# *Sustainable and Integrated use of Agave*


III
International
Symposium on Agave
ISA

# *Sustainable and Integrated use of Agave*

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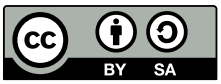



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# ÍNDEX

# PROLOGUE

The agave plants emblematic of Mexico have been used since ancient times and today are still used in several applications, the most known being the production of alcoholic beverages and in particular tequila. The agaves and their derivatives are a subject of investigation of many Mexican institutions as well as foreign ones. Over the years, research topics have been diversified, addressing a wide range of topics such as genetic improvement, micropropagation, diseases, agave fructans (characterization and application as an ingredient), agave distilled beverages other than tequila, exploitation of by-products, among others.

In its third edition, the International Symposium on Agave (ISA III) organized by the Centro de Investigación y Asistencia en Tecnologia y Diseño del Estado de Jalisco A.C. (CIATEJ) allowed to bring together researchers from all over the country as well as foreigners to share their research and discuss the different issues related to agaves and the diversification of their uses. During the symposium there were 36 oral presentations as well as 34 works in the form of posters.

Of the works, some of them were selected and are presented in chapters form in this work. They are divided into 5 major topics: scientific aspects related to the agave, science and technology of beverages obtained from agave, extraction/purification and biological effects of fructans and other byproducts, treatment and utilization of by-products and finally social and ethnobotanical aspects. The papers presented make clear the great potential of the agaves but also highlights the points that have to be taken care of and / or addressed as soon as possible like the conservation of the germplasm, the problems of phytopathology as well as the sustainable use of the endemic species of Agave. In the works related to beverages and fructans, the great variety of products available in terms of beverages and the importance of the microorganisms involved in the fermentations, and in the case of fructans the diversification of their applications in different foods are described.

The CIATEJ with this third organization of the International Symposium of Agave continues to demonstrate its great commitment to this sector and the scientific community to promote the dissemination of research work by promoting contact with industry and thanks all institutions, government agencies and companies that allowed to realize this new edition of ISA III as well as the publication of this work.

**Anne Christine Gschaedler Mathis, Ph. D.**
Director Zapopan Unit
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THEMATIC I Scientific trends on Agave

# Biological control of agave wilt by Trichoderma sp. associated with agave root

**López-Domínguez H.M[1], Vega-Ramos K.[2], Uvalle-Bueno J.[3] and Qui-Zapata J. A.[4]***

## ABSTRACT

One of the main strategies to control root diseases associated with *Fusarium oxysporum,* is the use of *Trichoderma* strains as biological control. However, most of the commercial products contain microorganisms isolated from others crops to those that are planned to protect and from environmental conditions that do not correspond to those where they will be used. Then, it is necessary to isolate strains associated to the root of the plants that have to be protected. This strategy was explored for the control of agave wilt, the main disease of blue agave (*Agave tequilana* Weber var. azul). The aim of this work was to isolate strains of *Trichoderma* associated with agave and to evaluate its protection against agave wilt. Samples of agave rhizosphere were taken on commercial crops of blue agave. Isolation of *Trichoderma* strains was made on selective media THSM. Subsequently, 20 isolates were selected to evaluate their protection capacity during infection of agave plantlets I with *F. oxysporum.* Plantlets were inoculated with a spore suspension (1X107 spores/ml) of each *Trichoderma* isolated. Subsequently, they were inoculated at the base of plant with a spore suspension of *F. oxysporum* (1X107 spores/ml). A scale of 1 to 5 for the disease index was performed, where 1 corresponds to a healthy plant and 5 a plant with severe symptoms of disease. For each *Trichoderma* strain, root colonization was evaluated by trypan blue staining. It was observed that several isolates of *Trichoderma* show a protective effect against *F. oxysporum*, unlike commercial strains that did not show a better protection. In addition, better protection was observed in those isolates that had higher colonization of agave root. It was possible to obtain isolates of *Trichoderma* that colonize the root of the agave and protect it against infection by *F. oxysporum.*

**Key words:** *Fusarium oxysporum*, Agave wilt, *Trichoderma*.

## INTRODUCTION

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The blue agave (*Agave tequilana* Weber variety azul) is the only raw material authorized for the production of tequila. This crop has great losses caused by pest and diseases that affect up to 30% of the total area of cultivation (CRT 2010). One of the major diseases of the crop is agave wilting that is associated with the fungus *Fusarium oxysporum* (Avila-Miranda et al. 2010). To control root diseases associated with *F. oxysporum*, antagonistic fungus *Trichoderma* sp. has been widely used in various crops of importance with high effectiveness (Fravel et al. 2003). Although there are studies related to the use of *Trichoderma* for agave wilting control, the results of these studies are inconclusive for field applications because of agave environmental and crop conditions. The fungus *Trichoderma* has different mechanisms of protection against infection by phytopathogenic fungi such as: mycoparasitism, competition for space and nutrients, antibiosis and association with the root of the plant to inducing plant defense mechanisms (Howell 2003). The root colonization could be one of the most important mechanisms for the protection against infection of *F. oxysporum* in the agave.

Strains included in commercial products have been isolated from various crops and also strains are enhanced by protoplast fusion techniques, as an example is the T-22 strain (Harman 2000). It is considered that for these reasons the commercial strains are not readily colonize the roots of agave, and that are not withstand the environmental conditions where the agave develop (Kedrics et al. 2003). For these reasons, it is necessary to isolate *Trichoderma* native strains with the ability to associate to the root of agave and to protect against infection from *F. oxysporum*. The aim of this work was to isolate strains of *Trichoderma* associated with agave root and evaluate its protection against agave wilt.

## METHODOLOGY

### Collection of rhizosphere samples

Twenty rhizosphere samples (30 cm deep) from the base of healthy agaves and wilt symptoms were collected from five sampling points located in the states of Jalisco.

### Isolation of *Trichoderma* strains

For isolation of *Trichoderma* strains, a serial dilution technique was followed and a 103 and 105 dilution of each sample was prepared. One ml of each solution was pipetted on to a THSM agar plate and incubated at 28 °C for 1 week (Williams *et al.* 2003). Colonies appearing on the plates were isolated and re-inoculated into a PDA plate. After 7 days, single spore colonies were obtained by subculturing at 28°C. Distinct morphological characteristics were observed for identification, and were compared to a taxonomic key for the genus *Trichoderma* and the plates were stored at 4 °C.

### Biological material

*Trichoderma* strains isolated from commercial products for comparison with native strains were used. Commercial strains were *Trichoderma harzianum* (T-22), *T. virens and T. viride.*
*Fusarium oxysporum* isolated from the collection of CIATEJ referred to as FPC was used as a pathogenic strain.

### Evaluation of protection against *Fusarium oxysporum* infection to agave

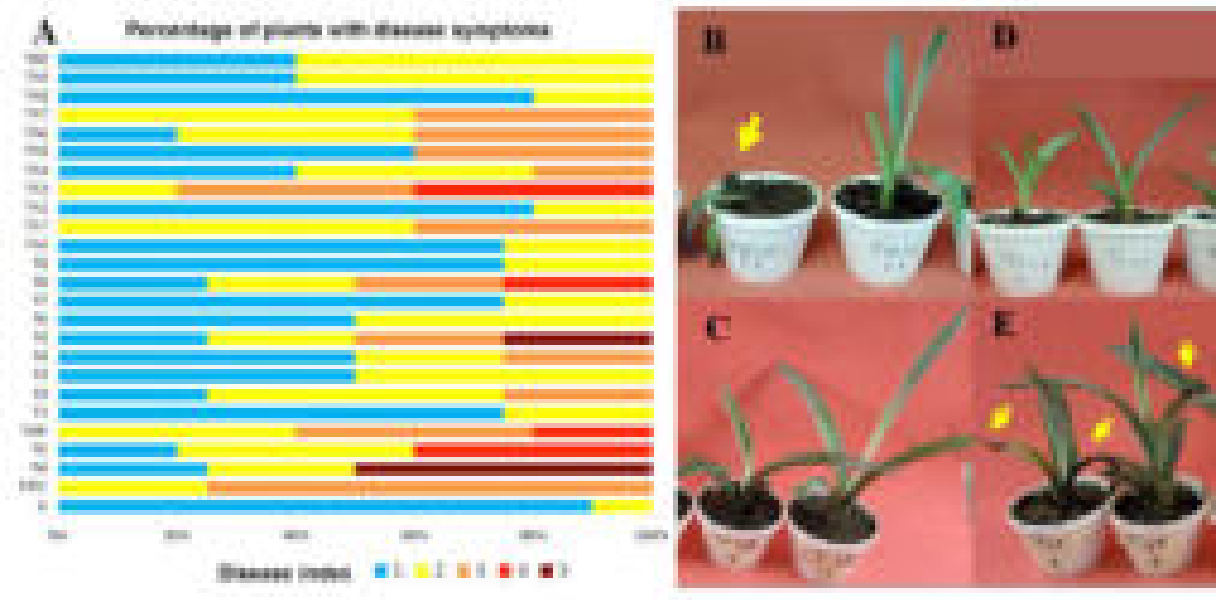
Twenty isolates were selected to evaluate their protection in agave plantlets against infection of *F. oxysporum*. Plantlets of agave from *in vitro* micropropagation of line PNOV were used (García-Vera 2011). Plantlets were inoculated with a spore suspension (1X10$^7$ spores/ml) of each *Trichoderma* isolated, 20 days before to inoculation of the pathogen. Subsequently, they were inoculated at the base of plant with a spore suspension of pathogenic *F. oxysporum* (1X10$^7$ spores/ml). Plantlets were incubated in plant growth chambers at 27ºC and photoperiod 16:8h light/dark. We used five replicates per treatment. photographic records were made every 15 days during four months of the experiment. The disease index (DI) was evaluated following an ordinal scale of 1 to 5, where 1 corresponds to a healthy plant and 5 a plant with severe symptoms of disease (García-Vera 2011).

### Agave root colonization

For each *Trichoderma* strain, root colonization was evaluated by trypan blue staining. Roots were cleared in 10 % KOH for 6 h, rinsed with water. Subsequently, they were stained with a 0,1 % trypan-blue solution in lactophenol overnight and fixed in 50 % lactic acid. Of each replication, 1 cm of five root segments was examined microscopically and the percentage of infected roots was calculated (Ahmad and Baker 1987).

## RESULTS AND DISCUSSION

Of the 20 isolates of *Trichoderma* selected, three of them had low protection against infection of *Fusarium oxysporum* (Fig 1A). While ten of the isolates showed outstanding protection. Commercial strains showed low effectiveness in protecting against infection of F. oxysporum under the conditions of the experiment (Fig 1B y E).




**Ø:** Control, **FPC:** *Fusarium oxysporum*, **TH:** *Trichoderma harzianum*, **TV:** T. *viride*, **TVR:** T. *virens* and **T1-T20:** Isolates of *Trichoderma* from the rhizosphere of agave. **A:** Percentage of plants with disease symptoms and disease index. **B:** Plantlets of agave inoculated with TH; **C:** Plantlets of agave inoculated with native *Trichoderma* strain T18; **D:** Plantlets of agave inoculated with native *Trichoderma* strain T7; **E:** Plantlets of agave inoculated with TVR. Arrows indicate disease symptoms.

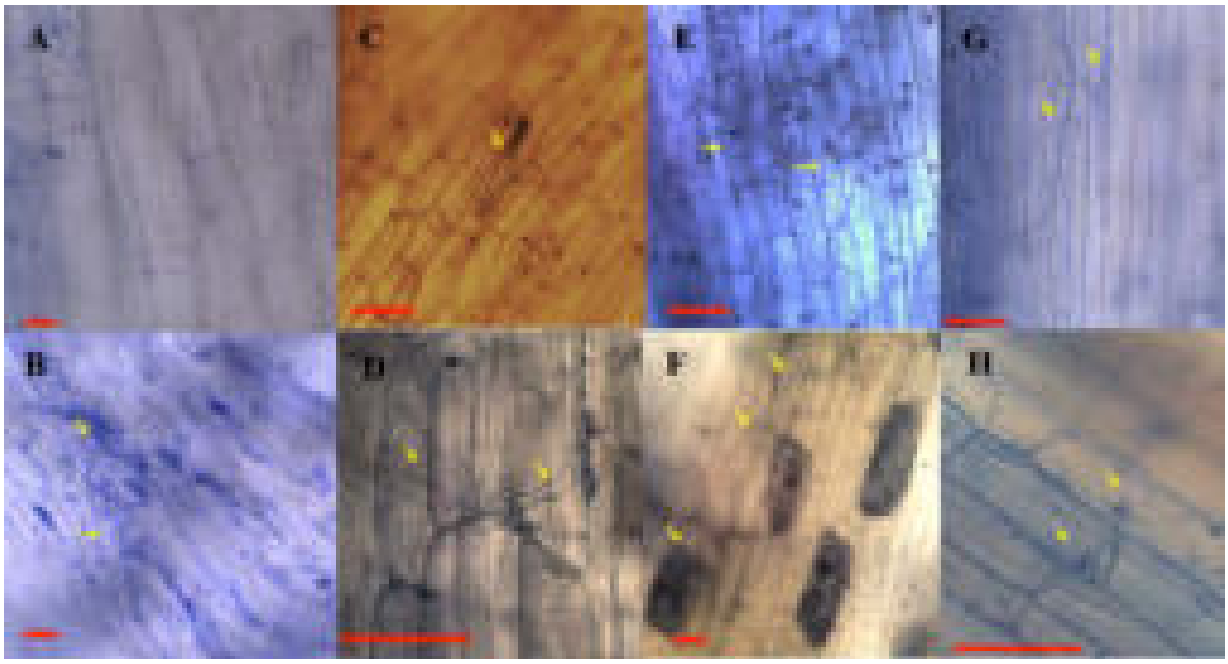


**Figure 2. Colonization of agave roots by *Trichoderma* strains. A:** Control, **B:** *Fusarium oxysporum*, **C, D:** *Trichoderma* sp. T7; **E, F:** *Trichoderma* sp. T18. **G, H:** *T. harzianum* T-22. Arrows indicate the presence of mycelium. Bar represents 50 µm.

Root-colonization *Trichoderma* strains, also showed greater protection against infection by *F. oxysporum* (Fig. 2). As for the commercial strains, its colonization was lower if compared to the native strains. It was noted that *Trichoderma harzianum* (T-22) its protection depended on its root colonization (Fig. 2G y H).

## CONCLUSION

It was possible to obtain isolates of *Trichoderma* that colonize agave roots and protect them against infection by F. oxysporum. These strains offer better protection than the commercial strains.

## ACKNOWLEDGEMENTS

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THEMATIC I Scientific trends on Agave

# Determination of chromosome number in two species of Agave collected in the state of Hidalgo, Mexico

**Rodríguez-Domínguez, J.M.[1], Gordillo-Tello, A.B.[2], Soto-Carrasquel, A.[3], Barba-González, R.[4] y Rodríguez-Garay, B.[5*]**

## ABSTRACT

Chromosome numbers of *Agave applanata* and *Agave salmiana* (two economically important species for the State of Hidalgo, México) are presented here. Metaphasic chromosomes were prepared according to various cytogenetic techniques: squash, splash, smear, steamdrop, and a modified steamdrop method; the best chromosome dispersion was obtained through steamdrop and modified steamdrop techniques in both species. The results showed different number of chromosomes: 2n=60 and 2n=150 for *A. applanata* and *A. salmiana* respectively. *A. applanata* showed a bimodal karyotype consisting of 10 large + 50 small chromosomes, on the other hand, *A. salmiana* also showed a bimodal karyotype consisting of 25 large + 125 small chromosomes. Considering the basic number x=30, A. applanata is a diploid species (2n=2x=60), whereas *A. salmiana* is a pentaploid species (2n=5x=150). This is the first report on the chromosome number of *A. applanata*.

**Key words:** *Agave applanata, Agave salmiana*, Asparagaceae, Cytogenetics, Ploidy level

## INTRODUCTION

*Agave* is a genus of the monocotyledonous family Asparagaceae, belonging to the subfamily Agavoideae. It is distributed from southern U.S.A. to Colombia and Venezuela, including the Caribbean Islands (García-Mendoza, 2002). The genus has a basic chromosome number x=30 showing different degrees of ploidy. Through chromosome counting and flow cytometry, it has been determined that in the genus *Agave* there are chromosomal numbers from 2n=2x=60 to 2n=6x=180 (Robert et al. 2008). The genus *Agave* has asymmetrical bimodal karyotypes (fifty short chromosomes and

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ten long chromosomes on the diploid cytotypes) feature that could be associated with a great morphological and ecological specialization (García-Mendoza, 2007). The chromosome number of plants can provide useful information for diverse fields of research including karyotaxonomy, genetics, cytogenetics, plant breeding, ecology, biogeography and molecular biology.

*Agave applanata* and *Agave salmiana* (Fig. 1) are two economically important species for the State of Hidalgo, México. *A. applanata* is an important host species for the edible worms "chinicuiles" or "gusano rojo del maguey" (*Comadia redtenbacheri*), which have been traditionally used as food in Mexican cuisine. On the other hand, *A. salmiana* is used to obtain "aguamiel" (honey water) and "pulque" (a fermented non-distilled beverage) and their leaves are used in the "barbacoa" industry a typical Mexican dish (Castro-Díaz and Guerrero-Beltrán, 2013).

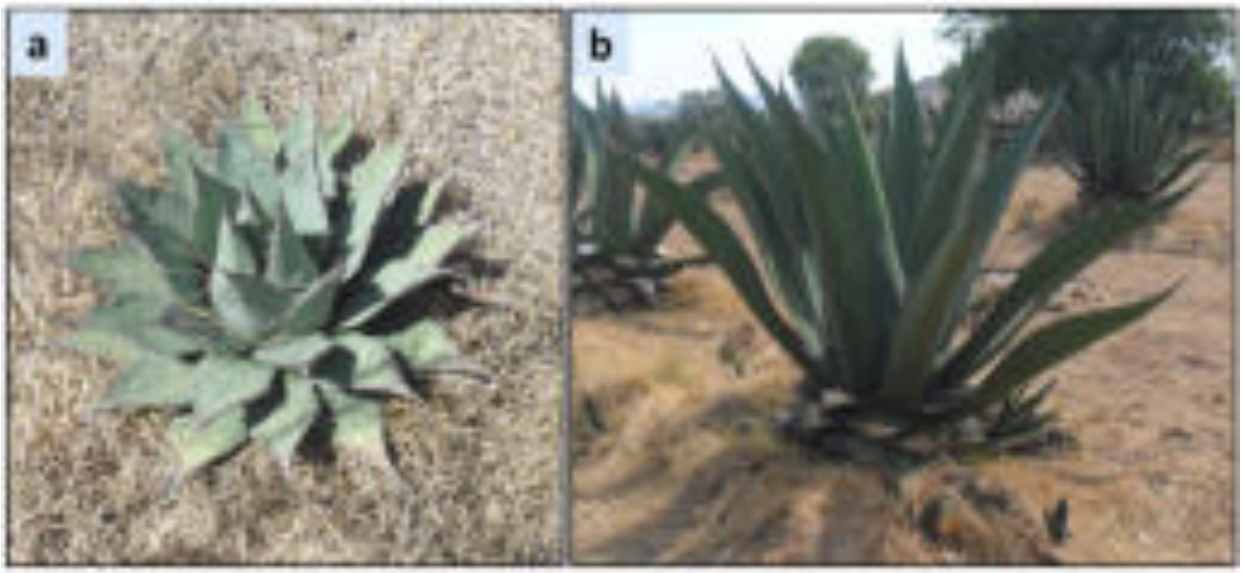


**Figure 1.** Plants of *Agave* growing in the State of Hidalgo. a) *Agave applanata*. b) *Agave salmiana*.

The aim of this work was the determination of chromosome number in plants of *A. applanata* and *A. salmiana* from the State of Hidalgo, Mexico.

## METHODOLOGY

Root tips were obtained from ten plants of *A. applanata* and *A. salmiana* collected in two different populations in the State of Hidalgo, Mexico. Metaphasic chromosome preparations were performed according to various cytogenetic techniques: squash (Belling, 1921), splash (Tapia-Pastrana and Mercado Ruaro, 2001), smear (Kurata and Omura, 1978), steamdrop (Kirov et al. 2014) and a modified steamdrop method (Rodríguez-Domínguez et al. 2016) to determinate which produced a greater chromosome spread; all samples were stained with acetorcein at 2%. The evaluation of metaphasic cells was carried out in a qualitative way. Data were evaluated trough nonparametric statistical analysis (Kruskal-Wallis and Mood tests) with a 95% confidence level. Chromosome preparations were observed with a Leica DMR-A2 microscope and the best ten spread metaphases of both, *A. applanata* and *A. salmiana* were captured with an Evolution QEi camera (Media Cybernetics Inc.,

Bethesda, Maryland, USA) with the Image Pro-Plus software and the images were sharpened with a High-Gauss filter.

## RESULTS AND DISCUSSION

Mitotic metaphases with a good chromosome dispersion were obtained through steamdrop and modified steamdrop techniques in both species, whose number of chromosome spreads was higher than those obtained by using the other mentioned techniques. The results showed different number of chromosomes: 2n=60 and 2n=150 for *A. applanata* and *A. salmiana* respectively (Fig. 2).

