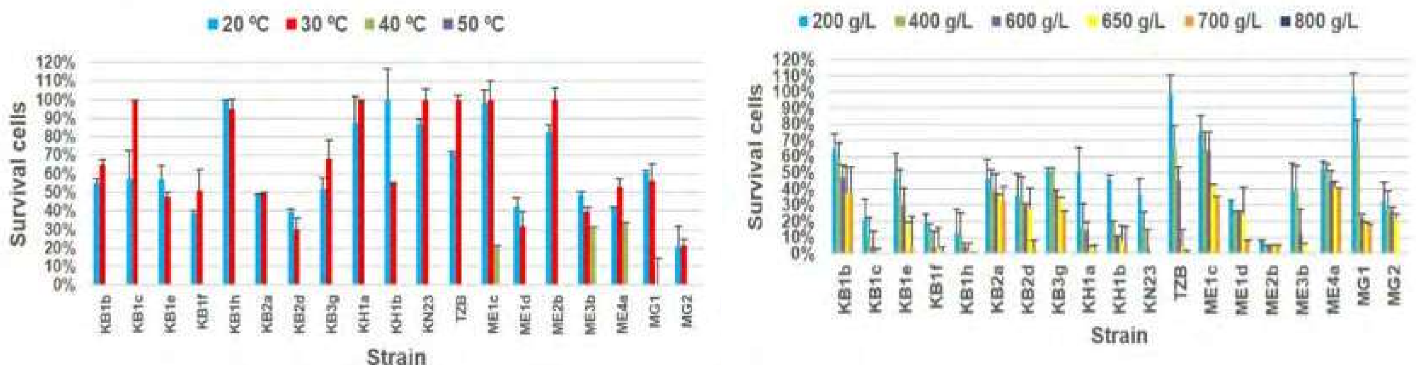

Figure 1. Left: Thermo-tolerance assay results in YPD agar at different temperatures (20ºC, 30ºC, 40ºC and 50ºC) with the 19 *Pichia kluyveri* strains. Right: Figure 2. Osmo-tolerance assay results with increasing concentrations of glucose (200 g/L, 400 g/L, 600 g/L, 650 g/L, 700 g/L and 800 g/L) with the 19 *Pichia kluyveri* strains.

Osmo-tolerance to glucose results are shown in Figure 1 Right, it is observed that the effect of glucose concentration on yeast cells is inversely proportional, as sugar concentration increases, cells survival decreases. Most of the strains, presented growth at glucose concentrations of 650 g/L, nine out of 19 strains presented growth at 700 g/L (some of them little concentration) and none of the strains presented growth at 800 g/L. Osmo-tolerance to fructose results are shown in Figure 2 Left, it is observed that the effect of fructose concentration on yeast cells like in the case of glucose, is inversely proportional, as sugar concentration increases, cells survival decreases.

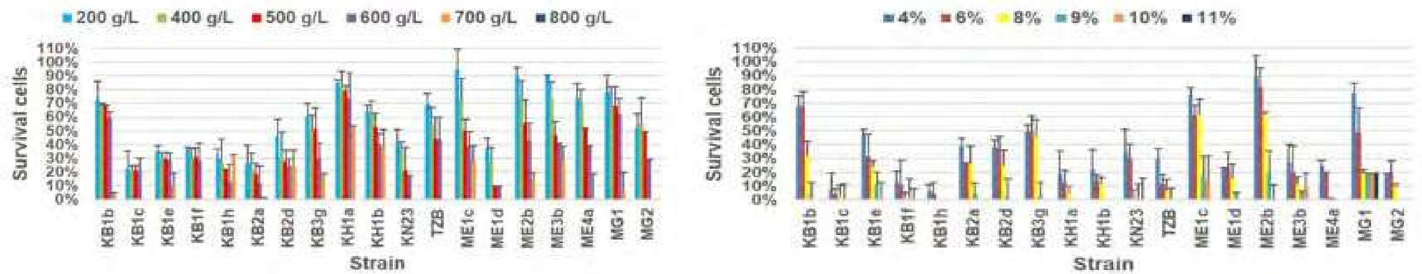

Figure 2. Left: Osmo-tolerance assay with increasing concentrations of fructose (200 g/L, 400 g/L, 500 g/L, 600 g/L, 700 g/L and 800 g/L) with the 19 *Pichia kluyveri* strains. Right: Ethanol tolerance assay in YPD media with increasing concentrations of ethanol (4%, 6%, 8%, 9%, 10% and 11%) with the 19 *Pichia kluyveri* strains.

Most of the strains, presented growth at fructose concentrations of 600 g/L, 11 out of 19 strains presented growth at 700 g/L (some of them little concentration) and none of the strains presented growth at 800 g/L. In comparison with glucose osmo-tolerance assay (Figure 1 Right) almost all the strains isolated from Mezcal and Cocoa, presented a higher growth with fructose as the carbon source and more strains were able to grow at a concentration of 700 g/L. This behavior may be due to the composition of the fermentation media of each of these processes, Mezcal is the product of the fermentation of agave juice from different plant varieties, it has been reported that agave juice is composed mainly by fructose (Srinivasan and Bhatia, 1952). Cocoa beans are fermented before separating the pulp that covers them, this pulp works as the fermentation media and its composed by a mixture of sucrose, glucose and fructose, being fructose predominant over glucose (Lefeber et al. 2011). Regarding to the strain isolated from Tejuino, it can be observed that it presented a higher growth in glucose, again this may be explained by the composition of the fermentation media, Tejuino is a traditional beverage product of fermentation of a mixture of maize, molasses and water, maize is composed by glucose. Ethanol tolerance assays are presented from Figure 2 Right to Figure 4. Figure 2 Right shows the results of ethanol tolerance assay in YPD media for all the *Pichia kluyveri* strains exposing them to increasing concentrations of ethanol, 4%, 6%, 8%, 9%, 10% and 11%. The results of ethanol tolerance assay in YPF media with increasing concentrations of ethanol (4%, 6%, 7%, 8%, 9%, 10% and 11%) is presented in Figure 3 Left

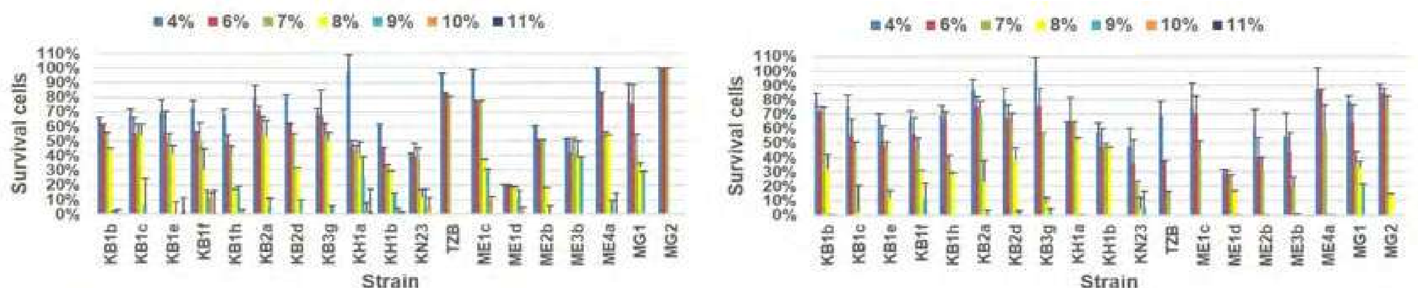

Figure 3. Left: Ethanol tolerance assay in YPF media with increasing concentrations of ethanol (4%, 6%, 7%, 8%, 9%, 10% and 11%) with the 19 *Pichia kluyveri* strains. Right: Ethanol tolerance assay in YPDF media with increasing concentrations of ethanol (4%, 6%, 7%, 8%, 9%, 10% and 11%) with the 19 *Pichia kluyveri* strains.

Analyzing Figure 2 Right and Figure 3 Left, it can be observed a similar effect to the one seen in osmo-tolerance, strains exposed to increasing concentrations of ethanol seem to tolerate it better when fructose is the carbon source rather than in presence of glucose. As it was discussed before, this may

be because of the composition of the substrates were these strains were isolated from. The results of ethanol tolerance assay in YPDF media with increasing concentrations of ethanol (4%, 6%, 7%, 8%, 9%, 10% and 11%) is presented in Figure 3 Right. The results of ethanol tolerance assay in YP media with increasing concentrations of ethanol (4%, 6%, 7%, 8%, 9%, 10% and 11%) is presented in Figure 4.

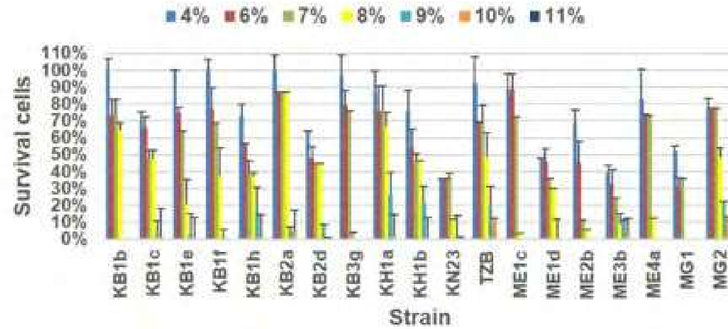

Figure 4. Ethanol tolerance assay in YP media with increasing concentrations of ethanol (4%, 6%, 7%, 8%, 9%, 10% and 11%) with the 19 *Pichia kluyveri* strains.

The results for ethanol tolerance in YPDF (figure 3 Right) and YP media Figure 4 show high cell growth under low ethanol concentrations, nevertheless, they tolerated up to 8% ethanol concentration, while results in YPD (Figure 2 Right) and YPF (Figure 3 Left) show growth in concentrations between 9% and 10%. Two Box-Plots were generated (data not shown) with all the data generated, one with osmo-tolerance results and one with ethanol tolerance results. It could be observed that ethanol tolerance had a greater impact on cell growth in comparison with osmo-tolerance. Cocoa strains showed a higher tolerance to ethanol in comparison with Mezcal strains, only ME1d from Mezcal group shows a greater ethanol tolerance. Most of the strains showed growth around 8% ethanol. A third Box-Plot (data not shown) was generated with the results of osmo-tolerance and ethanol tolerance assays is presented. It can be observed that strains KB1e, KH1a, KH1b and ME1d seem to be the most tolerant to the stress conditions evaluated. These results are useful since they can give a perspective of potential processes in which strains can be used as is mentioned by (De la Torre-González et al. 2016), especially if it is under stress conditions, for example, when it is exposed to high ethanol concentrations which as stated by (Pina et al. 2004) may be toxic for microorganisms at certain concentrations or in specific processes, like the one of "Vino cotto" studied by (Tofalo et al. 2009) where high sugar concentrations which many strains would not tolerate are present in the media.

CONCLUSION

It is interesting that yeast belonging to the same species and even to the same process and region they may present different response to external factors such as the carbon source or a toxic agent like ethanol.

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Yeast population associated with mezcal fermentation.

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ABSTRACT

Mezcal is a distilled alcoholic beverage produced in some regions of Mexico. To understand spontaneous fermentation, it´s necessary to identify the yeasts present in the mezcal musts from *Agave durangensis*. The sequence analysis of the amplified region 26s rDNA gene was used for the identification of yeasts. 26 isolates were identified in the traditional mezcal fermentation in Durango, Mexico. The identified yeasts were: *Saccharomyces cerevisiae, Kluyveromyces marxianus, Pichia kluyveri, Torulaspora delbrueckii* and *Hanseniaspora guilliermondii*. Although a high diversity of microorganisms was found at the beginning of the fermentation, *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* were the only yeasts species present at the end of fermentation..

Key words: Yeast, fermentation, mezcal, *Saccharomyces*, spontaneous fermentation.

INTRODUCTION

Mezcal is a traditional distilled beverage produced from the fermented juices of the cooked agave plant core in some regions of Mexico (Escalante-Minakata et al. 2008). Species of *Agave* plants such as *Agave angustifolia* in Oaxaca, *A. salmiana* in San Luis Potosi and Zacatecas, *A. potatorum* in Guerrero and *A. durangensis* is used in Durango, are used as raw materials (De León-Rodríguez et al. 2008). The process begins with the harvesting of the agave after 8 years of cultivation (Cedeño, 1995). The mezcal production process includes cooking in stone ovens to hydrolyze the inulin into fructose (De León-Rodríguez et al. 2008). At this stage, the polysaccharides are hydrolyzed to fructose syrup and some other organic compounds are generated by the Maillard reaction (Cedeño, 1995; De León-Rodríguez et al. 2008). The resulting juice is fermented through a spontaneous process by its own microorganisms and then distilled (Valdez et al. 2011).

This beverage involves a complex population of microorganisms during the fermentation, where yeasts are responsible for the production of various chemical compounds as alcohols with three or more carbons and ethyl esters, which can affect the characteristics of the final product (Cedeño, 1995; Valdez et al. 2011).

Studies have demonstrated that several species non-*Saccharomyces* are predominant at the beginning of spontaneous wine fermentations (Fleet, 2003), that appears to influence the generation of volatile compounds involved in the aromatic profile of the final product (Escalante-Minakata et al. 2008). The aim of this research was to study yeasts involved in traditional mezcal fermentation in Durango, Mexico.

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METHODOLOGY

Sampling, isolation and selection of yeast

Native yeasts were isolated at three times during fermentation stage (beginning, middle and ending) from one mezcal factory located in Nombre de Dios, Durango, México. The sample was diluted in sterile water and plated on YPD agar (Sigma-Aldrich, Darmstadt, Germany) and incubated at 28°C for 5 days. Colonies were selected based on morphology characteristics and isolates were purified by subculture for identification.

DNA extraction

Yeast DNA was extracted according by Wizard® Genomic DNA Purification Kit (PROMEGA, Madison, WI).

Yeast identification

The divergent D1/D2 domain of the LSU rRNA gene was amplified by PCR; each reaction contained 100 ng DNA, 50 mM MgCl2, 1U Taq polymerase, 10 mM deoxynucleoside triphosphate, 5mM of each NL1 (5´GCATATCAATAAGCGGAGGAAAAG) and NL4 (5´GGTCCGTGTTTCAAGACGG) primers (Kurtzman and Robnett, 1998). Amplification was performed for 35 cycles of denaturation at 94°C for 30 s, annealing at 61°C for 30 s, extension at 72°C for 1 min. Visualization of the amplified DNA was performed by electrophoresis in 1% agarose in 1X TAE (0.04M Tris-acetate, 0.001 M EDTA pH 8.0) and staining with ethidium bromide. The amplified fragment was purified with BigDye® XTerminator™ Kit v3.1 (Applied Biosystems, Foster City, CA). All sequences were obtained by ABI PRISM 3130 Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA). Aligment and edition were done with BioEdit v7.2.5 program (Carlsbad, CA). Edited sequences were aligned in the GenBank database using BLASTn program (Bethesda, MD).

RESULTS AND DISCUSSION

26 isolates were obtained from the different times during fermentation (beginning, middle and ending) which were identified as five different yeast species: *Saccharomyces cerevisiae* (11), *Kluyveromyces marxianus* (10), *Torulaspora delbrueckii* (1), *Hanseniaspora guilliermondii* (1) and *Pichia kluyveri* (3). *S. cerevisiae* was the dominant species, representing a 42.3% of the total yeast isolates in the distillery. This observation is concordant with previous studies on fermentations of agave in the region, where dominance of this yeast has been observed (Escalante-Minakata et al. 2008; Páez-Lerma et al. 2013). After *S. cerevisiae*, the next prevailing species is *K. marxianus*. The other yeast species were isolated only occasionally. Páez-Lerma et al. (2013) identified yeast species in a traditional mezcal fermentation process in Durango, finding *Saccharomyces, Kluyveromyces, Torulaspora, Candida, Pichia* and *Hanseniaspora*. We found similarities in some species. Table 1 shows the evolution of each identified yeast species, observing that some genera maintained throughout the fermentation. Even, there are yeasts only present in one fermentation stage (beginning, middle or ending).

Table 1. Characterization of yeast population.

Identified specie	Fermentation stage		
	Beginning	Middle	End
S. cerevisiae	✓	✓	✓
K. marxianus	✓	✓	✓
T. delbrueckii		✓	
H. guilliermondii	✓		
P. kluyveri	✓		

CONCLUSION

Fermentation process showed a high yeast diversity, probably because the factory maintains a traditional fermentation process. A large number of species non-*Saccharomyces* have been investigated to improve the sensory characteristics and attributes of the final product, which makes it essential to know the native yeasts that can be used as inoculant.

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Biological effects of Agave fructans and other by-products

Characterization of fructans extracted from *Agave mapisaga* leaves.

Plascencia, A., Gutiérrez-Mora, A., Rodríguez-Domínguez, M., Castañeda-Nava, J., Gallardo-Valdez, J. and Camacho-Ruíz, R.M.[1]*

ABSTRACT

Agave fructans are soluble fibers useful in human and animal health. Commercial agave fructans are produced from *Agave tequilana* stems, nevertheless, their recovery from agave leaves is interesting to be explored because leaves are a non exploited residue in tequila and mezcal industries. In the other hand, the characterization of fructans from agave species different to *Agave tequilana* is needed. *Agave mapisaga* Trel. is located predominantly in Michoacan, Morelos, Puebla and Zacatecas. This maguey is used for aguamiel, pulque agave syrup and *mixiote* production. Fructo-oligosaccharides (FOS) has been quantified in aguamiel extracted from *Agave mapisaga.* Nevertheless, there are no reports on agave fructans characterization from this agave. The extraction and molecular weight distribution of fructans extracted from *Agave mapisaga* leaves was performed. Water soluble carbohydrates were extracted from the bottom *Agave mapisaga* leaves. Molecular weight distribution of fructans were determined by HPLC-SEC. Soluble carbohydrates extracted from leaves were quantified, the 1.14% of the total weight were sugars. The extract was spray dried reaching drying efficiencies of 60%. The powder obtained contains 25% of mono and disaccharides and 75% of fructans. The fructans were distributed in low molecular weight or FOS (68%) and high molecular weight fructans (32%). Relative abundance of fructans according to polymerization degree was analyzed, low molecular weight fructans were most abundant than high molecular weight. The average molecular weight in number was 2214 g/mol. The feasibility to extract fructans from *Agave mapisaga* leaves was evidenced. Agave fructans extracted were mainly low molecular weight fructans with an average polymerization degree of 14 which are lower compared to commercial agave fructans extracted from stems of *Agave tequilana.*

Key words: Agave fructans, *Agave mapisaga,* Molecular weight distribution.

INTRODUCTION

Agave fructans are soluble fibers containing principally branched polymers known as agavins, composed of fructose units linked by β(2-1) and β(2-6) bonds (Lopez et al. 2003). Recent research has positioned agave fructans as an important source of soluble fiber with effect on health. The effect of agave fructans on mineral absorption, promotion of gut bacteria growth, activation of immune system, body weight reduction has been studied at *in-vitro* or pre-clinic stage (Castillo et al. 2018; Garcia-Vieyra et al. 2014; Moreno-Vilet et al. 2014). As natural polymer, agave fructans are composed of molecules that can be distributed according to their molecular weight, ranging from 180 g/mol to 6500 g/mol. Low molecular weight fructans have been studied, the results suggest relevant effects in health. The reductions in weight gain, liver steatosis and hyperglycemia in obese mice were attributed to low molecular weight

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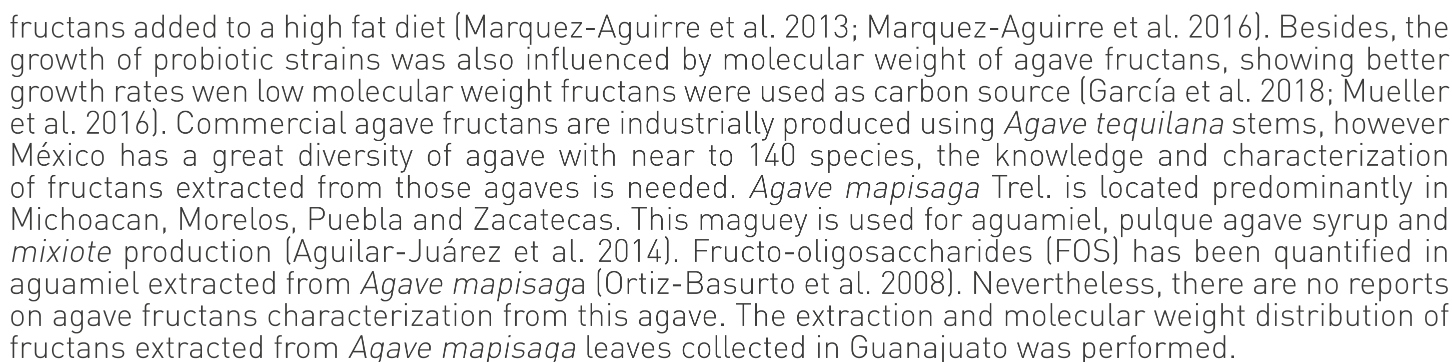

fructans added to a high fat diet (Marquez-Aguirre et al. 2013; Marquez-Aguirre et al. 2016). Besides, the growth of probiotic strains was also influenced by molecular weight of agave fructans, showing better growth rates wen low molecular weight fructans were used as carbon source (García et al. 2018; Mueller et al. 2016). Commercial agave fructans are industrially produced using *Agave tequilana* stems, however México has a great diversity of agave with near to 140 species, the knowledge and characterization of fructans extracted from those agaves is needed. *Agave mapisaga* Trel. is located predominantly in Michoacan, Morelos, Puebla and Zacatecas. This maguey is used for aguamiel, pulque agave syrup and *mixiote* production (Aguilar-Juárez et al. 2014). Fructo-oligosaccharides (FOS) has been quantified in aguamiel extracted from *Agave mapisag*a (Ortiz-Basurto et al. 2008). Nevertheless, there are no reports on agave fructans characterization from this agave. The extraction and molecular weight distribution of fructans extracted from *Agave mapisaga* leaves collected in Guanajuato was performed.

METHODOLOGY

Water soluble carbohydrates extraction

Leaves were collected from *Agave mapisaga* plants located on Ocampo, Guanajuato (N 21° 40' 44.2''; W 101° 29' 40.8''; Altitude 2266 m a. s. l.). Water soluble carbohydrates were extracted from the bottom of leaves using a hydraulic press, agave juice obtained was subjected to a thermal shock at 80°C by one hour and clarified by centrifugation at 4,000 rpm by 10 minutes at 4°C. Extracts were filtrated, spray dried and stored until their analysis.

Molecular weight distribution of agave fructans

Molecular weight distribution of fructans extracted were determined by High Performance Size Exclusion Chromatography (HPLC-SEC). Samples solutions at 25 g/L were prepared by triplicate and filtered by 0.45 µm. An Ultrahydrogel DP Water column coupled to a Waters chromatograph with refractive index detector was used. Distilled water was used as mobile phase at 0.36 mL/min at 61.7°C (column) and 50°C (detector). Calibration curve was performed with standards ranging from 180 to 12000 Da. The following parameters were calculated using the curve: weight average molecular weight (Mw), number average molecular weight (Mn), weight average degree of polymerization (DPw), number average degree of polymerization (DPn) and dispersity index (Moreno-Vilet et al. 2017).

RESULTS AND DISCUSSION

Agave leaves were used by Mexican communities for the production of fibers, in the gastronomy and for medicinal purposes, nevertheless recently agave leaves are studied for the recovery of fermentable sugars to bioethanol production (Avila-Gaxiola et al. 2018). The studies about extraction of agave fructans from the leaves are incipient, and in the case of *Agave mapisaga* there are no reports on the leaves fructans characterization.

Agave mapisaga leaves were sampled in Ocampo a municipality of Guanajuato state (Figure 1), in this locality the agave is used as living fence in a *milpa* field cultivating corn, bean and pumpkin.

Figure 1. Samples were collected in the locality of Ocampo, Guanajuato.

Soluble carbohydrates extracted from *Agave mapisaga* leaves (Figure 2) were quantified, the 1.14% of the total leave weight were sugars. The extract was spray dried reaching drying efficiencies of 60%. The powder obtained contains 25% of mono and disaccharides and 75% of fructans. The fructans were distributed in low molecular weight or FOS (68%) and high molecular weight fructans (32%) (Table 1). Commercial agave fructans, extracted from *Agave tequilana* stems, have different content of low and high molecular weight fructans, showing principally high molecular weight fructans 46-66% (Alvarado et al. 2014). Because their effects on health, described previously, low molecular weight agave fructans are of great interest in the industry.

Figure 2. ***Agave mapisaga*****, leaves were cut and the bottom of the leaves were used for carbohydrates extraction.**

Relative abundance of fructans according to polymerization degree was analyzed (Figure 3) evidencing that low molecular weight fructans (DP between 4 and 6) were most abundant than high molecular weight fructans (DP between 28 and 40). The average molecular weight in number was 2214 g/mol with an average polymerization degree of 14 (Table 1). The mono and disaccharides content was relatively high compared to commercial agave fructans, it is known that synthesis of polisaccharides occurs in the agave leaves and is common to found higher glucose and fructose contents than in the stem. The content and carbohydrate profile of fructans from *Agave mapisaga* leaves is not yet described.

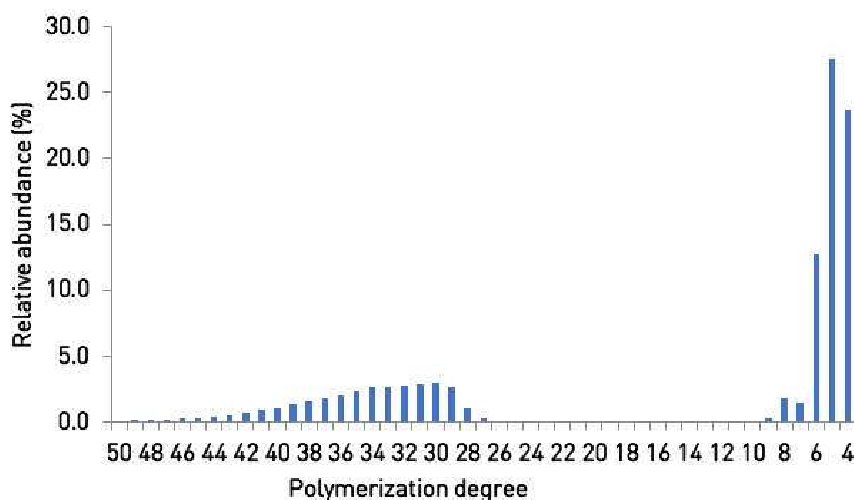

Figure 3. Relative abundance of fructans according to polymerization degree.

Table 1. Molecular weight distribution of agave fructans extracted from *Agave mapisaga* leaves.

Parameter	Content
Mn, g/mol	2214 ± 9
Mw, g/mol	4577 ± 9
Dpn	14 ± 0.5
Dpw	28 ± 0.5
P	2 ± 0.1
DP>10, %	32 ± 0.1
(FOS) 3<DP<10, %	68 ± 0.1
Sucrose, %	1.4 ± 0.5
Glucose, %	9.7 ± 0.5
Fructose, %	14.1 ± 0.5
Soluble sugars, %	1.14 ± 0.1

Mn: number average molecular weight, Mw: weight average molecular weight, DPn: number average degree of polymerization, DPw: weight average degree of polymerization, P: dispersity index, DP: degree of polymerization, FOS: fructooligosaccharides.

CONCLUSION

The feasibility to extract fructans from *Agave mapisaga* leaves was evidenced. Agave fructans extracted were mainly low molecular weight fructans with an average polymerization degree of 14 which are lower compared to commercial agave fructans extracted from stems of *Agave tequilana*. Studies related to economic feasibility are needed.

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Effect of the polymerization degree of agave fructans for the control of *Phytophthora capsici*.