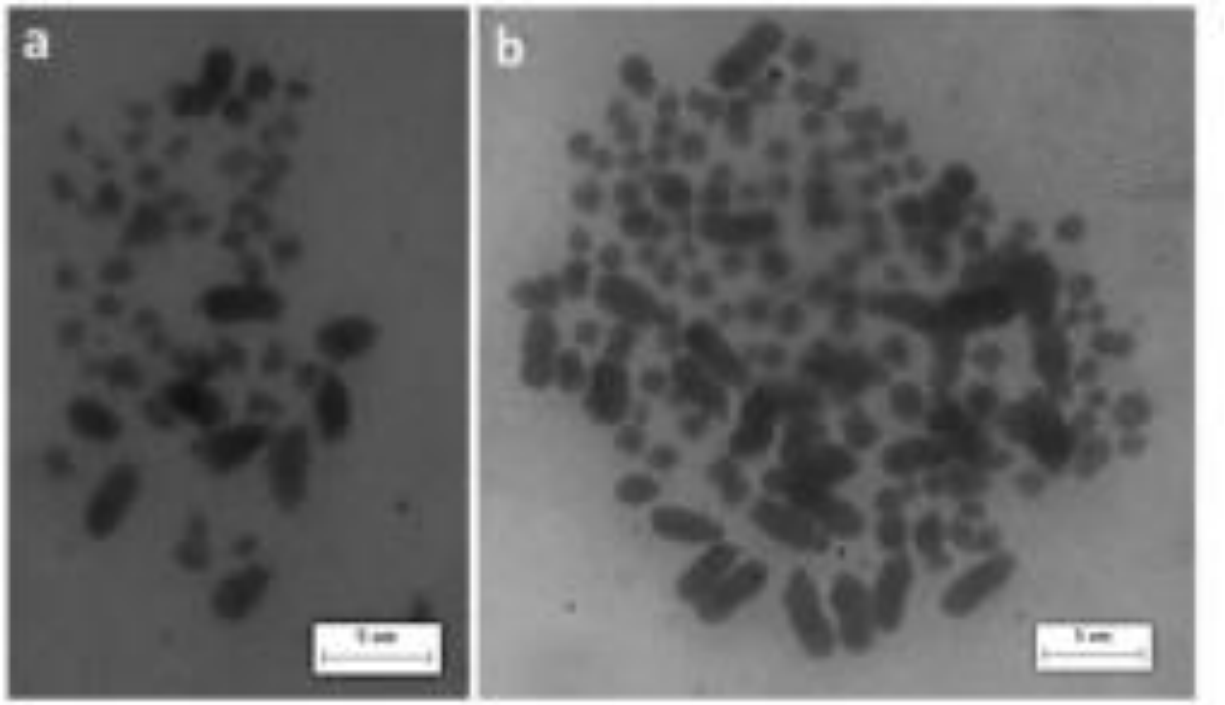


**Figure 2.** Mitotic chromosomes of *Agave applanata* (**a**) and *Agave salmiana* (**b**). Barr=5µm.

*A. applanata* showed a bimodal karyotype consisting of 10 large + 50 small chromosomes, being this is the first report on chromosome number for this species; on the other hand, *A. salmiana* also showed a bimodal karyotype consisting of 25 large + 125 small chromosomes. Regarding *A. salmiana*, tetraploids and hexaploids have been reported previously (Vignoli, 1936; Granick, 1944; García-Mendoza, 2007) our findings showed that the analyzed plants in the analyzed populations are pentaploids.

## CONCLUSION

Considering the basic number x=30, *A. applanata* is a diploid species (2n=2x=60), whereas *A. salmiana* is a pentaploid species (2n=5x=150).

## ACKNOWLEDGEMENTS

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THEMATIC I Scientific trends on Agave

# Effect of sodium nitroprusside (SNP) on in vitro shoot multiplication of *Agave angustifolia* Haw

**Romo-Paz F.J.[1] and Benjamín Rodríguez-Garay[2]***

## ABSTRACT

The effects of sodium nitroprusside (SNP) as nitric oxide donor on the micropropagation of *Agave angustifolia* Haw were investigated. 100% of explants in all treatments were able to form buds. The multiplication of axillary buds in the culture medium containing no SNP (Control) was observed to be slow, and the generation of new shoots per experimental unit was reduced (25.2), in addition to short shoots (7.62 cm) as compared to SNP treatments. On the contrary, the average number of shoots and shoot length was greater when the concentration of SNP increased (20 and 40 µM), showing that the addition of SNP significantly affects the development and regeneration of plantlets *in vitro*. The culture medium supplemented with 40 µM sodium nitroprusside was found to be statistically the best treatment, considering the high number of new shoots per experimental unit (46.6) and the average length of sprouts (12.56 cm).
However, the multiplication of axillary buds decreased when the concentrations of SNP were above 40 µM. This result shows that shoot multiplication is regulated by SNP in a dose dependent manner.

**Key words:** Nitric oxide, mezcal, Agave beverages, *in vitro* propagation, SNP.

## INTRODUCTION

The lack of an adequate supply of plant material for plantations, is a common problem for Agave agribusiness. Micropropagation is an efficient method to produce large quantities of clonal material in shorter periods of time than traditional methods, making efficient the continuous establishment of plantations (Richwine et al. 1996). Recently, sodium nitroprusside (SNP), a donor of nitric oxide (NO), has been used to promote regeneration of shoots in several plant species, such as *Vanilla planifolia* (Tan et al. 2013), *Dioscorea opposita* (Xu et al. 2009) and *Malus hupehensis* (Han et al. 2009). Plant hormones play an important role in the functioning, growth and development of plants, so that NO is now considered as a plant hormone (Leterrier et al. 2012). NO has the characteristics of

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a radical, which explains its high reactivity. The addition of NO through chemical donors such as sodium nitroprusside (NPS) is a topic of current interest in the in vitro plant propagation. The aim of this work was to study the effect of SNP in the micropropagation of *Agave angustifolia* Haw by improving the proliferation and quality of the produced shoots.

## METHODOLOGY

To determine the effect of SNP in the micropropagation, explants were placed on semi-solid MS basal medium and supplemented with 44.39 µM benzylaminopurine (BA), 0.11 µM of 2,4-Dichlorophenoxyacetic acid (2,4-D) (Castro-Concha et al. 1990), 3% (w/v) sucrose and diverse concentrations of SNP (0, 10, 20, 40, 60, 100 µM). All culture media was solidified with 0.8% (w/v) agar. The media were adjusted to pH 5.8 ± 0.2 and autoclaved. The SNP was added after autoclaving the media. Cultures were incubated at 27 ± 2°C under a photoperiod of 16-h light and 8-h darkness. Furthermore, all the culture containers were covered with red plastic (630 nm) to avoid degradation of SNP. The percentage of explants forming shoots and number of shoots per experimental unit were evaluated after 40 days of culture. The results were statistically analyzed by ANOVA and mean comparation by Tukey's test, $P \leq 0.05$.

## RESULTS AND DISCUSSION

100% of explants of *A. angustifolia* Haw formed buds. The multiplication of plantlets in the culture medium containing no SNP (control) was observed to be low, as the generation of new shoots per experimental unit was (25.2), in addition to short shoots (7.62 cm) as compared to other treatments ($P \leq 0.05$). The average number of shoots and shoot length was greater when the concentration of SNP increased (20 y 40 µM), finding that the addition of SNP as donor NO positively affected the development and regeneration of *in vitro* plantlets of *Agave angustifolia* Haw (Fig. 1), due to its direct effect on components of the cell wall that promote plant growth by decreasing cell-wall lignification and accelerating cell expansion (Wang et al. 2010).

The culture medium with 40 µM NPS, was the best statistically treatment ($P \leq 0.05$), taking into account the high number of new shoots per experimental unit (46.6) and the average length of sprouts (12.56 cm).

On the other hand, the multiplication of shoots decreased when the concentrations of SNP were above 40 µM. Some of the effects observed in explants treated with high concentrations of NPS (60 µM and 100 µM) was a low shoot production, tissue necrosis and leaf senescence, which was probably due to increased oxidative stress that inhibited the growth of the plantlets (Wang et al. 2010), similar to that reported by Guha and Rao (2012) in *Cymbidium* sp. who concluded that necrosis was due to oxidation imposed by SNP by generating a toxic $H_2O_2$ content. These facts demonstrate that shoot multiplication is regulated by the effect of SNP in a manner dependent on the dose, phenomenon observed by Han et al. (2009) where cell death and senescence increased as the concentration of NO increased.

Finally, the mechanisms by which NO improves efficiency in micropropagation in depth need to be elucidated. To our knowledge, this is the first report on in vitro axillary shoot multiplication of *Agave angustifolia* Haw using SNP as a nitric oxide donor.

**Figure 1.** Effect of different SNP concentrations on shoot multiplication of *Agave angustifolia* Haw. Bar = 5 cm.

## CONCLUSION

The effect of SNP seems to be of a dose dependent manner and inducing *in vitro* shoot proliferation at 40µM in *Agave angustifolia* Haw.

## ACKNOWLEDGEMENTS

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THEMATIC I Scientific trends on Agave

# Expression analysis of *CesA* gene related to fiber production in *Agave fourcroydes* Lem.

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## ABSTRACT

*Agave* Linnaeus, 1753 is a genus that includes about 166 species widely distributed in Mexico. Within this genus they have been described species with commercial importance, such as *Agave fourcroydes* Lem., which is characterized by high fiber content. To date is unknown the molecular factors that may be involved in that certain genotypes of *Agave* species have better fiber production and quality. However in some plant species such as cotton (*Gossypium hirsitum* L.) and flax (*Linum usitatissimum* L), it has been observed that the variation in the content of natural polymers (cellulose, hemicellulose and lignin) can be related to the quality of fiber. *A. fourcroydes* it is a species used in the production of fiber, but the behavior of genes related to the production of polymers is unknown. In this research we analyze the content of cellulose in *A. fourcroydes* Lem. plants with different sizes and also we analyzed the expression of *CesA* gene. The results indicated that larger plants have a higher cellulose content, which are correlated with increased expression of *CesA* gene. Meanwhile plants with low and medium size exhibited low expression of *CesA* gene (1 to 3 times less) compared to larger plants. The variations found by size plant, cellulose content and relative expression of *CesA* gene indicates that possibly environmental factors involved in growing play a major role in the development of plantations of *Agave* L. and possibly affecting their production and fiber quality.

**Key words:** *Cellulose synthase, fiber, A. fourcroydes Lem., CesA gene.*

## INTRODUCTION

*Agave fourcroydes* Lem. belonging to the Asparagaceae family is a plant widely used for fiber production and extraction of spheroidal metabolites (Infante, 2003). There are efforts trying improve the henequen industry in Mexico and global level. However it is to achieve this requires

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achieving competitiveness in production, through the transfer of technology, allowing producers to make efficient management of Agave and reduce costs, increase the volume of production and fiber quality. Within the context of competition, natural fibers are subject to competition from synthetic fibers. Therefore emphasized in increasing the productivity and quality of natural fibers. In some species of plants such as cotton (*Gossypium hirsitum* L.) and flax (*Linum usitatissimum* L.), it has been observed that high levels of polymers such as cellulose, hemicellulose, lignin is related to increased production and quality fiber. Additionally it has been observed a relationship with the expression of genes involved in the synthesis of these biopolymers.

Relative to the previous point and according to Guo *et al*. (2009), three genes coding for cellulose synthase enzyme isolated from *Gossypium hirsutum* L. have shown to increase its expression during anthesis (DPA), to 12, 15 and 18 days, reaching higher levels in the 18 day. This suggests the important participation of cellulose synthase gene for the production of cotton fiber. Also, Li *et al*. (2013), identified six isoforms of *CesA* gene in *Gossypium hirsutum* L. and *Gossypium barbadense* L. (Gh*CesA*5-Gh*CesA*10). Co-expression analyses indicated that *CesA*1, *CesA*2, *CesA*7, and *CesA*8 were isoforms that present a greater expression during the biosynthesis of secondary wall. Meanwhile the isoforms *CesA*3, *CesA*5, *CesA*6, *CesA*9, and *CesA*10 demonstrated to be involved in processes related to the formation of the primary wall for the initiation of cotton fiber and its elongation. Similar reports have been described in *Eucalyptus grandis* L., where the expression of three cellulose synthase genes (Egra*CesA*1, Egra*CesA*2, and Egra*CesA*3) in xylem cells has been related to the biosynthesis of wall cell, similar to that described in *Gossypium* spp. (Lu *et al*. 2008). As previous reports, support it possibly during the production of fiber in plants, the *CesA* gene, has a fundamental role that could be connected with the production and the quality of fiber in plants. Additionally the *CesA* gene proved to be involved in functions that involve primary and secondary cell wall formation.

Although that *A. fourcroydes* Lem. is widely used in the production of fiber, it is unknown the behavior of genes related to cellulose and hemicellulose production, reason by which this research analyze the cellulose content in plants of *A. fourcroydes* Lem. of different sizes and analyze the relative expression of *CesA* gene. The information generated in this work can in the future be used for the improvement of cultivars of *A. fourcroydes* Lem. in order to improve the productivity and quality fiber.

## METHODOLOGY

### Plant material.

Plant material was collected in hacienda Santa Teresa in Telchac village, select leaves of 5 years of different sizes. Samples was collected in 3 blocks, between each block a distance of 20m was considered. From each block were selected 3 sizes of plants (low, medium and larger), we collected samples by triplicate.

### Determination of fresh weight, dry weight and cellulose content in *Agave*.

The determination of extractable was performed according to the method T-207 cm-99 of the TAPPI. For the quantification of lignin used the Klason method (ANSI, 1977a) and for cellulose quantification was performed according to Rajev Kumar *et al*. (2009), additionally the parameters of leave length, dry weight, fresh weight and humidity were determined by triplicate in the samples.

**Relative expression of *CesA* gene.**

Extraction of RNA was performed by the method of TRizol® and proceeded to cDNA synthesis with Kit PROMEGA. For the design the primers was considerate the conserved domain of cellulose synthase. The qPCR was realized with One Step (Applied Biosystem). The gen 18S rDNA gene was used as reference gene according Tamayo-Ordoñez *et al*. (2016) and Tamayo-Ordoñez *et al*., (2015)

**Data Analysis**

Data corresponding to leave length, dry weight, fresh weight and cellulose content, were subjected to ANOVA analysis, using SAS statistical software package. 9.0 (2000) and the program Origin 9.1. Statistical tests were evaluated with $\alpha = 0.05$ and the means were compared using Tukey's test ($P > 0.05$).

## RESULTS AND DISCUSSION

Twenty seven plants of different sizes were collected and were classified according to their size in low, medium and larger, which show leave length of 139.37, 110.09 and 75.88 cm, respectively (fig. 1A). The values of fresh weight, dry weight, and percentage of humidity were analyzed and identify classes. Statistical analyses indicated three data classes that coincided with the different size of plants. Fresh weight and dry weight, data indicated that plants of larger size (139cm), present on average 773g fresh weight and 196g of dry weight. Medium-sized plants (110 cm), showed present 410g of weight fresh and 108g of weight dry. Low-sized plants (75 cm), presented on average 196g of weight fresh and 33g of dry weight (figs. 1B y 1C). The Tukey test indicated that the three classes of data presented differences significant, the larger plants present higher values of weight fresh and weight dry, values that decreasing according to size of plants analyzed.

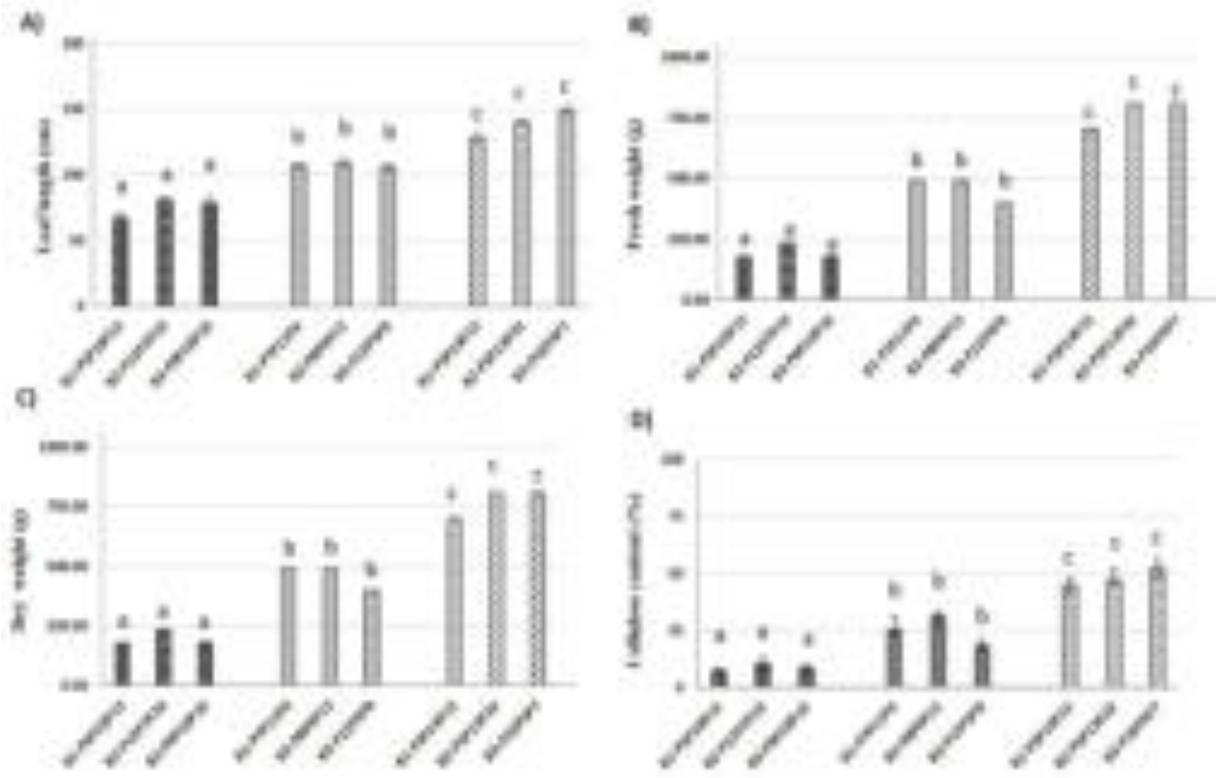

**Figure 1.** Schematic representation of parameters evaluated in *A. fourcroydes* Lem. plants of different size. A) Length leaf. B) Fresh weight, C) Dry weight and D) percentage of cellulose content.

Determination of cellulose content, indicated that the plants of larger, medium and low plants present 48%, 24% and 8% of cellulose content, respectively. The greater amount of cellulose was found in larger plants. (figure 1D).

The expression analysis indicated major expression of *CesA* gene in larger plants. These plants showed a relative expression of 1 and 3 times more in comparison to medium and low plants. This could be related to that large plant contain major number of cells and higher expression the gene related to biosynthesis for to maintenance and primary and secondary wall formation is required. (figure 2).

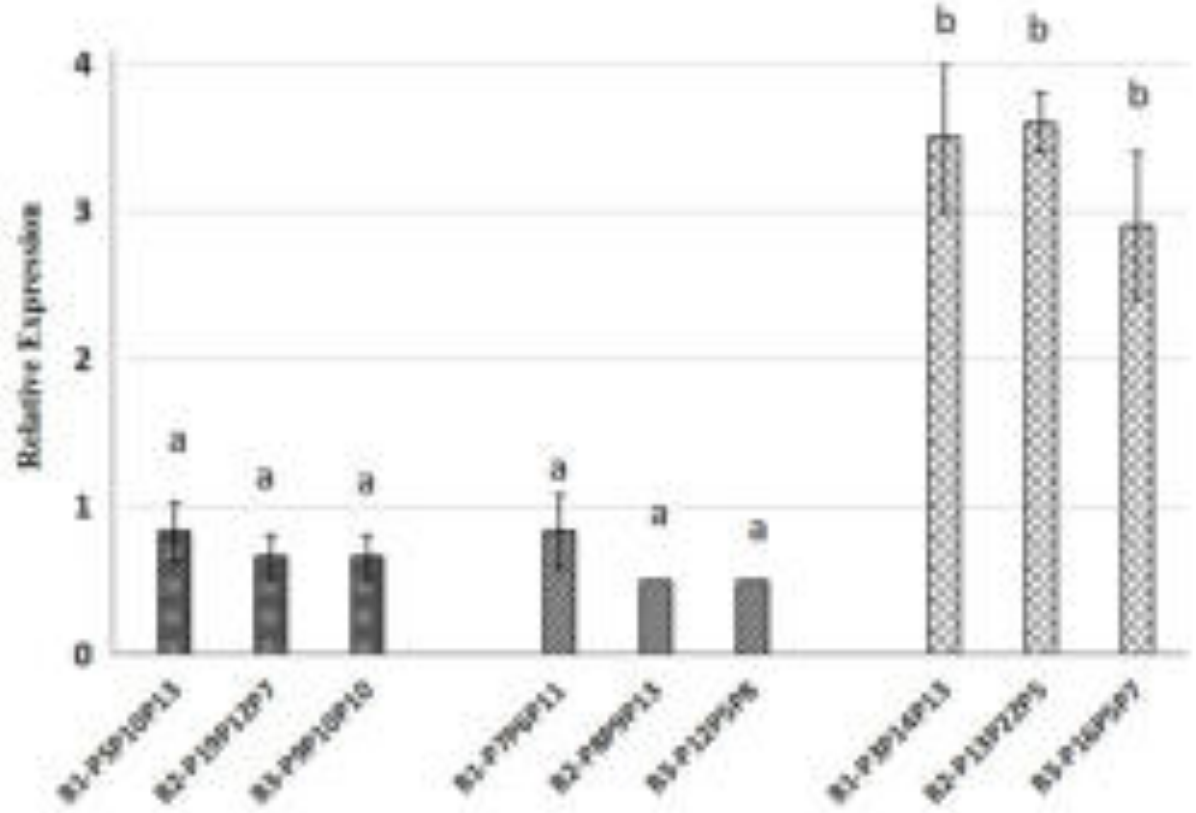


**Figure 2.** Relative expression of *CesA* gene in *A. fourcroydes* Lem. plants with different size.

In literature, highest values of cellulose content are reported in *A. fourcroydes* Lem. and *A. lechugilla* (≥76%). Results obtained in this study indicated that the larger plants showed higher percentage of cellulose content, however are 1.8 times more lower than those reported by Vieira *et al.* (2002), suggesting that in our plants collected the habitat where they grow plays an important factor in the gain in fresh weight, dry weight, and possibly cellulose content. Finally, respect to relative expression of *CesA* gene is possible that in large plants, the total number of cells is greater in comparison to middle and low plants, it is possible that in larger plants require more expression of genes involved in the maintenance and primary and secondary wall formation, coinciding with a greater expression of *CesA* gene in *A. fourcroydes* Lem. of larger size.

## CONCLUSION

The variations found in expression of *CesA* gene and cellulose content in *A. fourcroydes* Lem. plants with different size, indicates that environmental factors (Sims, 2003), could be affected the development of *A. fourcroydes* Lem. plants and could affect their productivity and quality of fiber.