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ABSTRACT

One of the strategies for the control of diseases is through the use of elicitors, whose function is to stimulate the plant to protect from the attack of pathogens. This study shows agave fructans impact as potential elicitors, depending on their structure and polymerization degree, on the control of *P. capsici* in chili pepper. Fructans from *Agave tequilana* var. Cenizo with three degrees of polymerization were used; low polymerization degree (LPD=2-10), intermediate polymerization degree (IPD=10-22), high polymerization degree (HPD=22-60). The fructans were prepared at 0.01%, 0.05%, and 0.1% concentrations and were applied in a foliar spray and in the root of 30 days old serrano chili pepper (cv. Camino Real). Ten days after the application of fructans, plants were inoculated with 10 mL of a zoospore suspension 1x10^6 zoospores mL^{-1} of *Phytophthora capsici*. The most susceptible plants were those in which the elicitor was applied by the foliar way, it is associated with the infection caused by *P. capsici* due to the necrosis that was observed in the *stem* of the infected plants and to the severe defoliation. Furthermore, the plants treated with fructans of low polymerization degree presented the greatest symptoms of the disease compared with the fructans of high polymerization degree. The protective effect was mainly reflected in plants with a high polymerization degree in both foliar and root application. A differential response was also found in root growth and plant height with the application of fructans of different polymerization degrees and the inoculation of *P. capsici*.

Key words: Diseases, elicitors, plant protection, pepper wilt.

INTRODUCTION

Fructans are compounds that are naturally present as storage carbohydrates in many plant species. An important source of fructans are agave plants; where Mexico has the most extensive biodiversity of the *Agave* genus, being the *Agave tequilana* Weber one of the most economically important species due to its use in tequila production (Sánchez-Madrigal et al. 2017; García-Gamboa et al. 2018). Agave fructans consist of a complex mixture of neo-fructans that have different polymerization degrees (PD). Since agave has high levels of fructan, several alternatives are being developed for its potential use, mainly as

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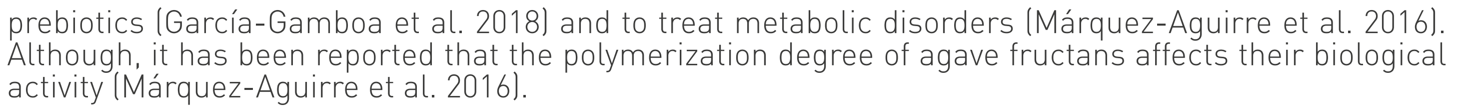

prebiotics (García-Gamboa et al. 2018) and to treat metabolic disorders (Márquez-Aguirre et al. 2016). Although, it has been reported that the polymerization degree of agave fructans affects their biological activity (Márquez-Aguirre et al. 2016).

Carbohydrates such as fructans have been described as elicitors of reactions events during biotic and abiotic stress, this reaction implies a complex communication system necessary for the coordination of metabolism with growth, development, and activation of responses to environmental changes and stresses (Sánchez-Madrigal et al. 2017). Activation of defense reactions related to biotic stress implies the essential step of the microorganism detection by highly conserved molecular patterns called PAMPs (Pathogen Associated Molecular Patterns) or MAMPs (Microbe-Associated Molecular Patterns) (Jones and Dangl, 2006).

The use of fructans in the protection of economically important crops has been poorly reported, generating an effective defense response against pathogens. Fructans as the Burdock fructooligosaccharides, extracted from roots of *Arctium lappa*, applied in tomato crops against *Botrytis cinerea* where it was observed the biosynthesis of volatile organic acids (Sun et al. 2013). Likewise, in cucumber, fructans were used against *Colletotrichum orbiculare* and an increase in the accumulation of lignin and antioxidant enzymes (polyphenol oxidase and superoxide dismutase) was observed (Zhang et al, 2009). Worldwide, Mexico ranks second in the production of chili pepper (*Capsicum annum* L.); in addition, it represents an important source of income for the country and a great source of employment. However, its production is severely affected by the wilt disease caused by *P. capsici*.

In order to reduce the use of agrochemicals for the management of disease, in this work we proceeded to evaluate the exogenous application of agave fructans for use as possible elicitors in protection against wilt disease in pepper plants, through two application methods (foliar and root), as well as the effect of three polymerization degrees and different concentrations.

METHODOLOGY

Reagents

Fructans from *Agave tequilana* var. Cenizo with three degrees of polymerization were used; low polymerization degree (LPD=2-10), intermediate polymerization degree (IPD=10-22), high polymerization degree (HPD=22-60), which were obtained by Aldrete-Herrera (2013) and García-Gamboa et al. (2018). The fructans were prepared at 0.01%, 0.05%, and 0.1% (w/v).

Biological and vegetal material

The *Phytophthora capsici* (CH11) strain was isolated by Sylvia Fernández Pavia of the Universidad Michoacana de San Nicolás de Hidalgo, Mexico.

Seeds of Serrano chili pepper (cv. Camino Real) were disinfected by submerging for 3 min in 0.2 M of sodium hypochlorite (NaClO), then rinsed with distilled water and placed for germination in trays with the sterile substrate Sunshine®-mix 3. The seeds were germinated in an acclimation room at 26 °C with a photoperiod of 16/8 h light/dark. Thirty days after seeding, the plants were transplanted into plastic bags with the sterile substrate

Evaluation of crop protection

At the time of the transplant, the defense induction was performed where the plants were distributed in treatments of twenty blocks with 10 repetitions each, including treatment with control plants (ø). The plants were treated with 10 mL of distilled water (control, ø), and the fructans (0.01%, 0.05%, and 0.1% (w/v) concentrations) were applied in a foliar spray and in the root. Ten days after the first induction, the second application of fructans was made with the same conditions, in parallel one block of plants was inoculated with 10 mL of the zoospores' suspension of *P. capsici* (PHC) The oomycete was grown in corn meal agar for seven days under dark conditions at 26 °C and subsequently cut into 1 cm2 fragments and

placed in a flood with sterile distilled water for 10 days for the generation of sporangia at 24 °C in the dark. After this time, they were left at 4 °C for 2 h for the release of the zoospores. These were quantified in a Neubauer chamber and resuspended in sterile distilled water at a concentration of 1×10^6 zoospores mL^{-1}. A photographic record was taken at the end of the experiment to determine the incidence of the disease through characteristic symptoms of the disease and the height, fresh mass, dry mass of the plants were recorded.

Tetrazolium chloride (TTC) assay

The root viability was considered by a modified triphenyl tetrazolium chloride (TTC) method. The reduction of triphenyl tetrazolium chloride (TTC) to 2,3,5-triphenyl formazan (TTF) was used for the visual determination of root viability. The root samples were taken at the end of the experiment and washed with distilled water. Their surfaces were dried carefully with absorbent paper. The roots were placed in a small beaker and incubated with 10 mL 1:1 (v/v) mixture of 1% TTC solution and 0.l M phosphate buffer (pH 7.0) at 37 °C for 1 h in the dark. The reaction was stopped by the addition of 1 mL of 1 M H_2SO_4. The TTF was evaluated by the change in the color in the roots. A red color indicated the viability of the roots.

RESULTS AND DISCUSSION

In the present study, the effect of fructans on the protection of chili pepper against *P. capsici* was demonstrated, as well as the polymerization degree, the application method and the concentration are decisive to reduce the incidence of the disease. It was observed that the treatments with the high degree of polymerization both in foliar and root application were those that showed a lower incidence of the disease was less than 10% of the infected plants (Figure 1). It was also determined that the most effective concentrations in the foliar application were 0.05 and 0.1% showing an effectiveness of 100% compared to the PHC treatment, and we demonstrated that the survival of the plants is given to induction by fructans because they did not show an effect on *in vitro* growth of *P. capsici* (data not shown). Sun et al. (2013) demonstrated that application of Burdock fructooligosaccharides (BFO) decreased the rate of disease in grape against *Penicillium expansum*, *Colletotrichum musae*, and *Botrytis cinerea*, and the effect of application induced an increase in the expression of *NPR1* gene, which is a key positive regulator of the SA- dependent signaling pathway, the main pathway for the induction of systemic acquired defense. Similar results in the BFO application were shown by Wang et al (2009) in which they found 4-fold increases in PR1 expression levels at 6 hours after application in tobacco infected with tobacco mosaic virus (TMV). In this work, the effect of agave fructans in control of wilt pepper could be comparable to that observed in other crops.

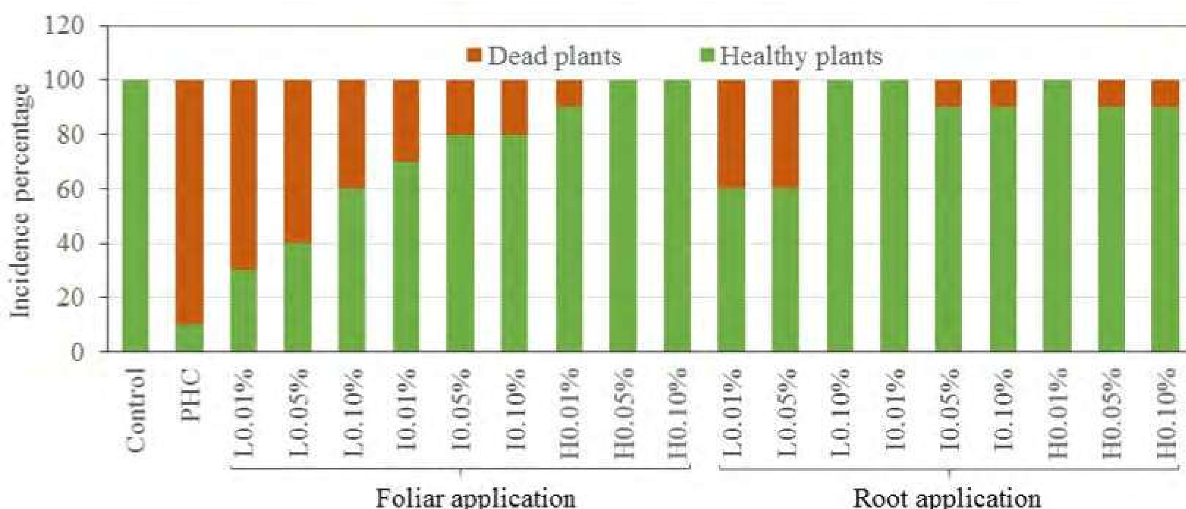

Figure 1. Incidence of the disease in chili pepper plants with agave fructans application. The treatments correspond to PHC: Plants infected with *P. capsici*, L: low polymerization degree, I: intermediate polymerization degree, H: high polymerization degree. All treatments with fructans were inoculated with *P. capsici*.

We also observed a positive effect in reducing tissue damage. The damage generated by *P. capsici* infection was assessed indirectly and qualitatively through the viability of the tissue, a similar effect was observed in the foliar and root application, having greater viability of living tissue compared with the PHC treatment. Along with the viability, we also observed that there was less damage to the root shown by the characteristics of the roots with abundant lateral roots (Figure 2). Burdock fructooligosaccharides induce defense mechanisms through the activation of the salicylate pathway, it has been shown by Zhang et al. (2009) that they induce the activation of PR proteins in addition to antioxidant enzymes such as polyphenol oxidase and superoxide dismutase, besides the accumulation of lignin, in addition, lignin accumulation is directly associated with an effective defense mechanism against pathogens.

In addition to the effect observed in the plant protection, it was observed that fructans increased the dry weight of chili pepper plants (Figure 3), this could be attributed to the effect of signaling and promotion growth. Sugars, in general, can act as signaling molecules and/or as global regulators of gene expression, acting like hormones and translating nutrient to the regulation of growth and the floral transition (Eveland and Jackson, 2011). Sugars such as glucose, sucrose, and trehalose have been shown to be involved in the development of various plant organs such as hypocotyl and cotyledons. In general, it has been shown that hexoses tend to have greater potential as signaling molecules to promote organ development and cell proliferation, whereas sucrose is mostly associated with differentiation and maturation (Koch, 2004). The use of agave fructans in the exogenous application in plants could open the door to new mechanisms of signaling and promotion of growth, therefore in later works, it would be interesting to explore these pathways.

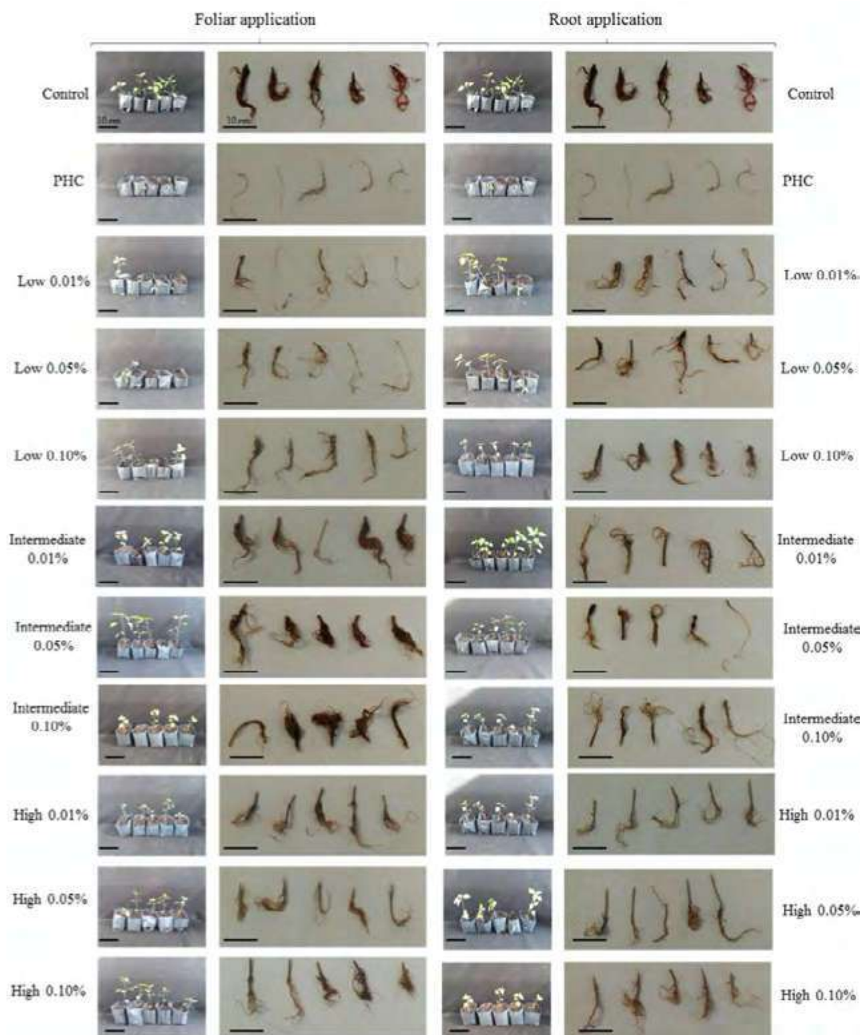

Figure 2. Effect of the agave fructans with low, intermediate and high polymerization degree and method of application on the control of *P. capsici* in chili pepper plants. PHC: Plants infected with *P. capsici*. All treatments with fructans were inoculated with P. capsici.

CONCLUSION

The application of agave fructans has a beneficial effect on the control of *P. capsici*, this is the first study that shows that agave fructans could be an alternative to be used as elicitors against plant diseases. The effect of fructans depends on the method of application, the polymerization degree, and its concentration. A relationship was found between foliar application, the intermediate polymerization degree and the highest concentration evaluated.

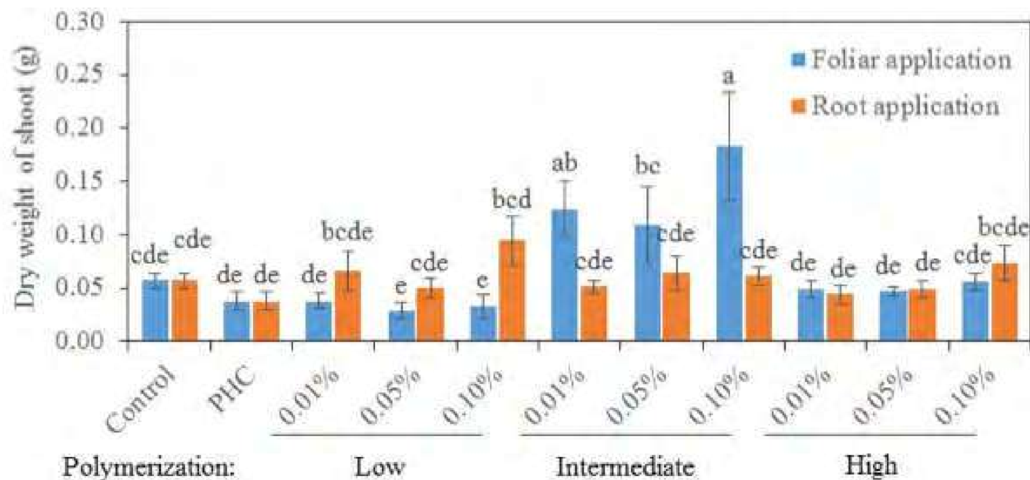

Figure 3. Dry weight of chili pepper shoot as influenced by different polymerization degree and application methods of agave fructans in the control of *P. capsici*. PHC: Plants infected with *P. capsici*. All treatments with fructans were inoculated with *P. capsici*

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Identification and quantification of phytosterols of an ethanolic extract obtained by microwave-assisted extraction.

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ABSTRACT

In this work a microwave-assisted extraction (MAE) method was developed for the extraction of phytosterols in a short time from *Agave angustifolia* Haw compared to the conventional method of maceration which uses hours for its extraction. The phytosterols were extracted from "piña" (leaves and stem) and leaves of *A. angustifolia* by MAE and maceration. The extraction conditions of MAE were: 200 W microwave power; also, ethanol was used as a solvent and four different extraction times (7'30",10',12'30"and 15'); for the maceration technique, ethanol as a solvent was used in the same way, but with 48 h of maceration time. Two phytosterols, β-sitosterol and β-sitosterol β-D-glucoside were determinated from the ethanolic extract by high-performance thin-layer chromatography (HPTLC). The quantification results of β-sitosterol β-D glucoside, obtained in this work showed the best extraction time, which was 7'30" by MAE as in the stem and as in the leaves. Besides, the best part of the plant for the extraction of β-sitosterol β-D-glucoside was the stem, which showed a greater amount of this metabolite in the four extraction times by MAE 7'30",10',12'30" and 15' (178.57, 146.83,121.43 and 118.25 mg / g dry weight respectively) compared to the quantification results obtained in the leaves in the same extraction times by MAE 7'30",10',12'30" and 15' (121.43, 119.84, 110.32 and 107.14 mg / g dry weight respectively). On the other hand, if we compare both extraction techniques, we can conclude, that the best extraction technique for β-sitosterol β-D-glucoside from *A. angustifolia* was MAE, by obtaining a greater quantity of β-sitosterol β-D-glucoside in the four extraction times (minutes), instead of hours, as it was done in the maceration method.

Key words: ***Agave angustifolia*** **Haw, phytosterols, extraction methods, microwave-assisted extraction, high performance thin layer chromatograph.**

INTRODUCTION

Currently, the extraction of natural products takes an important role in manufacturing processes of ingredients derived from natural products. Growing numbers of finished products formula contain botanical ingredients for their functional properties such as coloring, antioxidants, flavor, and pharmacology activity (Chemat and Strube, 2015). Among them we can name the genus Agave which is endemic of America, confirmed by the presence of approximately 200 species, of which 75% are found in Mexico (García-Mendoza, 2007). Evidence of pharmacological activity has been reported for this genus, where anti-inflammatory, antiparasitic, cytotoxic, against cancer and immunomodulatory activity are some of the most important (Lozoya et al. 1982; Argueta et al. 1994).

One example of this genus is the *Agave angustifolia* Haw which has been used in Mexican traditional

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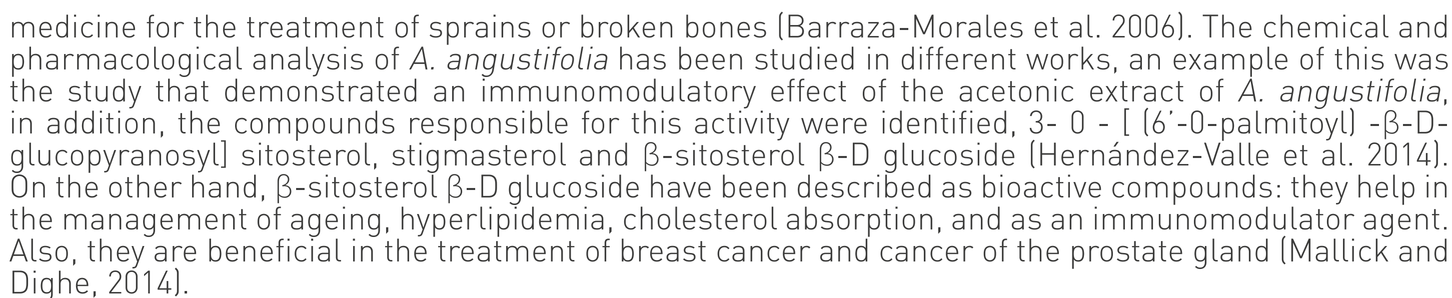

medicine for the treatment of sprains or broken bones (Barraza-Morales et al. 2006). The chemical and pharmacological analysis of *A. angustifolia* has been studied in different works, an example of this was the study that demonstrated an immunomodulatory effect of the acetonic extract of *A. angustifolia*, in addition, the compounds responsible for this activity were identified, 3- O - [(6'-O-palmitoyl) -β-D-glucopyranosyl] sitosterol, stigmasterol and β-sitosterol β-D glucoside (Hernández-Valle et al. 2014). On the other hand, β-sitosterol β-D glucoside have been described as bioactive compounds: they help in the management of ageing, hyperlipidemia, cholesterol absorption, and as an immunomodulator agent. Also, they are beneficial in the treatment of breast cancer and cancer of the prostate gland (Mallick and Dighe, 2014).

Nevertheless, the traditional extraction of phytosterols is done by a simple solvent soaking procedure, which is a laborious task which requires a long-time extraction and also hazardous because it uses toxic solvents (Mandal and Tandey, 2016). Alternatively, novel techniques available are microwave-assisted extraction (MAE), supercritical fluid extraction (SFE), and pressurized solvent extraction (PSE) are a new concept for natural product extraction methodologies (Chan et al. 2011). MAE has received important attention in different fields, among them research, but mainly in the research of medicinal plants, because it offers certain advantages such as: use of green solvents, less extraction time and a high yield compared to the conventional extraction techniques. The microwave is an electromagnetic wave, which consists of an electric field and a magnetic field that oscillate perpendicularly to each other in frequency that varies from 0.3 to 300 GHz. The heating of the microwave energy is applied directly on the molecules by ionic conduction and dipole rotation and, therefore, only the selective and specific materials can be heated depending on their dielectric constant. Different bioactive compounds have been extracted from MAE such as terpenes, alkaloids, glycosides in natural products (Chan et al. 2011; Xiao et al. 2013)

The objective of this research was to compare the potential of MAE for the extraction of phytosterols from *A. angustifolia* with the conventional maceration method.

METHODOLOGY

Plant Material

The "piña" (stems or central head) and the leaves from *A. angustifolia* of 5 years old were collected in the State of Morelos, Mexico, which are the depositories of the records of the identity of biological samples. The "piña" and the leaves were fractionated, after the plant material was placed in an oven at a constant temperature of 40°C for 48 hours. The dry plant material was ground to powder particles.

Microwave-assisted extraction (MAE)

The MAE experiments were performed in CEM, model Discovery System, a continuous microwave power supply system with an output operator selectable by the operator from 0 to 300 watts (+/- 30 watts) programmable in 1-watt increments. The dry plant material of "piña" and leaves was applied separately through the MAE method under the following conditions: 200 W of microwave power, 20: 1 ml / g of liquid / solid, ethanol as a solvent, 70°C of temperature extraction with different extraction times 7'30",10',12'30"and 15'. After all the samples, the solutions were filtered and concentrated at low temperature and reduced pressures.

Maceration method

The dried plant material of "piña" and leaves were macerated separately in ethanol for 48 hours. Finally, the ethanolic extracts were concentrated at low temperature and reduced pressure.

Chromatographic conditions

The sample solutions were spotted in the form of bands of width 6.0 mm with a Camag microlite syringe

on precoated, silica gel glass plate 60F254 (20 cm X 10 cm with 250 µm thinckness; purchased from Merck using a Camag Linomat V. The plates were prewashed in methanol and activated at 120°C for 20 min prior to chromatography. The mobile phase consisted of toluene: ethyl acetate: formic acid 5: 5: 0.5 and 35 ml of mobile phase was used per chromatography [14]. Linear ascending development was carried out in 20 cm X 10 cm twin through glass chamber satured. The optimized chamber saturation time for mobile phase was 20 min at room temperature (22°C approx.) at relative humidity of 48. The length of chromatogram run was 8.5 cm. Subsequent to the scanning, HPTLC plates were dried in a current of air with the help of an air dryer. Densitometric scanning was performed with Camag TLC scanner IV in the reflectance absorbance mode at 200 nm and 540 nm and operated by Vision Cats software (2.3 Camag) with the help of a deuterium and tungsten lamp. Subsequent to the development; HPTLC plate was dipped in 4-hydroxybenzaldehyde reagent followed by drying it in oven al 110°C for 5 min. The calibration curve for standard solution consisted of 5 different volumes (0.5, 1.0, 1.5 2.0 and 3.0 µL). For β-sitosterol β-D-glucoside determination on the plate was applied 4.0 µL of extracts obtained by MAE and maceration. The stock solution of β-sitosterol β-D-glucoside of 250.00 µg / ml was dissolved in methanol and for the experimental samples 5 mg of each ethanol extract of MAE and maceration were dissolved in 1 ml of methanol. The Evaluation was carried out by comparing peak area with linear regression.

RESULTS AND DISCUSSION

Figure 1 confirms the presence of β-sitosterol β-D-glucoside and a fraction of β-sitosterol in the ethanolic extracts obtained from the stem and leaves by MAE, obtaining the same Rf value of the references for β-sitosterol β-D-glucoside had a Rf 0.11 and the fraction of β-sitosterol presented a Rf 0.67.

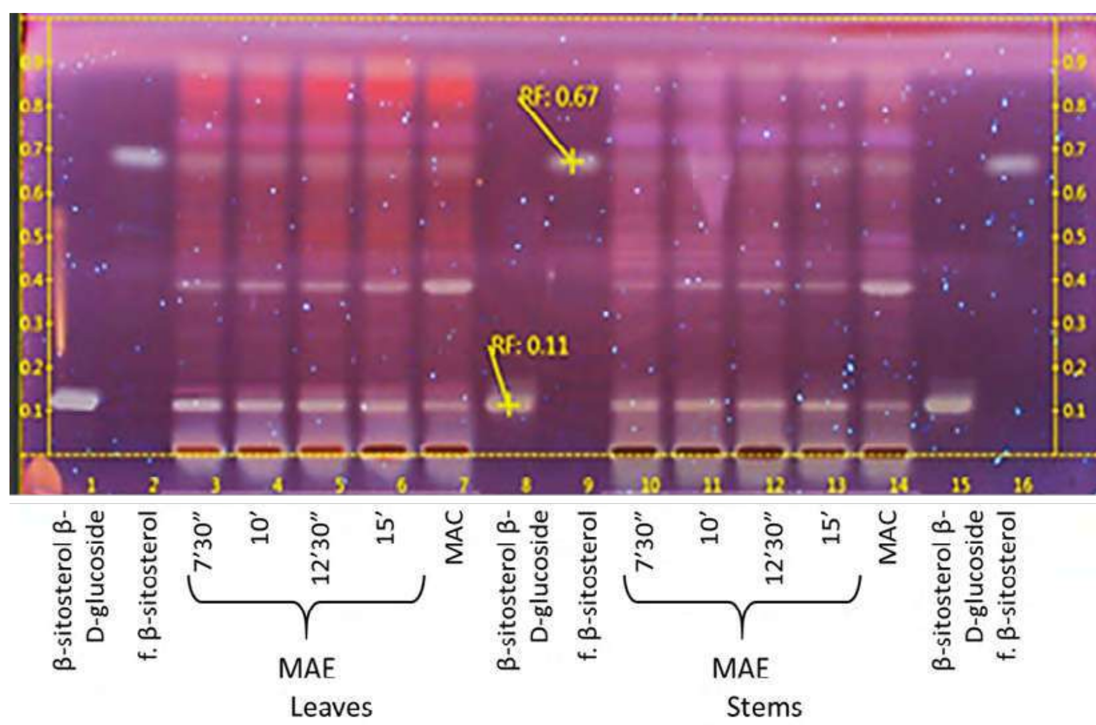

Figure 1. HPTLC derivatized plate of β-sitosterol β-D-glucoside and ethanol extracts by MAE and maceration (MAC) of *A. angustifolia*.