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THEMATIC I Scientific trends on Agave

# Genetic transformation of *Fusarium oxysporum* as a tool for the study of pathogenesis during agave infection.

**Navarro López D.[1], Dupré P.[2], Qui Zapata J.[3]***

## ABSTRACT.

*Agave tequilana* disease caused by *Fusarium oxysporum* has not been widely addressed at level of plant-pathogen interaction; therefore the knowledge provided by a histopathological interaction can generate a significant impact on the characterization of the disease. The aim of this work is transform *F. oxysporum* with the gene of the Green Fluorescent Protein (GFP) to evaluate the infection during compatible interaction of *F. oxysporum* with *A. tequilana* Weber var. azul. For transformation, a construct was generated with the expression vector pUE08 which contains the constitutive promoter of pyruvate kinase pki1 and the trpC terminator, in which was inserted in frame with the promoter the sGFP (variant S65T) gene obtained from pMKH1 plasmid, into the restriction sites EcoRI/BamHI resulting in a construct designated as pUE-sGFP. This construct was analyzed by sequencing and the results obtained show that the gene is inserted in frame with the promoter, so can be concluded that sGFP gene will be expressed correctly when it will be inserted into *F. oxysporum*.

**Key words:** *Fusarium oxysporum*, Green Flourescent Protein (GFP), wilt disease, *pki1* promoter, *trcC* terminator.

## INTRODUCTION

*Agave tequilana* Weber var. azul (blue agave) is one of the 272 endemic species of Mexico considered the exclusive raw material for the manufacture of tequila, with an established surface for planting of 89, 000 Ha, that comprises 224, 847 356 plants with yielding of 30 kg per plant (Vega-Ramos et al. 2013). One of the problems facing crop is the wilt disease (marchitez del agave) caused by *Fusarium oxysporum*, the most common species of the genus of great agronomic interest (Di Pietro et al. 2003). Little has been reported about the mechanisms of pathogenesis *of F. oxysporum* in *agave* to the establishment of the disease. An approach to the agave - *F. oxysporum* interaction was made in previous work by evaluating resistance at biochemical level (accumulation of reactive oxygen

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species (ROS), production of pathogenesis related (PR) proteins, phytoalexins and phytoanticipins (Bahena-Reyes, 2014; López-Velazquez, 2015). However, to date histopathological characterization of the interaction *of F. oxysporum* with its host has not been addressed.

In view to investigate the interaction, we propose to transform *F. oxysporum* using the green fluorescent protein (GFP) marker gene and to infect *A. tequilana* with the transformed strain. The characterization of this interaction hereafter will provide important knowledge for the development of strategies for disease control. We report here the construction of the plasmid that will be used in further experiment.

## METHODOLOGY

For the transformation *of F. oxysporum* strain, a vector containing the constitutive promoter from the gene encoding pyruvate kinase (*pki1*) from *Trichoderma reesei*, the terminator trpC from *Aspergillus nidulans* and the marker gene GFP was generated. Oligonucleotides were designed with the restriction sites *Bam*HI and *Eco*RI and a polymerase chain reaction was performed to obtain the sGFP fragment (S65T variant) from the plasmid pMKH1-sGFP. The fragment was digested with *EcoRI/BamHI* restriction enzymes and purified using the Minielute reaction cleanup kit (QIAGEN®). 30 ng of the purified fragment mixed with 10 ng of pUE plasmid and 5u of T4 DNA ligase (Promega®) were ligated in a 1:3 molar ratio vector insert protocol. The ligation was then purified with ten volumes of n-butanol, electroporated in *Escherichia coli DH5-α* strain and plated on LB medium supplemented with 25μ•ml$^{-1}$ of cloramphenicol. pUE plasmid contains the hygromicin B phosphotransferase (hph) gene, β-galactosidase (*lacZ*) gene for blue/white screening and chloramphenicol gene for bacterial selection marker. All cloning manipulations were made on DH5-α strain and a high yielding competent cells where prepared following the method of Tang et al. (1994).

To ensure the sGFP insertion was in frame with *pki*1 promoter, three oligonucleotides were designed, two upstream at the promoter region at 497bp and 261bp from the sGFP start site and one at the terminator trpC, then a PCR was performed and the results were visualized by electrophoresis assay in a 0.8% agarose gel.

The obtained sGFP fragment and the corresponding construction for the sGFP expression in *F. oxysporum* were sequenced and analyzed by alignment with the reference sequences obtained from NCBI database in the Snap Gene Software.

## RESULTS AND DISCUSSION

The fragment corresponding to sGFP gene from the pMKH1-sGFP plasmid was obtained with the BsGFPF-CGCGGATCCATGGTGAGCAAGGGCGAGGAG y BsGFPR-CCGGAATTCTTACTTGTACAGCTCGTCCATG oligonucleotides, generating a product with 720bp cloned in the expression vector pUE on the restriction sites *EcoRI/BamHI*, and the resultant plasmid was called pUE-sGFP (Fig. 1).

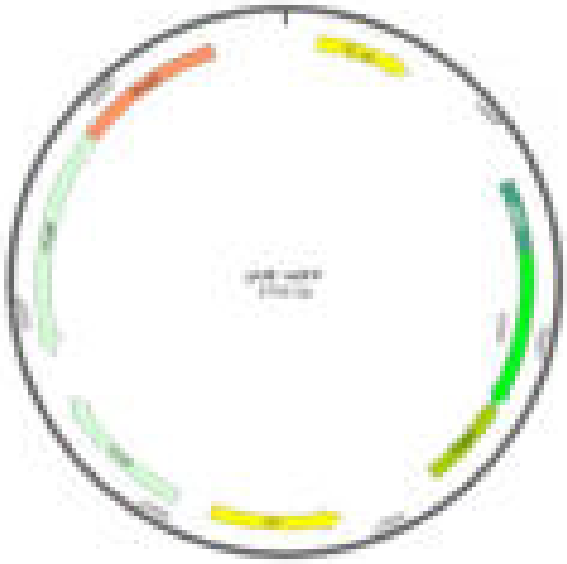

**Figure 1.** Schematic representation of plasmid map pUE-sGFP.

The pUE-sGFP plasmid firstly was analyzed by the resistance of DH5-α to chloramphenicol and blue/withe screening. Then, the polymerase chain reaction products analyzed in agarose gel showed the correct fragment sizes of 1377bp (obtained by the 1GFP-CGTTGCTGTGAG ACCATGAG and 2GFP-GCGGATTCCTCAGTCTCGTA oligonucleotides) and 1141bp (obtained by 3GFP-CGTTGCTGTGAGACCATGAG and 2GFP oligonucleotides), lines 2 and 5 respectively Figure 2. Beside this the fragment corresponding to 720bp sGFP was analyzed in constructions, pUE-sGFP and pGEM-sGFP with the and BsGFPR, lines 8 and 9 (Fig. 2).

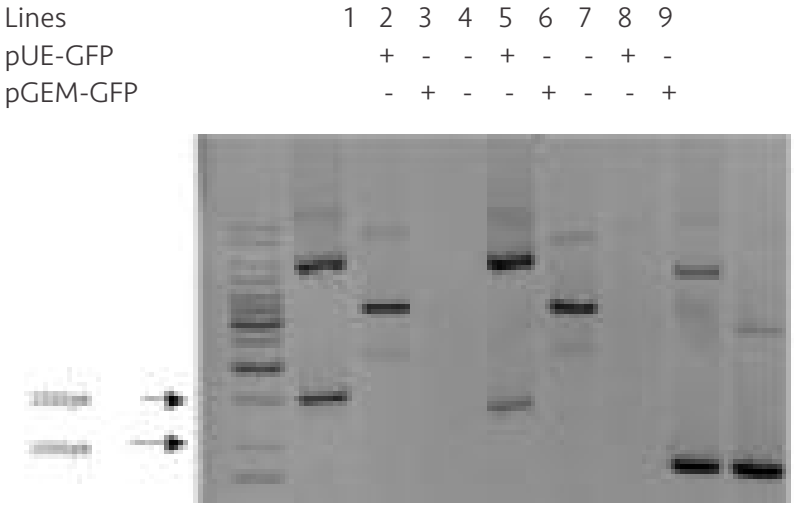


**Figure 2.** Electrophoresis assay of pUE-sGFP construction. Line 1 shows the molecular weight marker, line 2 and 5 shows the fragments of 1377bp and 1141bp obtained by PCR with 1GFP-2GFP and 3GFP-2GFP primers respectively. Lines 3 and 6 are the pGEM-sGFP plasmid employed as negative control of PCR reaction. Lines 8 and 9 shows the 720bp sGFP fragment obtained with the BsGFP-F and BsGFP-R from the pUE-sGFP and pGEM-sGFP.

Finally we analyzed the sequencing results by alignment with an *in silico* construction of *pki*-sGFP-*trp*C sequence in which the 880bp downstream and 269bp upstream from the sGFP start codon, with an exception of an unmatched sequence of 32bp which correspond to the multiple cloning site, match with the reference sequence as shows fig. 3.

**Figure 3.** Sequencing analysis of pUE-sGFP construction. A 32 bp segment doesn't anneal and correspond to the multiple cloning site of pUE08 plasmid.

## CONCLUSIONS

The pUE-sGFP is constructed in frame with the *pki*1 promoter whereby the resulting transformant of *F. oxysporum* strain, will express sGFP protein and will be used to evaluate the agave–*F. oxysporum* interaction.

## ACKNOWLEDGEMENTS

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THEMATIC I Scientific trends on Agave

# *In vitro* conservation of three species of agaves by using temporary immersion system.

**Herrera-Isidrón L.[1], Isidrón-Pérez M.[2], Herrera-Isidrón J. A.[3], Castro-López S.[4], Nieto-González A.[5], Núñez-Palenius H.[6]***

## ABSTRACT

Plants from *Agave* spp. are conserved at "UG-SAGARPA *in vitro* National Collection of Agaves, Camote de Cerro and Achiote" in the University of Guanajuato. At present, plants are kept in semisolid basic medium Murashige and Skoog (MS). Temporary immersion systems (TISs) offer significant advantages for plant conservation, like improvement in plant quality and survival, due to an efficient supply of nutrients and oxygen in the liquid medium. Here we describe efficient methodologies for the *in vitro* conservation of two economically relevant agaves (*Agave tequilana* and *Agave applanata*), and for the endangered species *Agave victoriae-reginae*. TISs were useful for the in vitro conservation of three important species of agave providing high levels of *viability* (70-85%) and survival (85-90%).

**Key words:** *Agave* spp., Temporary immersion systems, germplasm.

## INTRODUCTION

The *in vitro* National Collection of Agaves, Camote de Cerro and Achiote at University of Guanajuato (UG-SAGARPA), allows the production of plants which can be kept under minimum growth conditions. At present, plants are conserved in semisolid basic MS medium (Murashige and Skoog, 1962). Temporary immersion systems (TISs) offer significant advantages for plant conservation like improvement in plant quality and survival due to an efficient supply of nutrients and oxygen in the liquid medium (Etienne and Berthouly, 2002). Additionally, TISs reduce costs, manual labors and it facilities the medium change. Additionally, TISs decrease costs, because liquid media includes no agar, laboriosity is reduced when compared to solid media and it facilitates automation (Watt, 2012).

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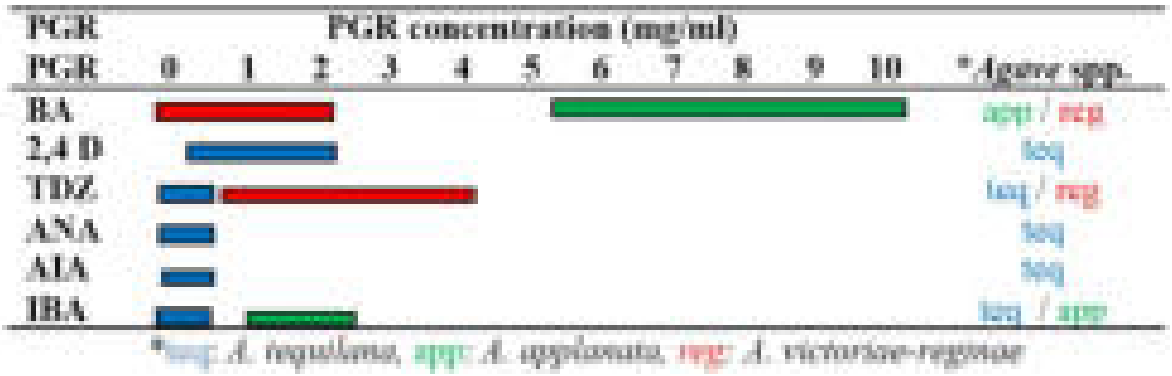
Previous reports have established *in vitro* regeneration protocols for *A. victoriae-reginae* and *A. tequilana* propagation (Rodriguez-Garay et al. 1996; Ramírez-Malagón et al. 2008; Martínez-Palacios et al. 2003), whether by indirect somatic embryogenesis or by direct organogenesis; while very few has been reported for *A. applanata* in tissue culture. In those cultures, plants grew in semi-solid substrate including a gelling agent, being agar one of the most expensive ingredients in the culture medium. Handling of vegetal material and its periodic transfer to renewed media is time consuming and causes contamination and tissue damage (Weathers and Giles, 1988).

To overcome the difficulties associated with semisolid media, system of propagation based in temporary immersion provides an interesting approach, consisting in the immersion of plant tissue during specific periods of time in the culture medium (Etienne and Berthouly, 2002). As a contribution to the current procedures of agave conservation, here we describe efficient methodologies for the *in vitro* conservation of two economically relevant agave species (*Agave tequilana* and *Agave applanata*), and for the endangered species *Agave victoriae-reginae*.

## METHODOLOGY

Agave *in vitro* seedling explants were inoculated into a SIT system containing MS half-strength liquid media and they were supplemented with plant grow regulators (PGR) at different concentrations (Table 1).

**Table 1.** Plant grow regulators (PGR) and range of concentrations used in SIT assays.




All cultures were kept at 25 ± 2 °C under a 16 h photoperiod. Data of plant viability (increment in the plant size and in the number of new leaves, shoot and root induction) were recorded after 10-24 weeks in culture, according to the agave species. The effect of the immersion frequency (1 min of immersion every 24, 48 or 72 h) and pre-acclimation on the plant survival was examined for *A. tequilana* and *A. victoriae-reginae*.

## RESULTS AND DISCUSSION

Viability of agave plants cultured in SIT varied from 66 to 86 % (Fig. 1). For *A. applanata*, 33% of plants showed necrosis signals, maybe due to the high PGR concentration used in some treatments being similar to Ramírez-Malagón *et al*. (2008).

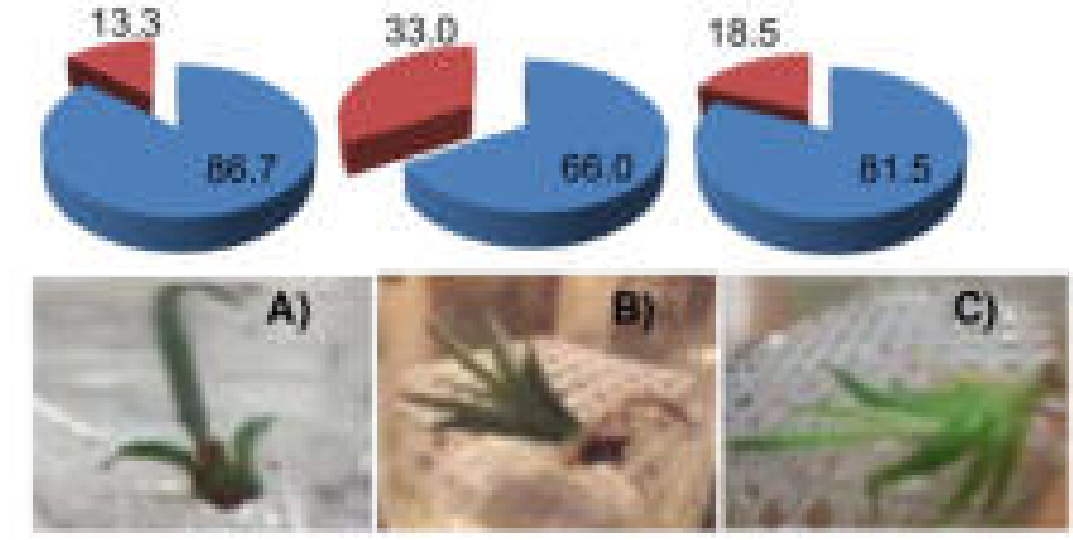



**Figure 1.** Viability percentage of three agave species cultured in SITs. **A)** *A. tequilana*, **B)** *A. applanata*, **C)** *A. victoriae- reginae. blue: viability, red: necrosis*.

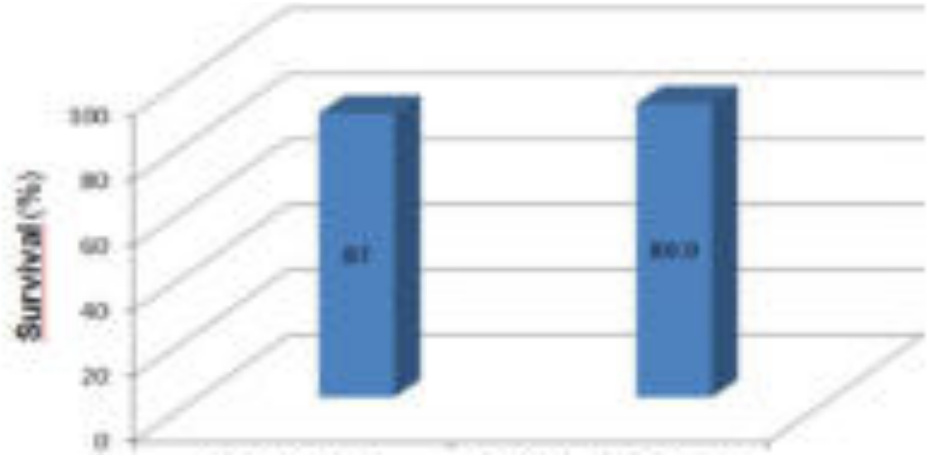



**Figure 2.** Survival of agave plants in the pre-acclimatization stage on peat moss substrate. Left: *A. tequilana, right: A. victoriae-reginae*.

One minute single immersions in liquid medium every 72 h were optimal for keeping sanity of agave plant cultured in TISs. This reduces laboring in regards to conventional semisolid medium for germplasm preservation, which is advisable. The use of *peat-moss* in the pre-acclimatization allowed 98% plant survival (Fig. 2).

## CONCLUSION

Temporary Immersion Systems in liquid media were useful for the in vitro conservation of *Agave tequilana* Weber var. azul, *A. applanata*, and *A. victoriae-reginae* three important species of agave providing high levels of viability (70-85%) and survival (85-90%).

## ACKNOWLEDGEMENTS

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THEMATIC I Scientific trends on Agave

# *In vitro* culture of *Agave* spp. in biorreactors under LED light.

**Herrera-Isidrón J.A.[1]*, Núñez-Palenius H.[2], Herrera-Isidrón L.[3], Isidrón-Pérez M.[4], Trejo-Rodríguez R.[5], Herrera-Altuve J. A.[6], Hernández-Francisco V.[7] y Morales-Luna R.[8]**

## ABSTRACT

Plants from four different Agave species (*A. tequilana, A. salmiana, A. durangensis and A. applanata*) were cultured *in vitro* by using Temporary Immersion System (TIS) under LED illumination. Plants received about 60 or 120 $\mu mol \cdot m^{-2} \cdot s^{-1}$ from LED lamps while controls received just 16.1 $\mu mol \cdot m^{-2} \cdot s^{-1}$ from fluorescent lamps. Plants were individually placed in homemade SIT and distributed in shelters according light intensity. Aluminum foils were used to cover the shelters in order to increase light intensity but avoiding the use of extra LED lamps. The growth chamber was maintained at 25 ±2 °C under a 16-h photoperiod. Temporary immersion cultures were established with immersion of explants for 1 min every 72 h. Primary results revealed that plants grew normally under our experimental conditions. The combination of LED lamps and aluminum foils might benefit *Agave* plants multiplication and conservation while reducing power consumption. However more immersion cycles are necessary in order to confirm this tendency.

**Key words:** *Agave* spp, LED light, Temporary immersion systems.

## INTRODUCTION

*Agave* is a succulent genus within the Asparagaceae family. These xerophytes plants have thick leaves which usually end in a sharp point and are indigenous to arid regions of Mexico (Escamilla-Treviño, 2012). They find application in liqueur industry, human food and forage, among others. *Agave* plants usually grew very slowly; they need several years to reproduce and to propagate. In order to shorter the propagation period, Plant Tissue Culture techniques (PTC) have commonly be used. Among PTC,

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the Temporary Immersion Systems (SIT) in liquid media is presently the preferred method for plant propagation because it produces pathogen-free and invigorated plants, while reduces costs and time (Watt, 2012). Plants receive short-period immersion in 50% culture media while most of the time they remain not immersed. Light is usually provided from fluorescent lamps but it has been recently reported the use of LEDs (Stutte, 2009) since they consume less energy and produce less heat than fluorescent counterparts. However, photosynthesis and photomorphogenesis are both affected by light quality, light intensity and photoperiod (Cope *et al*., 2014). As photosynthetic active radiation (PAR) differs from one light source to the other, the aim of this work was to address the capacity of *Agave* spp. plants to be propagated under LED light.

## METHODOLOGY

Explants from *A. tequilana* Weber var. azul, *A. salmiana* Otto ex Salm-Dyck, *A. durangensis* Gentry and *A. applanata* Koch ex Jacobi were cultured in TIS under conditions reported elsewhere (Mordocco, *et al*., 2009). Beginning at the leaf base, just above the apical main meristem, a 4–5 cm long basal portion of the leaf roll (including a small meristem) was excised and placed in sterile Murashige and Skoog (MS) half-strength liquid media (Murashige and Skoog, 1962) supplemented with 6-Benzylaminopurine (BAP) 1mg·$l^{-1}$, 2,4-D 10µg and vitamins. Illumination was provided by LEDs (Fig. 1, left) or by fluorescent lamps (control) with Photosynthetically Active Radiation (PAR) 16.1 ± 1.4 (mean ± SEM) µmol·m-2·$s^{-1}$ for 16 h and 8 h darkness. Immersion lasted 1 min every 72 h and temperature was kept at 28.7 ± 0.9 ºC. Several growth variables were recorded: plant height, canopy width, leaf number and necrotic areas. One-way breakdown ANOVA plus Tukey pos-hoc test was used to assess the difference between variables values measured at the beginning and after four weeks of culture.

## RESULTS AND DISCUSSION

Plants are more likely to absorb light in some wavelengths than in others, likewise lamps radiate different light intensity distribution across the 400-700 nm wavelength bandwidths. All *Agave* plants survived after the 4 weeks period and plants sanity was fine (Fig. 2, right). Most of the evaluated growth factors showed no difference among the plants which received LED or fluorescent light.