Table 1 shows the results of the quantification of β-sitosterol β-D-glucoside, we can appreciate that the best extraction time was 7'30" by MAE as in the stem and the leaves. Besides, the best part of the

plant for the extraction of β-sitosterol β-D-glucoside was the stem, which showed a greater amount of this metabolite in the four extraction times by MAE 7'30",10',12'30" and 15' (178.57, 146.83,121.43 and 118.25 mg / g dry weight respectively) in comparison with the quantification results obtained in the leaves in the same extraction times by MAE 7'30",10',12'30" and 15' (121.43, 119.84, 110.32 and 107.14 mg / g dry weight respectively). On the other hand, if we compare both extraction techniques, we can conclude, that the best extraction technique for β-sitosterol β-D-glucoside from *A. angustifolia* was MAE, by obtaining a greater quantity of β-sitosterol β-D-glucoside in the four extraction times (minutes), instead of hours, as it was done in the maceration method. The results of this work showed that the highest yield was obtained in lower extraction times, in comparison with the samples of maceration with other investigations, whose hexane extract of Sisymbrium irium had 0.00210 mg / g of β-sitosterol β-D-glucoside (Al-Massarani et al. 2017).

The calibration curve of β-sitosterol β-D-glucoside from ethanol extract from stem was linear in a range of between 100 and 500 ng/band, with a regression coefficient of R=99.75% and a regression equation, $y= 8.553x10^{-9}x$ and, as for the calibration curve of ethanol extract from leaves, it was linear a range of between 100 and 400 ng/band, with a regression coefficient of R=99.64% and a regression equation, $y= 1{,}058x10^{-8}x$.

Table 1. Concentrations of β-sitosterol β-D-glucoside obtained with different times of MAE and extraction by maceration (MAC) of 48 h, of leaves and steams of *A. angustifolia*. n = 3

Extraction time	mg of ß-sitosterol ß-D- glucoside / 1 g of dried extract			
	Leaves	Std Dev	Stems	Std Dev
MAE 7'30''	121.43	0.000011	178.57	0.000017
MAE 10'	119.84	0.000058	146.83	0.000058
MAE 12'30"	110.32	0.000058	121.43	0.000006
MAE 15'	107.14	0.000023	118.25	0.000058
MAC	40.48	0.000013	64.29	0.000012

CONCLUSION

It was possible to obtain better extraction yields of phytosterols from *A. angustifolia*, using ethanolic-MAE extraction to reduce from hours to minutes extraction time.

ACKNOWLEDGEMENTS

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Pancreatic lipase inhibitory activity of agave fructans.

Sánchez-Hernández, I.*, Torres-González, R. and Padilla-Camberos, E.[1]

ABSTRACT

Agave fructans have shown anti-obesity effects in animal models and clinical trials with obese individuals but the mechanism involved in those activities are still unknown. One of the possible factors is the pancreatic lipase inhibition, an enzyme involved in lipid metabolism. In this work, the porcine pancreatic lipase inhibitory activity was determined by a colorimetric assay using 4-nitrophenyl β-D-glucopyranoside (4-NP) as substrate. This enzyme hydrolyzes 4-NP to release p-nitrophenol, a colored product that can be monitored at 410 nm. Agave fructans were analyzed at the concentration used for previous clinical essays. Agave fructans were able to inhibit pancreatic lipase by 49%. This is a good value compared to 58% inhibition of the positive control, orlistat. Inhibition of pancreatic lipase is a viable strategy to treat obesity with safe natural products. Pancreatic lipase is a key enzyme in dietary triacylglycerol absorption, hydrolyzing triacylglycerol to 2- monoacylglycerol and fatty acids. It is well known that dietary fat is not directly absorbed from the intestine unless it has been subjected to the action of pancreatic lipase. These results suggest that Agave fructans prevents the dietary fat hydrolysis, possibly in the small intestine and reduces the subsequent intestinal absorption of dietary fat. The mechanism proposed of anti-obesity activity of Agave fructans is through pancreatic lipase inhibition. Whereby, it could be used as an alternative for lipids control.

Key words: Fructans, Pancreatic lipase, Agave, *In vitro*.

INTRODUCTION

Currently, overweight and obesity have become a worldwide problem. In 2016, according to the World Health Organization (WHO), 39% of men and 40% of women over 18 were overweight (WHO, 2019). Consumption of higher-energy dense products is one of the main causes of increased abdominal fat. Decreasing the consumption of these foods is beneficial for health, thus a lower lipid contribution in the diet could generate positive changes in medium- and long-term, that why drugs that help to reduce the lipid absorption in the diet is a viable strategy for the management of overweight and obesity (Bellisari, 2008).

The drugs used such as orlistat in the management of these conditions can be associated with some side effects, such as steatorrhea and vitamin malabsorption, among others (Morera et al. 2013). That is why alternatives have been sought whose long-term effects are less than those of commercial drugs (Sharma et al. 2005).

Fructans are fructose polymers forming the reserve carbohydrates of some plants, among them the Agavaceae family to which the agave genus belongs (Montañez et al. 2011). These present important

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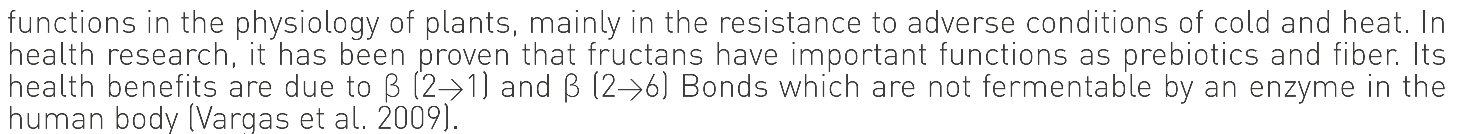

functions in the physiology of plants, mainly in the resistance to adverse conditions of cold and heat. In health research, it has been proven that fructans have important functions as prebiotics and fiber. Its health benefits are due to β (2→1) and β (2→6) Bonds which are not fermentable by an enzyme in the human body (Vargas et al. 2009).

Agave fructans have shown anti-obesity effects in animal models, decreasing fat mass, body, liver weight, and generate satiety in rodents (Urías-Silvas et al. 2008), while ingestion of agave fructans in clinical trials showed a decrease in body mass index, total body fat percentage, and a decrease in triglyceride levels (Padilla et al. 2018). However, the mechanisms associated with these activities up to now are unknown, therefore the aim of this study was to explore the pancreatic lipase inhibitory activity of Agave fructans.

METHODOLOGY

Agave fructans were collected in Jalisco and Michoacán states obtained from *Agave tequilana* (Weber). According to methodology of Marquez, juice was filtered, spray dried with an inlet temperature of 90 ˚C and an outlet temperature of 170–190 ˚C and processed with tangential flow filtration and passed through membranes of molecular weight cut off 3 KiloDalton (kDa) and 1 kDa, to obtain fructans with a degree of polymerization of less than 10 (Marquez et al. 2013).

The porcine pancreatic lipase inhibitory activity was determined according to Padilla et al. 2015. The enzyme under the reaction conditions hydrolyzes 4-nitrophenyl β-D-glucopyranoside (4-NP) to release p-nitrophenol, which is a colored product that can be monitored at 410 nm.

Briefly, in a test tube, the sample (or, Orlistat) was mixed with 0.5 mL lipase solution previously dissolved in a buffer phosphate saline (PBS) at 10 mg/mL, and then it was incubated at 37 °C for 30 min. Subsequently, 1 mL substrate 4-NP was added to it. After incubation, its absorbance was measured at 410 nm against a blank. All assays were realized for triplicate.

The percent inhibition was calculated using the following formula:

$$\% \text{ Activity} = (Ac - As) \times 100$$

Where Ac and As are the absorbance of control and sample, respectively. The control contained all constituents except a test sample. Orlistat was used as a positive control.

Agave fructans were analyzed at concentration of 96 mg/kg, similar as used for previous clinical assays (Padilla et al. 2018).

RESULTS AND DISCUSSION

The results obtained show that agave fructans can be used to reduce the levels of lipids in the blood. Agave fructans were able to inhibit pancreatic lipase by 49% showing good inhibition when it was compared to 58% of the positive control, demonstrating similar results (Figure 1). Pancreatic lipase inhibition is a viable strategy for managing overweight and obesity with safe natural products.

Fructans from Agave have been shown to have activity in the control of body fat, triglyceride, and glucose. In addition to serving as a liver and kidney protector *in vivo* studies, however, more studies are needed to know the mechanisms of action associated with these activities, as well as to know the possible relationship between the biological activities found.

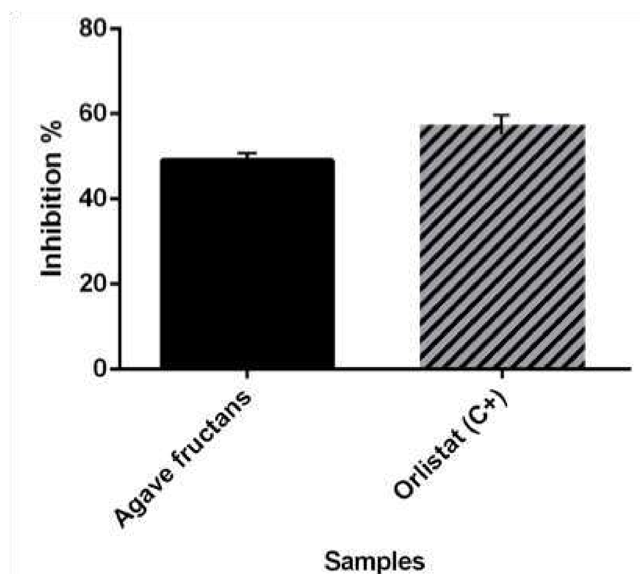

Figure 1. Percentage of inhibition of pancreatic lipase of Agave fructans in comparison whit orlistat.

In the present work, we suggest one of the possible mechanisms of action associated with the previously reported weight loss and triglycerides decrease, (Padilla et al. 2018; Urias et al. 2008).

This may be because the pancreatic lipase is a key enzyme in dietary triacylglycerol absorption, hydrolyzing triacylglycerol to 2- monoacylglycerol and fatty acids. It is known that dietary fat is not directly absorbed from the intestine unless it has been subjected to the action of pancreatic lipase (Han et al. 2001).

The results obtained are similar to those reported by Sharma and colleagues, obtaining an average pancreatic lipase inhibition percentage of 49.35% for the 75 plants tested in the study which, if compared with the 49% obtained by fructans, differs by only 0.35% (Sharma et al. 2005).

These results suggest that Agave fructans prevents the dietary fat hydrolysis, possibly in the small intestine and reduces the subsequent intestinal absorption of dietary fat. However, further studies are needed to evaluate whether enzyme inhibition is the only action way that fructans have.

However, although the results are similar to those of Roh and collaborators with other Korean plant extracts (Roh and Jung, 2012). It has been observed that, although orlistat has positive effects in the short and medium term *in vivo* studies, this weight loss can be recovered in a variable period of time (O'Meara et al, 2004), due to the similar effect of fructans compared to orlistat, more studies are needed to see the effects of fructans in the medium and long term.

CONCLUSION

The mechanism proposed of anti-obesity activity of Agave fructans is through pancreatic lipase inhibition. Whereby, it could be used as an alternative for lipids control, but further preclinical and clinical studies are recommended.

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Physicochemical and rheological properties of Aloe vera and agave fructans as wall materials on the microencapsulation of probiotics by spray drying.

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ABSTRACT

Heteropolysaccharide formulations such as Aloe vera mucilage and agave fructans are used in the food industry for their ability to modify the functional properties of food systems. The aim of this work was the study of the rheological properties of standardized mixtures of Aloe vera mucilage and agave fructans of high degree of polymerization (HDP) on their use as wall materials for the microencapsulation of probiotics by spray drying (SD). Mucilage of Aloe vera was obtained from plants cultivated in greenhouse, HDP fructans were obtained by membrane ultrafiltration technology, seven formulations of these two polysaccharides prepared by mechanical homogenization were spray dried, the physicochemical, morphological and rheological (DHR-1 Rheometer TA Instruments) properties were determined. A probiotic microorganism (*Lactobacillus plantarum*) suspended in the best formulation was spray dried and the survival measured. The obtained powders had a monomodal particle size distribution with a maximum humidity content of 10.7%, an a_w of 0.254 and a yield of 9.25%. SEM showed well-defined morphologies but some of the formulations presented collapsed structures. The viscoelastic properties of the reconstituted powders show the predominance of the viscous module over the elastic in low angular frequencies (ω <10 rad/s), this behavior is inverted at high angular frequencies which show that the structures of the formulations are random coil entanglements that can be deformed such in those of a weak gel. The reconstituted powers also showed a shear thinning behavior. The selected formulation was able to protect the 70 % of suspended probiotic. These findings support the use of new heteropolysaccarides for the microencapsulation of probiotics by SD and show a promising future for symbiotic formulations in foods.

Key words: Agave fructans, Aloe vera, Spray drying, Microencapsulation, Synbiotics.

INTRODUCTION

The use of vegetable biopolymers is a practice accepted for the formulation of wall materials on the protection of bioactive compounds and microorganisms (Coghetto et al. 2016; Robert et al. 2015). In

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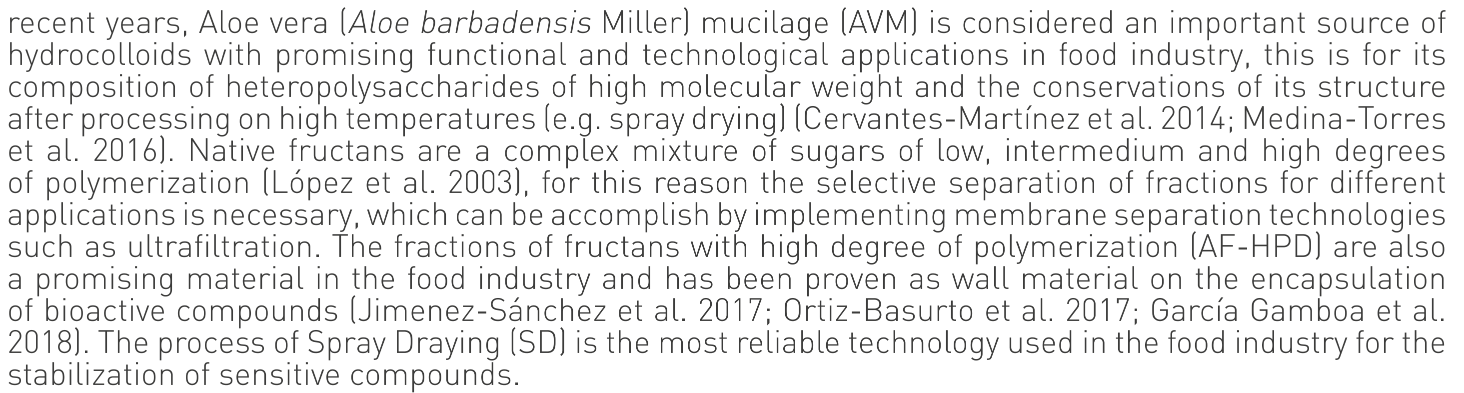

recent years, Aloe vera (*Aloe barbadensis* Miller) mucilage (AVM) is considered an important source of hydrocolloids with promising functional and technological applications in food industry, this is for its composition of heteropolysaccharides of high molecular weight and the conservations of its structure after processing on high temperatures (e.g. spray drying) (Cervantes-Martínez et al. 2014; Medina-Torres et al. 2016). Native fructans are a complex mixture of sugars of low, intermedium and high degrees of polymerization (López et al. 2003), for this reason the selective separation of fractions for different applications is necessary, which can be accomplish by implementing membrane separation technologies such as ultrafiltration. The fractions of fructans with high degree of polymerization (AF-HPD) are also a promising material in the food industry and has been proven as wall material on the encapsulation of bioactive compounds (Jimenez-Sánchez et al. 2017; Ortiz-Basurto et al. 2017; García Gamboa et al. 2018). The process of Spray Draying (SD) is the most reliable technology used in the food industry for the stabilization of sensitive compounds.

Therefore the aim of this study is to characterize the physicochemical, rheological and some morphological proprieties of formulations of AVM and AF-HPD when these are processed by SD, identify changes on the microstructures of the reconstituted powders and test the most stables formulations for the encapsulation of *Lactobacillus plantarum*.

METHODOLOGY

Biopolymers extraction and separation

Aloe vera mucilage was obtained from 3 years old plants. The cortex of the leaves was removed and the gel was stored at 4 °C, for processing in a household commercial extractor. Fructan commercial powder presentation Fiber Prime® Best Ground International SA de CV, Gdl. Jalisco Mx), was dissolved in water to concentration of 20 °Bx ultra filtrated in a Pellicon 2 system (Millipore, MA, USA), with a membrane of molecular weight cutoff 5 KDa.

Lactobacillus plantarum lp115 and biomass recovery

A lyophilized *L. plantarum* lp115 strain pallet was suspended (1% w/v) in 2 L of MRS broth and incubated at 37 °C for 24 hours. The separation of biomass from the broth was carried out by centrifugation at 10000 rpm for 10 min at 4°C.

Formulations, preparation of microcapsules and spray dry process

Table 1 shows the experimental design. Light homogenization with a braun mixer was applied for the microencapsulation of *L. plantarum* in a ratio of 5:1 (biopolymer – microorganism). All the treatments were spray dried in an industrial scale pilot spray dryer LPG5 (CIMA Industries, China) at the operational conditions: volume feed, 1.5 L/h; hot air inlet temperature, 150 °C; rotating speed, 27500 rpm.

Physicochemical proprieties of the powders

A gravimetric method (AOAC 2011) was used to determine the moisture content of the powders. Results were expressed in dry basis. Water activity (a_w) was measured with an Aqualab Series 3 (Meter Group, Inc. USA) device.

Morphology of powders

Micrographs were taken at 5kv and magnification of 1000x in a Jeol JSM 6300 SEM microscope and the particle size distribution with a particle analyzer Mastersizer 3000 (Malvern Instruments ltd., UK).

Rheological studies

Rheological measurements were carried out in a controlled stress Rheometer DHR-1 (TA instruments) with the methodology described by Medina-Torres et al. (2016).

RESULTS AND DISCUSSION
Physicochemical properties of formulations and powders

Percentages of total solids (w/w) of each biopolymer and pH of the different studied formulations (treatments) are shown (Table 1). In addition, the moisture content (MC), a_w and yield process (Y) for the spray dried of said formulations. For all five treatments, outlet temperature in the drier was 85±2 °C and adiabatic saturation temperature 55 ±2.2 °C. A change in pH value for treatments T2 and T3 is notable, this is for the non-reductive sugar nature of the AF-HPD fractions (López, et al. 2003), which can interact by hydrogen bonds with the acetylated mannose units of the acemannan polymer which is the predominant polymer in the AVM (Im et al. 2005; Rodríguez-González et al. 2011). Moisture and yield values are similar to those reported by (Cervantes-Martínez et al. 2014) and (Medina-Torres et al. 2016) when they SD fresh AVM, however the temperature used in this work is 20 and 40 °C lower than in theirs, this could ensure a minor degradation of the biopolymers and higher protection for the encapsulated agent. In addition the MC and a_w values are direct related to the percentage of AF-HPD added to formulations, this is a cause of the functional property of water entrapment (Ortiz-Basurto et al. 2017; Rajam and Anandharamakrishnan 2015).

Table 1. Composition, Physicochemical properties and yield process of the spray-dried formulations.

Tmt	*AVM (g/100g)*	*HPD-AF (g/100g)*	*pH*	*MC (d.b) (g/100g)*	a_w	*Y(g/100g)*
T1	80	20	4.43±0.07[a]	7.95±1.63[a]	0.195[a]	9.25±0.23[a]
T2	60	40	4.52±0.04[b]	9.5±2.01[b]	0.225[b]	8.58±0.37[b]
T3	50	50	4.6±0.09[b]	10.7±1.82[b]	0.254[b]	8.05±0.74[b]

[a]Values are means (n=3); means in the same column not sharing a common superscript later are significantly different ($P<0.05$)

Powders morphology and particle size distribution

On the micrographs (Figure 1 A, B and C), particles between 10 to 15 µm can be seen with smooth and uniform surface; most of them are deflated or collapsed but with a homogenous form. This could be the result of the SEM methodology of the vacuum applied to the samples when coated with gold (Dolly et al. 2011). The most stable particles are the ones of T1 (A), which can be the result of higher concentration of AF-HPD that change the glass transition temperature caused for the interaction of different polymers (Li et al. 2016). Figure 1D shows the particle size distribution for all treatments and is evident the monomodal character of the microparticles, also a Dv (50) between 15 and 16.8 was determined which confirms the size in the micrographs. During the drying process, the temperature is a key factor over the morphology and size, this is because the rate and quantity of water evaporation, (León-Martínez et al. 2011).

Rheological behavior in simple shear flow

The tests reveal a Newtonian behavior at low shear rates (long time) (Figure 2A), however a dependency to viscosity for shear rate is evident when the magnitude of the effort is increased (> 10 s^{-1}). This assert a non-Newtonian shear thinning pseudoplastic behavior. The first plateau or Newtonian behavior is seen in a longer range of shear rate for some of T1 and T7 (Figure 2B). The change in range and magnitude of the Newtonian and non-Newtonian behavior is the result of the arrangement and conformation of the network that the biopolymers have.

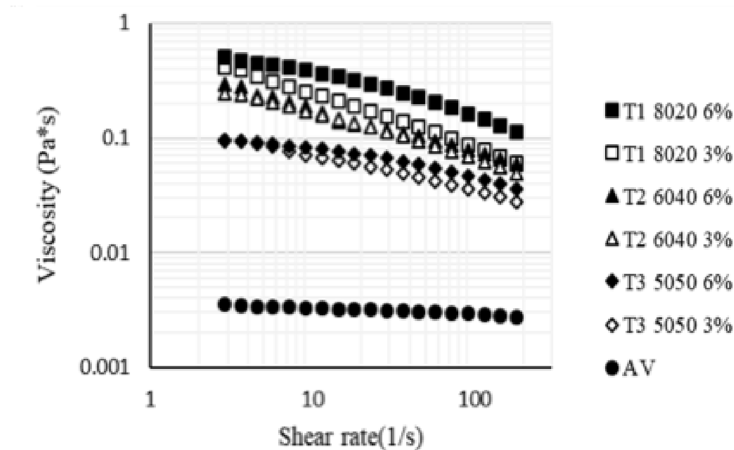

Figure 2. Flow curves of treatments and the effect of concentration.

Lactobacillus plantarum survival to SD process

The results of survival for *Lactobacillus plantarum* to SD process when encapsulated are shown in Table 2. Each treatment was inoculated with 8.23 log CFU/g of lactobacillus. The treatments that shown higher survival of *L. plantarum* were T4 and T7, corresponding to those added with arabic gum. Similar findings were reported by (Rajam et al. 2012) using calcium alginate and skimmed milk isolates as wall materials.

Table 2. Survival rate of *L. plantarum* for the selected biopolymer formulation as wall material.

Treatment	Encapsulated	Released	%S
	log CFU/g	log CFU/g	
T1(AVM - AF-HPD)	2.67	4.97	60.32
T4 (AVM - AF-HPD + GA)	3.43	5.75	69.76

CONCLUSION

It is possible to spray dry formulations of AVM and AF-HPD and obtain stable powders with homogenous surfaces and monomodal particle size distribution. The resuspended powders have a shear thinning behavior (n<1). The formulations of AVM-AF-HPD and arabic gum show promising expectations as wall materials for have shown a relative high survival rate of *L. plantarum* when spray dried.

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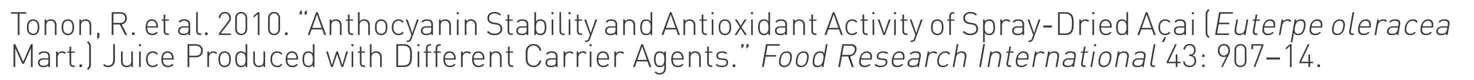

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Physicochemical characterization and carbohydrate profile of maguey syrup and aguamiel from the state of Hidalgo.

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ABSTRACT

In the state of Hidalgo, maguey syrup is produced by hand, obtained by aguamiel (AM) concentration of different agave species from the upper mezquital region, therefore the quality and composition of the syrup depends directly on the quality of the AM used. AM is the sap that contains the bud of the plants commonly known as magueyes; belonging to the family of the agaves. The collection is done every 12 h after scraping the stem of the agave, and the production lasts from 4 to 6 months. The main component of AM are carbohydrates, although the presence of amino acid, ascorbic acid and iron has also been reported. Differences in AM composition are still not clear, have been proposed as main factor: the region, agave species, weather, among other. In this work it was studied the physicochemical characterization and carbohydrate profile of maguey syrup from two zones (Zempoala and Cardonal) of the state of Hidalgo and AM from different agaves of Zempoala, Hidalgo. The pH was the physicochemical parameter with higher variations. The color of the AM is greatly obscured by the concentration process at high temperatures until the syrup is obtained at 70 °Brix. Large variations were found in carbohydrate profile, especially in simple sugars (glucose, fructose and sucrose) of AM and syrups evaluated.

Key words: Aguamiel, syrup, maguey, carbohydrates, Hidalgo.

INTRODUCTION

The maguey syrup is traditionally consumed as sweetener in the state of Hidalgo; it is produced by hand through the direct concentration of aguamiel (AM) of different agave species from the upper mezquital region. The quality and composition of the syrup mainly depends on the AM used and the concentration temperature. AM is the sap that contains the bud of the plants commonly known as magueyes; belonging to the family of the agaves, and is harvested exclusively from certain agave species known as ¨maguey pulquero¨, including *Agave americana*, *A. atrovirens*, *A. ferox*, *A. mapisaga* and *A. salmiana* (Escalante et al., 2008). The traditional method to obtain AM is from mature agaves (8–12 years), in which a cavity is made in the center by cutting the floral stem, where sap is manually collected by scraping. The collection is done every 12 h and the production lasts from 4 to 6 months. The main component of AM are carbohydrates, although the presence of amino acid, ascorbic acid and iron has also been reported

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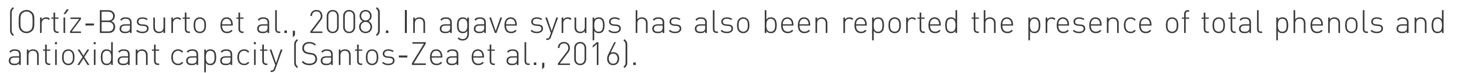

(Ortíz-Basurto et al., 2008). In agave syrups has also been reported the presence of total phenols and antioxidant capacity (Santos-Zea et al., 2016).

Since the production is an artisanal process, the consumption of agave syrup is mostly local. However, there is great potential in the high mezquital area to produce large quantities in industrial form, but one of the main problems to achieve this is the standardization of the raw material, in this case the AM, as the final quality of the product depends on it. The differences of the AM composition due to the region, the agave species, the weather, among others, are still not clear, however, the carbohydrate profile has been proposed as a possible authenticity marker between agave syrups of different species such as *A. tequilana* and *A. salmiana* (Mellado-Mojica and López, 2015).

In this work it was studied the physicochemical characterization and carbohydrate profile of maguey syrup from two different zones of the state of Hidalgo and AM from different agaves of Zempoala, Hidalgo, in order to evaluate the variability of the raw material for agave syrup.

METHODOLOGY

Materials

Nineteen samples of aguamiel were collected from *Agave salmiana* (var. salmiana or manso, var. ayoteco) and A. atrovirens (penca larga) in Zempoala, Hidalgo, through traditional methods applied by the producers. The samples were pasteurized at 90°C for 10 minutes in autoclave and stored in freezing until the analysis. Four samples of syrup from a different batch were obtained from the sale in two different zones of the state of Hidalgo (San Andrés Daboxtha, Cardonal, and Zempoala, Hidalgo.).

Physicochemical characterization

The pH was measured directly in AM samples or 20% w/v syrup:water solutions using a pH meter (PC1100 Horiba, Laqua, Japan).

The total soluble solid (TSS) was analyzed using a digital refractometer (PAL-α, Atago, Tokio). The color of samples was measured using a Mini Scan chromameter (Hunter-Lab, Japan). CIE L*a*b* coordinates were measured, where L* is the luminance component (ranging from 0 to 100), while a* and b* are color coordinates related respectively with the red/green and yellow/blue spectral ranges, with values varying from –120 to +120.34.

Carbohydrate profile

The carbohydrate profile was determined using a HPLC methods. The HPLC-SEC system consisted on a 1220 Infinity LC System HPLC coupled with a refractive index detector (Agilent, Alpharetta GA, USA). To quantify the amount of glucose, fructose and sucrose it was used an Aminex HPX-87C ion exchange (7.8mm d.i. × 300 mm, Bio-Rad Hercules, CA, USA) column, deionized water as mobile phase at 0.55mL/min and a calibration curve from 0 to 10 g/L. Samples were diluted between 10 - 15 g/L in deionized water and filtered through a 0.45μm polyethersulfone (PES) membrane. The presence of fructans and fructooligosaccharides (FOS) was determined using a size exclusion column according to methodology proposed by Moreno Vilet (2017).

Statistical analysis

The results were reported as a mean ± standard deviation from the triplicate analysis. Statistical analysis was conducted by one-way ANOVA and differences among means were calculated by Tukey's test using a level of significance of $p < 0.05$.

RESULTS AND DISCUSSION
Physicochemical characterization

Table 1 shows the results of the evaluated physicochemical parameters. The TSS of maguey syrups were between 67 and 72°Brix, while for AM from all varieties were from 10.1 -17.4 °Brix. The pH ranged from 4.2 to 7.2, in AM samples, while in syrups it was found a more stable pH of about 5. The color of the syrups was much darker compared to AM and this was quantified in the L* parameter corresponding to luminance. Although differences in the color of the AM samples can be observed, these differences were not attributed to the agave variety.

Table 1. Physicochemical characterization of maguey syrup and agave sap (aguamiel).

Sample	TSS	pH	Color		
	°Brix		L*	a*	b*
Syrup-Zempoala	70.57 ± 1.25[a]	4.76 ± 0.01[a]	7.14 ± 5.36[a]	3.93 ± 3.09[a]	7.30 ± 5.58[a]
Syrup-Cardonal	68.95 ± 2.22[a]	4.99 ± 0.1[a]	0.98 ± 0.45[a]	2.03 ± 0.95[a]	1.28 ± 0.72[a]
AM- Manso	13.74 ± 2.08[b]	5.57 ± 1.38[a]	41.70 ± 3.22[b]	1.81 ± 2.01[a]	12.41 ± 6.76[a]
AM- Penca larga	14.10 ± 0.85[b]	5.39 ± 0.56[a]	41.40 ± 2.05[b]	1.28 ± 0.17[a]	10.96 ± 2.07[a]
AM- Ayoteco	11.5 ± 0.01	7.04 ± 0.01	38.55 ± 0.02	2.45 ± 0.10	14. 20 ± 0.33

TSS: Total soluble solids. Different letters in each column mean significant statistical differences between results ($p < 0.05$).

Carbohydrate profile

Table 2 shows the average results of carbohydrate profile in dry basis obtained from HPLC-SEC analysis. The amount of fructans DP> 10 varied between 1% and 12.6%, FOS varied between 1% and 12%, sucrose varied from 12.4 to 97% and monosaccharides (glucose and fructose) varied from 0% to 80%. The greatest concentration variation was found in simple sugars (glucose, fructose and sucrose); however, not statistical difference was found between groups due to the samples variability. The fructan content, especially of FOS, is important because it is associated to the beneficial prebiotic properties that stimulate the selective growth of probiotic bacteria in the microflora of consumers. To maintain a high content of these polysaccharides in the syrup, it is advisable to process it quickly or else, inactivate the enzymes responsible for the hydrolysis process through pasteurization.

Table 2. Carbohydrate profile of maguey syrup and agave sap (aguamiel) analyzed by HPLC-SEC.

	Fructans (DP >10) (% d.b.)	FOS (DP <10) (% d.b.)	Sucrose (% d.b.)	G + F (% d.b.)
Syrup-Zempoala	5.84 ± 1.13	2.42 ± 0.58	60.57 ± 2.25	31.17 ± 3.97
Syrup-Cardonal	7.70 ± 0.30	5.55 ± 0.03	28.46 ± 2.22	58.29 ± 1.95
AM- Manso	3.87 ± 3.22	3.23 ± 3.36	72.49 ± 25.18	20.4 ± 23.14
AM- Penca larga	3.33 ±1.15	3.48 ± 1.80	38.27 ± 18.46	54.92 ±15.65
AM- Ayoteco	4.99 ±0.12	2.60 ± 1.2	12.47 ± 0.08	79.88 ± 1.0

FOS: Fructo-oligosaccharides, G+F: sum of glucose and fructose percentage, **d.b.** dry basis.

In the case of sugars like glucose, fructose and sucrose they represent up to 90% of the carbohydrates in AM and syrup. From those sugars, the main component for *A. salmiana* syrups was sucrose in accordance with the reports of Mellado-Mojica and López (2015), on the other hand, for AM or syrup from *A. tequilana* or *A. mapisaga* the profiles report a higher concentration of fructose, followed by glucose and a very low concentration of sucrose (Ortíz-Basurto et al., 2008; Mellado-Mojica and López, 2015). Different carbohydrate profiles have been reported in syrups from different agave species; however, in this work samples with different glucose, fructose and sucrose profiles were found in the same species, but of different varieties. Figure 1 shows clearly the sugar concentrations of AM samples and shows a trend in the samples of *A. salmiana* var. manso different to *A. salmiana* var. ayoteco and penca larga (*A. atrovirens*); however, more specific studies are needed focused on evaluating the AM from different varieties of agave in the region in order to achieve this affirmation.

Clear differences in sucrose and G+F concentrations in syrups were observed due to the region where they come from. Syrups from Zempoala present a high concentration of sucrose in accordance to the most AM samples from the same region and syrups from Cardonal present a higher concentration of glucose and fructose (Table 2).

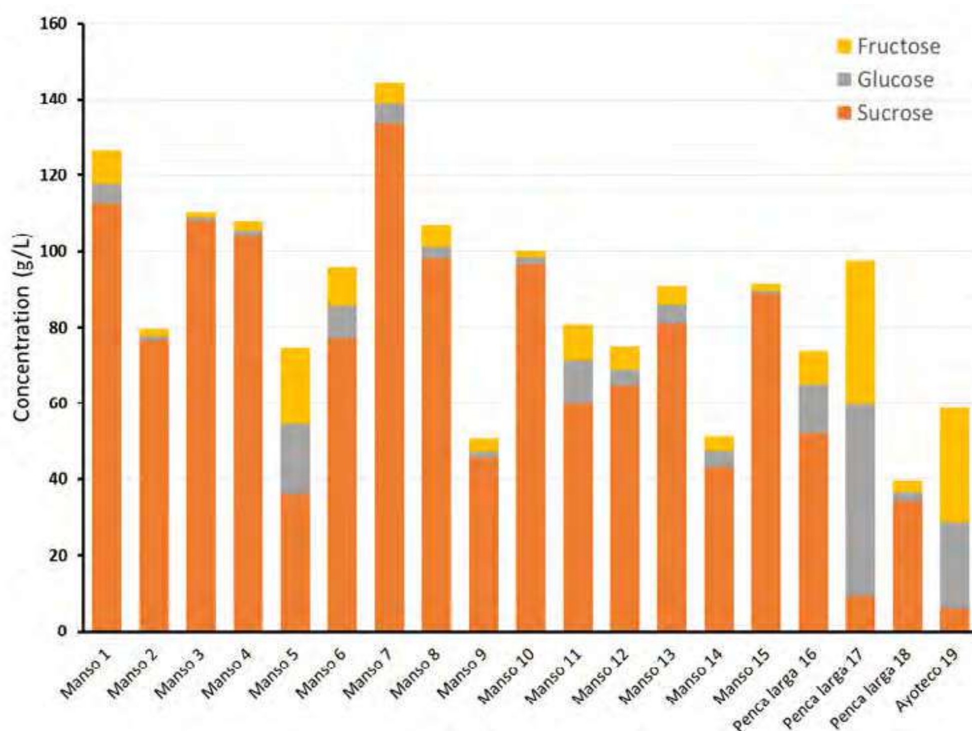

Figure 1. Sugar concentration in aguamiel (AM) from different agaves

CONCLUSION

The pH was the physicochemical parameter with higher variations; however, these variations do not depend on the species or variety of the agave. The color of the AM is highly obscured by the concentration process at high temperatures until the syrup is obtained at 70 °Brix.

Great variations were found in carbohydrate profile, especially in the simple sugars of AM and syrups that were evaluated, which may affect the sensory and nutritional quality of maguey syrup.

Studies about AM composition based on agave species, growing areas or harvest periods as well as the standardization of syrup processes, are needed.

ACKNOWLEDGEMENTS

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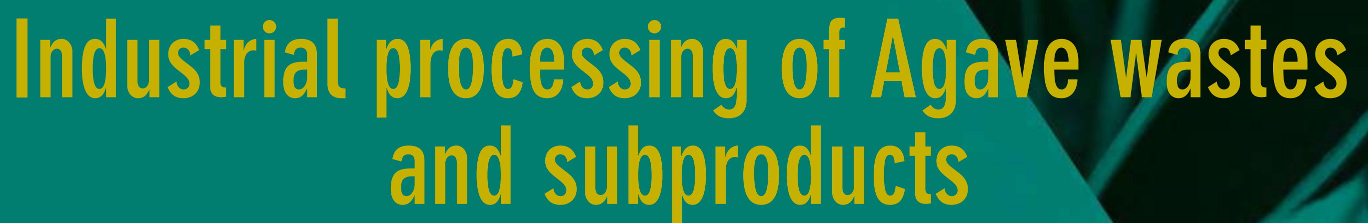

Industrial processing of Agave wastes and subproducts

Bio-hydrogen production from tequila vinasse depending on tequila production process 100% agave.

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ABSTRACT

In Mexico, the production of tequila is one of the most important businesses in the agro-industry. In the last 20 years the production of tequila has increased by 203% which has motivated the development of new technologies for its production. For example, the process without cooking, where sugars are extracted from the pineapple of the Agave plant in 1/6th of the time it takes to perform the traditional cooking process. Regardless of the process of extraction of sugars, for each liter of tequila 10-12 L of vinasses are obtained as waste. Vinasses are acid effluents whose high concentration of organic components makes them attractive to recover resources from waste streams. The objective of this work was to characterize vinasses of the tequila process with cooking and without cooking to evaluate its hydrogen production potential. As a result, from this study the tequila production process does have an impact on the physicochemical characteristics of the tequila vinasse and, therefore, on its hydrogen producing potential.

Key words: Vinasse, hydrogen, tequila production, physicochemical characteristics.

INTRODUCTION

In Mexico, tequila producers are among the most important companies in the agroindustry. In the last 20 years the production of tequila has increased by 203%, reaching a production volume of 273.3 million liters in 2016, for which 941.8 thousand tons of agave were used (CRT, 2017) this has motivated the development of new technologies for its production, for example, the process without cooking. Which optimizes the traditional process where the pineapple is cooked in ovens and then passed to grinding, fermentation, distillation and bottling or aging; in the process without cooking the pineapple is torn and passed through a diffuser which extracts the juices that are subsequently cooked. The production of the tequila has as by-products the bagasse (solid waste) and the vinasse (liquid waste) which are waste with high impact to the environment (Monlau et al., 2014; Gschaedler, 2015; Bautista-Justo, 2001 & Villanueva, 2015).

Vinasses are the main liquid residue obtained from the distillation process of tequila, which are acid effluents with a high concentration of organic matter and a large amount of solids. Its composition includes acids, phenols, carbohydrates and unsaturated components with high chemical oxygen demand (COD) and Biological Oxygen Demand (BOD) (Salgado et al., 2010). The study of the physicochemical characteristics of the tequila vinasses from different tequila production processes (with cooked and without cooked pineapple of A. tequilana) will allow the evaluation of the potential to use them as a

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substrate in the production of hydrogen and thus, to carry out the prospecting yield of resource recovery from these wastewaters for future exploitation.

Hydrogen is considered an ideal energy vector because it is recyclable, efficient and clean. The production of hydrogen by fermentation of organic waste and recyclable materials is carried out freely without contamination, is friendly to the environment and in most cases is cost effective (Li & Fang, 2007). This work is based on the study of the effect of these two tequila production processes on the physicochemical characteristics of the vinasse and its hydrogen producing potential, which we present as a resource recovery system using the tequila vinasses, which are acid effluents. with high organic load to produce hydrogen through anaerobic digestion. Some of the most attractive characteristics of hydrogen is its energy content per unit of weight (143 GJ / ton), it is a carbon-free fuel that only generates water when it is combusted or used in conventional cells for the generation of energy. In addition, biohydrogen is a gas of low solubility that can be easily separated from water, purified and used as an energy carrier. (Dávila-Vázquez et al., 2009; Buitrón et al., 2010; Mansouri et al. 2017 & Das Debrabrata, 2008).

METHODOLOGY

Physicochemical characterization

The vinasses were obtained from tequila producing houses in Tequila, Jalisco from both production processings, with and without cooking. The analysis methods used in the physicochemical characterization are referenced in Table 1.

Table 1. Analytical methods for the physicochemical characterization.

PARAMETERS	METHOD
Acidity	NMX-AA-036
pH	Electrode
COD (mg/L)	HACH 8321
BOD (mg/L)	BOD TRAK
Total organic carbon	SHIMADZU
Phenols	Folin/Ciocalteu et al. 1956
Total sugars (mg/L)	Dubois et al. 1956
Total solids (g/L)	NMX-AA-034
Total volatile solids (g/L)	NMX-AA-034
Total dissolved solids (g/L)	NMX-AA-034
Total nitrogen (mg/L)	HACH TNT 828
Nitrates (mg/L)	HACH TNT 835
Nitrites (mg/L)	HACH TNT 839
Total phosphorus (mg/L)	HACH TNT 844
Phosphates (mg/L)	HACH TNT 844

Hydrogen production

The biological production of hydrogen was carried out under the internal guidelines of Specific Hydrogen Production of the Latin American Biohydrogen Network, which aims to standardize the methodology to determine the specific production of hydrogen in batch reactors. The batch fermentative assays for hydrogen production were carried out in an automatic methane potential test system (AMPTS II) provided by Bioprocess Control (Lund, Sweden). The different samples of total vinasse and centrifuged vinasse as well as an endogenous blank were used as substrate. All assays were performed in triplicate for each sample.

Inoculum

Granular anaerobic sludge was thermally pretreated for the selection of hydrogen-producing microorganisms. The sludge was dried for 24 hours in an oven at 105 °C. Then it was crushed in a mortar to reduce the particle size to approximately 850 μm, obtaining a texture similar to that of a powder. Prior to use, it was dried at 105 °C for 2 hours and cooled in a desiccator to ensure its water-free weight.

Essay bottles

For each bottle, 4.3 grams of inoculum were weighed to maintain a substrate / inoculum ratio of 2.7 in a volume of 360 mL. To each bottle were added 22.6 mL of mineral medium and buffer. Then 337.4 milliliters of vinasse was added to complete a volume of 360 mL. During the trials a working volume / headspace ratio of 1.5 was maintained, in accordance with the recommendations of the AMPTS II manual. The initial pH of each test was adjusted to 7.5, adding by dripping solutions of 5 N NaOH or 5 N HCl. The water bath was adjusted to a temperature of 37 °C. The headspace of the bottles was displaced with nitrogen for approximately 30 seconds to achieve anaerobic conditions. The program started according to the software manual of the AMPTS II, the stirring was adjusted to 150 rpm.

Chromatographic analysis of gas composition

The composition of the gases obtained from the head space of the batch reactors were analyzed by gas chromatography. The analysis was performed on a Clarus 580 Perkin Elmer chromatograph equipped with a thermal conductivity detector and a packed column of 3,048.

RESULTS AND DISCUSSION
Physicochemical characterization

The results of the analysis for both samples of tequila vinasses (with cooked and uncooked processing of the pineapple) are displayed in Table 2.

The values of solids, total sugars, phenols, total phosphorus and phosphates; are higher in the vinasse sampled from the traditional cooking process, this could be due to the thermal treatment for saccharification of sugars from the *A. tequilana* pineapple and the grinding of cooked agave fibers.

Table 2. Physicochemical characterization results.

PARAMETERS	COOKED VINASSE	UNCOOKED VINASSE
Acidity	6,276.50	2,398
pH	3.53	3.48
COD (mg/L)	59,683	90,900
BOD (mg/L)	7,325	9,625
Total organic carbon	30.92	10.93
Phenols	1,465.50	886.42
Total sugars (mg/L)	18,225.28	5,263.02
Total solids (g/L)	39.91	19.48
Total volatile solids (g/L)	37.61	20.17
Total dissolved solids (g/L)	37.84	20.72
Total nitrogen (mg/L)	103.33	143.67
Nitrates (mg/L)	244.33	115.53
Nitrites (mg/L)	1.19	3.24
Total phosphorus (mg/L)	213.33	17.40
Phosphates (mg/L)	40.5	4.12

Hydrogen production

As seen in Figure 1, the batch reactors that used the cooked vinasse as a substrate began to produce hydrogen until the second day of the experiment, presenting a Lag Phase of 48 hours. This can be attributed to the content of phenols and furan compounds, these inhibitors negatively affect the function of the cell membrane, as well as the growth and glycolysis of the fermentative bacteria (Behera et al. 2014). On the other hand, in the reactors where the uncooked vinasse was used as substrate, the biological production of hydrogen started after the first 24 hours. The final biohydrogen volume produced was higher in the reactors that used as a substrate the vinasse without the cooking process, obtaining a standardized average of 794.67 milliliters of H_2. The result obtained from the reactors with vinasse from the cooking process as a substrate had an average of 685.95 standard milliliters of H_2.

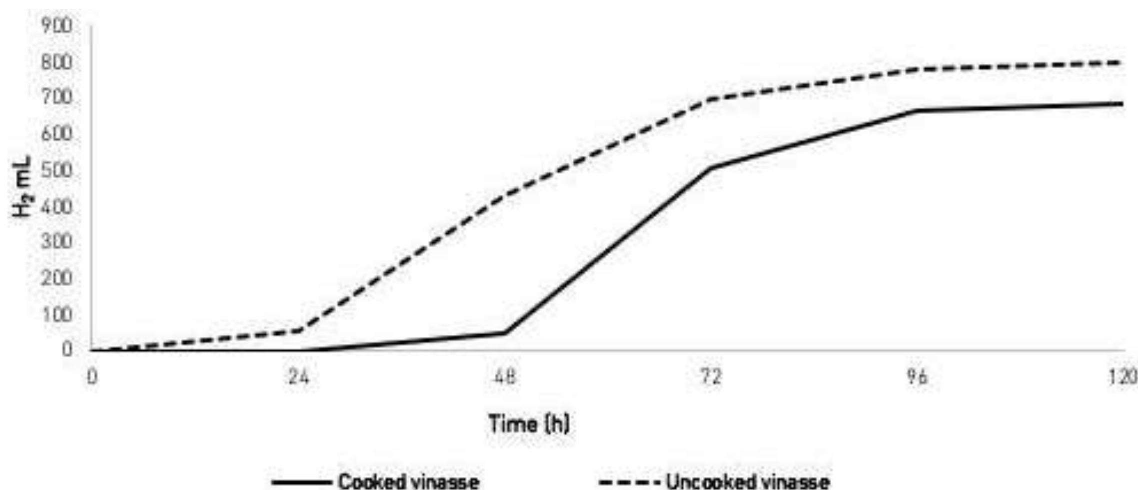

Figure 1. Hydrogen production results

Chromatographic analysis of gas composition

The percentages of hydrogen were lower in the reactors where the cooked vinasse was used as substrate, with an average of 13.57% of H_2 and 67.27% of CO_2 at 72 hours. The remaining percentage of gas were not identified because the standards tested were only for hydrogen, methane and carbon dioxide. At the end of the experiment (120 hours) the percentage of hydrogen increased to 23.63% and the carbon dioxide also increased to 75.02%. In the reactors using vinasse from the process without cooking, a greater concentration of hydrogen was produced at 72 hours 26.48%, and 72.19% of CO2. After 120 hours of the experiment, an increase in hydrogen concentration was detected at 41.39% and a decrease in the concentration of CO_2 with a value of 57.63%.

CONCLUSIONS

The tequila production process has an impact on the physicochemical characteristics of the tequila vinasse and, therefore, on its hydrogen producing potential. The vinasse from the cooking process has a higher yield in NmL H_2/g COD, however the vinasse without cooking produced a greater total volume of H_2. The vinasse without cooking has greater advantages for the recovery of resources such as hydrogen, this also considering the expansion of the process without cooking in the tequila industry and the increase of this agroindustrial effluent.

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Effect of application of tequila vinasses on the rhizosphere of maize plant.

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ABSTRACT

Vinasse is a residue generated from alcohol production from sugarcane, beet, agave (for tequila production), and from the distillation of other beverages. Some characteristics of this wastewater are high organic matter (2.2-3.9%), acid pH (3.5 to 4.2) and high cation concentration (6.3 ± 0.13 mg/L), among others. The large volumes of vinasse generated represent a challenge to natural environment because its irrigation to soil is a common practice and little is known about its impact on the rhizosphere of plants. Two experiments were conducted in a greenhouse to evaluate the effect of tequila vinasse application on the rhizosphere of maize: 1) A native consortium (NC: *Claroideoglomus etunicatum, Funneliformis geosporum* and *F. mosseae*) and a commercial inoculum (CI: *Glomus intraradices*) in symbiosis with *Zea mays* were used to evaluate the effect of the irrigation of two tequila vinasse concentrations (30% and 60%), with two acid pH levels (3.5 and 4.5), and water as control. 2) Maize crops with a weekly irrigation of vinasse (at 25%, 50%, and 75% concentration), or water (W) with native AMF (as a control) was used to evaluate the effect of vinasse application. Height, biomass, spore density, relative abundance (RA) and colonization of AMF, soil pH, electrical conductivity (EC), total phosphorus (PT) and phenols were evaluated in both experiments. The results showed that vinasse application promoted an increase in pH, EC and concentration of phenols in soil, and a decrease in AMF colonization regardless of vinasse concentration applied. At the end, the application of vinasse did not favor plant growth.

Key words: Agricultural amendment, growth, development, *Zea maize*, HMA.

INTRODUCTION

During the production of tequila, 10 L of vinasse are generated per liter of tequila (Retes-Pruneda et al. 2014). Vinasse disposal to the soil as a fertilizer is a common practice because of its high organic load (Moran-Salazar et al. 2016). However, there are negative and positive effects reported on the soil, and little is known about their effects on the rhizosphere (Sanchez-Lizarraga et al. 2017). The primary objective of this investigation was to evaluate the effect of tequila vinasse application on maize rhizosphere a) assessing how vinasse pH and organic load affect commercial and native arbuscular mycorrhizal fungi (AMF) and b) comparing different vinasse concentrations.

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METHODOLOGY

Sampling site, soil and vinasses characterization

The soil was sampled from an agricultural site located in El Arenal, Jalisco, México (20º 46´ 7˝ N, 103º 41´ 18˝ W) at the northwest of the State. This site has been an agricultural field with sugarcane cultivation (*Saccharum officinarum*) for 30 years. The experimental site has two areas, 1) a field (2.4 ha) irrigated with tequila vinasses for at least 5 years and, 2) a field (1.8 ha) that has never been applied with vinasses.

The soil samples were randomly collected from both areas, which divided in three plots. A total of 21 subsampling points were obtained from each plot (n = 9). The soil samples were homogenized and sieved (5 mm mesh) to remove vegetation and stones. Vinasses were collected from two tequila distilleries in order to have enough vinasse for the development of the entire experiment. Both use a traditional agave production process which includes steam cooking of agave leaves. Vinasses were stored at 4º C during the experiment.

The soil was characterized for pH, water holding capacity (WHC), cation exchange capacity (CEC), EC, total organic carbon (TOC), total nitrogen, total and available phosphorus, texture and particle size distribution using the methodology proposed by Dane and Topp (2002). The vinasses was characterized for pH, EC, biological oxygen demand (BOD), chemical oxygen demand (COD), arsenic, cadmium, copper, chrome, mercury, lead, nickel, zinc and iron, volatile suspended solids (VSS), settled solids (SS), and available P and total N by The Standard Methods for the Examination of Water and Wastewater™ (APHA,1992).

Isolation of AMF

AMF spores from each plot (*n* = 9) were isolated three times, using a wet sieving method with sucrose at 50% (w/v) and four sieves with 600, 150, 75 and 45 µm mesh (Brundrett et al. 1994). Spores amount were registred in each treatment. The collected spores were propagated *in vivo* in pot traps, containing a mixture of sand, sampled soil and sand (1:1:2), using sorghum (*Sorgum vulgare*) and alfalfa (*Medicago sativa* L.) as host plants to promote the proliferation of AMF during 6 months. The pots were irrigated with distilled water only once a week, which promoted dryness periods. At the end of this time, the spores were collected with the technique before mentioned, the abundance was estimated and five grams of the mixture (soil: sand with AMF) were used as native inoculum (NI).

Greenhouse experiment

Experiment I

In this experiment the two factors evaluated were inoculum (2) and treatment (5). Pots with sterile soil of area 1 and corn seedlings were irrigated with water (A) or vinasse at two pH levels (3.5 and 4.5) and two organic matter concentrations (30% and 60%) for 90 days. Either a commercial (CI) or native (NI) AMF inoculum was added.

Experiment II

In this experiment maize plants, soil and treatment were evaluated. Pots with soil of area 2 and corn seedlings were irrigated with different vinasse concentrations (25%, 50% and 75%) or water (0%) for 120 days. Height, biomass, spore density, relative abundance (RA) and colonization of AMF, soil pH, EC, PT and phenols in the rhizosphere were recorded in both experiments.

Statistical analyses

The data obtained was evaluated through an analysis of variance (ANOVA) and a Tukey's honestly significant difference (HSD) post hoc test. The STATGRAPHICS® Centurion XVI software and XLSTAT was used.

RESULTS AND DISCUSSION

Vinasse and soil characterization

The vinasses showed an acidic pH (3.49 to 4.24), high concentrations of total phosphorus (10.23-652.66 ± 1.9-25.48 mg L^{-1}), total nitrogen (140.55-586.67 ± 4.55-11.55 mg L^{-1}), biological oxygen demand (BOD) (13,500-40,005 ± 458-3,481 mg L^{-1}), chemical oxygen demand (COD) (40,433-57,246 ± 846-2148 mg L^{-1}), solids (28,000-133,833 ± 577-1,485), and total phenols (320.7-1,052.7 ± 0.0-41.6 mg L^{-1}). Heavy metals (As, Cd, Cu, Cr, Pb, Ni, Zn, Fe) were not detectable.

The soil of area 1 was moderately acidic (pH of 5.88 ± 0.24) and had an electrical conductivity of 0.234 ± 0.040 mS cm^{-1}. A high content of phosphates (28.34 ± 9.18 mg kg^{-1}), an average content of total nitrogen (1033.3 ± 656.3 mg kg^{-1}) and high content of organic matter (70.00 ± 0.00 g kg^{-1}) was found. The soil of area 2 was classified as phaeozem, with sandy loam texture, a 223 mS cm^{-1} electrical conductivity (EC), 41.57 g kg^{-1} total organic carbon, 0.998 g kg^{-1} total nitrogen, 42.7 mg kg^{-1} total phosphorus and 29.62 mg kg^{-1} available phosphorus.