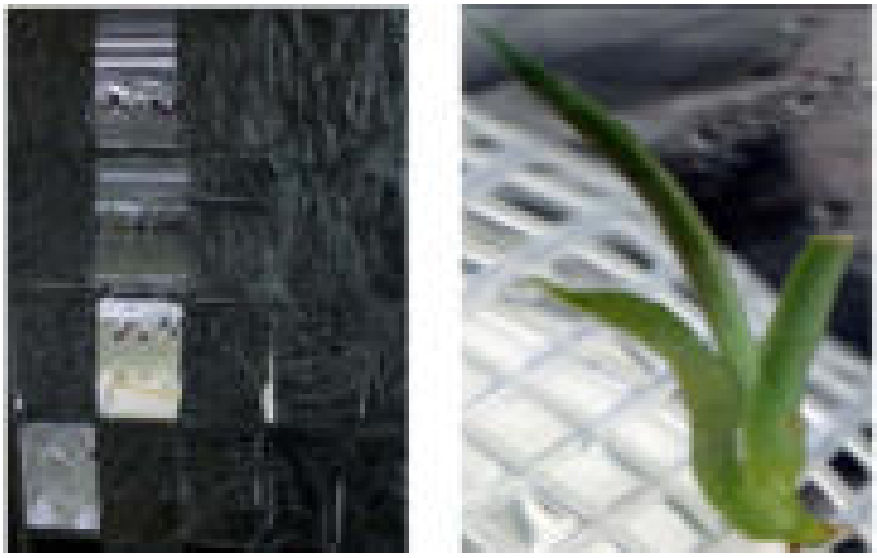

**Figure 1.** Single plants within SITs located on four shelves. LED PAR: A) 62.8±1.9, B) 60.2±1.6, C) 117.7±3.4, D) 118.9±2.5 µmol·$m^{-2}s^{-1}$. Aluminum foils increase light intensity. Right. *A.durangensis* plant after 4 weeks under LED illumination.

Table 1 depicts values recorded for *A. durangensis* plants, the rest of the species showed similar results (data not shown). The plant height was the only factor which increased more in LED illuminated plants than in the ones which receive fluorescent light. No difference was observed in plants under LED intensities L1 and L2, even when L2 was almost twice of L1. The incorporation of blue or red LED light during *in vitro* propagation of *Rehmannia glutinosa* has reported a beneficial way to increase the medicinal values of the plant (Manivannan *et al*., 2015). However, additional data from longer experiments should be analyzed before replacing the light sources.

**Table 1.** Effect of light intensity over four growth variables in *A. durangensis* plants. Data indicates differences between variables at the beginning and after four weeks of culture.

| Factor | L1 (LED) | L2 (LED) | fluorescent |
|---|---|---|---|
| Height (cm) | 0.42±0.06[a] | 0.47±0.08[a] | 0.34–0.04[b] |
| Canopy width (cm) | 0.19±0.06[a] | 0.19±0.06[a] | 0.2 –0.04[a] |
| Leaf number | 0.21±0.09[a] | 0.25±0.09[a] | 0.23 0.05[a] |
| Necrotic areas | 0.12±0.07[a] | 0.23±0.06[a] | 0.40 0.10[a] |

L1: includes data from shelves **A)** and **B)**, L2: shelves **C)** and **D)**. Data are presented as mean ± SEM. Means with different letter within rows do statistically differ according to one-way breakdown ANOVA and Tukey pos-hoc test. p: 0.05, n = 9.

About 80 W were needed in order to reach 16 $\mu mol \cdot m^{-2}s^{-1}$ PAR by using fluorescent lights, whereas only 10 watts were necessary to obtain the same PAR when LEDs lamps were used. Therefore, the use of LEDs in PTC, specifically in TIS, was a sustainable way to reduce energy consumption in *Agave* propagation protocols, without affecting the plant proliferation rate and viability

## CONCLUSION

Plants from *A. tequilana, A. salmiana, A. durangensis* and *A. applanata* species growing under LED light did not differ regarding plant height, canopy width, leaf number and necrotic areas from plants growing under fluorescent light in our experimental conditions.

## ACKNOWLEDGEMENTS

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THEMATIC I Scientific trends on Agave

# Offshoots induction in *agave* mezcalero (Agave spp) by applicating phytohormone, nitrogen and using strangling method.

Quezada Díaz A.[1], Orea LaraG.[2]*, Hernández Vargas V.[3], Loera Gallegos H.M.[4]

## ABSTRACT

The natural reproduction of Agave mezcalero (*Agave* spp) in the southeast of Durango state is not enough to satisfy the produce necessary for the the mezcal industry. The main means of propagation is by seed, obtaining plants with broad genetic variability. Only some plants (3 to 4%) form tillers, limiting its spread to obtain uniform plants that supplies raw materials to the mezcal industry. In this research it was evaluated the effect of ethrel 240 as fitohormona, application of nitrogen, and throttled of apical meristem of plant in the incitement of tillers for agave mezcalero. It was used plants of Agave mezcalero with a height between 25 and 30 cm. They were provided with plastic mulch and drip irrigation. The treatments consisted of the application of ethrel 240 in concentration of 20, 40, 60 and 80 ppm; two nitrogen sources CO $(NH_2)_2$ and $KNO_3$; and removing and not removing the apical meristem. The parameter evaluated were the number of leaves, the length of the leaf, the canopy of plant, and the number of offshoots. The results showed that the concentration with the most number of offshoots obtained were with 60 and 40 ppm ethrel. The best source of nitrogen was potassium nitrate ($KNO_3$) for the incitement of offshoots in agave. The strangling of apical meristem did not make any statistical difference for the incitement of offshoots. In fact, it limit the growth of the plant. The distance among each plant should be greater than 30 X 30 cm. Otherwise, the quick growth of plants will saturate the 30 X 30 cm space, and it will not allow counting the number or the development of tillers. The use of mulching system should be avoided since plastic prevents the growth of buds, causing weakness and death of tillers.

**Key words:** spread, buds, stem, bud.

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## INTRODUCTION

In arid and semiarid areas of southeastern of Durango state, the Mezcalero Agave is an important plant in the development of the economic activity. The Mezcalero Agave (*Agave* spp) is mainly used to produce mezcal. This industry has depended on the extraction of natural populations of plants (Valenzuela, *et al*., 2003). Because the Agave mezcalero has a 94-96% germination, it mainly reproduces by seed. However, this method of reproduction has high genetic variability. In the other hand, the reproduction by tillers creates uniform plants, but this method is very limited because only 3% of population produces tillers (Orea, *et al*., 2014). The spread of Agave could be sexual by seed or asexual by apomixis, rhizomes, or tillers. Most agaves used to produce mezcal come from clones. They are reproduced by rhizomatous, shoots and bulbils from the inflorescence. Other species of Agave rarely or never proliferate tillers (Nobel, 1998). Vegetative propagation practices such as strangling, severe pruning, drought, fix branches to the groustrand, remove flower buds, flowers and fruit removal can stimulate vegetative growth. (Salisbury, 2000). The use of organic compounds such as growth regulators or plant hormones can inhibit or modify the physiological process of the plant (Weaver, 1976). Application of ethylene stimulates germination of various grains, shoot growth in bulbs, hardwood cuttings, and root; premature leaf abscission, young fruits and other organs (Weaver, 1976; Hernandez, 2009). The application of ethylene concentrations around 10 ppm causes the production of roots, stem, tissues of leaves, and roots develop preexisting in the stems (Azcon, 1993). In pineapple, ethylene stimulates the generation plant shoots applied at concentrations 20 ppm, with 18.52 g/L of urea, and pH 8.5 (Panama, 2003). The applications of nitrogenous in plant banana; reinvest the reproductive growth to vegetative growth; and stimulating budbreak (Cruz, *et al*., 2012). The demand for the mezcal industry requires to establish commercial plantations similar to the parent plant materials. However, because its reproduction by tillers is scarce, it is necessary to seek alternative vegetative propagation. The purpose in this investigation is to incite offshoots in Agave Mezcalero (*Agave* spp) by application phytohormone, nitrogen, and using strangling method.

## METHODOLOGY

The experiment was conducted at the Centro Interdisciplinario de Investigación para el Desarrollo Integral Regional Unidad Durango, del Instituto Politécnico Nacional (CIIDIR IPN). It was used Mezcal maguey plants as vegetative material from a nursery garden with age of 2.5 years, and height between 25 and 30 cm. The experiment was set in beds of 1.20 m width and a system of plastic mulch and drip irrigation (Hernández, 2013). After six months from planting, the treatments were applied. Treatments consisted in the application of ethrel 240 with concentration of 20, 40, 60 and 80 ppm; two sources of nitrogen $CO(NH_2)_2$ or $KNO_3$ each with a concentration of 18.14 g/L; adjustment of pH to 8.5 (Panamá, 2003); and strangling of the apical meristem for a set of plant. Following, two applications of 640 mL of each treatment mentioned above were applied to each plot. They were applied directly to the base of the leaves with one day of separation. Both applications were provided during afternoon time. The experimental unit consisted in one plots of 1.20 x 1.20 m with one meter apart between plot. In each plot, Agave plants were replanted with a distance of 30 x 30 cm between plants with a total of 16 plants. A sample was taken each month for a period of seven months. In the seventh and last sample the extraction of the plants were performed. The parameters evaluated were: total number of outbreaks, number of leaves, length of leaf and canopy. The experiment was designed under random conditions with a factorial arrangement AxBxC, and four repetitions (Montgomery, 2004). The results were analyzed using Statistical Analysis System (SAS) program (SAS Institute, 1996) using analysis of variance and mean test using Tukey method $\alpha \leq 0.05$.

## RESULTS AND DISCUSSION

The effect of different levels of ethrel 240, nitrogen source, and strangling of the apical meristem in some plants, did not show statistical differences on the parameter under study- Number of leaves (NH), leaf length (LH), leaf width (AH), plant's canopy (DP), and number of tillers incited (NB). Through the first six samples and with exception of the AH parameter in sample six (data not reported), treatment 16 made a statistically difference with lower AH value (10.69 cm) with respect to other treatments. Table 1 shows the results obtained in the seventh sample and shows that the parameters NH, LH, AH and DP did not made statistically difference. However, for the NB parameter, treatments made statistically different.

According to Table 1, for the buds parameter, two treatments showed statistical differences with respect to the others. The first treatment consisted in a concentration of ethrel 240 of 60 ppm, strangling of apical meristem, and application of $KNO_3$. On the other hand, the other treatment consisted in a concentration of 40 ppm without strangling the apical meristem and application of $KNO_3$. These two treatments showed the highest values with 3.56 and 3.50 number of incitement of offshoots, respectively.

**Table 1.** Response of Agave mezcalero (*Agave* spp) plants to the application of ethrel 240 (20, 40, 60, 80 ppm), nitrogen ($CO(NH_2)_2$ and $KNO_3$), and strangling of the apical bud in some plant for the incitement offshoots.

On contrary, the treatment that consisted of 40 ppm of ethrel 240, urea as a nitrogen source, and strangling obtained the lowest value of buds incitement. In addition, this treatment was the one with the lowest average value with respect to the control having a value of 0.69 induced offshoots. The pattern in the number of leaves (NH), leaf length (LH), leaf width (AH), and plant's canopy (DP) did not showed a significant difference. This suggests that the experiment was developed under homogeneous conditions (Figs. 1 and 2) because the vegetative growth was not affected by the incitement of tillers. In fact, this can be verified with the NH variable, as shown in fig. 2.

**Figure 1.** Initial phase of the experiment, one month after planting the agave.

**Figure 2.** End of experiment, seven months after planting the agave.

Additionally, fig. 3 shows the behavior of the agave plantation as response of the application of ethrel 240, nitrogen, and strangling for the incitement of offshoots during seven months of experimentation. observation showed that during the first three samples, the incitement of tillers was not perceptible. nonetheless, an increase in the incitement of tillers was shown after the fourth sample. This increment remained constant until the last sample was made.

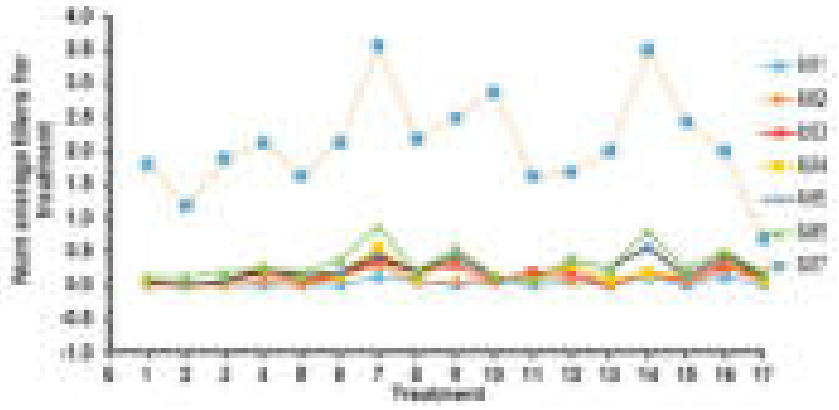

**Figure 3.** Tillers development in agave mezcalero plants.

Although there were no statistical differences in the studied parameters in the first six samples, the behavior of the variable-NB remain similar. However, in the seventh sample, a considerable increase of NB was observed. This result was obtained because in the last sample, plants were extracted.

This allowed to count all tillers formed. In contrast to previous samples where it was not possible to quantify all tillers completely because they were covered by the plastic mulch, and also because of the quick growth of the plants covered all the space among them. Moreover, it should be noted that the difficulties to quantify the tillers appeared in the third collection of data (after 3 months). The spaces among plants left at the time of planting (30 x 30 cm) on each plot was quickly covered by the growth of plant's leaves. And significantly hindered the counting of tillers formed by each treatment (Figs. 4 and 5).

**Figure 4.** Distance among each plant (30 x 30 cm) at the beginning of treatments application.

**Figure 5.** Growth of plants after three months of applying treatments. (Third sampling).

Several of the formed tillers had considerable damage because the plastic mulch did not allow them to emerge and thus the tillers continued their development in the absence of solar radiation. These tillers showed a weak growth while others led to decomposition and death of the stem due to the high temperature and humidity prevailing underneath plastic mulch. When realising about this problem, the plastic mulch was broken around the plant to avoid the problem. However, this practice was not good enough and still several tillers were damaged or lost as shown in figs. 6 and 7.

**Figure 6.** Breaking plastic mulch to allow the surface of tillers.

**Figure 7.** Breaking plastic mulch to allow the surface of tillers.

Based on the above results, it is considered that, the use of plastic mulch should be avoided for the production of tillers, even if plastic mulch could provide the advantages of efficient water use, quick growth of plants, and good quality (Hernández, 2013).

## CONCLUSIONS AND RECOMMENDATIONS

1). The application of nitrogen and ethrel 240 incited plant of *Agave* spp to form offshoots
2). Most induction of offshoot was in treatments of 60 and 40 ppm ethrel, $KNO_3$, with and without strangling
3). In the production of offshoot, mulching is not recommended because the plastic does not allow the emergence of offshoot
4). Because there was not found statistical differences in the incitement of offshoots by strangling plants and without strangling plants, it not recommended practicing strangler because it affects their development.

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THEMATIC II **Science and technology of Agave beverages and other derivatives**

# Characterization of microbial population dynamics associated with different juices of *Agave tequilana*

**Quezada R.[1], Gschaedler A.[2], Kirchmayr M.[3]**

## ABSTRACT

Currently tequila distilleries use diffusers for the extraction of sugars from the agave plant, which make the global production process more efficient. It is possible to observe great changes in the color and quantity of organic matter in the agave juices according to the method of extraction (traditional or diffuser); in the case of the process by diffuser, the hydrolysis treatment of sugars also generates an impact on the appearance of the agave juices.

The concentration of bacteria increased in the traditional processes as compared to the diffuser processes; while the concentration of yeast increased in the diffuser processes. Only one genus of yeast (*Saccharomyces*) and bacteria (*Lactobacillus*) were present in all fermentations. S. *cerevisisae* populations were detected in the presence of 2 or 3 different genera of *non-Saccharomyces* yeasts in all agave juices obtained by traditional and by diffuser processes.

**Key words:** Tequila, fermentation, microbial population dynamics.

## INTRODUCTION

The methods for identification of microorganisms can range from the use of morpho-physiological and biochemical techniques to the use of molecular techniques such as sequencing, PCR-DGGE, PCR-RFLP and PCR real time (Segura García et al., 2010) and now mass spectrometry MALDI-TOF can be included as another reliable alternative for microorganism identification.

A wide range of alcoholic beverages derived from agave plants where the manufacturing processes varies between each region and the microflora involved in the fermentation step is highly diverse (Lappe-Oliveras et al., 2008). Among these, Tequila is the beverage most widely recognized in Mexico and in the world. The traditional production process involves the "jima" of agave plants, cooking

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in an oven or autoclave, grinding, fermentation and distillation. Currently, in the Tequila industry, it is common to use diffusers that make the extraction of fructans more efficient; in this process, the cooking step is replaced by the extraction of sugars from the raw agave and afterwards the hydrolysis of the fructans through a thermal and acidic treatment. The objective of this study was to determine the impact of the different practices on microorganisms involved in the fermentation process.

## METHODOLOGY

Sampling was performed in four tequila distilleries in Jalisco state with a total of six processes, taking into account the two types of processes: traditional (A1, A2, B, C1) and with use of diffuser (C2, D). Cell concentrations were determined by counting using a Neubauer counting chamber and recovery of microorganisms was achieved by plating serial dilutions onto agar plates; for yeast Wallerstein Nutrient agar (WL, SIGMA-ALDRICH) with chloramphenicol and for bacteria De Man, Rogosa and Sharpe (MRS, SIGMA-ALDRICH) agar was used. Identification was performed by MALDI-TOF MS using the Maldi-Biotyper 3.1 software (BRUKER DALTONICS).

## RESULTS AND DISCUSSION

Clear differences could be observed between the agave juices obtained by traditional processes and diffuser processes; the amount of solids in the juices obtained by the traditional process was higher meanwhile the color was lighter in the juices obtained by diffuser, as show in fig. 1.

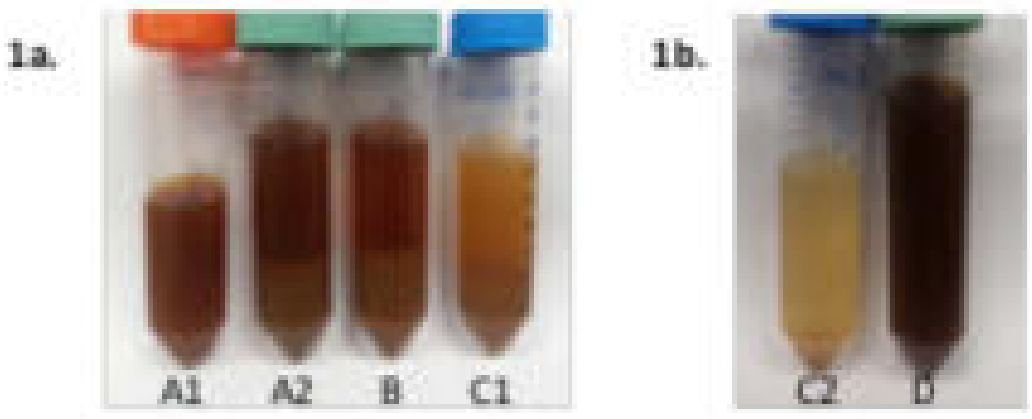


**Figure 1.** Juices of *Agave tequilana*. 1a. Traditional process and 1b. Diffuser process

In the fig. 1b, it is possible to observe that the aspect of both agave juices is different although both were obtained by diffusor; this is because the process of hydrolysis of fuctans is different. In the case of distillery C2 the hydrolysis is carried out by thermal and acidic treatment; and in the distillery D the hydrolysis is by treatment in an autoclave causing darkening of the juice through caramelization of the sugars.

**Table 1.** Microbial cell counts in Neubauer's Counting Chamber

| Tequila Distilleries | Bacteria / ml | Yeast / ml | Ratio bacteria/yeast |
|---|---|---|---|
| A1 | 2.55E+07 | 2.80E+06 | 9.1 |
| A2 | 1.10E+08 | 2.75E+06 | 39.8 |
| B | 6.70E+07 | 2.05E+06 | 32.7 |
| C1 | 4.40E+07 | 1.65E+06 | 26.7 |
| C2 | 4.20E+06 | 6.30E+06 | 0.7 |
| D | 3.45E+07 | 1.75E+07 | 2.0 |

As shown in Table 1, the concentration of bacteria was higher in the traditional processes as compared to the diffuser processes while the concentration of yeast was increased in the diffuser processes. When comparing the concentrations of bacteria and yeast between both processes the ratio ranged from 9 to 40 in traditional juices and from 0.7 to 2 in diffuser juices.

Table 2 shows the different genera of yeast (y) and bacteria (b) isolated from the different fermentations. Only one genus of yeast and bacteria, *Saccharomyces* and *Lactobacillus*, were present in all fermentations. Just in one traditional process acetic acid bacteria could be detected while in one diffuser process *Streptococcus* could be observed, being the first report of this genus in alcoholic fermentation carried out with agave juice.

**Table 2.** Identification of microbial populations by MALDI-TOF.

| Microorganisms identified | Tequila Distilleries | | | | | |
|---|---|---|---|---|---|---|
| | A1 | A2 | B | C1 | C2 | D |
| *Clavispora*[y] | | | ✓ | | ✓ | |
| *Hanseniaspora*[y] | | ✓ | ✓ | | | |
| *Issatchenkia*[y] | | | | | ✓ | ✓ |
| *Kluyveromyces*[y] | ✓ | ✓ | | | | ✓ |
| *Lachancea*[y] | | ✓ | | ✓ | | |
| *Pichia*[y] | | | | ✓ | | |
| ***Saccharomyces***[y] | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| *Torulaspora*[y] | | | | ✓ | | |
| *Zygosaccharomyces*[y] | | ✓ | | ✓ | | |
| *Acetobacter*[b] | | ✓ | | | | |
| *Gluconobacter*[b] | | ✓ | | | | |
| ***Lactobacillus***[b] | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| *Leuconostoc*[b] | ✓ | ✓ | ✓ | ✓ | ✓ | |
| *Streptococcus*[b] | | | | | ✓ | |
| *Weissella*[b] | | ✓ | ✓ | ✓ | | |

In Table 3, it is possible to observe the evolution of each identified yeast specie, showing that some genera maintained its population throughout the fermentation; also, there are yeasts only present in some stage of fermentation (beginning, middle or ending).