Experiment I

The final average height of the plants was 63 cm. A decrease in height and biomass was observed in the plants treated with vinasse against the control. The application of vinasse increased pH, EC, PT, and phenols in soil. The colonization was decreased in both inocula, but this was higher in the CI (Figure 1), and spore density decreased in CI only (Figure 2). The population composition of AMF in NI was disturbed.

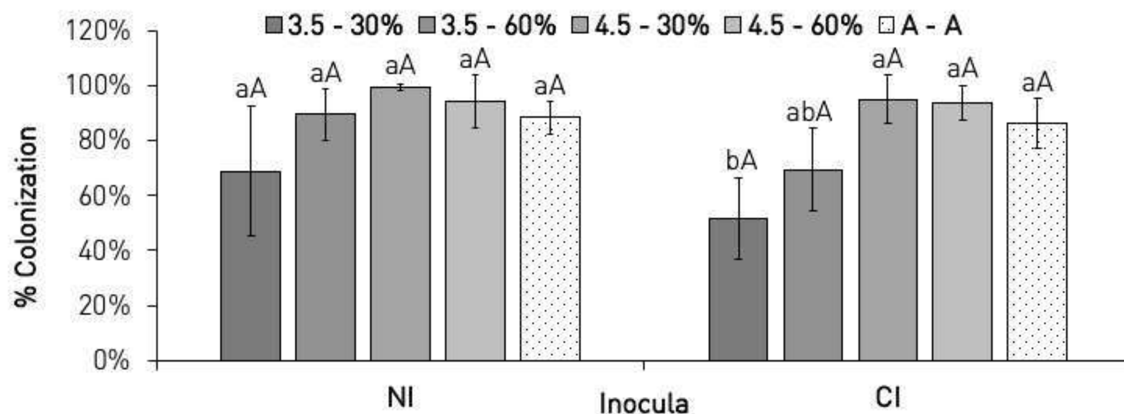

Figure 1. Comparison of mycorrhizal colonization percentage between the five vinasse treatments (pH 3.5 and 4.5, concentration of vinasse 30% and 60% and water A), and two AMF inocula after three months. Different lowercase letters indicate significant difference between treatments, for the same inoculum; Different uppercase letters indicate significant difference between inocula for the same treatment for a p-value <0.05. The bars represent the standard deviation. n = 3.

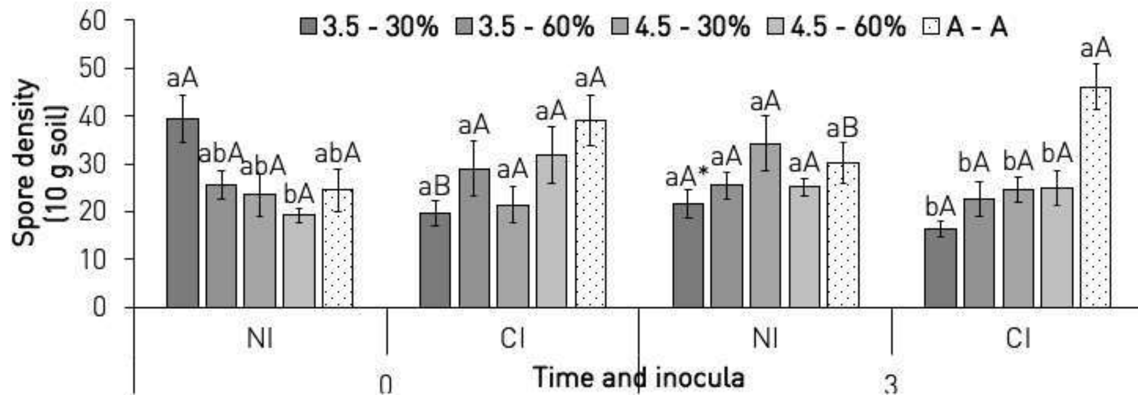

Figure 2. Comparison of spore density in soil (spores per 10 g of soil) between the five vinasse treatments (pH 3.5, 4.5, concentration of 30% and 60% and water), and two inocula of AMF, at initial time (t0) and after three months (t3). Inoculum I is a commercial inoculum, inoculum N is a native inoculum. Different lowercase letters indicate significant difference between treatments, for the same inoculum and time; Different uppercase letters indicate significant difference between inocula for the same treatment and time; asterisk indicates a significantly lower value between times for the same treatment and inoculum for a p-value <0.05. The bars represent the standard error. n = 6

Experiment II

The final average height of the plants was 63 cm. Soil pH, EC (Figure 3) and phenols increased. No benefit from the use of vinasse in plant height or biomass (Figure 4) was observed. A decrease in root colonization (5-10 %) and RA (10-20 %) in some AMF species was observed.

In both experiments, the results showed that vinasse composition has negative effects in soil characteristics and AMF, independently of the inoculum used. On the one hand, a high concentration of salts inhibits the growth of corn plants (Ertani et al. 2013), while increases in salinity negatively affect the AMF (Campagnac et al. 2014). Besides, some acids had beed found in tequila vinasses such as acetic acid (about 50–180 mg L^{-1}) and butyric acid (about 8–39 mg L^{-1}) (Rodríguez-Félix et al. 2018) disturb AMF populations (Arimi et al. 2014), which is further detrimental to plants because AMF help alleviate stress

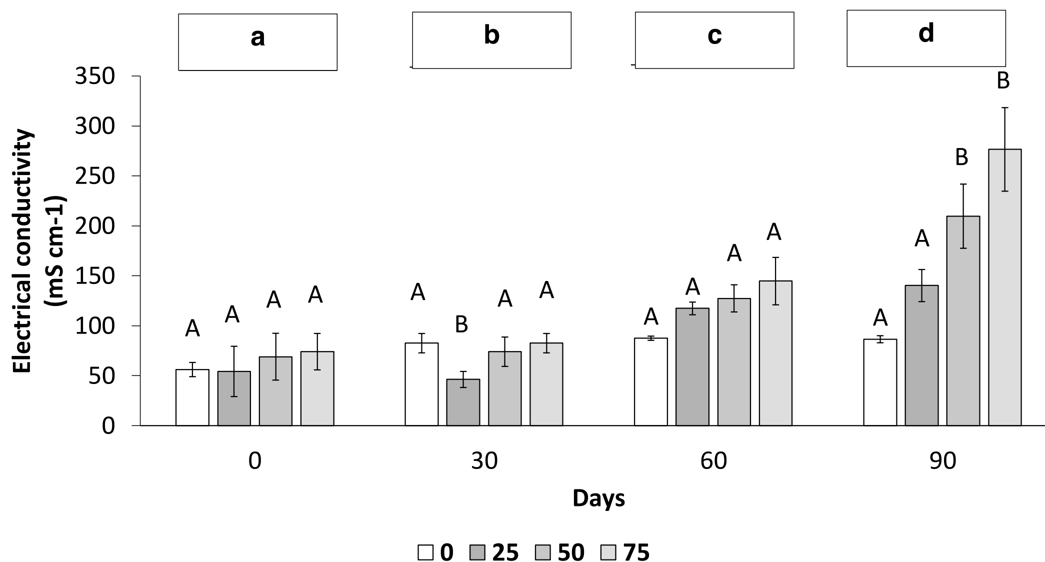

Figure 3. Electrical conductivity of the soil after vinasse irrigation (at 25%, 50% and 75%) and water (0%). Different uppercase letters indicate significant difference between the same treatment at different time for a p-value <0.05. Different lowercase letters indicate significant difference between treatments trough the time for a p-value <0.05. The bars represent the standard error. n = 6.

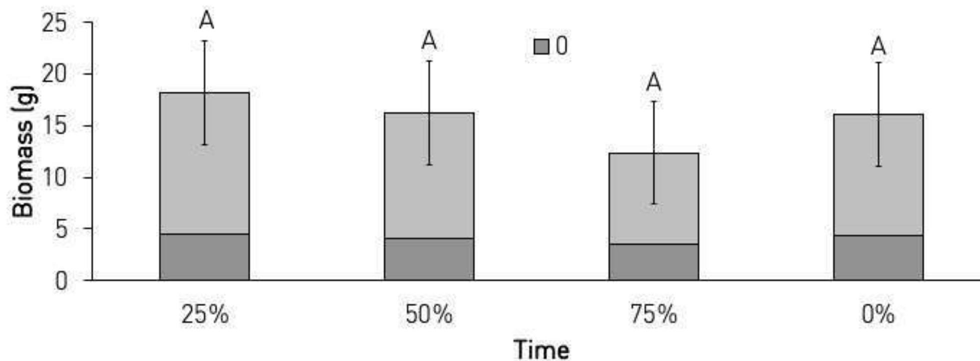

Figure 4. Total plant biomass (g) with the three vinasse treatments (25%, 50% and 75%) and water (0%) at initial (0) and final (90 day) of experimentation. Different uppercase letters indicate significant difference between treatment for a p-value <0.05. The bars represent the standard error. n = 6

CONCLUSION

The results showed that vinasse application promoted an increase in pH, EC and concentration of phenols in soil, as well as a decrease in AMF colonization and spore density of non-native AMF, regardless of vinasses concentration applied. At the end, the application of vinasse did not favor plant growth.

ACKNOWLEDGEMENTS

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Effect of ozone pretreatment on physicochemical characteristics and phenolic compounds generation on agave bagasse hydrolysates.

Castellanos-López, E. and Aguilar-Juárez O.[1] *

ABSTRACT

Agave bagasse is the fiber produced as a byproduct of the tequila industry. It represents aproximately 40% of the total weight of agave processed. Anually, around of 1.78x10^8 kg are generated (Saucedo-Luna, 2010). The main interest on this feedstock is due to its high sugar content and low lignin content (Contreras-Dávila et al. 2017). The lignin makes it difficult for the enzymes to access to the cellulose and hemicellulose during the enzimatic hydrolysis, therefore, different pretreatments have been studied for the biomass delignification. One of the most promising pretreatments is the ozonolysis offering selective lignin degradation with minimal effects on hemicellulose and cellulose contents.

On the pretreatment of lignocellulosic material, fermentation inhibitory compounds are generated, such as phenolic compounds, furans and AGV. The amount of compounds that can be found on this material depends on the composition of the biomass and the conditions of the pretreatment that will be used to obtain fermentable sugars. In this work some physicochemical characteristics and the presence of inhibitory compounds were compared between two hydrolyzed agave bagasse, one with O_3 treatment and a second one without pretretment with the objective of identifying the presence of inhibitory compounds for hydrogen production.

Key words: Agave bagasse, pretreatment, ozone.

INTRODUCTION

The agave bagasse is the residual fiber that remains after cooking, cutting and extracting the fermentable juice of the agave for tequila production (Saucedo-Luna, 2010). The large volumes generated annually from this lignocellulosic biomass have become an environmental and economic problem (González et al. 2005).

Due to the composition of this material and its renewable nature, it has become a substrate of interest for biofuels production. In order to use this biomass as substrate, a pretreatment must be carried out, during this pretreatment the biomass lignocellulosic structure is open, realizing sugar polymers from their ligations to lignin, improving the accessibility to the polysaccharides to produce fermentable sugars (Kaur et al. 2015).

Ozonolysis is one of the most promising pretreatments offering selective lignin degradation with minimal

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effects on hemicellulose and cellulose contents (Travaini et al. 2016). This chemical pretreatment consists of the use of ozone to oxidize, solubilize and degrade the lignin present in the biomass, resulting in a pretreated material with favorable characteristics for enzymatic hydrolysis (Travaini et al. 2013).

In the pretreatment of lignocellulosic biomass, in addition to the production of fermentable sugars, compounds that act as inhibiting agents in fermentation such as phenolic compounds, furans and AGVs can be generated (Oliva, 2003).

METHODOLOGY

Agave bagasse was pretreated with O_3 and after that, enzymatic hydrolysis was performed. The pretreatments are described below, the feedstock was provided by (León, 2018):

1. SAMPLE A: Ozone bubble-diffuser reactor was used to perform the ozonation of agave bagasse at 1.44 L/min during 62 min, at room temperature (27 °C ± 2). Reactor was fed with AB at 45% w/w of humidity, using a particle size less than 0.6 mm.

Pretreated Agave Bagasse (5% w/v) was subjected to enzymatic hydrolysis in a complete-mix reactor (3 L working volume) at 50 °C, 0.1 M sodium citrate buffer (pH 4.8), 120 rpm using Celluclast® (5.7% w/w).

2. SAMPLE B: Agave Bagasse (5% w/v) was subjected to enzymatic hydrolysis in a complete-mix reactor (3 L working volume) at 50 °C, 0.1 M sodium citrate buffer (pH 4.8), 120 rpm using Celluclast® (5.7% w/w).

3. SAMPLE C: Enzymatic hydrolysates of agave bagasse with O_3 pretreatment. Ozone bubble-diffuser reactor was used to perform the ozonation of AB at 1.44 L/min during 62 min, at room temperature (27 °C ± 2). Reactor was fed with AB at 45% w/w of humidity, using a particle size less than 0.6 mm.

Pretreated Agave Bagasse (5% w/v) was subjected to Enzymatic Hydrolysis in a complete-mix reactor (3 L working volume) at 50 °C, 0.1 M sodium citrate buffer (pH 4.8), 120 rpm using Celluclast® (0.2% w/w) and Viscozyme® (5% w/w).

4. SAMPLE D: Enzymatic hydrolysates of avage bagasse without O_3 pretreatment. Agave Bagasse (5% w/v) was subjected to Enzymatic Hydrolysis in a complete-mix reactor (3 L working volume) at 50 °C, 0.1 M sodium citrate buffer (pH 4.8), 120 rpm using Celluclast® (0.2% w/w) and Viscozyme® (5% w/w).

The hydrolysates were characterized in terms of Chemical Oxygen Demand (COD), total nitrogen, total sugars and total phenolic content.

To identify the inhibitory compounds, the analytic techniques of (Rodríguez, 2017) and UPLC were used.

RESULTS AND DISCUSSION

The samples used are described on Table 1, two enzyme cocktails were used for the hydrolysis, the hydrolysis was performed on both samples separately, the ozonolysate bagasse and the untreated bagasse.

Table 1. Processed Samples.

Sample	Pretreatment	Enzyme
A	With O_3	Celluclast®
B	Without O_3	Celluclast®
C	With O_3	Viscozyme®+Celluclast®
D	Without O_3	Viscozyme®+Celluclast®

The effect of the ozonolysis is mainly observed on the COD results, as shown on Table 2. Some phenolic compounds might act as inhibitory agents during the fermentation, this phenolic compounds are mainly generated during lignin degradation, phenolic content is less on the bagasse treated with O_3, for samples C and D which would mean an increase in the final yield of hydrogen production. However, compared to concentrations obtained in enzymatic hydrolysis, both samples have low concentrations.

In regards of total sugars on each sample, the hydrolysates of bagasse that were pretreated with O_3 have a greater amount of sugars, regardless the enzyme cocktail used for the hydrolysis both samples (A and C) have more than 6 g/L, the sugar content con samples that were not pretreated with O_3 is considerably low. This could be attributed to the efficiency of the ozonolysis, but also the bagasse composition.

Table 2. Characterization of hydrolysates (mg/L).

Sample	COD	NO_3-	Total Sugars	Total phenolic content
A	33.97	40	6445.79	235.64
B	20.20	25.5	1741.21	110.64
C	40.37	42.2	6412.46	346.75
D	29.10	25.3	3005.44	427.31

The potentially inhibitory compounds found on the samples are shown on Table 3. Sample B presents a smaller amount of inhibitory compounds. Differences between samples can be attributed to the treatment conditions.

Table 3. Potentially inhibitory compounds (mg/L)

Sample	Eugenol	Gallic acid	4- hydroxybenzoic acid
A	0.54	16.1	11.9
B	<0.5	<0.5	5.8
C	0.68	<0.5	12.3
D	0.89	24.9	14.6

CONCLUSION

To take advantage of the agave bagasse, and other lignocelullosic biomass, is important to determine not only parameters such as COD or the amount of sugars, but also the presence of compounds (weak acids, HMF, phenol, etc.) that, during the fermentation, might affect the total biofuel production.

Ozone pretreatment effectiveness was observed on the sugar content of the samples (A, C). Due to the conditions of enzymatic hydrolysis, and the bagasse composition, the amount of potentially inhibitory compounds present on samples might not affect significatively the fermentation.

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Effect of the phenolics remotion over the bio-hydrogen production using tequila vinasses as substrate.

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ABSTRACT

Tequila vinasses are a potential substrate for hydrogen production via dark fermentation due to its high volume of generation and residual sugar concentration. However, tequila vinasses have also the presence of phenolic and furanic compounds that can potentially inhibit the process.

Due to the polarity of these inhibitory compounds they can be removed using activated charcoal. This work studied the effect of the phenolic and furanic compounds removal with activated charcoal adsorption over the bio-hydrogen production.

Dephenolization of tequila vinasse with activated charcoal removed 98% of the total phenolics gallic acid eq., and 100% of the hydroxymethylfurfural present in the vinasse. 39% of total sugars were retained in the activated charcoal. Dephenolizatión procedure decreased the maximum cumulative bio-hydrogen production by 13%, the lag phase by 23%, and increased the Rmax by 22%. The adsorption treatment increased the molar yield by 11% but, decreased the volumetric yield (LH_2/Lvinasse) by 18%.

The results showed that activated charcoal adsorption is not a suitable method to detoxify the tequila vinasses, since it removes fermentable sugars which affect hydrogen production.

Key words: Bio-Hydrogen, Vinasses, Phenolics, Inhibition, Fermentation.

INTRODUCTION

Hydrogen can be a clean alternative for fossil fuels, as it has high energy values, null generation of contaminant gasses, and can be produced by biological processes. Dark fermentation has been highlighted due to its higher production rates, and lower energy investment in comparison with other biological processes (Arimi et al. 2015). Dark fermentation can be defined as an anaerobic process in which a mixed culture of microorganisms transforms sugars and other organic compounds in a mixture of short chain fatty acids as the acetic and butyric, producing hydrogen and carbon dioxide as byproducts (Dávila-Vázquez et al. 2008). It has also the advantage of using residual organic matter or sugar-rich wastewater as stillage waters (Buitrón et al. 2014).

Tequila production generates large volumes of vinasses, (close to 11 L per liter of tequila produced (López-López et al. 2010). Tequila vinasses had been studied as a potential substrate for the biohydrogen production due to its high generation rate and residual sugar concentration. Although, tequila vinasses also have a high concentration of phenolic compounds that can potentially inhibit the fermentation process (Rodríguez-

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Félix et al. 2016). Detoxification using activated charcoal adsorption had been reported to increase the hydrogen production rates and yields when acid hydrolysates of water hyacinth were used as substrates Cheng et al. 2015). When Tequila vinasses were treated using adsorption resins, the maximum hydrogen cumulative production was increased by 118% (Rodríguez-Félix et al. 2017). However, there are no reports of the effect of an A.C. treatment to the tequila vinasses over the hydrogen production. Thus, the main objective of the present work was to study the effect of the application of an adsorption pretreatment using activated charcoal to remove the phenolic compounds of the tequila vinasses on biohydrogen production.

METHODOLOGY

Sampling and characterization

Tequila vinasses were collected in Tequila Jalisco, they were generated from a traditional tequila production process (with agave pine coking). Tequila vinasses were characterized (Table 1) and stored at 4 °C.

Table 1. Parameters measured in the characterization of tequila vinasses.

Parameter	Method
DQO	Standard methods (APHA, 1999)
pH	potentiometry
Total phenolics	4-aminoantipyrine
Total Sugars	phenol-sulfuric
Reducing sugar	DNS
Total solids	NMX-AA-034-SCFI-2001-12/13
Total volatile solids	
Volatile fatty acids	HPLC-IR
Individual phenolic and furan compounds	UPLC-UV, QDA (Rodríguez-Félix et al. 2017)

Chromatographic methods

The volatile fatty acids were measured by high-performance liquid chromatography in a Varian Prostar model 200 with a Bio-Rad AMINEX HPX-87H column coupled to a refraction index detector. The mobile phase was sulfuric acid 5 mM, the oven temperature was 65 °C and the flow rate was 0.6 mL/min.

Individual phenolic and furanic compounds concentrations were measured via an ultra-performance liquid chromatography, in an Acquity class 1 equipped with a Waters Bech C18 (1000 x 2.1 mm) packed with 1.7 µm particles. The system was coupled to two detectors (UV-QDA) using the conditions reported by Rodríguez-Félix et al. (2017).

Dephenolizatión procedure

Dephenolization was carried out in a 1.7 cm internal diameter glass column. The column length was 25 cm and was packed with 23 g off activated charcoal (Darco SIGMA-Merck 20-40 mesh). 1 L of tequila vinasses was passed through using a peristaltic pump at a flow rate of 3 mL/min for 5.5 hours in each run.

Batch fermentation tests

Batch tests were performed in the automatic methane potential tests system (AMPTS II). The experimental design was a randomized block, studying the type of vinasse (raw tequila vinasses (RTV) and dephenolized vinasses (DTV)) as study factor, and the initial vinasse concentration (5, 15 and 30 gDQO/L) and the inoculum substrate ratio (I/S) as block factors.

22 mL of buffer and nutrient solution composed by (g/L) NH_4Cl : 41.6, MES : 19.52, $MgCl_2 \cdot 6H_2O$: 2.0, $FeSO_4 \cdot 7H_2O$: 1.6, $CoCl_2 \cdot 6H_2O$: 0.04, $MnCl_2 \cdot 4H_2O$: 0.04, KI : 0.04, $NiCl_2 \cdot 6H_2O$: 0.008, $ZnCl_2$: 0.008 were added to each bottle. An anaerobic granular sludge coming from a TV treatment plant was thermally treated to inhibit methanogens and used as inoculum (Moreno-Andrade et al. 2011).

The gas composition was analyzed qualitatively to discard methanogenesis via gas chromatography in a Clarus 580 Perkin Elmer with a HayeSep D (3mx3.2mm, mesh 100/120; Perkin Elmer Clarus NOCI) column coupled to a Thermal conductivity detector. Using the conditions reported g.

The modified Gompertz model (eq. 1) was fitted to experimental data obtained using r2018a Matlab.

$$H = Hmax\ exp\left\{-exp\left[\frac{2.71828 * Rmax}{Hmax}(\lambda - t) + 1\right]\right\}$$

Eq. 1. Gompertz modified model where H (mL/L) is the hydrogen volume per liter of reactor, Hmax (mL/L) is the maximum cumulative production, Rmax (mL/L*h) is the máximum production rate and λ (h) is the lag phase.

Statistical analysis

All experiments were carried out in triplicates, a 3-way analysis of variance and a multiple range test by least significative difference were made in Statgraphics Centurion 16.

RESULTS AND DISCUSSION
Characterization of tequila vinasses

The characterization of RTV and DTV is shown in Table 2. The dephenolization procedure removed 98% of total phenolics. No significative xylose and glucose losses were observed, although, the total and reducing sugars concentration were reduced by the treatment in a 39 and 30% respectively.

Table 2. Physicochemical characterization of tequila vinasses.

Vinasse Parameter	RTV g/L	DTV g/L
COD	38.00	33.00
pH	3.60	3.60
Glucose	0.88	0.82
Xylose	2.99	2.85
Total solids	29.40	20.95
Total volatile solids	26.66	20.30
Total Sugars	14.49	8.81
Total reducing sugars	6.54	4.57
Total phenolics	0.90	0.02

Exception made of 5-hydroxymethylfurfural (HMF), individual phenolic and furanic concentrations in the RTV were lower than the minimal inhibitory concentration as reported by (Siqueira and Reginatto 2015), (Table 3). Also, they were closer to those in traditional process vinasses (Rodríguez-Félix et al. 2017). No individual phenolic or furanic compounds was detected in DTV.

Table 3. Concentration of individual phenolic and furanic compounds.

Compound	RTV concentration (mg/L)	Minimal inhibitory concentration (mg/L)*
3-hydroxybenzoic acid	1.0	150
4-hydroxybenzoic acid	6.0	150
Furfural	1.8	250
5-Hydroxymethylfurfural	119.0	100

*Minimal concentration studied by Siqueira and Reginatto (2015)

Batch fermentation tests

All the Gompertz parameters calculated are presented in Table 4, all the correlation coefficients (r^2) were between the ranges 0.95-0.99. The pretreatment affected all the parameters studied ($P<0.05$). The cumulative hydrogen production (Hmax) was decreased by 13% on average, this could be partially attributed to the sugar loses in the adsorption treatment as sugars are the main substrate for fermentative processes. The maximum specific production rate was increased in 22% on average, it was previously reported that HMF can decrease the Rmax in concentrations up to 100 mg/L (Siqueira and Reginatto 2015) so the HMF removal can explain the results obtained for this parameter in the batch tests. The lag phase was reduced by 23%, low weight phenolic and furan compounds can easily trespass the cell membrane and affect enzymes of the metabolism that can produce prolongated lag phases that can explain the reduction in the lag phase when phenolic and furan compounds are removed (Siqueira and Reginatto 2015; Bundhoo and Mohee 2016).

The hydrogen molar yield was increased by 11%, this indicates a low inhibition and is similar to the findings of Gutiérrez-López et al. (2015), in that work, no total phenolics inhibition was observed in enzymatic hydrolysates of agave bagasse. As is mentioned before, the individual inhibitory compound concentration was under the values reported inhibiting the fermentative processes that explain this result. The volumetric yield (LH_2/Lvinasse) was decreased by 18% with the adsorption pretreatment, this was expected to happen in terms of the low molar yield increase and the considerable sugar loss caused by the detoxification treatment.

Table 4. Mean constants calculated of the Gompertz model fitting.

Vinasse	I/S	I.C.	Hmax	Rmax	Lag phase	Volumetric yield	Molar yield
		(gDQO/L)	(mL H_2/L)	(mL H_2/L*h)	(h)	($LH_2/L_{vinasse}$)	($molH2/mol_{T.S.}$)**
RTV	2.7	5	425.3 ± 0.3 **Aa**	63.5 ± 21.9 **Aa***	17.1 ± 0.8 Aa	3.2 ± 0.0 Aac	1.8 ± 0.0 Aa
		15	1,347.6 ± 2.2 **Ab**	64.3 ± 8.1 **Ab***	21 ± 2.8 Ab	3.5 ± 0.1 Aa	1.9 ± 0.0 Aa
		30	1,879.0 ± 79.5 **Ac**	42.2 ± 7.3 **Ab***	35.8 ± 2.5 Ac	2.4 ± 0.1 Ac	1.3 ± 0.1 Aa
	7.5	5	406.9 ± 0.4 **Aa**	49.1 ± 8.8 **Aa***	16 ± 1.2 Aa	3.09 ± 0.0 Aac	1.7 ± 0.0 Aa
		15	1,412.8 ± 2 **Ab**	70.2 ± 19.2 **Ab**	24.86 ± 2 Ab	3.58 ± 0.0 Aa	2.0 ± 0.0 Aa
		30	2,000.0 ± 1.6 **Ac**	91.7 ± 25.9 **Ab**	23.12 ± 2.3 Ac	2.53 ± 0.0 Ac	1.4 ± 0.0 Aa

Vinasse	I/S	I.C. (gDQO/L)	Hmax (mL H_2/L)	Rmax (mL H_2/L*h)	Lag phase (h)	Volumetric yield ($LH_2/L_{vinasse}$)	Molar yield ($molH2/mol_{T.S.}$)**
DTV	2.7	5	317.6 ± 13.9 **Ba**	40.4 ± 8.4 **Ba***	14.72 ± 0.1 Ba	2.1 ± 0.1 Bac	1.9 ± 0.1 Ba
		15	1,067.5 ± 39.6 **Bb**	75.2 ± 0.6 **Bb***	16.3 ± 0.8 Bb	2.3 ± 0.1 Ba	2.1 ± 0.1 Ba
		30	1,742.2 ± 120.9 **Bc**	59.5 ± 33.5 **Bb***	21.3 ± 3.1 Bc	1.9 ± 0.1 Bc	1.7 ± 0.1 Ba
	7.5	5	292.1 ± 55.6 **Ba**	64.2 ± 0.8 **Ba**	15.05 ± 0.5 Ba	1.93 ± 0.4 Bac	1.8 ± 0.3 Ba
		15	853.6 ± 163 **Bb**	120.8 ± 28.4 **Bb**	16.5 ± 0.7 Bb	2.02 ± 0.1 Ba	1.8 ± 0.1 Ba
		30	2,128.5 ± 82.3 **Bc**	86.9 ± 15.5 **Bb**	17.41 ± 2.8 Bc	2.39 ± 0.0 Bc	2.2 ± 0.0 Ba

Capital letters in the same column indicate significative differences between vinasses.
Lower letters in the same column indicate significative differences between initial concentrations.
* Indicate significative differences between I/S ratios n=3.

CONCLUSIONS

The pretreatment improved parameters of the biohydrogen production in terms of maximum production rate, lag phase, and molar biohydrogen production yield, this result could be helpful to optimize expenses and space by building smaller reactors for the biohydrogen production using tequila vinasses.

In contrast, the negative effects over the maximum cumulative biohydrogen production and its subsequent decrease over the productivity calculated on the volume of vinasses basis showed that the treatment with activated charcoal is not a suitable method to detoxify the tequila vinasses.

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Leaf-silage of *Agave tequilana* Weber var. blue as forage for ruminants.

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ABSTRACT

Tequila is considered an emblematic beverage of Mexico. Its production generates waste, such as plant leaves in the field, and these represent a high amount of biomass that could be used to feed livestock. According to some authors, the *A. tequilana* leaves represent about 49% of the total plant weight, which could be used as several thousand tons of fresh biomass at any time of the year through silage techniques. The objective of this study was to evaluate some characteristics of *Agave tequilana* Weber var. Blue's leaf silage that can be indicators of good quality food for ruminants. Leaves were obtained from a plantation (approximately 5 years of age), and they were left to dry in the field for12 days. A forage chopper, with a 13 HP gasoline engine, was used for chopping. The silos were made in black plastic bags, weighing 40 kg each. Then, 800 g of urea and 800 g of molasses were added in order to increase the Nitrogen and sugar content, respectively. The ensiled was made using a commercial vacuum cleaner of 1 HP to extract the air. The leaf humidity at the ensiling time was 85%, and a pH of 5.34 was observed. After 30 days, the humidity was reduced to 77.37% and the pH to 4.25; likewise, the protein was increased from 2.64% to 31.59%. The nitrogen-free extract was 30.33 and an in vitro digestibility of 75.31 was determined. Silage characteristics are considered acceptable in terms of color, odor, pH and nutritional content.

Key words: Urea, sheep, molasses, digestibility, biomass.

INTRODUCTION

The production of alcoholic beverages, such as mezcal and tequila, generates liquid wastes such as vinasses and solid wastes such as bagasse and leaves, after cooking and harvesting, respectively. Considering that tequila's demand, Nationally and worldwide, is significantly increasing, it is expected that *Agave tequilana* residues at field (leaves) will increase as well (Sánchez-Carmona et al., 2017).

Tequila is the emblematic beverage of Mexico, and according to the Tequila Regulating Council (CRT, its Spanish acronym), in 2018, 309.1 million liters were produced. 72.5% of that production was exported to more than 120 countries (224.3 million of liters). For this production, 1,138,800 t of *A. tequilana´s* raw material was consumed. For the elaboration of this drink, only the pineapple of the *Agave tequilana* Weber var. Blue plant is used, leaving the leaves in the field without any use. According to Montañez et al. (2011), the leaves represent 48.5% of the total weight of the plant. The Agave Tequila Product System reports a planted area of 90,000 hectares, throughout the Tequila Denomination of Origin (DOT, its Spanish acronym) area. Likewise, the Agri-Food and Fisheries Information System (SIAP, its

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Spanish acronym) reports an area of 13,439 hectares planted in the Guanajuato State for 2017, with an approximate pineapple yield of 59 t ha^{-1}. Guanajuato State ranks second nationally in *Agave tequilana* planted surface, which represents a great quantity of left biomass (leaves) in the field after harvest.

This amount of leaf biomass could be used for animal feed using forage conservation techniques, such as silage, which allows forage to be stored, conserving its quality and palatability. So, it is possible to increase the animal load/ha and replace or supplement with concentrates (Garcés et al. 2004) This would help to reduce the negative impact of food lack in the year's dry season, turning a waste product into income for agave and livestock producers. Therefore, the objective of this study was to evaluate some characteristics of *Agave tequilana* Weber var. Blue's leaf silage for indicators of good quality as food for ruminants.

METHODOLOGY

Location

This study was carried out at the San Carlos ranch, located at km 61 + 100 of La Piedad de Cavadas-Pénjamo road, Pénjamo County, Guanajuato State, México. This municipality is located between parallels 20°38' and 20°11' N Latitude and meridians 101°35 'and 102°06' of W Longitude, and 1 600 and 2 500 mas. Pénjamo County is bordered by Jalisco State and the municipalities of Manuel Doblado and Cuerámaro to the north, the municipalities of Cueramaro and Abasolo and the Michoacán de Ocampo State to the east, the Michoacán de Ocampo State to the south, and the states of Michoacán de Ocampo and Jalisco to the west. Pénjamo County has a temperature range from 16 to 22 ºC, a rainfall from 700 to 900 mm per year, and a sub-humid semi-warm climate with summer rainfall of lower humidity (63.4%), sub-humid temperate with summer rainfall of medium humidity (23.6%), semi-warm sub-humid with summer rainfall of medium humidity (11.8%), and sub-humid tempering with summer rainfall of lower humidity (1.2%) (INEGI, 2009).

Plant material

The plant material was collected from a plot in the Pénjamo municipality, Guanajuato State. To reduce size, a forage chopper with a 13 HP gasoline engine was used to achieve particle size reduction (as shown in Figure 1). The leaves, which came from 5-year-old plants, were harvested then left in the field to dry for 12 days.

Figure 1. Collection, transfer and chopping of *Agave tequilana* leaves.

Silos preparation

For the silos production, 400-gauge polyethylene black bags were used. They were filled with 40 kg. of the chopped plant material, then 800g urea and 800g molasses were added to each bag using a backpack sprayer, but chemicals were previously dissolved in tap water for proper dispersion (Figure 2). The extraction of air was carried out using a commercial 1 HP vacuum to promote an anaerobic fermentation. Bags were tied up with plastic straps and stored for 30 days for further analysis.

Figure 2. Preparation and storage of *Agave tequilana* leaf silage.

RESULTS AND DISCUSSION

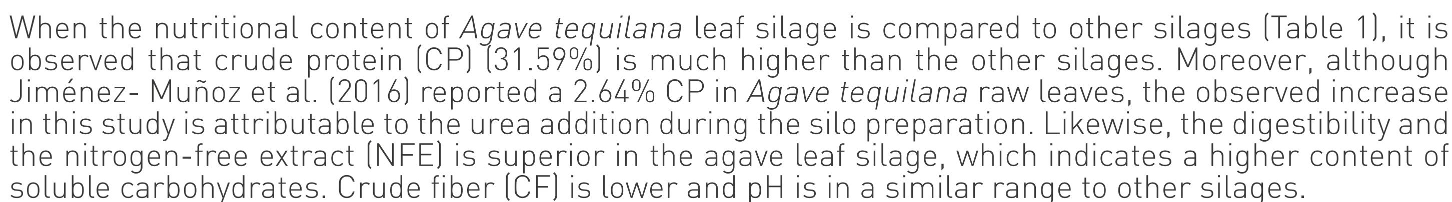
The *Agave tequilana* leaf silage presented a light green color, a pleasant, slightly sour smell. Its pH was 4.3, parameters that according to Wagner et al. (2012) are indicators of good silage (Table 1). The agave leaves had an 85% humidity at the time of ensiling, which is above to that reported by Montañez et al. (2011). These authors reported a 79.5% humidity on the leaves of *Agave tequilana*. The silage humidity found in this study was 77.4%, a value that differs from that reported by Alvarez et al. (2009), who found a 90.2% humidity in an *Agave salmiana* silage. The digestibility was determined in 75.31% and is different from that reported by Ramírez-Cortína et al. (2011), who determined a value of 36% for the *Agave tequilana* bagasse.

Table 1. Comparison of nutritional content of different silages.

NUTRIENT	*Agave tequilana* leaf	Corn*	Sorghum*	Oat*
	%			
Dry material	22.63	20-25	20-25	20-25
Ashes	13.06	6.31	8.74	9.58
Crude protein	31.59	8.41	8.79	10.10
Crude fiber	23.69	27.00	38.20	38.2
NFE	30.33	20.8	5.09	2.90
pH	4.25	3.91	4.09	4.50
Digestibility	75.31	70	55	65

*SFDAN (Spanish Federation for the Development of Animal Nutrition) Tables.

NFE: Nitrogen-free extract.



When the nutritional content of *Agave tequilana* leaf silage is compared to other silages (Table 1), it is observed that crude protein (CP) (31.59%) is much higher than the other silages. Moreover, although Jiménez- Muñoz et al. (2016) reported a 2.64% CP in *Agave tequilana* raw leaves, the observed increase in this study is attributable to the urea addition during the silo preparation. Likewise, the digestibility and the nitrogen-free extract (NFE) is superior in the agave leaf silage, which indicates a higher content of soluble carbohydrates. Crude fiber (CF) is lower and pH is in a similar range to other silages.

CONCLUSION

The bromatological analysis of the silage of *Agave tequilana* leaves indicated that this is a good alternative as livestock feed, particularly in the year dry season, when the pastures do not grow naturally and the cost of feed input. Therefore, *Agave tequilana* growers could obtain extra income by selling the leaf-silage or by feeding their own animals.

An evaluation of the silage quality with animals, would help define and validate its use in livestock.

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Use of adsorbents for the detoxification of hydrolyzed agave bagasse.

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ABSTRACT

Tequila industry is one of the main agroindustries in Mexico, its production generates different subproducts, where we can find vinasse and bagasse in large quantities in relation with the tequila liter, wastes that have a high environmental impact. Wastewater from food industries can be treated with biological processes to generate biogas rich in methane or hydrogen, these biofuels are obtained from renewable sources that can replace part of fossil fuels which have limited reserves and damage the environment. Hydrogen as a fuel can be obtained by biological digestion with lignocellulosic wastes such as agave bagasse, this has a great potential to release sugars through a pretreatment and a hydrolysis, however, these processes also generate inhibitors which affect the yield of the hydrogen production as fuel. These inhibitors can be eliminated by physical processes, such as adsorption.

The purpose of this work is characterizing the adsorption process of, at least, two different adsorbents by obtaining its isotherms and kinetics, based on the saturation time of the adsorbents-adsorbates by quantification of total phenols, as an inhibiting agent, and reducing sugars, as a conversion agent, in the hydrolyzate wich allows to obtain the parameters referring to Langmuir and Freundlich, useful to know the adsorbents maximum capacity and their affinity to the adsorbates, as well as the necessary contact time for these materials to reach the equilibrium with total phenols and reducing sugars. These parameters will be useful to obtain a detoxified hydrolyzate at the ideal adsorbent concentration and contact time for its possible biological production of hydrogen.

Key words: Agave bagasse, inhibitors, resin, carbon, hydrogen.

INTRODUCTION

Agave cultivation has a great industrial importance due to its agro-ecological requirements and the tequila production. Processing this beverage results in a waste called bagasse, which is mostly water and lignocellulosic fibers (Rendón et al. 2009). Using this waste is limited and causes environmental pollution, so alternatives have been sought for its use, such as the biofuels generation. Hydrogen use as a clean and renewable fuel has advantages over hydrocarbon fuels in terms of energy efficiency and relation with the environment, with water being the only resulting by-product, also is widely used as an energy source in industry, which generates its growing demand and its need to be produced in a sustainable and economic way (Kapdan and Kargi, 2006).

Biological methods are used to produce hydrogen which carry out a fermentation by microorganisms. The genus *Clostridium* is one of the most popular in biofuel production from wastes, using glucose and other simple carbohydrates as a conversion substrate, so that more complex pre-treated substrates can

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be used as lignocellulosic fibers. This genus has a much higher yield than other microorganisms as well as producing other by-products of interest such as acetic acid, butyric acid and some alcohols (Reith et al. 2003).

It has been proposed for the use of agroindustrial wastes in hydrogen production and for obtaining a good yield of biofuels, these wastes must be previously treated by means of processes to remove fermentation inhibiting compounds. Different alternatives such as the use of adsorbents, which have been used in the treatment of industrial waste (Paredes, 2011). Chemical compounds adsorption can be carried out with the use of different materials. In the inhibitory compounds adsorption process of different bagasse types, the resins have proved to be an effective material for the treatment of this waste type, which has allowed obtaining clearer liquors and of greater degree of purity. These treatments types can be optimized by using different resins types, temperature conditions, adsorbent concentrations and contact times (Napoles et al. 2005).

METHODOLOGY

Materials

Hydrolyzed agave bagasse

Agave tequilana Weber var. azul hydrolyzate comes from tequila houses in the municipality of Tequila, Jalisco. The hydrolyzate sample was stored at -4 °C during its physicochemical characterization and adsorption isotherms and kinetics determination.

Activated carbon

Carbón Activado Darco® (CA) with a particle size (mesh) of 20-40, surface area of 600 m^2 g^{-1} and pore volume of 0.95 mL g^{-1} was provided by the Sigma-Aldrich company.

Amberlite XAD4 resin

Resina Amberlite XAD4® (XAD4) with a particle size (mesh) of 20-60, surface area of 750 m^2 g^{-1} and pore volume of 0.98 mL g^{-1} and styrene-divinylbenzene matrix was provided by the Sigma-Aldrich company.

Adsorption isotherms determination

To characterize the adsorption process it is necessary to know its isotherms, so the two adsorbent materials, activated carbon (previously washed with distilled water and dried in an oven at 105 ºC for 24 h) and resin (previously washed and hydrated with distilled water) were placed in batch with 10 mL of hydrolyzate (previously centrifuged at 10 000 rpm for 5 min) at different adsorbent concentrations, 0, 0.5, 1, 1.5, 2 and 2.5% (w v^{-1}), maintaining a constant temperature of 23 ºC and 240 rpm agitation for 24 h contact time until reach equilibrium. Later detoxified hydrolyzate was characterized to determine the total phenols (FT) and reducing sugars (AR) concentration, by using the colorimetric method of 4-aminoantipyrine and the technique of Miller DNS, respectively, to make a profile of adsorbent concentration against adsorption capacity at equilibrium, this allowed obtaining the parameters referring to the isotherms of Langmuir and Freundlich, models which are shown below.

$$q_e = \frac{k_L \; q_m \; C_{eq}}{1 + k_L \; C_{eq}}$$

Where q_e is the component amount adsorbed (mg adsorbate g^{-1} dry resin), C_{eq} is the equilibrium concentration of the liquid phase (mg L^{-1}), k_L and q_m are the Langmuir constant (L mg^{-1}) and the saturation capacity (mg adsorbate/g dry resin) respectively.

$$q_e = k_F \ C_{eq}^{n^{-1}}$$

Where k_F is the Freundlich constant which is the maximum capacity (mg g^{-1}) and n^{-1} (mg L^{-1}) is the adsorption intensity.

Adsorption kinetics determination

To characterize the adsorption process it is necessary to determine the adsorbents saturation time, so the two adsorbent materials, activated carbon and resin, were placed in batch with 10 mL of hydrolyzate at different contact times, 5, 15, 60, 360 and 1440 min at constant concentration of 1.5% (w v^{-1}) maintaining the same conditions that were used with the isotherms. Subsequently, the treated hydrolyzate was characterized to determine the total phenols and reducing sugars concentration, to make a profile of adsorbate concentration against time to reach equilibrium when there were no statistically significant differences in the time intervals, this allows obtaining the ideal time to perform the detoxification process in the hydrolyzate. Data analysis and model parameters calculations were performed by using non-linear regression methods with the MATLAB software.

RESULTS AND DISCUSSION

The behavior of the adsorption process based on the values of the parameters of the isotherms is shown in Figures 1 and 2 and Table 1, where the adjustment was made for the average obtained from the experimental replicas. According to Saffarionpoura et al. (2016) and Xunjun, (2015) the higher constant k_F or q_m numerical value, the higher capacity saturation (maximum capacity), and the higher constant k_L or n-1 value the higher adsorption intensity (affinity), so when comparing the values of k_F for these adsorbents it is observed that the CA has a greater saturation capacity for both adsorbates, but a lower affinity towards FT, unlike the XAD4, which shows greater affinities differences in FT and AR, the highest affinity difference for both adsorbents-adsorbates is at concentration of 1.5% (w v^{-1}). The values of R^2 are in 0.9500-0.9900 range, which shows more validity for the Freundlich model.

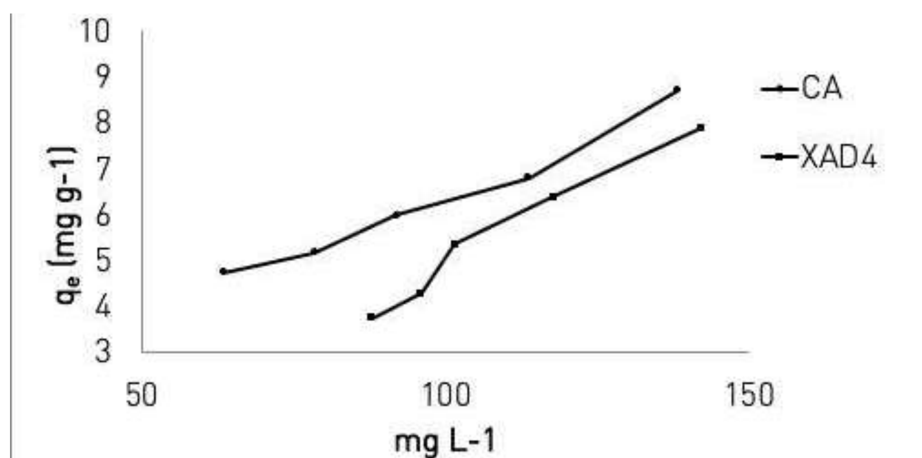

Figure 1. Total phenols adsorption behavior.

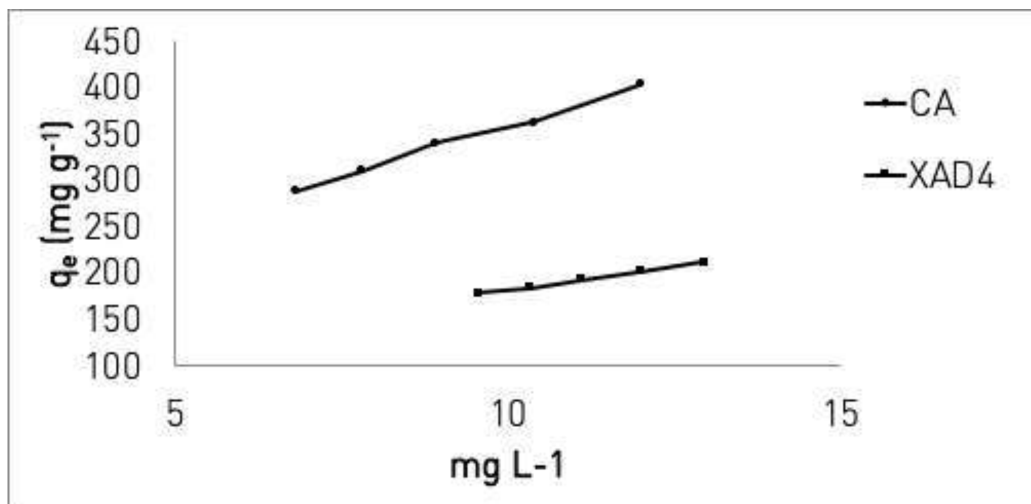

Figure 2. Reducing sugars adsorption behavior.

Table 1. Isotherm parameters.

Constant	Adsorbent			
	CA		XAD4	
	FT	AR	FT	AR
Langmuir				
k_L (L mg^{-1})	0.0020	2.8595E-5	1.5933E-5	0.0652
q_m (mg g^{-1})	38.94	341.11	0.7560	460.35
R^2	0.9610	9.4532E-5	0.8665	0.9925
Freundlich				
k_F (mg g^{-1})	0.1464	92.7890	0.0061	48.0300
n^{-1} (mg L^{-1})	1.2164	1.6964	0.6900	1.7304
R^2	0.9674	0.9933	0.9631	0.9945

Table 2 shows remotion differences of FT and AR with CA and XAD4 over time, looking for the ideal time where the adsorption equilibrium is reached. This point is in the interval time where there is no significant difference (> 5%) in remotion percentages. For XAD4 its adsorption equilibrium is in 60-360 min for both adsorbates, while for CA its adsorption equilibrium with FT is in 60-360 min and AR in 15-60 min. Once having these data, it is considered use 360 min as exposure time for both adsorbents, since greater inhibitor removal is desired. At this exposure time, a maximum removal was achieved, of 47.47% for FT and 26.92% for AR with CA, and of 42.20% for FT and 19.87% for AR with XAD4, values close to those reported by Ranjan (2009) of 26% for fermentable sugars with CA.

Table 2. Kinetics remotion differences (%).

Adsorbent	XAD4	CA		
Time interval (min)	FT	AR	FT	AR
0-5	2.64	0.32	1.32	1.6
5-15	3.96	2.24	3.96	4.17
15-60	7.91	6.09	7.91	15.38
60-360	32.97	11.22	29.01	5.77
360-1440	0	0.96	1.32	3.21

CONCLUSION

It was possible to characterize the adsorption process by means of isotherms and adsorption kinetics that were obtained through detoxification by means of the adsorbents applied to the hydrolysate. As result, it was obtained a notable decrease in the concentration of both inhibitors and sugars; with which the elimination percentage for each substrate concentration and contact time of each adsorbent, the ideal ones when finding the point where the difference between the phenols and the sugars was higher and there were no significant differences of concentrations between the time intervals.

Activated carbon Darco® was identified as the adsorbent that has a better detoxification behavior of this type of substrate with respect to the Amberlite XAD4® resin, this is based on its higher loading capacity and on the adsorption time, the price and availability. Although the resin has a greater affinity towards phenolic compounds and a lower affinity towards sugars, this difference is not significant.

ACKNOWLEDGEMENTS

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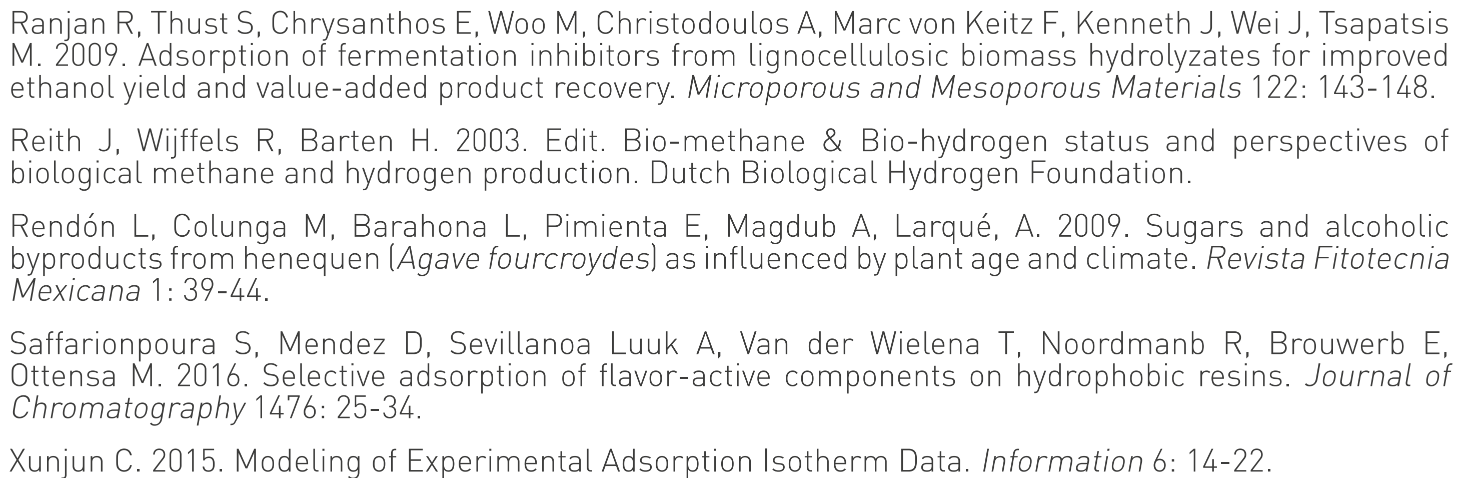

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Social and ethnobotanical aspects

AGAVE SPIRIT

Social and ethnobotanical aspects

Artisanal preparation of comiteco from the producers perspective.

Valdivieso-Solís, D. G., Verdugo-Valdez A. G. *, Sánchez-Cortés, M. S., Orantes-García., C. [1]

ABSTRACT

Comiteco is an artisanal beverage derived of agave, this is obtained for distillation of aguamiel mixed with sugar cane and water, and the organoleptic characteristics of beverage depend of proportions to raw material used by each producer. In this chapter we describe the importance of the beverage for artisan producers of the Meseta Comiteca, Chiapas, Mexico. For this, we apply semi-structured interviews to primary producers (farmers who get aguamiel called aguamieleros) and secondary producers (comitequeros) in addition to visits to places where the beverage is produced and to field where agave plants are; in march and april of 2017 and 2018 to generate complementary information about the process. The producers of aguamiel and comiteco have knowledges acquired through different experiences; principally related to their ancestors that are reflected in their environment social, family, economic and cultural. This is reflected in the value and interest that producers give for the beverage, that for some, it begins to be one of its main economic activities. In turn, the production has been influenced by situations that involve political and economic interests. We found some limitations to the realizations of this study: the current situation of the comiteco influences the distrust of the producers to authorities. This caused many producers to avoid talking and sharing their story, which explain the low number of interviews achieved. The comiteco has a promising future for their characteristics, artisan producers are those who keep the tradition alive and those who face the actual problematic. Work on different aspects that influence in this it is essential.

Key words: *Comiteco, agave, artisanal process, producers, aguamiel*.

INTRODUCTION

The people of the Meseta Comiteca has learned to harness the agaves as a natural resource for the comiteco, which is a beverage produced in Chiapas from mid XIX century as an economic activity on a small scale (Reynoso-Santos et al. 2012) Comiteco is a fermented and distilled beverage that is made from *Agave americana* L. and *Agave salmiana* Otto ex Salm-Dyck (Reynoso-Santos et al. 2012) The history of comiteco begins with the arrival of the Dominican friars and with them the distillation, its elaboration is strongly linked to the traditions of the Meseta Comiteca, and its production continues to preserve the artisan level because the industrial production is limited to a few producers (Moreno-Terrazas et al. 2017). This beverage is a distillate of the fermentation of agave mead, "honey" of agave or "piloncillo" of sugar and water that can be found in different degrees of aging and flavors. Since this is a chiapaneca beverage with great potential, this paper aims to describe the artisanal elaboration of comiteco and their productive importance for artisans of the Meseta Comiteca and to record the current problem of it.

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METHODOLOGY

Study zone

This work was carried out in the municipalities of Comitán de Domínguez and Tzimol, Chiapas; both places are part of the Meseta Comiteca, which cover 10.12% of the territory of the state of Chiapas, Mexico.

Research technique

We carry out a qualitative study through semi-structured interviews in March both 2017 and 2018, modified from the work of Erlwein et al. (2013) about pulque, to know the importance that grant to comiteco the artisanals productors of aguamiel and comiteco. The interviews contained questions about the process, generalities, economy, production time, knowledge focused on production and market. As well as complements we stay with producers in convivence experience in the months of important production (March-April) also in both years 2017 and 2018. These with the objective of realize an analysis and the description of the answers obtained. At the same time we tried to know the current socioeconomic status among the producers and how this influenced the interest to continue the production activity, in addition to other reasons that have influence in the development of the production of the comiteco beverage.