Lachance in 1995 identified yeast species in a traditional tequila fermentation process (Lachance, 1995), finding *Brettanomyces, Candida, Hanseniaspora, Kluyveromyces, Pichia, Saccharomyces, Torulaspora* and *Zygosaccharomyces*; in this study, as shown in Table 2 and Table 3, there are similarities in some genera but additionally the genera *Clavispora, Debaryomyces, Issatchenkia* and *Lachancea* were identified, however *Candida* was not identified.

In the beverages derived from agave the presence and concentration of volatile compounds such as acids, alcohols, esters and terpens have a great impact for the quality of the beverage. In spontaneous fermentation of wine, the first period of aroma production is carried out by the *non-Saccharomyces* yeast which can produce high concentrations of some compounds with a remarkable influence on the sensory quality (Escalante Minakata *et al*., 2008). For example, *Hanseniaspora* and *Pichia* carry out the esterification of alcohols increasing the concentrations of esters (Rojas et al., 2001); and *Kluyveromyces marxianus* produces compounds such as alcohols and esters with fruity aroma (Wittmann *et al*., 2002). In this study it was possible to observe the presence of these genera in both traditional processes and processes with diffuser.

**Table 3.** Characterization of yeast population dynamics by PCR-DGGE.

| Distillery | Microorganisms identified | Fermentation stage | | |
|---|---|---|---|---|
| | | Beginning | Middle | End |
| A1 | *Saccharomyces cerevisiae* | ✓ | ✓ | ✓ |
| | *Hanseniaspora vinae* | ✓ | | |
| A2 | *Saccharomyces cerevisiae* | ✓ | ✓ | ✓ |
| | *Hanseniaspora vinae* | ✓ | | |
| | *Lachancea fermentati* | ✓ | | |
| B | *Saccharomyces cerevisiae* | ✓ | ✓ | ✓ |
| | *Hanseniaspora vinae* | ✓ | ✓ | ✓ |
| C1 | *Saccharomyces cerevisiae* | ✓ | ✓ | ✓ |
| | *Hanseniaspora vinae* | ✓ | ✓ | ✓ |
| | *Debaryomyces hansenii* | ✓ | | |
| | *Lachancea fermentati* | ✓ | ✓ | |
| | *Dekkera bruxellensis* | ✓ | ✓ | |
| C2 | *Saccharomyces cerevisiae* | ✓ | ✓ | ✓ |
| | *Dekkera bruxellensis* | ✓ | ✓ | |
| D | *Saccharomyces cerevisiae* | ✓ | ✓ | ✓ |
| | *Kluyveromyces marxianus* | ✓ | | |

## CONCLUSION

A clear impact of agave juice preparation practices on microbial population composition in differen-Tequila fermentations could be observed regarding the relative abundances of bacteria and yeast

populations; however further sampling campaigns are needed to correlate the observed differences in microbial diversity to the agave juice extraction methods.

## ACKNOWLEDGEMENTS

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THEMATIC II Science and technology of Agave beverages and other derivatives

# Co-culture yeast-bacteria as candidates for tequila production

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## ABSTRACT

Tequila production is a complex process carried out by different yeast, classified in two important groups, *Saccharomyces* and non-*Saccharomyces*, where the major aromatic contribution in the fermentation process is realized by non-*Saccharomyces* species. In tequila fermentation process is possible to find different yeast species; some of them are able to establish positive, neutral and negative interactions. During cider and wine fermentations positive impact of interactions are reported, like reduction of the time of alcoholic fermentation or increasing of esters levels. The aim of this work is the study of direct yeast-bacteria interactions using *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* yeasts, and *Leuconostoc sp*. bacteria in co-culture fermentation. According to the results, it was possible to classify the yeast-bacteria interactions and identify the possible candidates for tequila fermentation process.

**Key words:** Interaction, LAB, Tequila, Non-*Saccharomyces*, Mixed cultures.

## INTRODUCTION

Tequila is a distilled beverage obtained by alcoholic fermentation process from the sugar of cooked agave plant cores; the tequila production involves the alcoholic fermentation process and also two distillation steps, first (ordinario) and the second (rectificado) where tequila is obtained (Prado-Jaramillo et al. 2015). In wine fermentation process yeast-yeast and yeast-bacteria interactions had been studied and different reports show that the interactions has an impact on the composition of volatile compounds and that the alcoholic fermentation is realized in minor time (Cheraiti et al. 2005). In agave fermentation process the yeast-yeast interaction has a positive impact in volatile composition and ethanol yield (Lopez et al. 2014), moreover the LAB presence in agave juice had been reported, but their function, interaction and impact is unknown (Narváez-Zapata et al. 2010). The aim of this

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work was to evaluate the co-culture fermentation in similar conditions as agave juice (concerning sugar concentration).

## METHODOLOGY

### Yeast and bacteria

The yeast used *Saccharomyces cerevisae* and *Kluyveromyces marxianus* and also *Leuconostoc sp* bacteria are from CIATEJ collection strains bank.

### Fermentations

The growth the LAB and yeast was in MRS culture media enriched with 130 g/L of sucrose, incubated at 30°C for 96 hours in 100 ml flask.

### Yeast and bacterial cell counts

The cell count was determinate by flow cytometry every 5 hours during three days and 24 hour the latest two days.

### Sugar consumption

Sugar consumption was determinate using the DNS method, the results were expressed in g/L (Nehme et al. 2008).

## RESULTS AND DISCUSSION

Cell population and substrate consumption were studied in the co-cultures *Saccharomyces cerevisiae-Leuconostoc sp, Kluyveromyces marxianus-Leuconostoc sp* in a period of 96 hours (Figure 1, 1A). In the co-culture *Saccharomyces cerevisiae-Leuconostoc sp*, yeast had a similar behavior with the single culture during exponential phase but after 20 hours of growth in co-culture *Saccharomyces cerevisiae* showed a decrease in cell population. This phenomena coincides with the increment of the bacterial population at the same time, this effect had been reported by Cañas et al. (2015). The substrate consumption rate was minor in contrast with single culture up to 60 hours; after that time the co-culture and single of the yeast culture consumed the substrate totally at 96 hours (Figure 1, 1B). This behavior allows to identify that no substrate competition is occurring between yeast-bacteria species according with Duarte et al. (2011). On the other hand, the bacteria *Leuconostoc sp.* growth with similar rates as the co-culture with *Kluyveromyces marxianus* as bacteria single culture until 72 hours, where the cell population increased (Figure 1, 2A). However the yeast in co-culture showed a delay in 15 hours and a poor development in cell population during the fermentation period, this phenomena were reported by bacteria inhibition in yeast according to Rossouw el al. (2012). During the co-culture fermentation, the substrate consumption rate was affected compared with the yeast (single culture Figure 1, 2B), being a disadvantage in fermentation of alcoholic beverages processes (Cañas et al. 2015).

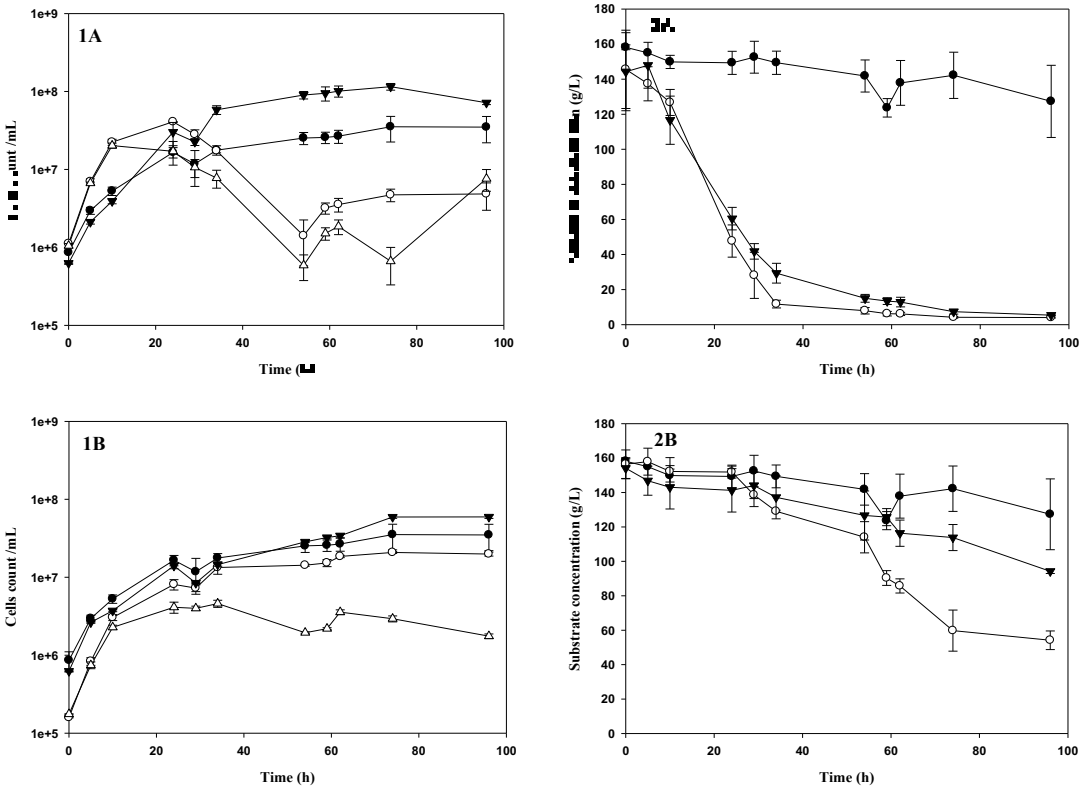


**Figure 1.** Comparison of single and co-culture yeast-bacteria fermentations. 1A: *Saccharomyces cerevisiae-Leuconostoc sp*, 1B: *Kluyveromyces marxianus-Leuconostoc sp*; cell growth pure culture of *Leuconostoc sp* (filled circle), pure culture of *Saccharomyces cerevisiae* (circle), *Leuconostoc sp.* co-culture (filled triangle) and *Saccharomyces cerevisiae* co-culture (triangle). 2A: substrate consumption *Saccharomyces cerevisiae-Leuconostoc sp*, 2B: substrate consumption *Kluyveromyces marxianus-Leuconostoc sp.* bacteria (filled circle), yeast consumption (circle) co-culture consumption (filled triangle).

## CONCLUSION

With the results obtained in this study it was possible to determine the neutral, positive and negative microbial interactions in co-culture fermentation process. In case of *Saccharomyces cerevisiae-Leuconostoc* sp co-culture, the bacterial test suggests a cell promotion effect without detriment in yeast population, which indicates a positive interaction between yeast-bacteria. On the other hand *Kluyveromyces marxianus-Leuconostoc* showed an inhibition effect in yeast population and decrease in the substrate consumption rate. In the co-culture fermentations is necessary to evaluate gas and liquid chromatography to determine the effect in the quality of the final product in order to identify potential candidates for tequila fermentation process.

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THEMATIC II **Science and technology of Agave beverages and other derivatives**

# Determination of sotol qualityproduced in Durango

**Cifuentes D.L.A.[1]*, Gómez Ortiz S.[2], Velasco González O.[3]**

## ABSTRACT

The States of Durango, Chihuahua and Coahuila have the designation of origin of the sotol which has awarded them the Mexican Institute of the Industrial property, on June 13, 2002. It product that protects, is a drink alcoholic originally of the area geographical that covers all and each one of them municipalities that make up these three States, since have with a production considerable, what wants to say is that these States are them unique entities in the country and them unique places in the world that can develop a drink and call the Sotol.

Due to the great boom that has had this alcoholic beverage is that, in 2006, producers of these three States signed the Constitutive Act of the Council regulator Mexican of the Sotol, with the aim of boosting production at home and abroad, as well as implementing the technology, because this drink is still processed in a rustic way.

The analytical control of these so-called alcoholic products is necessary at a rate of risk involving exaggerated health consumption. The Norma Oficial Mexicana stipulates only certain tests for each type of drink, depending on its nature.

This project focuses on the analyses physicochemical of one of the most important drinks alcoholic that occur both in Durango, as in other States in the North of the country called sotol, which is obtained from the fermentation of the juice of the plant *Dasylirion* spp., whose common name is sotol, a species of sea grass leaves long, fibrous plant lanceolate-shaped, green, whose usable part for the elaboration of the sotol is the pineapple or the head and that is native to the arid zones of the North of Mexico. To determine of the quality of sotol that takes place in the Durango state expires with the stated parameters of the NOM-159-SCFI-2004.

**Key words:** Sotol, Durango, Vinateros, Denomination of Origin, *Dasylirion* spp

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## INTRODUCTION

The States of Durango, Chihuahua and Coahuila have the designation of origin of the sotol which has awarded them the Mexican Institute of the Industrial property, on June 13, 2002. It product that protects, is a drink alcoholic originally of the area geographical that covers all and each one of them municipalities that make up these three States, since have with a production considerable, what wants to say is that these States are them unique entities in the country and them unique places in the world that can develop a drink and call the Sotol.

Due to the great boom that has had this alcoholic beverage is that, in 2006, producers of these three States signed the Constitutive Act of the Council regulator Mexican of the Sotol, with the aim of boosting production at home and abroad, as well as implementing the technology, because this drink is still processed in a rustic way.

Here the importance of investigating the quality of the sotol that occurs in this region to determine whether it is really within the parameters to brand the Norma Oficial Mexicana which sets the features and specifications that must meet all the members of the productive, commercial and industrial chain of the Sotol. This Norma Oficial Mexicana is applied to all processes and activities related with the production of sotol, as well as packaging, marketing and commercial practices linked to the alcoholic beverage called Sotol.

The analytical control of these so-called alcoholic products is necessary at a rate of risk involving exaggerated health consumption. The standard official Mexican stipulates only certain analysis for each type of drink, depending on its nature.

This project is focused in the analysis physico-chemical of an of them more important drinks alcoholic that is produce both in Durango, as in others States of the North of country called sotol, which is retrieved of the fermentation of the juice of the plant. *Dasylirion spp*., whose common name is sotol, a species of sea grass plants of long, fibrous, leaves of lanceolate shape, green, whose usable part for the elaboration of the sotol is the pineapple or the head and that, is native to the arid zones of the North of Mexico.

During the 18TH and 19TH centuries, in the Mexican rural landscape, dominated the Haciendas, self-sufficient communities that produced what they needed to eat: meat, grains, handicrafts and indigenous beverages made from grape, sugarcane or agave, depending on the region, products that were served with pride at the table of the landowners. Its quality depends on much of the skills and knowledge of the producers of sotol either handmade or produced industrially.

## METHODOLOGY

The sotol the vinateros produce is obtained from cooking, grinding, fermentation and distillation of the juice of the plant species of the genus Dasylirion spp., whose common name is sotol, sea grass floor of long, fibrous, leaves of lanceolate shape, green, whose usable part is pineapple or head assessed beverages produced in the Nombre de Dios, Cuencamé, and Mezquital, Durango, for being the main producers  analytical determinations: percent of alcohol in volume at 20 °C, dry extract (g/l), methanol mg/100 ml of anhydrous alcohol, aldehydes (such as acetaldehyde) mg/100 ml of

anhydrous alcohol, higher alcohols mg/100 ml of anhydrous alcohol, esters (such as ethyl acetate) mg/100 ml of anhydrous alcohol, furfur aldehyde mg/100 ml of anhydrous alcohol.

## RESULTS AND DISCUSSION

**Table 1.** Physico-chemical analyses on samples of sotol. % Av % alcohol volume; e.s. extract dry (g/l); A.S. higher alcohols (mg / 100ml of anhydrous alcohol); Mt methanol (mg / 100 ml of alcohol anhydrous); To the aldehyde (mg / 100 ml); Ester (mg / 100 ml); Furfural (mg / 100ml).

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | | | | | |
| | | | | | | | |
| | | | | | | | |

According to the produced sotol (Figs. 1-7), the vinata physico-chemical specifications of San Miguel de Temoaya, Mezquital, Dgo, has a content of methanol, esters and aldehydes above the limit allowed by the standard. Similarly, Torrecillas, Cuencamé, Dgo., vinata not conforms to your content % of Alcohol, higher alcohols, methanol, esters, and Furfural is located at the bottom and on top of the minimum and maximum of the standard.

The producers do not have a control in the production process of sotol. Do not know the existence of the norm, much less of the importance of having a Denomination of Origin.

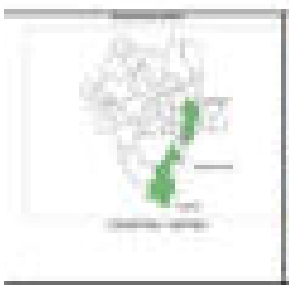

Figure 1. Representation of the sampling points.

Figure 2. Cutting of sotol.

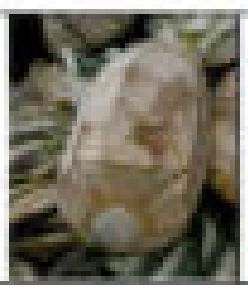

Figure 3. Raw heads

Figure 4. Floor furnace

Figure 5. Heads cooked sotol

Figure 6. Fermentation Batteries

Figure 7. Destilador

## CONCLUSION

According to the literature review carried out for the implementation of this project, it can be concluded that there have been little or no studies on the various stages of the process of elaboration of the sotol. So I think that it is of great importance, the study of all stages of the production process and in particular fermentation, distillation and rectification since they originate compounds which have an impact on the composition of distillate end, as well as its organoleptic characteristics.

Producers do not have control in the process and this so I could see that from the selection of raw material, they do not have very clear the exact moment in which sotol plants should be cut, and sometimes even if they have a clear cut plant that does not comply with the requirements looking only to complete the charge to get in cooking.

Based on the experience of the producers of the vinatas performance of the production of sotol low during the time waters, covering the period between the months of June to September (which coincides with the sampling of this project) which leads us to conclude that during this period the sugar content is bosses and therefore yield in production.

And that possibly sugars reducers are greater in times of drought, even though this would be another objective of study to be able to check it.

Analysis of dry extract of the Nombre de Dios and Temoaya, Mezquital, vinatas shed a value that lies within the parameters that it marks the NOM-159, although as we could observe the sotol turrets is located well above the permissible parameter, noting once removed the sign from the oven, the formation of candy, which leads us to think that this sotol suffered adulteration and that added some kind of sugar During its development.

In accordance with the results obtained with regard to esters, specifically ethyl acetate, you can see that the vinatas of Nombre de Dios, as well as the turrets are within the range allowed by the NOM-159, while the vinata from Temoaya, Mezquital above passes the parameters permitted by the said standard, although I cannot determine accurately that to this, as is you can be attributed both to the type of matter prima, as well as to the process of production.

Analysis of methanol to the different samples and noting the results in such tests I can conclude the sotol produced in the vinatas of Nombre de Dios., and Torrecillas, Cuencamé, Dgo., have levels of methanol permitted by the NOM, while that of Temoaya, Mezquital, Dgo., is above the margin permitted by this standard which leads me to conclude that this sotol is not suitable for human consumption and that can cause a poisoning in who ingests it.

The analysis of the dry extract of the vinatas of the Nombre de Dios and Temoaya, Mezquital, show a value that is within the parameters that we mark the NOM-159, although as we were able to observe the sotol of turrets is well above the parameter allowed, noting once removed from the sample of the oven, the formation of caramel, which leads us to think that this sotol suffered an adulteration and you have added some type of sugar during its elaboration.

In the analysis of methanol to different samples and observing the results obtained in these analyzes

can I conclude that the sotol produced in the vinatas of Nombre de Dios, Dgo., and Torrecillas, Cuencamé, Dgo., have levels of methanol allowed by the NOM, while that of Temoaya, Mezquital, Dgo., is above the permitted margin by this standard, which leads me to conclude that this sotol is not suitable for human consumption and which can cause poisoning in who ingests it.

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THEMATIC II Science and technology of Agave beverages and other derivatives

# Effect of temperature and glucose concentration on ethanol and ethyl acetate production during fermentation using *Kluyveromyces marxianus.*

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## ABSTRACT

*Kluyveromyces marxianus* have been isolated from a wide variety of habitats; this yeast appears to have a great future in biotechnological applications due to its characteristics such as: ability to grow on a wide range of substrates; thermotolerance; high growth rates and a better tendency to ferment when exposed to higher sugar concentrations. The potential of *K. marxianus* also has shown a higher volatile compounds production during agave juice fermentation for the tequila production. The aim of this study was to evaluate the temperature and glucose concentration effect on ethanol and ethyl acetate production.

Fermentations at 45°C caused slow growth of yeast and it was not able to consume all the available glucose in the medium. In fermentations at 25°C and 35°C, the growth was higher and increased almost 3 times. The highest ethanol production was observed at 35°C. Furthermore, low temperature fermentation (25°C) increased the ethyl acetate production, similar effect has been observed in *S. cerevisiae* yeast used in wine fermentation.

In conclusion, fermentations at 25°C and high glucose concentrations using *K. marxianus*, increased the ethyl acetate and ethanol production, those preliminary results could be used for agave juice fermentation.

**Key words:** *K. marxianus*, fermentation, volatile compounds.

## INTRODUCTION

During alcoholic beverages fermentation *S. cerevisiae* is not the only microorganism that can contribute to the aroma and flavor of the final product. Non-*Saccharomyces* yeasts, which occur

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naturally in all wine fermentations, are metabolically active and these metabolites may impact the quality of the alcoholic beverages (Jolly et al. 2014).

In recent years, re-evaluation of non-*Saccharomyces* yeasts role in winemaking has demonstrated that mixed fermentations using controlled inoculations of *S. cerevisiae* started cultures and non-*Saccharomyces* yeast represent a feasible way to enrich the aromatic quality of alcoholic beverages (Ciani and Comitini, 2011).

*Kluyveromyces marxianus* have been isolated from a wide variety of habitats, and appears to have a great future in biotechnological applications due to its characteristics such as: ability to grow on a wide range of substrates; thermotolerance; high growth rates and a better tendency to ferment when exposed to higher sugar concentrations (Fonseca *et al*. 2008; Lane and Morrissey, 2010). The potential of *K. marxianus* also has shown a higher volatile compounds production during agave juice fermentation for the tequila production (López-Álvarez et al. 2012; Amaya-Delgado *et al*. 2013; Segura-García et al. 2015).

The aim of this study was the evaluation of the temperature and glucose concentration effect on ethanol and ethyl acetate production during fermentations using *K. marxianus* (DU3) yeast.