RESULTS AND DISCUSSION

From the interviews realized it was possible to obtain an overview of the importance that artisanal producers give to the comiteco, which allowed us to know what are the reasons to continue the production or not. Also to know the issues involved in the current situation that has been lived in the region around this beverage.

In the Meseta Comiteca region, the primary producers (aguamieleros) expressed their concern for the cost and effectiveness of the activity.

> Don Arturo: "... the time we should take care of the plants until it mature in which we can begin to utilize is long. We need to protect the plants of the pests. We have to clean the planting before, along and after. I spend the whole day among my crops; I try to take care of all my plants equally. I take care of the agaves all the year even if they only produce four months".

> Don Fer: "These practices require a lot of money... and work. I dedicate myself totally to the cultivation and care of the agaves because I live from this. I produce aguamiel all year".

Álvarez-Duarte et al. (2018) mention the producers must wait at least eight years to start using the maguey. This has been part of the disinterest in cultivating it. For pulque, it is pointed out that there are tlachiqueros (mead extractors) that are dedicated only to the obtaining and sale of mead. Meanwhile other mead extractors sell it and also dedicate themselves to other agricultural activities. However they give good management to their crops (Álvarez -Duarte et al. 2018). The mead producers of "Maguey Comiteco" give the same a management to their crops due to lack of instruction. There is no documented management maybe due to lack of interest, there are no crop rescue programs among the comiteco producers. Erlwein, et al. (2013) indicates that among the practices of the tlachiqueros of Apan, Hidalgo, there is no previous layering of the plants. Due to this they are currently facing with shortage of plants and displacement by other crops. Aguilar et al. (2014) mentioned that in the Valley of Mexico do not exist a manual for these practices, but exist a lot estudies for this. The existence of a plague of a coleoptera that they call "picudo" represents a big problem due to the destruction of a large number of plants. Although efforts have been made to eradicate it, it has not the expected success.

> Don Fer and Don Arturo mentioned: "We know only three uses of mead: production of comiteco, agave "honey" and use in food".

Reynoso-Santos et al. (2012) who found that the "Maguey Comiteco" has been used as a live fence, construction, obtaining mead, production of pulque, in addition to the elaboration of comiteco. The use and exploitation of different parts of the agave can be an alternative for producers as it has been in other places (Flores et al. 2009; Vela, 2014; Nieves et al. 2011). In some states the producing communities market the aforementioned material and utensils in "tianguis" and popular markets, which could also be implemented by the Meseta Comiteca producers (Figure 1).

Now, regard to the other group of actors in the comiteco production the "comitequeros":

> All: "The artisanal production is currently limited. The activity still lacks support for the recognition of the beverage"
>
> Don Daniel and Don Augusto: "We have more years of experience because acquired the knowledge of our parents".
>
> This has allowed them to make improvements over time and as they get to know the market and acquired a deeper conception of the meaning of the comiteco: The most part have not passed the knowledge in to their family and they are generally very jealous of their recipe and their new knowledge. This situation is linked to the state of production. Historically, other fermented and distilled beverages derived from agave have had a long trajectory before be able to position each one in the level that they have now. It has had to be improving the product, looking for a market, and even then the production, recognition and income. The economic situation with tequila is not yet reached by other products that continue to maintain part or all of an artisanal process (Erlwein, 2013, Lappe-Oliveras et al. 2008; Montes, 2014; Álvarez-Ainza, 2017, Moreno -Terrazas, 2017; Salazar and Mungaray, 2009; Carrillo, 2007).

Figure 1. Topics in interviews; left) aguamielero, rigth) comitequero

The comiteco has a long tradition and has great cultural importance in the region of the Meseta.

> Don Martín: "The increase of distribution and sale is in the informal trade directly in bulk among existing customers".
>
> Don Javier and Don Daniel: "Not having a permit sale and a registered trademark, makes improvements on a larger scale difficult to reach on the product, which translates into the lack of ease for sales and little production".

Montes (2014) and Salazar and Mungaray (2009) mentioned that the trajectory of the scopes achieved by others beverages derived of agave, such as mezcales, has been long and complex. They contain themselves a long tradition of inheritance and adaptation to the changing historical conditions and demands. The comiteco have a different process but have a large history and culture too, have many

possibilities to grow in this field. Moreno-Terrazas et al. (2017) sustain that the industrialization of comiteco production is still incipient and the technological challenges are broad both applied to the beverage and the species of agave used.

Socioeconomic conditions have a great influence on the state of production of artisanal beverages such as the comiteco. In the region of comiteco production the commercialization of the products that already have the brand and the sales permit has grown both locally and externally. Artisan producers cannot afford laboratory studies for their products, the payment of trademark registration or any other necessary action, this condition could be like Salazar and Mungaray (2009) describe about bacanora that currently it is estimated that the producers have developed technologies and organizational forms adapted to the logic of a source of complementary income, but in general terms lacking that have a scheme of social organization of work, integration practices and productive specialization, formal mechanisms of knowledge dissemination, supply of raw materials and supplies, machinery, equipment and logistics services, among others.

The comiteco, like all the artisanal production beverages, keeps a quite extensive cultural, ecological and social repository (Moreno-Terrazas et al. 2017). This presents a unique flavor in each product, In addition to the culture that keeps the recipe, each producer has provided his "magic touch", this drink has been kept alive among the inhabitants and the people who consume it and know it for its flavor, culture and its traditional process, in terms of these characteristics. Carrillo (2007) argues that mezcales have a great diversity of flavors, colors and smells that are attributed to the production technique used, agave species, various cultural and natural elements of the area, this could be applied to comiteco, because is a derived of agave beverage.

CONCLUSION

The comiteco has a promising future. The market for distilled beverages derived from agave has been increasing and has expanded internationally, and this entails a greater responsibility, giving ecological, biological and cultural importance to the raw material and with this situation look for increase productivity and the quality of the final product. The sale of comiteco is relatively small compared to other beverages. Even the local bulk sale is larger and more accepted by its consumers. The handicraft production of Comiteco implies a history that has been built over the years, which has allowed them to have their own characteristics. Currently the artisanal producers are going through a difficult situation, there is a concern for prices in the raw material, the uncertainty in sales and the profitability of the activity, this also influences the interest in maintaining the tradition, trust in the secrets and share the acquired knowledge. The location of producers takes time and requires confidence, the producers with whom it was treated were Don Alberto, Don Martin, Don Fernando, Don Arturo, Don Daniel, Don Javier and Don Antonio, the contact with one of them propitiated the confidence to others, their productions are handmade, some with more history than others, but with great availability and interest. The sale of both aguamiel and comiteco in bulk to known and established customers. Artisanal beverages protect generations of knowledge, passion, dedication and risk.

The path traveled by other artisanal beverages should serve as an example and guide to achieve good results, without neglecting artisanal production, importance and culture, to reach this point it is necessary to make improvements and expand knowledge about them.

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Cost analysis of agave mezcalero in Oaxaca, challenges and perspectives.

Vázquez-Elorza. A. [1] *

ABSTRACT

Currently, there is a concern in small producers of agave mezcalero in the state of Oaxaca, mainly because they lack elements for marketing, among them, the identification of profits and financial budget to make useful decisions to sell their products at reasonable price. Although most of the traditional mezcal communities elaborate their product more by culture and tradition, which, for the sake of profit, activities are generally not remunerated (to a greater extent among women or children), in addition, they do not assign any economic value to indicators such as the cost of maguey, firewood, rent of plots, water or labor (man hours cost used throughout the process). In this context, this research aims to measure the level of costs and profits obtained by small producers of *agave mezcalero* considering a baseline for a plantation of six years. To thise purposes, several workshops and forums were held to generate costs for small and medium producers within the framework of the National Project of Problems 1406 CONACYT CIATEJ. In general, in the field work in the municipalities of Matatlán, San Juan del Río, Sola de Vega, among other municipalities, it was observed that, in practice, small agave producers lack records on costs, income and profitability. Among the main localities with the highest levels of profitability, that is, greater profits are Matatlán and Tlacolula, which on average a producer can obtain a daily income of $ 577 real pesos, being Yautepec a less competitive (data from average income per producer in a productive cycle).

Key words: Costs, primary production, agave, budgets, profitability.

INTRODUCTION

The generation and identification of costs in the primary sector is fundamental to improve agricultural decision-making, so it will be necessary to generate a training system to help the decision-making process and primary production; the above, independently that in the small producer population by tradition they elaborate their product more by culture and tradition, since the economic and natural resources are limited in every society. On the other hand, the accelerated growth of micro and small units of local production to satisfy the demand of mezcal consumers is driving producers to acquire new challenges in terms of sustainability; in addition, new dares to face the overexploitation of wild plants; new innovations arise to increase the value added to by-products for the use of different traditional businesses.

The study carried out in the field includes the respectful of knowledge and traditional culture. It should be noted that, in most cases, producers do not make the costs to determine prices, which translates into a lack of information to offer their products that include economic costs. Ideally, intangible costs should also be included, such as the value of their ancestral knowledge, culture and aspects related to the environment and nature that are related to the protection made by producers. To the extent that knowledge

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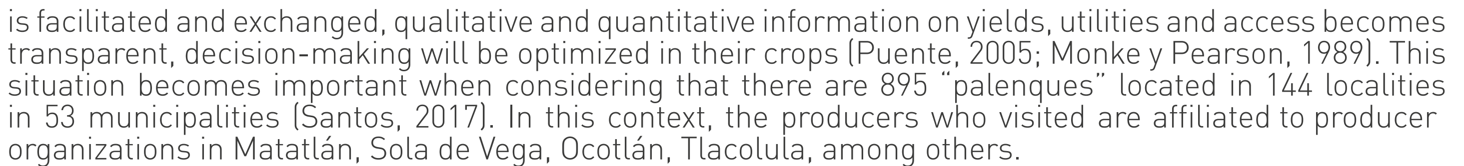


is facilitated and exchanged, qualitative and quantitative information on yields, utilities and access becomes transparent, decision-making will be optimized in their crops (Puente, 2005; Monke y Pearson, 1989). This situation becomes important when considering that there are 895 "palenques" located in 144 localities in 53 municipalities (Santos, 2017). In this context, the producers who visited are affiliated to producer organizations in Matatlán, Sola de Vega, Ocotlán, Tlacolula, among others.

METHODOLOGY

For the preparation of the cost indicators it was necessary to work with small producers in different municipalities of the state of Oaxaca (Ocotlán, Sola de Vega, Yautepec, Tlacolula y Matatlán). The variables include the opportunity cost of renting land in conjunction with its production, inputs, domestic factors (seed / labor / cultural work), machinery and equipment, government revenues, services and supplies, among others. It is important to bear in mind that, in most of the producers interviewed in Oaxaca, they lack classification systems on budgetary costs and revenues and cultural activities that contextualize projections of future profits in the plantations. However, working groups were organized with small representative producers. Through the participation of all of them it was possible to analyze three types of production scenarios of the plant (55 kg, 40 kg and 20 kg) considering 2,500 plants per hectare. In the first scenario observed an ideal situation because there are no problems in the production of agave; the second scenario shows the realities of plantations with affectations in the plantations causing a loss in the raw material and, the third scenario represents the most pessimistic.

Although, only the tangible costs were analyzed it is necessary to point out that the production process mentioned the intangibles related to the a) valuation of the environmental cost, considering that nature subsidizes the primary process; b) the valorization of the cultural cost that mezcal masters incorporate in the knowledge generation process through the years to produce agaves and mezcales, and c) the social cost that implies the medium and long term relationships for the generation of pollutants , the loss of biodiversity. which translates into a reduction of economic efficiency over natural resources and efficiency in the use of resources not used in the by-products generated for the production of mezcal. The focus groups helped to obtain the information, however, it was a challenge to carry out the construction of the indicators, although these were created with the active participation of the producers who have the information but do not register it in daily practice. Through the participation of the group, it was possible to generate financial costs and budgets in several regions of the entity. It is important to note that the information from SAGARPA (2018), is useful to contextualize the value of production, although more elements are required in the calculation of the cost to make better decisions in the productive units.

RESULTS AND DISCUSSION

Among the important elements observed in the field is the lack of valorization in the work that the family contributes to the direct and indirect activities on the production of *agave mezcalero*. That is, the work provided by family members represents an intangible contribution considered as a family activity in production, which is typical of tasks, although without obtaining an express payment for carrying it out. However, the benefits acquired by family members materialize when the sale of the plant is made, which can be reached up to six, seven or more years of maturity depending on the variety. The most important financial production costs of Matatlán, Oaxaca, are the plant, the rent of the land that is an opportunity cost to make use of the natural resource, although in practice it is little valued by the producers, the cutting plant of agave and, market freight that accounts for more than half of the costs in general terms.

The results on income and expenses in an average hectare, considering that an agave obtains a weight of approximately 55 kg and an amount of 150 tons (t) in one hectare (ha) in six years; In addition, during the second half of 2017, average prices were recorded between $ 12 and $ 15 pesos per kilogram for the purchase of the agave plant. When analyzing the expenses and income in the region of Matatlán, Oaxaca, it is obtained that an average producer reaches an income of $ 732 pesos per day. However, when there are reductions in production due to various external factors, the consequences translate into a reduction

in kilograms of agave, in this case 40 and 20 kg were established (according to field data), whose income was reduced, to know, from $ 257 to $ 60 pesos at current prices. In addition to the above, by transforming income into real prices, the purchasing power of money obtained in plantations is further reduced.

The most important production costs of Ocotlán - Zimatlán - Miahuatlán, Oaxaca are the plant, the land rent, the electric power, the cutting activities, the transport of cargo and the transport, which also represent more than half of the costs in general terms. In this region, it is estimated that the agave obtains an approximate weight of 55 kg and the estimate of 2,500 plants per hectare, which would obtain an average amount in the area of 138 tons (t) in six years. The price fluctuated on average to $ 9 pesos per kilogram. When analyzing the expenses and income, it is obtained that an average producer reaches an income of $ 355 pesos per day. However, when the weight of the agave is reduced, revenues decrease from $ 199 to $ -9 pesos at current prices (that is, loss of money translating into higher costs than revenues), which shows that, in some cases, the activity would lose productivity in the sector.

The most important financial costs of production in Tlacolula, Oaxaca are the plant, land rent, electric power, pineapple cutting, cargo transportation and transportation. The latter, compared to the other regions analyzed above, represent lower costs. The results explains why in this area there important producing companies of mezcal are that reduce transaction costs when they carry out the mobilization of the plant and, later, the drink. When we examine the expenses and income, a producer reaches an average income of $ 641 pesos per day. However, when pineapple weight is reduced, revenues decrease from $ 537 to $ 189 pesos at current prices.

Among the most outstanding costs in Sola de Vega are: the plant, the rent of the land, environmental services, mainly. This region is completely dedicated to the production of *agave mezcalero*. Therefore, the marketers come to buy the mezcal, in different palenques with relatively small productions, at very low prices, so the mobilization is transferred to buyers of large volumes of the product in the region. For the most part, producers are unaware of the destination of their product consumption and the city where it is consumed. The price fluctuated on average at $ 5 pesos per kilogram. When examining the expenses and income, a producer reaches an average income of $ 284 pesos per day. However, when raw material (of agave) weight is reduced, revenues decrease, from $ 140 to $ 25 pesos at current prices.

Figure 1 summarizes the income that an average producer would achieve at real prices according to the region and the projections of reduction in the number of kilos per hectare caused by different factors. In this sense, the region of Matatlán and Tlacolula stand out with higher levels of daily income for the producer.

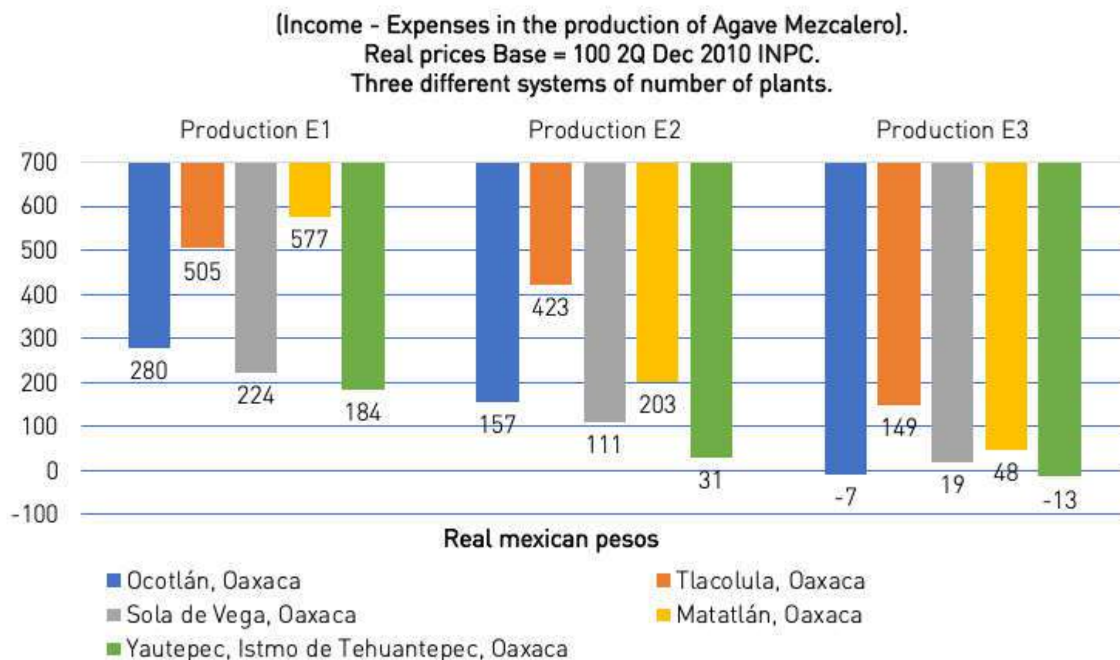
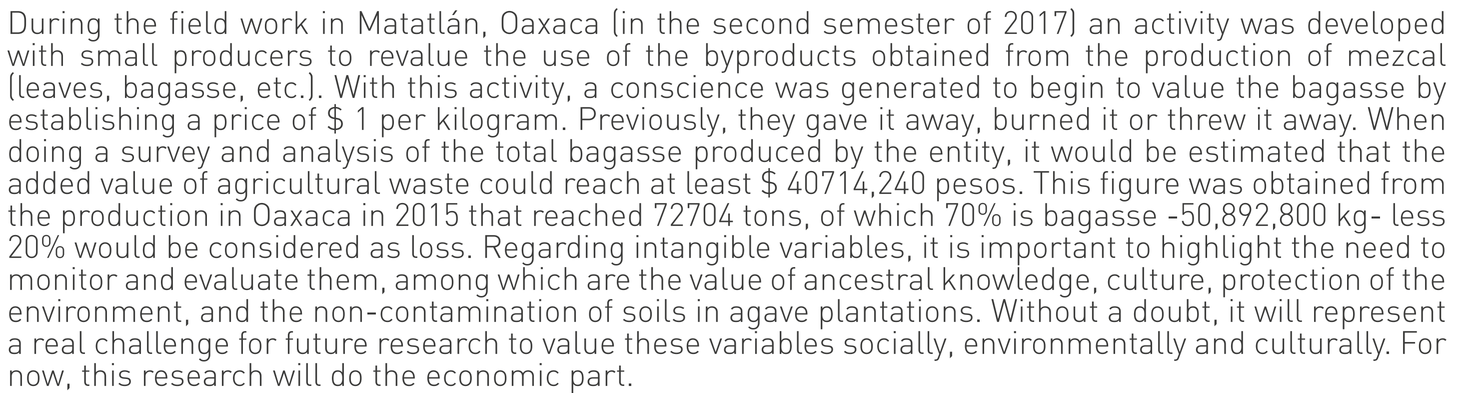


Figure 1. Income per capita per producer in various regions of Oaxaca.



During the field work in Matatlán, Oaxaca (in the second semester of 2017) an activity was developed with small producers to revalue the use of the byproducts obtained from the production of mezcal (leaves, bagasse, etc.). With this activity, a conscience was generated to begin to value the bagasse by establishing a price of $ 1 per kilogram. Previously, they gave it away, burned it or threw it away. When doing a survey and analysis of the total bagasse produced by the entity, it would be estimated that the added value of agricultural waste could reach at least $ 40714,240 pesos. This figure was obtained from the production in Oaxaca in 2015 that reached 72704 tons, of which 70% is bagasse -50,892,800 kg- less 20% would be considered as loss. Regarding intangible variables, it is important to highlight the need to monitor and evaluate them, among which are the value of ancestral knowledge, culture, protection of the environment, and the non-contamination of soils in agave plantations. Without a doubt, it will represent a real challenge for future research to value these variables socially, environmentally and culturally. For now, this research will do the economic part.

CONCLUSION

There are areas in Oaxaca that are more profitable given the characteristics of daily income that producers from Matatlán and Tlacolula can obtain, mainly. In the majority of rural producers, they do not know the methodologies and tools to generate the structure of production costs in production systems although they have the wealth of knowledge and information to carry them out, as they were obtained in field activities. This reality has as a consequence the lack of accurate information to optimize resources, establish prices in the sale of the product considering the cultural value and traditions, which, due to the need to obtain profits, must incorporate a value not only economic to the product, but cultural and environmental. There are success stories of small producers that have managed to balance the productive elements that are part of the ecosystem, production costs, and higher sales prices in the national and international market, in contrast to the local one.

It is important to bear in mind that marginalized producers should receive prices that represent the true value of work in the sector. In other words, small agave producers, for the most part, live in rural areas where infrastructure, information, capacities and social innovations are lacking, which must be encouraged by obtaining an overpriced product. They protect the recovery of the environment; however, they present a low level of social valorization of their activities that incorporate a lot of history, culture, life experiences which go unnoticed. Unfortunately, other actors focused on obtaining profits (intermediaries) commercialize ancestral knowledge and culture and, for the most part, deprive small rural producers of their social value.

ACKNOWLEDGEMENTS

Project "Protection, sustainability and use of the ecosystem in the biodiversity of the agave: the productive chain of mezcal in the states of Aguascalientes, the municipalities of Pinos and Villa Hidalgo in Zacatecas and the state of Oaxaca". National Problems 1406 CONACYT - CIATEJ.

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How is a traditional product defined? Case study of mezcal.

Vázquez-Elorza. A.[1] and Erick García-Barrón. [2] *

ABSTRACT

Currently, consumption of mezcal has increased significantly. This change in consumption habits could reflect a change in the meaning of the mezcal concept, since until a decade ago, it was considered a low quality drink. The determination and understanding of the meaning that consumers have of a food product, that is, the images, values and terms associated with the product in question is crucial for the food choice, even more so when these consumers have different cultural profiles. Therefore, this work was oriented to compare the words that define a traditional product such as mezcal in two representative Mexican populations, both in production and consumption of beverages representative of the country. Through a directed and representative sampling of the populations, 226 consumers of Mezcal in Oaxaca and the Metropolitan Area of Guadalajara were surveyed during May and June of 2018. An instrument was designed to show a list of 25 concepts generated based on a theoretical framework robust. From this, the respondents were asked to indicate which concept or concepts were related to the "Mezcal" based on their own opinion. The mentioned frequencies were obtained and analyzed by means of a *Chi*-square test. The Chi-square test showed that globally there were significant differences between the words that were used in each city ($p < 0.0009$). We identified 3 groups of consumers that differ by their socio-demographic characteristics and consumption habits. Each of the groups presented a characteristic image of what mezcal represents to them.

Key words: Mezcal, traditional product, concepts, culture, consumers.

INTRODUCTION

In the last 5 years, the Mexican market for alcoholic beverages has experienced an increase in production and consumption. Within this trend is the mezcal, a traditional alcoholic distilled beverage, with cultural and economic importance for the producing regions. The change in the consumption habits of a product is often considered as a sign that the perception towards the product is changing (Gómez-Corona et al., 2016). The determination and understanding of the meaning that consumers have of a food product, that is, the images, values and terms associated with the product in question, is crucial for the estimation of acceptance and / or preference by consumers, more even when said consumers have different cultural profiles (Guerrero et al., 2010). In each culture, there are norms and values that are used to define what is considered as adequate or inadequate food (Trichopoulou et al., 2007), reflected in the meaning of a concept for the sociocultural groups (Jodelet, 1984). In that sense, García et al. (2017), defined the concept "Mezcal" between populations with different cultural profiles. This work showed that the meaning of mezcal is related to the region of residence of the participants. In this context, this work aims to compare the use of terms obtained by García et al., (2017) to define the mezcal in two Mexican cities: Guadalajara and Oaxaca.

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METHODOLOGY

Cities

Two cities were considered: Guadalajara and Oaxaca. Guadalajara presents an incipient consumption and is part of the region of origin of tequila, a beverage considered a national symbol and the city of Oaxaca as the state capital, leader in the production and number of mezcal manufacturers (Consejo Regulador del Mezcal, 2019). In addition to other differences due to local culture, cuisine, geography.

Consumers

The study involved 226 consumers, 113 from Guadalajara and 113 from Oaxaca, from May to June 2018. The inclusion criteria were: Being from the region where the study was carried out; be of age; consume mezcal at least once a month and have wishes to participate in the survey. The sociodemographic characteristics are showed in Table 1.

Survey

A survey was applied, which was divided into two parts, the first consisted of the CATA (Check-All-That-Apply) test, where they were given a list of 25 concepts obtained by García et al. (2017) (Figure 1) participants should indicate which one or more of the concepts in the list describe the mezcal from their point of view. The second part consisted of compiling the sociodemographic characteristics of the participants, as well as aspects related to the consumption of mezcal.

Analysis of Data

CATA Test

The number of times each term was selected in the study cities was analyzed by the Chi-square test (Fisher's exact test $p < 0.05$), thus obtaining the characteristic frequency profile by city.

Sociodemographic data

To establish a relationship between consumption habits and sociodemographic characteristics, a Multiple Correspondence Analysis (MCA) was applied. In order to identify segments of consumers, a Hierarchical Cluster Analysis (HCA), Ward method, was applied on the first 8 dimensions that explained 50% of the variability of the data. Later to compare the consumer segments and the terms used to describe the concept of "Mezcal", the citation percentages per segment were analyzed by correspondence factor analysis.

RESULTS AND DISCUSSION

Sociodemographic characteristics of the participants

The characteristics of the participants are shown in table 1.

Table. 1 Socio-demographic characteristics of the participants.

	Variable	Guadalajara	Oaxaca
Gender	Men	62	61
	Women	51	52
Age	18-24 years	17	26
	25-34 years	42	44
	35-44 years	45	32
	45-54 years	8	9
	55 or more years	1	9
Level of studies	No university	15	38
	University	98	75
Occupation	Self-employee	51	21
	Employee	49	83
	Student	13	9
Income per month	$1-2,699	9	6
	$2,700-6,799	10	28
	$6,800-11,599	21	38
	$11,600-34,999	54	34
	$35,000-84,999	19	7
Consumption frequency	Every day	0	11
	Once a week	22	22
	Every third day	11	22
	Every fifteen day	20	21
	Once a month	50	36
	Least than once a month	10	1
Way of consumption	Mixed	10	2
	Mixed and alone	2	1
	Alone	101	110

Source: Own elaboration based on fieldwork

Overall it is observed that there was a greater participation of men than women. The majority of participants are in the age range of 25 to 45 years. The majority of participants in Guadalajara have university studies. The level of income is at a medium level. There is a higher frequency of consumption by consumers in Oaxaca than in Guadalajara, which could indicate a different perception about the product. Finally, the form of consumption in both cities is similar.

Analysis of citation frequency (CATA Test)

When comparing the frequency with which the words were used between each city, a significant difference was observed (X^2 (24) = 46.44, p <0.004). Figure 1 shows a characteristic profile of each city.