## METHODOLOGY

The *Kluyveromyces marxianus* (DU3) was isolated from spontaneous mezcal fermentation (CIATEJ collection). Fermentations were performed in Erlenmeyer flasks containing 500mL of mineral medium previously described (Tibayrenc et al. 2011), the temperatures were 25°C, 35°C and 45°C, the sugar concentration 100 g/L, 150 g/L and 200 g/L, and 100 rpm were fixed for mix. Volatile compounds were quantified by gas chromatography connected with head-space equipped with a flame ionization detector (FID) and a HP-INNOWAX column was used. Quantifying reducing sugars was performed by DNS, the reaction is based on the reduction of 3,5-dinitrosalicylic acid in 3-amino-5-nitrosalicylic when the aldehyde groups are oxidized by the carboxyl groups. The optical density was determined at 600 nm absorbance using a spectrophotometer.

## RESULTS AND DISCUSSION

Fig. 1 shows the growth of yeast *K. marxianus* (a) and reducing sugars concentrations (b) during fermentations at different temperatures. It is observed that the maximal growth was obtained with fermentation temperatures of 25°C and 35°C after 48h of culture. Fermentations at 45°C caused slow growth and no statistically significant differences between the high and low concentration of glucose are detected ($p < 0.05$). In addition, the yeast was not able to consume the available glucose after 48 h of fermentation at 45°C (see fig. 1b).

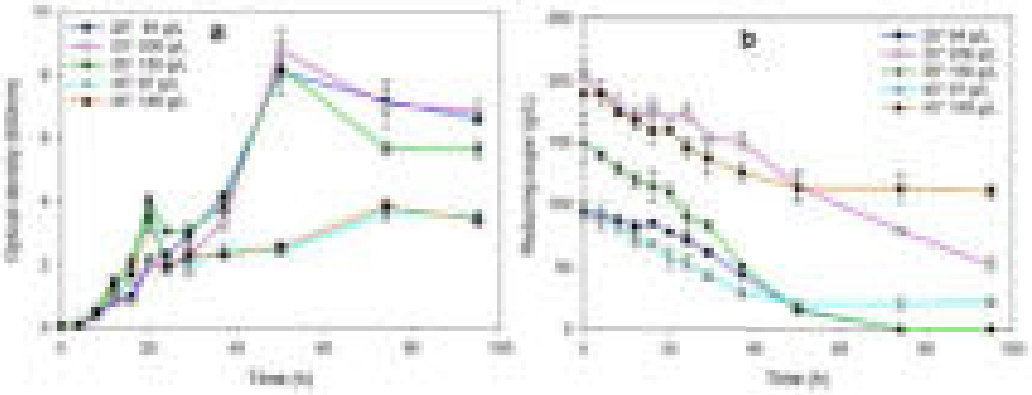

Figure 1. a) Optical density and b) reducing sugar concentrations during K. *marxianus* fermentation at different temperatures.

Fig. 2a shows that increased ethanol production was achieved with temperatures of 35°C (62.08 mg/L ± 0.98) and 25°C with high initial glucose concentration in the medium (59.42 mg/L ± 3.11). On the other hand, fig. 2b shows that during the condition 25°C and high glucose concentration in the culture medium, the highest production of ethyl acetate (92.25 mg/L ± 11.01) was observed.

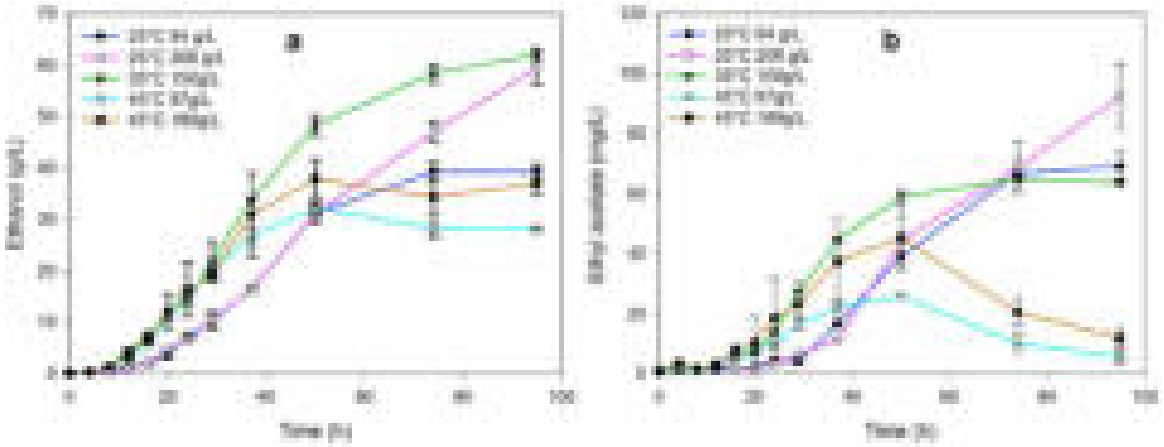

Figure 2. a) Ethanol and b) ethyl acetate production during K. marxianus fermentation at different temperatures.

Although it has been observed that low temperatures favor esters production during fermentation of wine with *S. cerevisiae* (Molina et al. 2007), the yeast *K. marxianus* and in particular the strain DU3 produces more ethyl acetate that *S. cerevisiae*. In high temperature (45°C) an interesting phenomenon in which the concentration of esters in the medium decreases is observed and ethanol concentration is maintained stable.

## CONCLUSION

The results obtained in this study show that fermentation temperatures ranging from 25°C to 35°C favor ethanol production using the yeast *K. marxianus*; it also showed that it is possible to obtain high concentrations of ethyl acetate in fermentations at 25°C. These preliminary results may be used for fermentations agave juice in order to increase the aromatic quality of the final product.

## ACKNOWLEDGEMENTS

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THEMATIC II Science and technology of Agave beverages and other derivatives

# Effect of thermal hydrolysis time of agave and their impact on fermentation process

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ABSTRACT

During the tequila process it is necessary hydrolyze the agave fructans to obtain fermentable carbohydrates. The most common system to hydrolyze the agave fructans is using the thermal hydrolysis. However, each tequila factory controlled the time and temperature of agave cooked. Those conditions have an important impact on the fermentation stage, because during these phase yeast inhibitors are produced as furfural and 5-hhydroxymethylfurfural by Millard reactions and caramelization process. The aim of this work was demonstrate the effect of the thermal hydrolysis on the fermentation process. The agave juice was obtained using four different times of thermal hydrolysis (12h, 24h, 36h and 48h). The results obtained shown than the agave cooked at 24h and 36h produce the highest ethanol concentration using the yeast *Saccharomyces cerevisiae* (AR5), those results are related to the low concentrations of some compounds that are inhibitors of the fermentation process like furfural and 5-hydroxymethylfurfural and the high fermentable sugar concentration. However, when the fermentation was carried using the *Kluyveromyces marxianus* (SLP1) the results were better, because the inhibitors not influenced in the fermentation capacity and the ethanol production was highest than the obtained with the *S. cerevisiae.*

**Key words:** Fermentation, *Saccharomyces cerevisiae, Kluyveromyces marxianus,* Inhibitors Compounds, Ethanol Production.

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## INTRODUCTION

*Agave tequilana* Weber blue var. is used for the production of tequila, agave syrup and fructans. Most of these processes except fructans, require an hydrolysis step to produce monosaccharides and disaccharides, which is made by cooking either oven floor, masonry oven, autoclave and even by acid hydrolysis juice crude extracted by diffusion (Cedeño, *et al*. 1999). It is necessary to consider that sugars during cooking are subject to a series of reactions, with complex mechanism, it is a function of pH, cooking time, temperature and composition of the raw material. The products obtained generally contribute to the flavor and aroma of alcoholic beverages produced from agave. Among the main reactions are caramelization, Maillard reactions, oxidation-dehydration and production of volatiles such as methanol. Some research mentioned the presence of compounds obtained by Maillard reactions in tequila as 3-methyl-1-butanol, phenethyl alcohol (Jiménez et al. 2016), 5-hydroxymethylfurfural (5-HMF), methyl-2-furonate, 2-3-dihydroxy-3,5 dihydro-6-methyl-4 (H) -pyran-4-ione (López, 1999), 2-acetyl-5-methylfuran, furfuryl alcohol 3-3,4,5-trimethylpyrazole and (Waleckx, et al 2010). Furfural and 5-hydroxymethylfurfural (5-HMF) are yeast inhibitory compounds. During sugar degradation, furfural is mainly derived from pentose dehydration and 5-HMF is formed from dehydration of hexoses. Those compounds could damage the yeast during fermentation, reducing enzymatic and biological activities, breaking down DNA, inhibiting protein and RNA synthesis (Gschaedler *et al* 2015). Most yeasts, are susceptible to various inhibitory, however, yeast strain as the *S. cerevisiae* (AR5) and *Kluyveromyces marxianus* (SLP1) were isolated from agave juice in presence of furfural and 5-HMF, and could be resistant to those compounds.

The aim of this work was demonstrate the effect of the thermal hydrolysis on the fermentation process using yeast natives from agave juice.

## METHODOLOGY

### Culture of Strain

#### *Used Strains*

Two yeast strains were used *Saccharomyces cerevisiae* (AR5) and *Kluyveromyces marxianus* (SLP1), they were isolated from tequila and mezcal juices. They are located on the strains bank of the Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco A.C. (CIATEJ).

#### *Solid Medium*

The yeasts were maintained and grown in a solid medium prepared with 3 g glucose, 1.5 g yeast extract, 3 g of peptone and 5.25 g agar, dilute to 150 ml, this was placed in an Erlenmeyer flask with capacity of 1 L. The medium was sterilized at 121°C and at 1.5 kg/cm$^2$ pressure during 15 minutes. It was poured into sterile Petri dishes 10 mm x 10 mm, in a laminar flow hood, subsequently left to gel and stored at 4°C for later use. Once the gelled medium is inoculated with a baked yeast strains obtained from the bank, incubated at 30°C for 48 h in an incubator

#### *Liquid Medium*

The liquid medium was prepared with 6.6 g of peptone, 6.6 g glucose and 3.3 g yeast extract, dilute to 330 ml, the medium was placed in an Erlenmeyer flask with a capacity of 1L. The medium sterilized at 121 ° C and at 1.5kg/cm2 pressure for 15 minutes. The medium was inoculated with cells previously

grown on solid medium, incubated in an orbital at 250 rpm for 12h at 30 ° C. Have elapsed 12h, the medium was centrifuged and was obtained a tablet, which was performed two washes with sterile physiological solution (NaCl) 0.08%. After the tablet was re-suspended in 20 ml of physiological solution, direct counting was performed under microscope and the necessary milliliters were calculated to inoculate agave juice with 10 million cells per milliliter.

### *Batch Fermentation*

The fermentation was carried out in Erlenmeyer flask. They were prepared using 250ml of agave juices and adjusted at 12°Bx. The agave juice was obtained using four different times of thermal hydrolysis at 98°C (12h, 24h, 36h and 48h) (Jiménez *et al.*, 2016). Each juice was sterilized at 121°C and at 1.5 kg/cm$^2$ pressure 15 minutes (figure 1).The flask were inoculated using native agave yeasts *Saccharomyces cerevisiae* (AR5) and *Kluyveromyces marxianus* (SLP1). The fermentation process was carried out by duplicate on each agave juice for 72h.

### *Sugar Consumption and Ethanol Production*

Sugar consumption and ethanol production were determinate by liquid chromatographic analysis, it was used Agilent Zorbax® SB-AQ column of 250 for 4.5 mm operating at 30°C, having as mobile phase phosphate buffer solution 20 mM at pH 2 (acidified with phosphoric acid) and acetonitrile in relation 99/1 (v/v), with a flow rate of 0.5 ml / min and an injection volume of 20 µl. The machine which conducted the analysis was an HPLC Agilent Technologies 1260, coupled to UV detector at a wavelength of 210 nm and a refractive index detector. Calibration curves for quantification were performed at different concentrations with the following standards: glucose, fructose, sucrose and ethanol.

## RESULTS AND DISCUSSION

The agave juices obtained after cooking were used for fermentation applying two yeast species, *S. cerevisiae* (AR5) and *K. marxianus* (SLP1). The results obtained for *S. cerevisiae* are shown in the fig. 1, the sugar concentration in each juice were different although were adjusted at 12°Bx, however, the sugars were consumed almost completely but at 48 h of cocking time juice the sugars remained were more than 10%, indicating the fermentation inhibition. The ethanol production was better on agave juice obtained at 24h and 36h of agave cooking; it could be possible because the fermentable sugars were higher. On the juices obtained after 12 h of thermal hydrolysis the ethanol production was lower, it is possible because the juice could maintain oligofructans, which are not fermentable. The agave juice obtained after 48h of cooking time, have yeast growth inhibitors as the furfural and 5-hidroxymethylfurfural. Those compounds could affect the yeast fermentation and decreased the ethanol production.

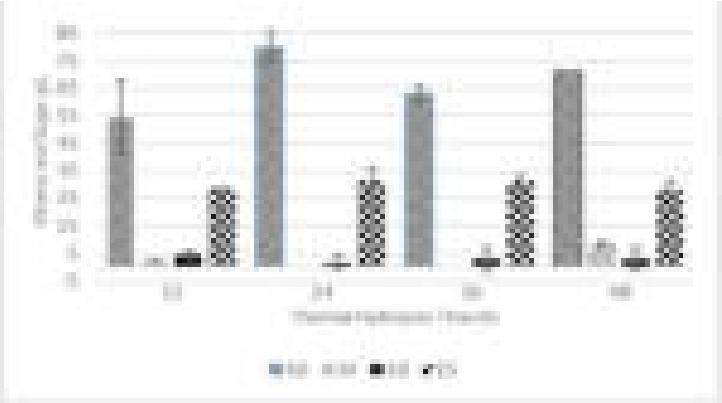

Figure 1. Ethanol production and sugar consumption using *S. cerevisiae* yeast (AR5). Initial sugars concentration (S0), final sugars concentration (SF), initial ethanol concentration (E0) and final ethanol concentration (EF).

The results obtained using the *K. marxianus* yeast are shown in fig. 2, where can be observed that inhibitors and the oligofructans compounds did not decreased the ethanol production as the *S. cervesiae* yeast. Unlike the ethanol production was higher using the *K. marxianus* yeast.

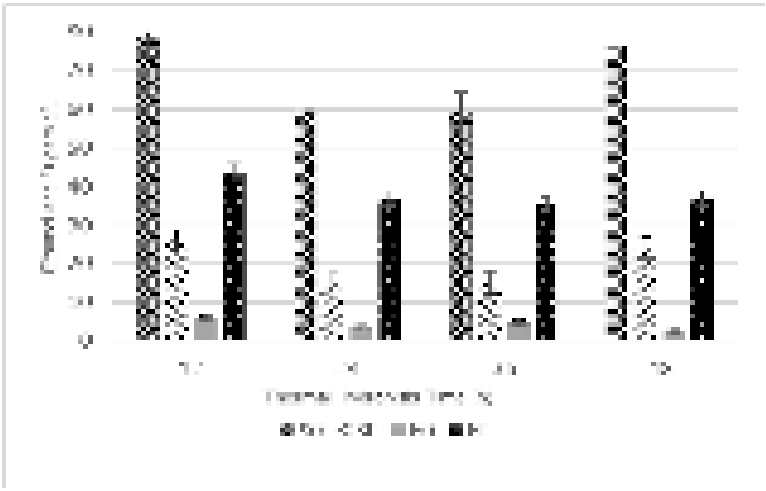

Figure 2. Ethanol production and sugar consumption using K. marxianus yeast. Initial sugars concentration (S0), final sugars concentration (SF), initial ethanol concentration (E0) and final ethanol concentration (EF).

## CONCLUSION

The thermal hydrolysis times of agave at 24h and 36h gives the best ethanol production using the *S. cerevisiae* yeast; those results are related to the low concentrations of some compounds that are inhibitors of the fermentation process like furfural and 5-hydroxymethylfurfural. Those compounds that are inhibitors not influenced in the fermentation capacity when was used *K. marxianus* yeast. It can be proved observing that ethanol production was better at thermal hydrolysis times of agave at 12h and 48h the using the *K. marxianus*.

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THEMATIC II Science and technology of Agave beverages and other derivatives

# In-line and at-line monitoring of yeast cultures for a rapid evaluation of the state of the cell

**Arana A.[1], Barbosa E.[2], Preziosi L.[3], Grousseau E.[4], Herrera E.[5], Arrizón J.[6], Cervantes J.[7], Gschaedler A.[8*]**

## ABSTRACT

Cell physiology in microbial cultures is an important parameter to evaluate in pursuance of achieving process efficiency. The aim of this work was to test rapid acquisition techniques to assess viability, cell size and morphology of *Kluyveromyces marxianus* SLP1, in the course of batch and continuous cultures using in-line and at-line approaches. During a batch culture, the *K. marxianus* SLP1 strain was subjected to a thermal shift, from 30ºC to 43ºC, where the in-line permittivity slightly decreased. When the temperature was raised from 43ºC to 52ºC, permittivity diminished drastically. Also, a continuous culture varying temperature and specific growth rate was carried out in order to register the physiological changes of *K. marxianus* SLP1 when in a low and high β-fructofuranosidase production state. Cell volume and morphology during the two physiological states were different from each other, being the cell smaller and elongated during the high enzyme production status. The tools used in this work demonstrated to be of fast elucidating cell stress response and viability which, together with image analysis, could be of great help to reveal the physiological state of the cell.

**Key words:** Yeast, Cultures, Rapid monitoring, Physiology.

## INTRODUCTION

In practice, microorganisms in all industrial fermentations are taken to their physiological limits. High product concentration, nutrient limitation and rough environmental conditions are some of the classic stress generating circumstances that affect the cell state and even its survival. The idea of evaluating the physiological state of the microorganisms has been a recurrent subject in bioprocesses

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engineering (Konstantinov, 1996) and it is considered essential to design effective control strategies in order to accomplish process optimization.

Among the key parameters to monitor during a cellular culture (substrate concentration and metabolites, temperature, pH, gas partial pressures, protein concentration) the concentration and state of the cells is of paramount importance (Justice et al. 2011) resulting in valuable physiological information (specific yields), making the biomass determination a common and needed parameter to evaluate (Schuster, 2000). Moreover, the size and morphology of the cell are known as good references to determine a metabolic state (Tibayrenc et al. 2010). Recent publications have pointed out that cell size, number of cells and viability could be obtained through on-line image analysis (Wei et al. 2007). Image microscopy, together with at-line measurements of the cell size dispersion could be an appropriate approach in order to obtain additional information on the physiological state of the cell.

Regarding cell viability, dielectric spectroscopy has been utilized to monitor microbial growth kinetics (Olguín et al. 2009), to elucidate cell concentration, conductivity on the culture medium, the cytoplasmic conductivity and the volume change of the vacuole in yeast cells (Asami, 2002) as well as for following the evolution of the membrane capacitance of *Saccharomyces cerevisiae* during ethanolic fermentation and physicochemical stress by the effect of acetic acid on the cytoplasmic conductivity recorded in real time (Tibayrenc et al. 2011).

Inside the bioprocesses, the mainstream of information is obtained from off-line measurements, consequently remaining insufficient for the development of control strategies in real time for optimizing the potential of the microorganisms by designing high yield processes. The evaluation of the physiological state of the cells in real time is quite important for the understanding and improvement of the cellular metabolism to achieve an efficient control to improve the metabolites of interest.

## METHODOLOGY

### Batch culture

A 16L bioreactor with sterilized chemically defined medium (Tibayrenc et al. 2010) and sucrose (100g/L) was inoculated with a pre-culture of *Kluyveromyces marxianus* SLP1 up to an on-line optical density value of 0.08. This culture was kept at a pH of 4.5, a temperature of 30ºC, an agitation from 400rpm to 800rpm and an aeration from 4L/min to 8L/min (to avoid oxygen limitation).

### Continuous culture

Experiments were performed in a 2L bioreactor with a working volume of 1.4L using chemically defined medium and *K. marxianus* SLP1. Several parameters were evaluated (temperature from 22ºC to 30ºC, specific growth rates from 0.04$h^{-1}$ to 0.35$h^{-1}$ and input substrate concentration from 25g/L to 40g/L). The pH was kept constant at 4.5 and dissolved oxygen above 20%.

### In-line and at-line measurements

Growth and viability were followed in real time using an in-line permittivity probe (Fogale, France) which was previously calibrated and sterilized. Cell size was obtained at-line using the CASY automated cell counter (Roche Innovatis, Germany) diluting the samples in CASYTON®. Cell images

were acquired with a digital camera mounted on a microscope at a 40X magnification.

## RESULTS AND DISCUSSION

In-line permittivity slightly decreased when the first thermal stress was applied. Then, when the temperature was raised to 52ºC, viability diminished drastically, probably due to cell lysis, while the in-line optical density remained almost constant (Fig. 1A). Changes in cell size dispersion (Fig. 1B) was only noticeable when the second thermal shift was performed (43ºC to 52ºC) showing the thermo-tolerant capabilities of this particular strain.

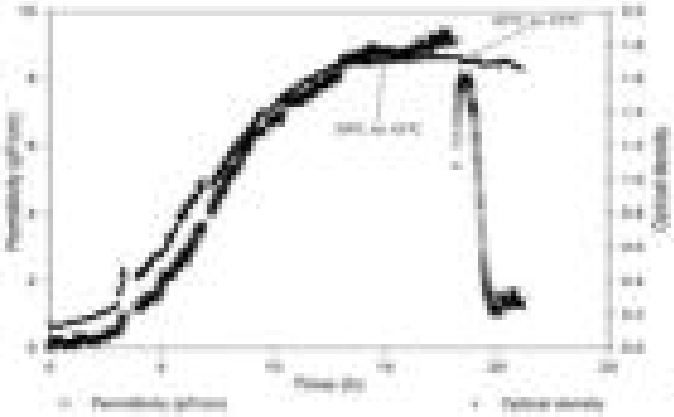

**Figure 1. A.** In-line permittivity and optical density of K. **marxianus** during thermal stress in batch culture.

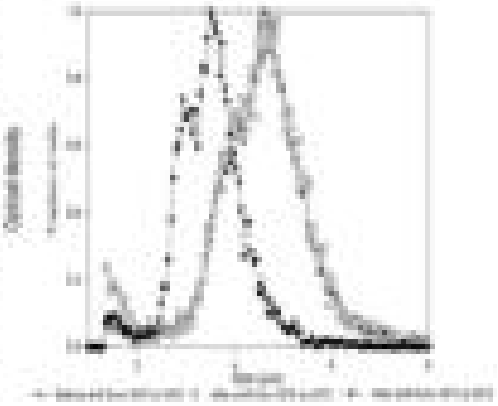

**Figure 1. B.** Cell size dispersions with the CASY automated cell counter.

Fig. 2A shows the differences in size and morphology of the cell when in a low/high enzyme production. The difference from microscope manual size measurements (Fig. 2A) and the CASY automated cell counter (Fig. 2B) was 2.38% in the low enzyme production state (LEP) and 9.23% for the high enzyme production state (HEP). Variability between size measurements may be attributed to cell morphology. In the LEP, several cell size dispersions could be observed (Fig. 2), being one of them close to the dispersion found during the HEP, which may explain that during the LEP only a fraction of the cell population was producing the enzyme.

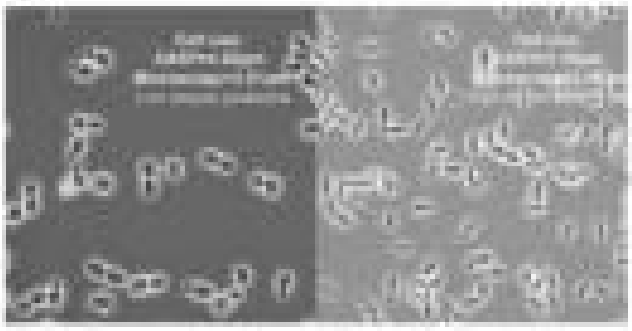

**Figure 2. A.** Cell size dispersion and shape during low/high enzyme production in a K. marxianus continuous culture.

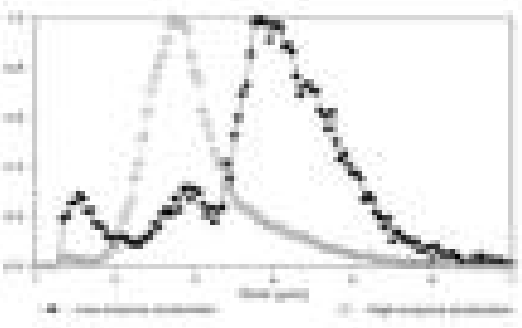

**Figure 2. B.** Cell size dispersions of the two physiological states with the CASY automated cell counter.

## CONCLUSION

The use of an in-line dielectric spectroscopy probe, demonstrated to be an excellent real time tool to elucidate cell stress response and viability when thermal shock was applied. This tool, together with an automated cell size meter could be used as an indicator of a desired physiological state for improving the production of a metabolite of interest and to avoid viability loss due to environmental factors.

## ACKNOWLEDGEMENTS

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THEMATIC II Science and technology of Agave beverages and other derivatives

# Isoamyl acetate production by *Pichia fermentans* isolated from alcoholic fermentation of Agave juice

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## ABSTRACT

The study of the fermentation parameters for *Pichia fermentans* resulted in the selection of the conditions, which allowed the growth and the isoamyl acetate production. Temperature of 28°C, pH 5.0, 1 vvm air flow and agitation speed of 120 RPM allowed obtaining the higher production of isoamyl acetate, which was 18 mg/L. Modifying cane molasses determined that the composition of the medium culture plays an important role in the production of isoamyl acetate in *P. fermentans*. The addition of isoamyl alcohol since the beginning of the fermentation allowed the increased of the production with regard to the use of the amino acid L-Leucine. Otherwise, the increase in the concentration of reducing sugars reduced the synthesis of the isoamyl acetate, however, increasing the content of assimilable nitrogen unto 250 mg $N_2$/L was obtained an increase of the aroma production of 0.53 ± 0.01 to 0.93 ± 0.03 g/L. Evaluation of other components of the molasses by the Plackett-Burman design, established that the $MgSO_4$, $Cl_2Ca$ and isoamyl alcohol have significantly influence in the synthesis of isoamyl acetate.

**Key words:** aroma, cane molasses, isoamyl alcohol, L-leucine, Plackett-Burman

## INTRODUCTION

Isoamyl acetate is a compound of great importance in the aroma industry, due to its characteristic banana aroma. It is widely used in the food, cosmetic and pharmaceutical industries. The current legislation considers the aromas from microbial or enzymatic processes as natural products. Thus, it has increased the importance of the production of this compound by biotechnological processes

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(FAO, 2012). Hernandez-Carbajal *et al*., (2013) demonstrated that the yeast *Pichia fermentans* ITD-00165, isolated from spontaneous fermentation of juice *Agave durangensis* (Paéz-Lerma et al., 2013), showed greater potential for isoamyl acetate production. However, this yeast has been poorly studied. Then, the objective of this work was to study the metabolism and the parameters that influence the synthesis of isoamyl acetate in *P. fermentans* ITD-00165.

## METHODOLOGY

This work was performed using the strain *Pichia fermentans* ITD-00165, belonging to the collection Laboratory of Microbial Biotechnology at the Instituto Tecnológico de Durango. The influence of the temperature (20, 28°C), pH (3.5, 5, 6.5), air flow (0.5, 1 VVM) and agitation speed (0, 120 RPM) on the synthesis of isoamyl acetate were evaluated. The effect of the concentration of sugars was performed by the hydrolysis of cane molasses with the method reported by Jaramillo *et al*., (2014). The effect of the nitrogen content was performed by adding $NH_4Cl$ and yeast extract in two concentrations, 150 and 250 mg N/L (Barbosa *et al*., 2009). Isoamyl alcohol (1 g/L) and the amino acid L-leucine (4 g/L) were evaluated as isoamyl acetate precursors.

The analysis of the nutritional elements was made using the placket-Burman (PB) design at two levels (Table 1). The duration of fermentations was 36 h for all experiments. Samples were taken every 4 hours. All experiments were performed in duplicate. The quantification of isoamyl acetate was performed using the method reported by Hernández-Carbajal *et al*., (2013). The data analysis was made using analysis of variance one-way, with a range comparison test, using the method of Duncan with a confidence level of 95% (p = 0.05).

**Table 1.** Complete Plackett-Burman design.

**Table 2.** Reducing sugars and nitrogen.

## RESULTS AND DISCUSSION

The best conditions for yeast growth and isoamyl acetate production were: 28° C, pH 5.0, air flow of 1 vvm and agitation speed of 120 RPM. These conditions allowed producing the highest isoamyl acetate concentration (18 mg/L). The addition of precursors increased isoamyl acetate production (Table 3, fig. 3). Nevertheless, the highest concentration of isoamyl acetate was obtained by the addition of isoamyl alcohol at the beginning of the fermentation.

Table 3. Isoamyl acetate production with addition of isoamyl alcohol and L-leucine.

| Compound | Time (h) | Isoamyl acetate (g/L) | $P_{max}$ | $Y_{P/S}$ | $Y_{P/X}$ | $Y_{P/X}$ |
|---|---|---|---|---|---|---|
| L- leucine | 0 | 350 ± 0.40$^{a}$ | 0.208 ± 0.00$^{a}$ | 0.70 ± 0.01$^{a}$ | 0.21 ± 0.04$^{a}$ | 0.02 ± 0.00$^{a}$ |
| | 12 | 430 ± 1.30$^{a}$ | 0.179 ± 0.00$^{a}$ | 0.91 ± 0.01$^{a}$ | 0.24 ± 0.01$^{a}$ | 0.01 ± 0.00$^{a}$ |
| Isoamyl alcohol | 0 | 860 ± 1.10$^{a}$ | 0.234 ± 0.00$^{a}$ | 0.82 ± 0.00$^{a}$ | 0.96± 0.02$^{a}$ | 0.04 ± 0.00$^{a}$ |
| | 12 | 730 ± 2.00$^{a}$ | 0.21± 0.00$^{a}$ | 0.97 ± 0.11$^{a}$ | 0.92 ± 0.07$^{a}$ | 0.02 ± 0.00$^{a}$ |

The increase in the concentration of reducing sugars in cane molasses (Table 2) did not increase the synthesis of isoamyl acetate in *P. fermentans* (Fig. 1). Low content of nitrogen present in the molasses possibly was the limiting factor in synthesis isoamyl acetate.

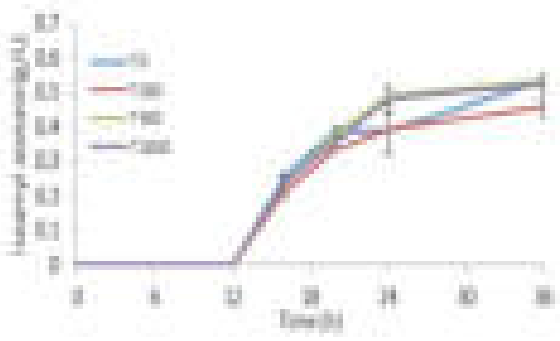

Figure. 1. Effect sugars on isoamyl acetate production.

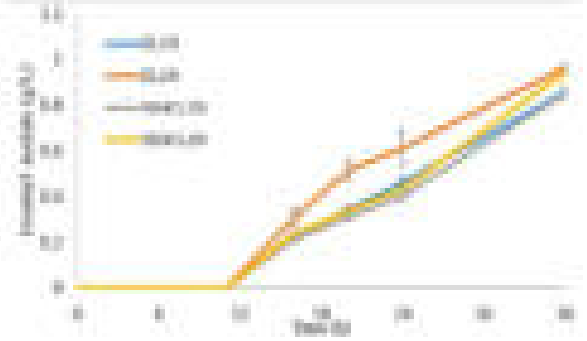

Figure. 2. Effect nitrogen on isoamyl acetate production.

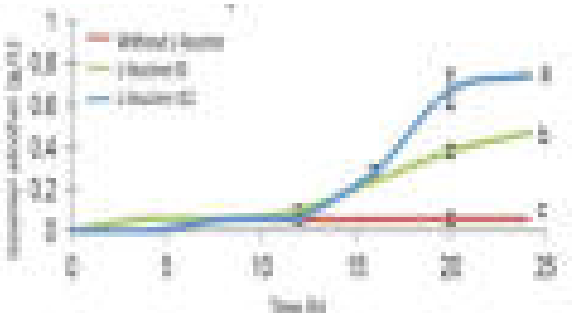

Figure. 3 Production of isoamyl alcohol.

Figure. 4. Principal effects on isoamyl acetate production.

Statistical analysis showed that a medium having a high sugar content (132.59 g/L) is not influenced by the type of nitrogen source ($NH_4Cl$ or yeast extract). However, the initial concentration of assimilable nitrogen proves to be significant for the synthesis of the aroma. Increasing the initial concentration of assimilable nitrogen (250 mg N/L) allowed increasing aroma production up to 96 and 930 mg/L, using $NH_4Cl$ and yeast extract, respectively (Fig. 2). The analysis of Plackett-Burman design (PB) determined that the $CaCl_2$, $MgSO_4$ and isoamyl alcohol were the most influential components on isoamyl acetate production in *P. fermentans* (Fig. 4). The $CaCl_2$ and $MgSO_4$ were used as indicators of the viability and vitality on yeasts (Rees and Stewart, 1997). Akita *et al*., (1990), established that the presence of magnesium is required for the activity of the enzyme alcohol acetyltransferase (AATase) responsible for the synthesis of acetate esters. Finally, it has been described the importance of isoamyl alcohol as precursor of the isoamyl acetate (Quilter *et al*., 2003).

## CONCLUSION

Isoamyl alcohol is the better precursor for isoamyl acetate production. The sugar content in the cane molasses has not an influence in the synthesis of isoamyl acetate, while isoamyl alcohol addition, initial nitrogen content, $CaCl_2$ and $MgSO_4$ are important factors for the aroma production.

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THEMATIC II **Science and technology of Agave beverages and other derivatives**

# Methanol in Agave Distillates. A Review.

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## ABSTRACT

Mezcal, Bacanora, Tequila and Raicilla are agave distillates with an ancestral tradition in México. First three distillates have denomination of origin and all have an important economic and social impact. Agave distillates sector generates about 30 thousand direct employees and about the same quantity of indirect employees. Agave distillates are complex, non-ideal alcoholic mixtures composed mainly by water, ethanol and a large amount of chemical compounds called *congeners*, as alcohols (i.e. methanol, n-propyl, isobutyl and isoamyl alcohols), esters, organic acids, terpenes and others compounds belonging to different chemical groups. Different sensory congeners show small concentrations in agave distillates and are not regulated officially but methanol is part of the regulated compounds. Methanol is a harmful chemical compound that can be ingested in some quantity by humans and may produce headache, vomiting, blindness and, in extreme cases, death. Toxicity of methanol is derived from the formaldehyde and formic acid formation and ingestion of hazardous amounts of methanol may be due to adulteration of agave distillates or due to uncontrolled production processes, because of this, official norms for agave distillates in México include the specification for maximum concentration of methanol in finished products. This review intends update information around this important congener because of its relevance on human health and on economics of agave distillates producers. Relevant factors as origin of methanol, effect of methanol consumption on health consumer, specifications for methanol content in agave distillates regarding to different prestigious distillates as well as different strategies for controlling methanol along elaboration processes of agave distillates are reviewed considering the official specifications.

**Key words:** Methanol, Mezcal, Bacanora, Tequila, Raicilla.

## INTRODUCTION

In Mexico, distilled spirits industries that use different species of agave as a raw material, have a significant social and economic importance. It is estimated that the production of Mezcal generates a total of 5,000 direct jobs and about 25,000 indirect (COMERCAM, 2015). Meanwhile, the Tequila

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industry generates about 29 thousand direct jobs (INEGI, 2013); however, Raicilla and Bacanora industries present today great growth opportunities. Agave distillates such as Mezcal, Raicilla, Bacanora and Tequila, like other world recognized drinks such as rum, whiskey, cognac, brandy, etc. are complex mixtures of two major compounds, water and ethanol, and a wide variety of chemical compounds in small concentrations called *congeners*. The type and concentration of these congeners are a result of the type of raw material and of the process under each distillate is manufactured and among them are compounds belonging to different chemical groups such as alcohols, esters, organic acids, furans, aldehydes, among others. The group of alcohols congeners found in agave distillates comprise a group ranging from a single carbon alcohol (methanol) to 16 carbons, such as hexadecanol (Benn and Peppard, 1996).

According to the official standard NOM-006-SCFI-2012, the agave authorized to produce Tequila is *Agave tequilana* Weber; to produce Mezcal, the official standard NOM-070-SCFI-1994 indicates the use of *Agave angustifolia* Haw, *A. esperrima, A. jacobi, A. weberi cela, A. potatorum zucc, A. salmiana* Otto, *A. durangensis* and *A. cupreata* (Aguirre-Dugua and Eguiarte, 2013; Villanueva-Rodriguez and Escalona-Buendía, 2012). For the preparation of Bacanora is used *A. angustifolia* (De Leon et al. 2008, NOM-168-SCFI-2004) and for Raicilla, producers use *A. maximiliana* (De Leon et al. 2008) and *A. inaequidens* (Valenzuela -Zapata et al. 2011). Tequila, Mezcal and Bacanora are distilled beverages that have denomination of origin and Raicilla is still marketed under various trademarks. Elaboration of agave distillates consists of a series of steps in common that are made with varying degrees of modernization: a) harvesting and jima of agave, b) agave cooking to hydrolyze fructans (water soluble carbohydrates that are the energy reserve of the plant), c) grinding of cooked agave to obtain reducing sugars, d) fermentation of sugars, e) double distillation and, f) product finishing processes and final filtration.

## ORIGIN OF METHANOL IN AGAVE DISTILLATES

Methanol is a chemical compound found in all agave distillates, which could be considered as one of the key compounds to verify their authenticity; at room temperature is a volatile, colorless liquid with a characteristic odor which also contributes to its sensory profile (Villanueva-Rodriguez and Escalona-Buendía, 2012). These same researchers point out, for the processes of Tequila and Mezcal, methanol as an important component that has its origin in the enzymatic demethylation of pectin. this point coincides with that indicated for manufacturing different distilled beverages using other raw materials such as fruits (Bauer-Christoph et al. 2003), such as apple and pear. In beverages made from the fermentation of mashes of crushed fruits, methanol and higher alcohols are major compounds that have an important influence on the quality and safety of distillates and can cause adverse effects on consumer health (Zhang et al. 2011).

Different researchers have reported the origin of methanol in manufacturing processes of alcoholic and non-alcoholic beverages (Revilla and Gonzalez-San José, 1999; Zhang et al. 2011, Glatthar et al. 2001)) indicating as the source of methanol the enzymatic hydrolysis of fruit pectin thanks to the action of enzyme pectin methyl esterase or PME (EC 3.3.3.11), during alcoholic fermentation. Pectins are a heterogeneous group of structural polysaccharides of varying degrees of polymerization and branching, that exist mainly in the middle lamella and the primary cell wall of cells of higher plants (Ming-Chang et al. 2011); are secreted in the cell walls in highly methyl esterified forms and are a highly heterogeneous group of polymers including homogalacturonans and rhamnogalacturonans I

and II. The content of pectin has been quantified analytically in *A. tequilana* with a methoxyl degree that can rise up to 40%, depending on the age of the agave (CIATEJ, 2016).

PME is an important enzyme for remodeling of tissues in plants and for growth and ripening of fruits (Lewis et al. 2008). Pectolytic enzymes are usually classified into two groups according to the statement by Baron (1990) and Brillouet et al. (1990), according to González-Revilla and San Jose (1998). The first group are the pectin methyl esterases, which hydrolyze methoxy groups. The second group is composed of the depolymerases that hydrolyze chemical links (1-4) of pectins and lyases. These promote the elimination of a water molecule when disintegrate pectin (Revilla and González-San José, 1998).

Micheli (2001) describes the reaction of demethylation catalyzed by the PME on pectins (Fig. 1):

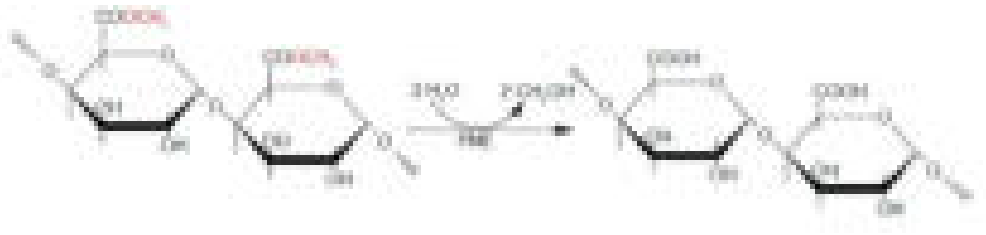

Figure 1. Demethylization of pectins and methanol formation.

Tellez (1998) made measurements of methanol during the cooking step of *A. tequilana* by using an industrial retort. She determined the methanol concentration in sampled juices along the agave cooking, finding concentrations up to 0.6 g/L in 24 hours, also indicating as important factors acidity and temperature. Apparently, cooking is the main step for methanol generation in agave distillates processes.

Officially, maximum methanol concentration in agave distillates is 300 mg/100 mL pure ethanol, in international spirits is allowed a different maximum concentration, as is shown in Table 1. It can be seen that methanol specification for agave distillates is lower than for other spirits.

**Table 1.** Official specifications for different distillates

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | |

*Methanol Content, mg/100 mL pure alcohol. *CEE nº 1576/89. **Karadeniz and Birincioglu, 2011. m: Mexican official norms.*

## METHANOL AND CONSUMER HEALTH

Methanol is rapidly absorbed by ingestion and inhalation, and to a lesser extent through the skin (Zocca, 2007). In the body, methanol is metabolized in the liver and converted formaldehyde first and then formate (HCOO-). Formate, can cause severe damage to health and even death of those who consume it. Methanol poisoning can cause serious damage to the central nervous system. Methanol has been recognized for its neuropathological effects such as subcortical bilateral putamen necrosis (Gonzalez, 2012), and cerebellar lesion in the hypothalamus, plus demyelination (Sandhir and Kaur, 2006). In different drinking levels, methanol can be toxic. For example, ingestion of 4 g of methanol may cause symptoms like headache, nausea, vomiting and dizziness. Ingestion of more than 10 g can cause blindness and more than 24 g produces lethal effects (Ming-Chang et al. 2011). For illustrative purposes, an agave distillate 40% ethanol v/v with a concentration of 250 mg methanol/100 mL of anhydrous ethanol contains 1 g Methanol/L of distillate.

## CONTROL STRATEGIES OF METHANOL IN SPIRITS OF AGAVE.

While production of Mezcal certified by the Quality Regulatory Board (COMERCAM) in different states has been increasing, there are still producer regions that need to launch a series of strategies to ensure compliance with the official specification of methanol. In a similar situation are producing sectors of Raicilla and Bacanora. In contrast, virtually all production of Tequila is certified by the Tequila Regulatory Council.

Regarding to strategies to reduce the content of methanol in agave distillates, may be mentioned those based on raw materials and on the process. With respect to the raw material, the usual practice is to process pineapple agave without leaves, ie, process agave pineapples completely shaven, with a white appearance to the naked eye. An agave prepared in this way has the potential to produce distillates with lower methanol content of 300 mg / 100 mL pure ethanol. Also, is mentioned the need to process not damaged agave pineapples. In the agave cooking step, the tequila industry uses mainly internal pressure vessels, but in recent years, some companies ferment sugars obtained by hydrolysis of the fructans from the agave leaching with hot water under temperature conditions ranging from 75 to 85 °C, this avoids the heating of agave for long times and allows to obtain a lesser methanol content in the product.

On the step of traditional grinding with roll mills, it is said that the higher the pressure of mills, the higher the methanol content in the resulting distillate. This point has not been tested experimentally.

A strategy for reducing the PME activity can contemplate their inhibition by different techniques. Kana et al. (1991) mention that, according to Lee (1975), the content of methanol in a fermented mash can be reduced when musts are heat treated before fermentation. This makes sense when one considers that most enzymatic reactions in different processes is stopped by applying heat for a period of time. In addition to the use of temperature as a PME inhibitor mentioned, Lewis et al. (2008) have used epigallocatechin gallate (EGCG) and gallocatechin gallate (GCG) as inhibitors of the action of PME.

As for distillation, if this is done in two steps, strategy to reduce the amount of methanol in the product is to perform tails cuts at each stage distillation when alcoholic strength is relatively high,

for example, higher than 25% ethanol v/v. in the second distillation. This strategy is based on that higher concentrations of methanol are present at the end of distillation, when the fraction call *tails* is received (Prado-Ramirez et al. 2005, Prado-Ramirez, 2014). Other alternative to reduce the content of methanol in a batch of distillate product is to make mixtures according to previous calculations, using for this purpose the methanol concentration of each batch and the desired concentration in the final mixture. Finally, Lachenmeier et al. (2006) report compliance with the official specification on the content of methanol as the most problematic for volatile compounds in agave distillates, being a critical point for Bacanora and Mezcal. This is understandable in view of the technologycal level of that is observed in their manufacturing processes.