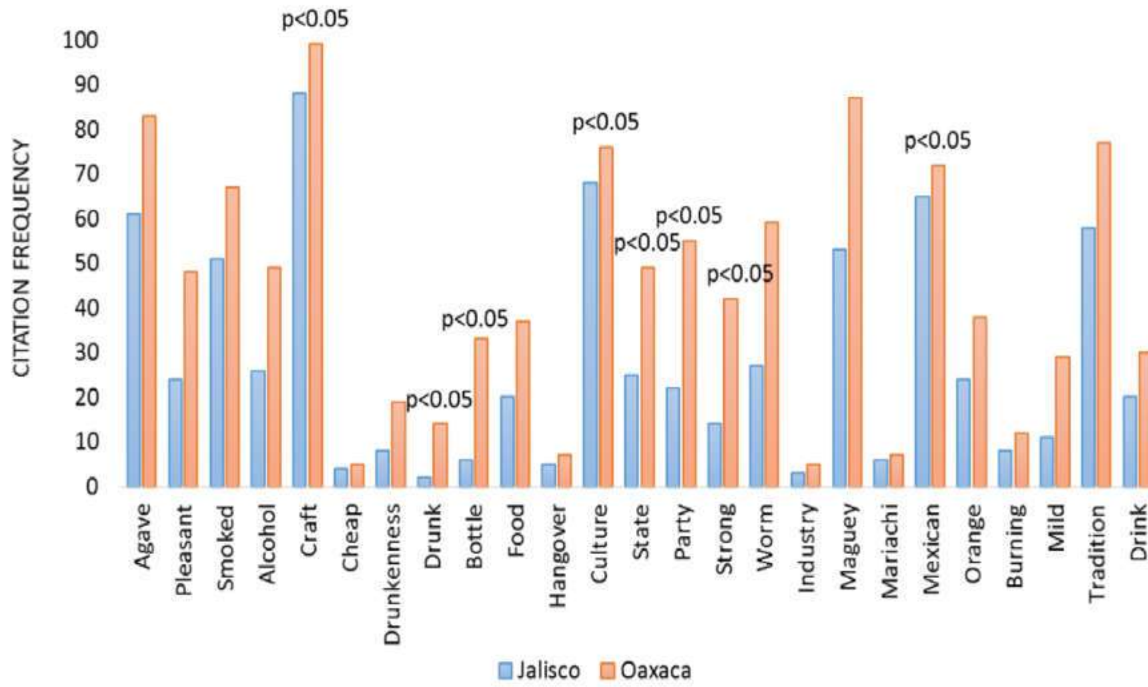

Figure 1. Frequency of citation with which each of the terms was marked in each city (p <0.05).

The results showed that Oaxaca participants used words more frequently than Guadalajara consumers. However, eight concepts presented significant differences. These words were: artisan, drunk, bottle, culture, state, party, strong and Mexican. According to Guerrero et al. (2000), the citation frequency is related to the strength or importance of a concept in the mind of consumers, so these 8 words can contribute to the formation of the image that consumers have about mezcal, particularly in Oaxaca, taking into account this result, mezcal is defined as an artisanal product, symbol of culture and identity (Mexican), present at parties, which is a strong drink (due to the high alcohol content) and consequently gets drunk. This result coincides with that observed by García et al. (2017), confirming the definition of the mezcal concept that they obtained using the word association test. In addition, it is confirmed that even when both cities are from the same country, there are differences in the meaning of a traditional product. This information is useful in the sense that allows to know the image of consumers about a traditional product and that from this it is possible to transmit messages that help to spread or position the product.

Identification of clusters based on sociodemographic data

Based on the multiple correspondence analysis and the hierarchical analysis, 3 different groups of consumers were identified (Figure 2).The analysis shows that factor 1, explains segment 3 is formed mainly by young students, with low incomes and who consume mixed mezcal and consume it with a lower frequency. In contrast, cluster 1 is made up of people with higher incomes and who are older, from Guadalajara and have a higher level of education. On the other hand, segment 2 is comprised of consumers from Oaxaca, who consume mezcal alone and whose incomes are at a medium level. This result shows the relationship between consumption habits and the demographic characteristics

of mezcal consumers, which shows that at a higher level of studies and income, participants consume mezcal more frequently and prefer to drink alone.

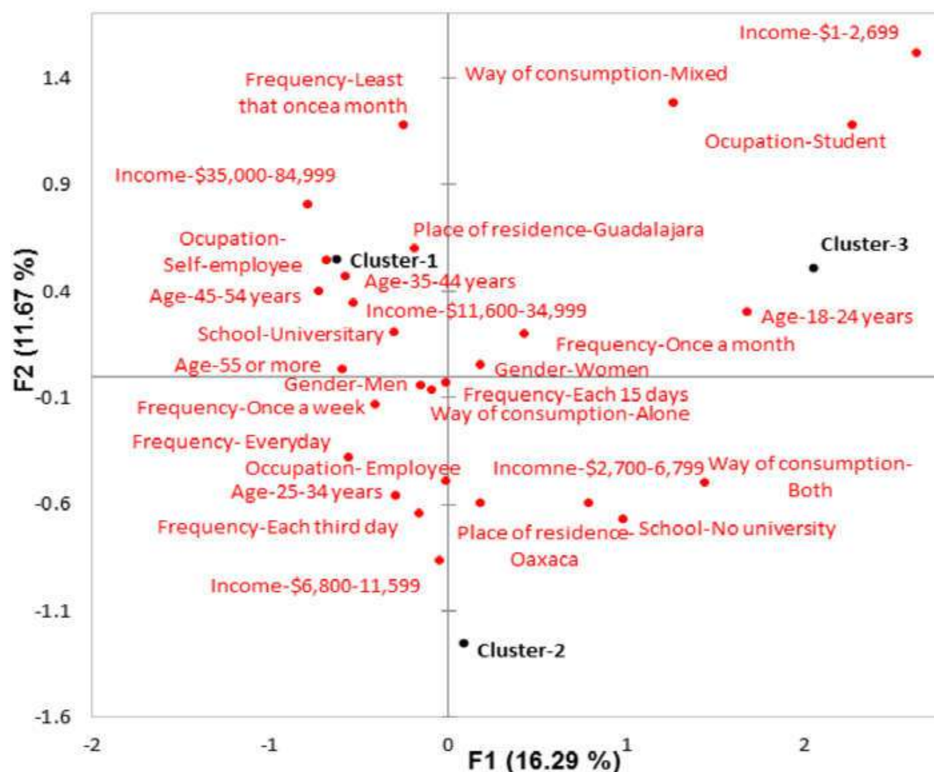

Figure 2. Representation of the 3 consumer segments obtained through MCA and HCA.

Comparison of the concepts used by segments

Figure 3 shows the factor analysis of correspondence on the citation percentages with which the concepts were marked in each segment. It is observed that the participants of cluster 1 used the words tradition, craft, culture, which shows that for this segment the mezcal is a symbol of tradition and culture. In contrast, cluster 2 describes mezcal as a product of experiences, based on the words used as drunkenness, hangover, drink, worm and strong, as well as party. Regarding group 3, consisting mainly of young consumers, they used the words industry, bottle and soft. With this result it is shown that the concepts that define a traditional product such as mezcal are related to consumption habits, as well as demographic features. It is important to mention that the image of a product developed within a group defines its members (Jodelet, 1984) that in this case the cultural identity is what guides the consumers of segment 1, experiential aspects in group 2 and composition and processing to segment 3.

CONCLUSION

In this work, the words that describe a traditional product such as mezcal were compared. Using the CATA technique, differences were observed among consumers in two Mexican cities with different levels of exposure and familiarity with mezcal. Since consumers more familiar with the product (Oaxaca consumers) tend to use concepts that define the drink more frequently. Through the use of multivariate statistics tools, it is observed that the concepts used are related to consumption habits and

sociodemographic characteristics, in addition to reflecting the image of the product developed within each group. As a future perspective, it is necessary to deepen the role of sociocultural variables in the formation of the concept.

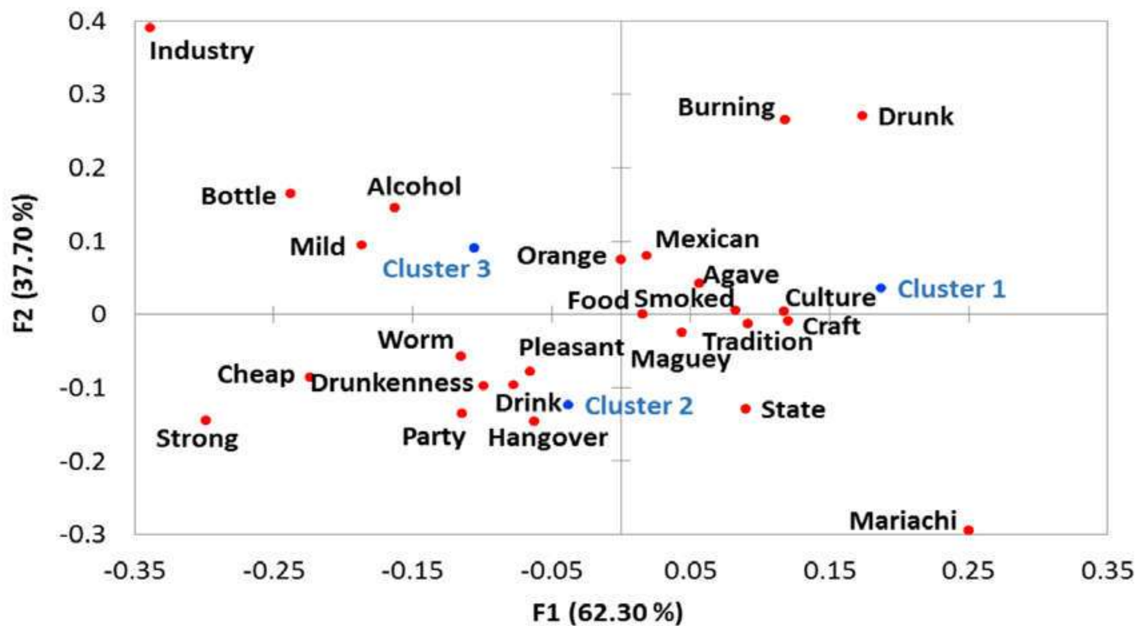

Figure 3. Representation of the concepts used by the 3 segments to define the mezcal.

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Project "Protection, sustainability and use of the ecosystem in the biodiversity of the agave: the productive chain of mezcal in the states of Aguascalientes, the municipalities of Pinos and Villa Hidalgo in Zacatecas and the state of Oaxaca". National Problems 1406 CONACYT - CIATEJ.

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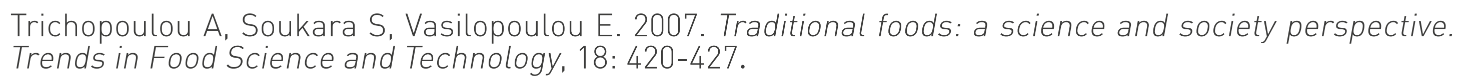

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Mezcales with name and last name. Diversity and typification as identity elements.

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ABSTRACT

Currently, with the resurgence and consolidation of mezcal as an emblematic and traditional drink of Mexico, a great demand has been generated in the national and international markets. However, this phenomenon has made it an object and reason for struggles and disagreements before the incorporation of several states that were originally excluded from the Appellation of Origin Mezcal (AOM), a legal figure that gives exclusivity to a territory for its elaboration, particularly those attached to the Regulatory Council of Mezcal (CRM). Such si the case of the state of Oaxaca in whose territory more than 80% of this drink is produced nationwide, and whose producers claim their paternity. However, historically, mezcal has been produced in more than 20 states, most of them not included in the AOM. All the mezcal produced in Mexico have particular characteristics that make them different from each other, however, few consumers know, hence it is important that the consumer, in addition to the place of origin of the mezcal, have information about the process under the which is elaborated, the type of agave that is used and the distillation techniques, which can be achieved by means of a typification based on some of these and other characteristics, associating them with a name and a surname, which reflect their origin and identity.

The objective of this work is to provide those elements through which the different mezcales produced in the territory protected by the DOM can be differentiated, assigning them a proper name according to their origin and characteristics.

Key words: *Appellation of Origin Mezcal, regionalization, identity, mezcal province, artisanal process.*

INTRODUCTION

The discord over the drink's paternity has given rise to confrontation and estrangement between government institutions and producers, since on one hand, the expansion of the territory protected by the Appellation of Origin Mezcal (AOM) enacted in 1994 (DOF, 1994) affected economic interests of the entrepreneurs and, on the other hand, by being a drink for consumption, its entry into globalized markets has had to abide by policies and specifications that have gradually diluted its essence. One of these is the modification of the Mexican Official Standard, NOM-070-SCFI-1994, to become NOM-070-SCFI-2016 (DOF, 2017), which establishes the characteristics and specifications for its production, packaging and marketing.

In this context, mezcal is currently catalogued according to its production process, classifying it in categories and classes, seeking to justify the existence and development of "artificial mezcal" with which ingredients (colorings and in some cases flavoring) can be added, distorting the physicochemical,

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organoleptic and sensorial characteristics that characterize genuine mezcal and through which the mezcal produced in different regions of the country can be distinguished, considering also the type of agave, the cooking process and even the distillation system, which is intimately linked to the cultural practices of the people and mezcal communities, which NOM doesn't consider for classification purposes.

The mezcal produced in Mexico have particular characteristics that differentiate them from each other, however, it is important that the consumer knows besides the process, type of agave and distillation techniques, their region of origin, which can be achieved by making a typification based on historic elements, cultural and geographic factors, associated with a name and surname that reflects based on some elements of historical, cultural and geographic factors, associated with a name and surname that reflects its identity, on which this proposal is based.

METHODOLOGY

Through a documentary analysis, the different species of agave used in the production of mescal are identified, their geographical distribution, uses and exploitation by the mezcal communities (indigenous and rural population), whose link with the plant becomes a very important cultural element, having a connotation of identity. Parallel to this, both the AOM as well as the NOM, which defines its physicochemical characteristics as its category and class, which arguably are key elements for their classification, are insufficient since it does not reflect their origins and identity. Similarly, suggesting the use of raw materials of any type of agave, provided that it is cultivated or is wild within the territory covered by the WCO, further dilutes a possible typification. Derived from this analysis, some elements can be identified. A trait of identity can be assigned to the mezcal, associated with the geographic origin (origin), gentilic (identity), features (distinctive), process (technique), specificity (Type of agave), among others, which represent and show the diversity of attributes offered by the mezcal.

RESULTS AND DISCUSSION

At the sight of the expansion of the territory covered by the AOM, reflecting the claim of the mescal regions which were excluded in the first version of the declaration (Figures 1 and 2), there is a need for mezcals to be differentiated by giving them a specific name that, on one hand, identifies their region of origin and, on the other hand, highlights their identity associated with the region and its inhabitants identified through gentilics so that the consumer has the option to choose according to their preference. In both cases there are different criteria, regardless of the type of agave used and the techniques that give it its physicochemical and organoleptic characteristics, regardless of their category and class defined by the NOM.

Figure 1. Territory covered by la AOM (1994).

ID	ESTADO	MUNICIPIOS
1	Durango	Todo el estado
2	Guerrero	Todo el estado
3	Oaxaca	Todo el estado
4	San Luis Potosí	Todo el estado
5	Zacatecas	Todo el estado
6	Guanajuato	San Felipe
		San Luis de la Paz
7	Tamaulipas	11
8	Michoacán	29
9	Puebla	115

Figure 2. Expansion of the territory covered by the AOM (2018).

Producers, consumers, advocates, researchers and other types of experts (Abdelmassih, 2016; Hernandez, 2018), have suggested that mezcal be classified according to their origin, taking into account its territorial source, since the name 'mezcal' is a generic term which only refers to a beverage distilled from agave and proves insufficient for their typing. In the same way, before the changes manifested by the mezcal sector and strategies undertaken by entrepreneurs for commercialization considering the classification imposed by the new version of the NOM by defining categories (Mezcal, Mezcal Artisanal and Ancestral Mezcal) and classes (white or young, Reposado, Añejo, etc.), in this regard, it is considered that these elements are equally inadequate for typification, coinciding with Hernández (2018) referring hat, artisanal and ancestral mescal are products with an important cultural background and not only merchandise, so it should not be relegated to history, culture and geography (biocultural aspects) in the consideration of a typification that defines its identity, so it is recommended to establish regional geographical designations ahead of the enormous expansion of the AOM. Previously, had already pronounced his support of a classification of mezcal based on mescal provinces and gentilics to assign a name and surname with which its origin and provenance could be identified. The issue has been resumed, suggests that a classification of the mezcal could be created considering a generic category (Mezcal) and grouped into subcategories according to their region of origin.

In this context, it is proposed, the definition of a name and surname giving identity to the mezcal produced in the territory of the AOM, based on an analysis of the elements involved in its preparation and most representative aspects, which is likely to achieve this objective.

The proposal concurs with the mescal provinces and the gentilics are the main indicators of the origin of the mezcal produced in the NOM, whose recognition would palliate the frictions generated before the expansion of the AOM.

Elements of identity of mezcal: All mezcals are possessors of properties and features that gives them an identity, these qualities are attributed to the type of agave which is used in its production, its process, techniques and implementation, cultural aspects that are the result of traditions, usages and customs developed in the communities where these are made, which reflect and synthesize ancient practices given the bond developed with the plant.

Variety of Agave: Agave, raw material used in the production of mezcal, is due to a natural geographic distribution, the agro-ecological criteria (traditional agroecosystems) that each community applies in terms of its management (wild or cultivated), is a result of their historical and cultural heritage. Similarly, the diversity of agaves is a consequence of different adaptations to environmental conditions.

In table 1, 18 of the main species of agave, used in the production of mezcal, are listed, highlighting the state of Oaxaca, where the greatest diversity is concentrated.

Table 1. Main varieties of agave used for the production of mezcal in the territory of the AOM.

Variety of Agave (Diversity)	Colloquial name	Distribution (Mezcal Province)
A. americana	Arroqueño, Coyote, Sierra Negra	Valles Centrales
A. americana var. oaxacensis	Americano, Teometl, Serrano, Coyote,	Valles Centrales
A. cupreata	Papalote, Chino	Cuenca del Balsas
A. angustifolia Haw	Espadín, Espadilla	Valles Centrales, Mixteca
A. marmorata Roezl	Tepeztate, Curandero	Mixteca, Valles Centrales
A. potatorum Succ.	Tóbala, Papalometl, Maguey de Monte	Valles Centrales, Mixteca
A. inaequidens	Alto, Lechuguilla, Largo	Cuenca del Balsas
A. salmiana, var. crassispina	Verde, Manso, Bronco, Cimarrón, Potosino	Altiplano Potosino-Zacateca-no-Guanajuatense
A. duranguensis	Cenizo	Nombre de Dios
A. rodacantha	Cuixe, Mexicano	Sierra del Sur, Mixteca, Valles Centrales
A. marmorata	Pichometl	Mixteca
A. tequilana Weber	Azul	Ciénega
A. karwinskii	Tobasiche, Barril, Cirial, Madrecuixe, Martine-ro	Valles Centrales
A. maximiliana	Lechuguilla, Manso, Masparrillo	Nombre de Dios
A. univittata subsp. lophanta	Estoquillo, Lechuguilla	Sierra de San Carlos
A. semanniana	Chato	Valles Centrales, Mixteca, Sierra del Sur
A. convallis	Jabalí	Valles Centrales
A. bovicornuta	Masparrillo	Nombre de Dios

Source: National Commission for the Knowledge and Use of Biodiversity. Mezcal and Diversity Map. 2006.

Process: Another distinctive element for the typing of mezcal would be the process or the production technique, which also records variants, most of which are within the artisanal classification although there are marked differences in terms of processes, technique and the elements used in the different mezcal regions, which allows to identify standard products, with standardized physicochemical and organoleptic characteristics that according to the NOM (Table 2).

Mezcal Provinces: Corresponds to large geographic regions within which different mezcal producing communities are located, where different species of agave and varied elaboration processes are used (Gallardo, 2016) whose territory extends even outside the AOM.

Table 2. Distinctive Elements and Variants of mezcal produced in the territory of the AOM.

Processing Technique (Distinctive)
From Campanilla (Mexquitic SLP.)
From Hacienda (Altiplano Potosino-Zacatecano-Guanajuatense)
From Sierra (Serrano)- Guerrero, Tamaulipas
From Rancho (Guanajuato, SLP, Zacatecas)
From Chorrera (San Luis Potosí)
From Olla (Oaxaca, Guerrero)
From Montera (Michoacán, Durango)
From Cascomite (Puebla)

Source: Own elaboration based on the references consulted

Cultural area: Term (noun or adjective) that denotes the origin or relation with a geographical place. In the case of mezcal, the term refers to the name of a mezcal produced in an area with a mezcal tradition (state), whose scope can be more specifically as a municipality or community (Gallardo, 2016), hence for this proposal two variants are considered: A) Origin - Provenance and B) Link - Region (Table 3).

The mezcal regions identified within the AOM and the most representative gentilics where there is a greater number of production units (Palenques, Vinatas, Factories, etc.) indicated in Table 3, is not exhaustive and could be excluding some communities, particularly for the state of Oaxaca. Reference is made to the districts, without specifying particular populations that may have very strong differences such as the case of the Miner mezcal that takes place in Santa Catarina Minas and other communities, for which there is no name.

Table 3. Mezcal provinces and regions (A y B), criteria to typify and give name to the mezcal produced in the territory of the AOM.

Territorial Region (Mezcal Province)	Region-A (Origin-Provenance)	Region-B (Locality –cultural area)	Territorial Region (Mezcal Province)	Region-A (Origin-Provenance)	Region-B (Locality-Cultural area)
Altiplano	Potosino	Mexquitic	Valles Centrales	Oaxaqueño	Ejutleco
		Charcas			Zimatleco
	Zacatecano	Pinense			Etlense
	Guanajuatense	Jaraleño			Ocotlense
Cuenca del Balsas	Michoacano	Etucuareño			Tlacoluleño
		Morelense			Zaachilense
	Guerrerense	Chilapeño			Yoganense
		Mazatleco			Mitleco
		Zihuaqueño			Matatleco
		Mochitleco			Zapoteco
		Tierra caliente	Sierra del Sur	Oaxaqueño	Suchilteco
		Costeño			Yauteco
		Serrano			Solteco
	Poblano	Mixteco			Miahuatleco
		Atlixqueño			Amatleco

Territorial Region (Mezcal Province)	Region-A (Origin-Provenance)	Region-B (Locality –cultural area)	Territorial Region (Mezcal Province)	Region-A (Origin-Provenance)	Region-B (Locality-Cultural area)
Sierra de Mil Cumbres	Michoacano	Chareño	Mixteca	Oaxaqueño	Mixteco
		Querendense		Poblano	Mixteco
Ciénega	Michoacano	Sahuayense	Cañada	Oaxaqueño	Ixcateco
Nombre de Dios	Duranguense	Dioseño	Cañones	Zacatecano	Del Teúl
Sierra de San Carlos	Tamaulipeco	Serrano			

Source: Own elaboration based on the references consulted.

CONCLUSION

Given the current scenario that the Appellation of Origin Mezcal has imposed on the sector, typifying the mezcal by assigning a name and surname according to its origin would set the tone for the consumer to receive information about its provenance, a condition that the current Official Mexican Standard NOM -070-SCFI-2016 does not consider in its recent modification. Although it is true that the domestication of species induced its cultivation in areas outside its habitat, it is also worth highlighting the existence of endemic or exclusive species of a region that are used in the elaboration of mezcal (*A. cupreata*, *A. inaequidens*, *A. salmiana*, among others), whose development is limited by certain environmental conditions. Establishing mezcal categories based on the type of agave used, its process, materials, instruments or technologies used, puts at a disadvantage many of the mezcal regions that for historical reasons established a pause in their production (continuity), with the state of Oaxaca, where a local bioculture has been developed, generating a false expectation that the mezcal is originally from that state, which has slowed the inclusion of new territories to the AOM. Given this scenario, the concern has arisen to form regional regulatory councils that consider territories outside the AOM that cannot call their product mezcal, relegating it to the category of a distillate, being at a considerable disadvantage to be exempt from the policies and the regulation and control mechanisms imposed by the current Regulatory Council based in Oaxaca. Given the biological richness and cultural diversity of the mezcal regions, it is proposed to assign them a name and surname that reflects their identity.

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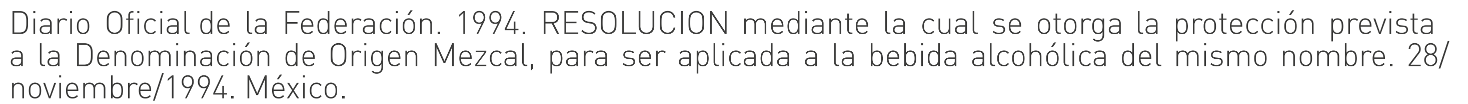

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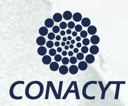
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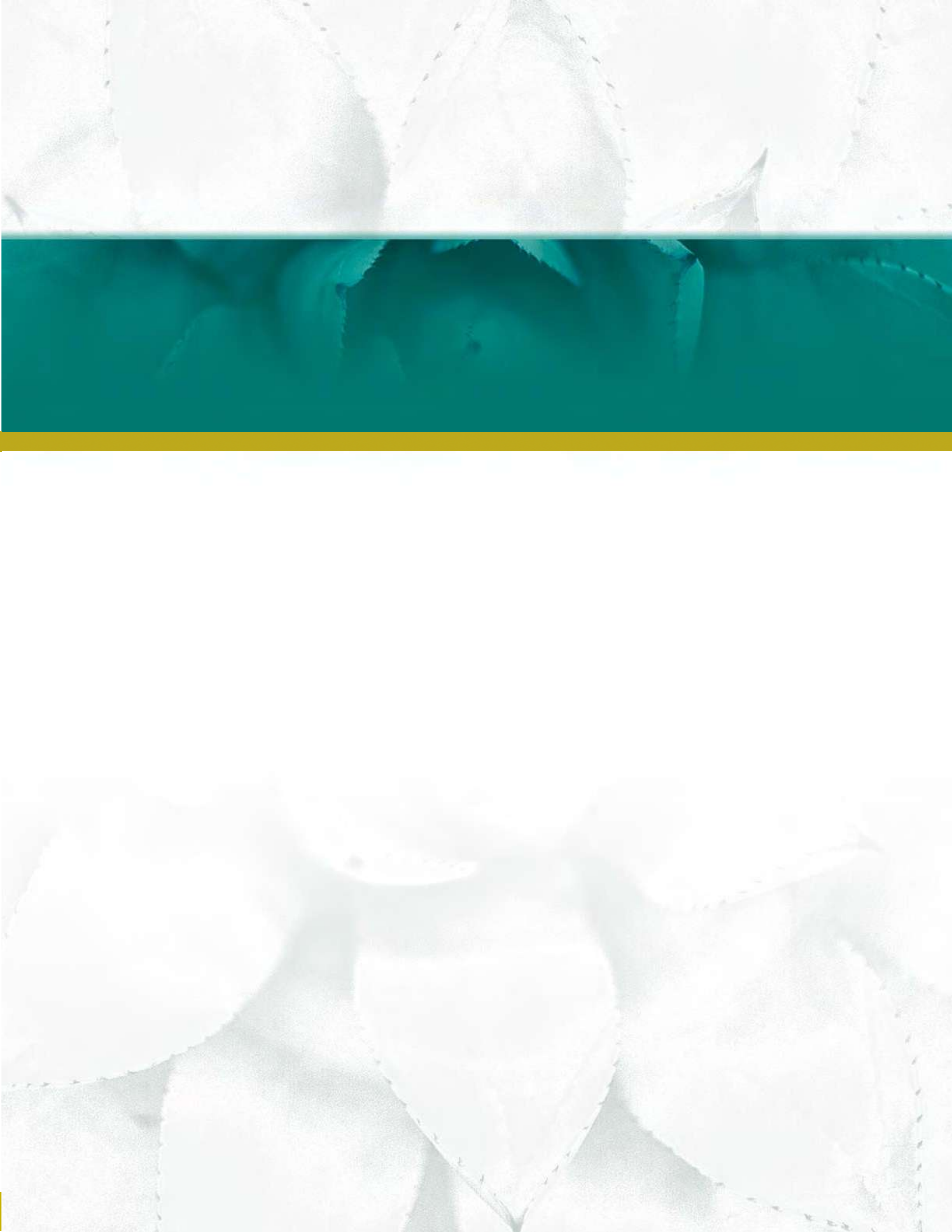

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