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THEMATIC II Science and technology of Agave beverages and other derivatives

# Tolerance to phenolic compounds by mezcal yeasts

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## ABSTRACT

The phenolic compounds and other inhibitors of fermentation present in vegetable hydrolyzates pose a significant challenge for the sustainable lignocellulosic materials refining industry and other end products. For this reason, there is a continous search of microorganisms tolerant or better addapted to such compounds. The aim of the present work was to characterized six selected yeasts strains (*Saccharomyces* and non-*Saccharomyces*) belonging to the LBI-CBG mezcal´s yeast collection. The four *Saccharomyces cerevisiae* strains tested showed a high tolerance to vanillin and furfural, up to 1.5 g $L^{-1}$. We also observed a delayed growth due to the effect of toxicity of vanillin and furfural for the majority strains, however the growth was similar after of 72 hours except for the highest tested concentration (2 and 3 g $L^{-1}$, respectively), that could be possible a phenomena of adaptation or biotrasformation of the inhibitors.

**Key words:** Phenolic compounds, Oxidative stress, Agave, Yeasts, Tamaulipas.

## INTRODUCTION

Mezcal industry, as well as any activity involving alcoholic fermentation, requires the action of yeasts, however the composition of the agave must basically depends on the raw material, which varies in turn with the agave species, agronomic and process conditions. Encouraged by the high potential of using biofuels and biochemical to produce biofuels and biochemicals from lignocellulosic materials, which are natural, abundant, and renewable. However, during the degradation of the lignocellulosic materials, inhibitor compounds, including organic acids, furans, and phenolic compounds, are formed with the release of sugars. These inhibitors stunt the growth and block the metabolism of the microorganisms and induce the accumulation of ROS in *S. cerevisiae*, reduce enzymatic and biological activity, break down DNA, and inhibit protein and RNA synthesis (Shen et al., 2014). It is well known that agave musts also contain growth inhibitors such as furfurals, saponins and vanillin, the latter

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is one of the major phenolic aldehyde compounds produced by the degradation of lignin, and all of which cause stress in the yeast during fermentation (García et al., 2011). The characterization and selection of vanillin and furfural tolerant yeast is an important pre-requisite for efficient bioethanol production from lignocellulosic biomass, among other end products. The aim of this work was to establish the mezcal yeast´s tolerance to oxidative stress, at different concentrations of compounds like vanillin and furfural, measured by the production of carbon dioxide along of fermentation and their growth capacity.

## METHODOLOGY

The six yeast strains used belong to the LBI-CBG (Tamaulipas´ mezcal) yeast collection and are conserved in 60% glycerol at -70 °C, and the commercial wine strain *Saccharomyces cerevisiae* Fermichamp (DSM Food Specialties B.V., The Netherlands) was used as control in this study. The strains used are representative of the two species of yeast that have showed high tolerance to hydrogen peroxide and sulphur compounds, furthermore they have shown fermentative contrasting characteristics such as *Saccharomyces cerevisiae* (Sc3Y8, ScMsc3, Sc3D4) and *Torulaspora delbrueckii* (Td1AN2 and TdAN9). Inocula were grown in YPD medium incubated for 24 h at 30 °C, with shaking at 200 rpm. All experiments were carried out using exponentially growing cells, inoculating to an initial $OD_{600}$ of 0.05 (approximately $1x10^6$ cells $mL^{-1}$) in Corning minibioreactors, with a working volume of 20 mL (medium M2 with relationship 1:9 Glucose/Fructose at 100 g $L^{-1}$ of total sugars) shaking at 300 rpm at 30°C. Different concentrations of vanillin and furfural in a range from 0.5 to 3 g $L^{-1}$ were added. Kinetics of the production of carbon dioxide was monitored as weight loss. Each experiment was done in triplicate for the statistical analysis. The yeast growth was followed by counting in a Neubauer chamber, by counting the colony forming units (CFU $mL^{-1}$) as described for the microdrop technique (De la Torre et al., 2016) in YPD plates after incubation at 30°C, for 24 h.

## RESULTS AND DISCUSSION

Strains evaluated in this experiment were tolerant to most conditions tested, except for the higher concentrations of vanillin and furfural (2 and 3 g /L, respectively) as shown in fig. 1. We observed that for all strains that $CO_2$ production was delayed when vanillin and furfural were added. However, the final production of $CO_2$ was similar in the majority of the evaluated concentration levels. Furthermore, at lower concentrations of vanillin and furfural the production of $CO_2$ in some strains were higher than in the control. This phenomenon was also observed by García et al. (2011) when the production of ethanol increased by the presence of furfural, and this result can be extrapolated to our results as $CO_2$ and ethanol kinetics are closely linked during the first stages of the ethanolic fermentation. Hence, we can attribute the tolerance to vanillin and furfural due that some non-specific aryl-alcohol dehydrogenase enzyme(s) that can convert vanillin to the less toxic compound vanillyl alcohol in *S. cerevisiae* (Shen et al., 2014) or converting the furfural to furfuryl alcohol under anaerobic conditions (Sarvari et al., 2003). It is also known that genes encoding the ergosterol biosynthetic enzymes could be associated with vanillin tolerance and to other inhibitor compounds, because the ergosterol is important for maintaining the fluidity and stability of the membrane. However, knowledge about the toxicity of vanillin is still limited, and the mechanism by which the *S. cerevisiae* resist the intracellular vanillin is still not clear (Shen et al., 2014).

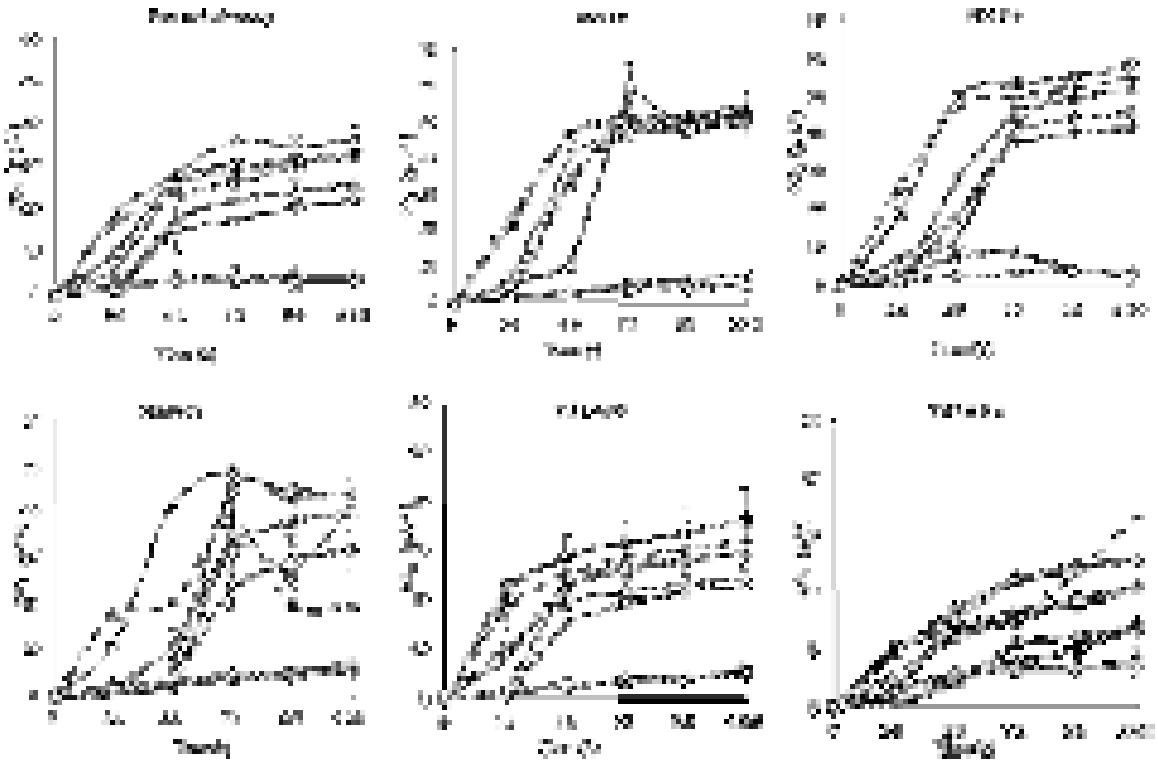

Figure 1. Kinetics of carbon dioxide production of the strains tolerant with different levels of inhibitors. ----- Control; vanillin concentrations: -■- 0.5, -▲- 1, -♦- 1.5, -●- 2 (g $L^{-1}$); furfural concentrations: -Δ- 1, -◊- 1.5, -○- 3 (g $L^{-1}$).

On the other hand, the oxidizing effect of the compounds it was also observed due to inhibition of cell growth (Fig. 2). The growth at 1.5 g $L^{-1}$ vanillin and furfural were delayed, however, the final biomass reached similar values to non-stressed conditions, but 48 hours later for vanillin and 72 hours later in the case of furfural. It is known that resistance to oxidative stress increases during the stationary phase (Arellano et al. 2013), similarly to observed it in this work. The effect of toxicity of vanillin had been observed previously so like the delay growth was observed by Nguyen et al., (2014) to vanillin concentrations (0- 6 mM) was lower than in this work.

## CONCLUSIONS

The seven yeast tested tested were tolerant up to 1.5 g.$L^{-1}$ for both, vanillin and furfural, which is a high tolerance, as agave must usually contain around 1 g $L^{-1}$ of furfural. However, the results obtained show a different behavior of the yeast strain, but not all strains can be produce carbon dioxide like in normal conditions due to a mechanism not clear.

## ACKNOWLEDGEMENTS

The authors thank the financial support of Instituto Politécnico Nacional (projects SIP2016-1919 and 2016-1090) and the IPN scholarship and BEIFI financial supports to PVL.

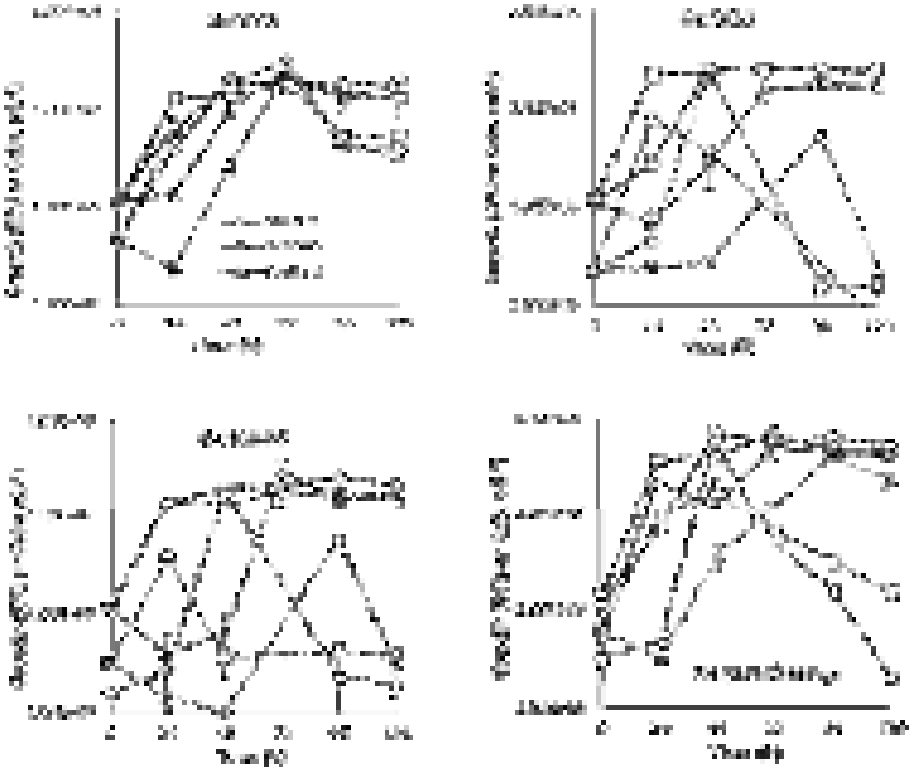

Figure 2. Growth kinetics of the strains in the presence of 1.5 g $L^{-1}$ of vanillin or furfural, monitored by cell concentration (continuous lines) and by viable counts (dotted lines).

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THEMATIC II Science and technology of Agave beverages and other derivatives

# Validation of a method for the determination of volatile compounds that could be markers of authentic tequila.

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## ABSTRACT

Beverages made from agave, such as tequila, have a denomination of origin, the majority compound are controlling by specification of official rules like NOM-006-SCFI-2012. Validation of analytic techniques are the unique way to demonstrate the veracity of results. The principal families found in this spirits are furans, terpenes, esters and some acetyls, highlight the family of terpenes, which is believed to come from the plant, being the most abundant linalool, α-terpineol and trans α-farnesol, this are responsible for the floral, sweet, citrus and woody aroma. Gas chromatography with flame ionization detector is probably the technique most used for volatile compound identification. Validation of analytic technique that identified the specific compound by Mexican rules and other minority compound such as 2-methyltetrahydrofuran, ethyl octanoate, 2-acetyl furan, linalool, 5-methylfurfural, 4-terpineol, ethyl decanoate, menthol, furfuryl alcohol, alfa-terpineol, ethyl dodecanoate, 2-phenylethanol, and alcohol perillyl that could be used as quality control to determine the authenticity of agave beverages. The parameters of precision, linearity, percentage of recovery, limit of identification and quantification were validated using CCAYAC criteria. This work presents an analytical technique to identify and quantify minority compound that could be use such a marker of authentic agave beverage.

**Key words:** agave, spirit beverages, authenticity.

## INTRODUCTION

The spirit beverages such as tequila can be easily adulterated and some of them can be sales as tequila agave 100% or tequila. The Mexican official standard NOM-006-SCF-2012 evaluate some components for the quality control of tequila such as ethanol (35-55 % v/v), superior alcohols as propan-1-ol, 2-methyl-1-propanol, 2-methyl-1-butanol, 3-methyl-1-butanol, butan-1-ol and butan-

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2-ol (20-500 mg/100 mL AA), methanol (30-300 mg/100 mL AA), aldehydes as acetaldehyde (0-40 mg/100 mL AA), esters as ethyl acetate (2-250 mg/100 mLAA) and furfural (0-4 mg/100 mg AA). All these compounds are present in the tequila in high amounts and accordance with the law; this compounds can be identified and quantified by gases chromatography coupled flame ionization detector (GC-FID). Studies about different stages of the production process of tequila haven been identified 327 volatile compounds and they were classified in 12 functional chemical group, however, in the final product (second distillation) haven been identified 187 compounds (Prado-Jaramillo et al. 2015). Most of these volatile compounds are present in small content and have an impact on the aroma and flavor of tequila and present very low concentration (Escalona-Buendía et al. 2004).

The Mexican regulations just evaluate the major compounds that can be identify and quantify by Gas Chromatography using Flame ionization detector (GC-FID), therefore is important to find minor compounds that can be classifiers from agave origin with simple techniques and robust analysis such GC-FID.

## METHODOLOGY

### Chemicals

Compounds used as standards purchased from Sigma-Aldrich (Toluca, México). The HPLC-grade ethanol was obtained from Sigma-Aldrich (Toluca, México).

### Tequila Samples

The analytical method was evaluated with authentic silver tequila samples obtained from Cámara Nacional de la Industria Tequilera (CNIT)

### Sample preparation

Sample preparation was carry out in agree with the NOM-006-SCF-2012, using glass material class A, the sample was prepared in 25 mL with 0.5 mL of IS1 and 0.5 mL of IS2, they are taken 2 mL in a vial and injected in gas chromatography.

### Chromatographic conditions and validated method

Chromatographic method was performed by a gas chromatography flame ionization detection (FID) and autosampler (Agilent Technologies, 6890N), equipped with an Innowax capillary column (60m*0.25 mm id*0.25µm film thickness). The oven temperature was programed at 40°C and maintained by 6.5 min, increased until 165°C at a rate of 10°C min-1, 30°C min-1 to 220°C and finally maintained at 220°C for 10 min. Temperature of the injector was 220°C and detector 240°C. The gas carrier was helium at 0.7mL min-1. Sample injection was 0.5 µL and it was 3:1 as split ratio.

The chromatographic method was validated for linearity, precision, limit of detection and quantification and accuracy. It was injected ten levels of calibration curves according to the linear range previously stablish; precision was evaluated by obtaining the repeatability and intermediate precision using seven injections of each level, both parameters were expressed as relative standard deviation (%RSD). The linearity was obtained by the percentage of relative coefficient ($R^2$) and the limits of detection (LOD) and quantification limit (LQD) were estimated according to The International Conference on Harmonisation guidelines 1994 and the accuracy was obtained using the analysis of recovery. The tequila silver was spiked at, low, medium and high concentrations (80%, 100% and

120% of the concentration of the calibration curve) this was evaluate by three times, the results tell us how recover what added.

## RESULTS AND DISCUSSION

Performance characteristics, such as trueness, precision, linearity and sensitivity of the GC-FID method was evaluated, the results are shown in Table 1. The recovery percentage was obtain to the measure of additional tequilas using the same ten levels of calibration curve, results obtained was according to establish at the CAYACC between 80 – 120 %. Repeatability was evaluated using a level of calibration curve and this was injected seven time in same condition, precision was evaluate using 3 levels of calibration curve en the acceptance requirements was CV< 5% to majority compound and 10% for the propose such as possible markers, percentage of variety coefficient was the reported, accepting 3% for all compound in parameter intermiade presicion. Gas chromatography allow to detect concentration around 0.01 mg*L-1 in some compounds, this concentration was an excellent result for the propose of this job.

Linearity of all compound was measure using the criteria percentage of coefficient correlation equal or most than 99%. The parameters measured in this validation, indicate that analityc technique can be use for analysis of agave beverage, the validation present the evidence necessary to demonstrate de veracity of obtain results.

**Table 1.** Validation parameters

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## ACKNOWLEDGEMENTS

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THEMATIC II **Science and technology of Agave beverages and other derivatives**

# Volatile profile of artisanal Raicilla elaborated in the Jalisco state.

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## ABSTRACT

Raicilla, artisanal beverage produced in Jalisco and Nayarit states from different species of agave, was characterized in its volatile profile by GC-FID and GC-MS. The majority volatile profile allowed to determine that the parameters in which some products exceeded the limits regulated by Official Mexican Standards were in esters, higher alcohols and furfural. On the other hand, 86 minor volatile compounds were quantified by direct injection into a GC-MS system, of wich ketones, acids and miscellaneous compounds were the most abundant.

**Key words:** Raicilla, volatile compounds, agave distillate.

## INTRODUCTION

Raicilla is a regional alcoholic beverage produced in Jalisco and Nayarit, Mexico. In its processes is permitted to use 100% of the maguey *A. maximiliana, A. inaequidens, A. valenciana, A. angustifolia* and *A. rhodacantha*, among others, except *A. tequilana* Weber var. Azul. Raicilla is distilled from fermented juices spontaneously or with cultured yeasts, extracted from mature heads of the previously cooked maguey (PROY-NOM-199-SCFI-2015). The Raicilla elaboration process basically comprises the same stages of the artisan process to produce mezcal, bacanora and other important mexican distilled beverages elaborated from the genus Agave, so its chemical composition is equally complex. This product is still elaborated under traditional processes, currently its consumption has increase and it is looking for international markets, nevertheless information on the volatile composition related to their quality characteristics and identity is scarce. The aim of this study was to characterize the volatile profiles of Raicilla from different regions of the Jalisco state, as the composition and quality approach.

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## METHODOLOGY

### Raicilla samples and analytical reagents.

Eleven samples of different Raicillas "Silver 100%", were kindly provided by local producers in the municipalities of Mascota and Cabo Corrientes, Jalisco in July 2016. The samples were stored at 20 ± 2 °C until analysis. Ethanol, acetaldehyde, ethyl acetate, ethyl lactate, ethyl nonanoate, methanol, 2-butanol, 1-propanol, isobutanol, 1-butanol, isoamyl alcohol, 1-pentanol, 2-pentanol and furfural were purchased (GC grade, purity ≥ 99 %) from Sigma-Aldrich (St. Louis, MO, USA).

### Chromatographic analysis

The major volatile compounds regulated by Official Mexican Standard, were determined by GC-Flame Ionization Detector (FID), standardizing the samples and calibration levels as described in the Mexican Standard NMX-V-005-NORMEX-2013. The samples and standard disolutions were analyzed by auto-injecting 1 µL (split 50:1) in a gas chromatograph 7890B Agilent technologies. Separation was done in a HP-INNOWAX column (No. part 19091N-136 Agilent). Carrier gas was helium at a flow rate of 1 mL/min. Oven initial temperature was 50 °C for 7 min, then increased at 10 °C/min to 165 °C, and finally increased at 20 °C/min until 220 °C, held for 5 min. Injector and detector temperatures were 220 and 250 °C, respectively. Quantification was based on the internal standard method according to NMX-V-005-NORMEX-2013. Each sample was assayed in duplicate.

Minor volatile compounds analyses were carried out by direct injection in a GC-MS. The column and gas chromatograph used for this determination were the same described above, but GC detection was coupled to an Agilent 5977A MSD. Samples were automatically injected (1 µL, splitless mode), helium flow at 1.5 mL/min. Oven temperature program was 60°C to 180 °C at 3 °C/min held 2 min, then increased to 230 °C at 15 °C/min, held for 30 min. Injector and detector temperatures were 250 and 260 °C, respectively. Compound ionization was in EI mode at 70 eV and spectral signals were obtained on scan mode. Tentative identification of detected compounds was based in a mass spectrum comparison with those obtained in the NIST16L library and confirmed by using pure standards when possible. Quantification was made with methyl nonanoate as reference standard, analyzed under the same conditions that samples. Each sample was assayed in duplicate.

### Alcoholic content (% alc. Vol.).

The alcoholic content in samples was measured according to Mexican Standard NOM NMX-V-013-NORMEX-2013. % Alcohol volume was used for obtain the compounds concentration in anhydrous alcohol base.

## RESULTS AND DISCUSSION

### Majority volatile profiles

The profiles of the major compounds in different samples analyzed are shown in Fig. 1, whereas the detailed quantitative results are shown in Table 1.

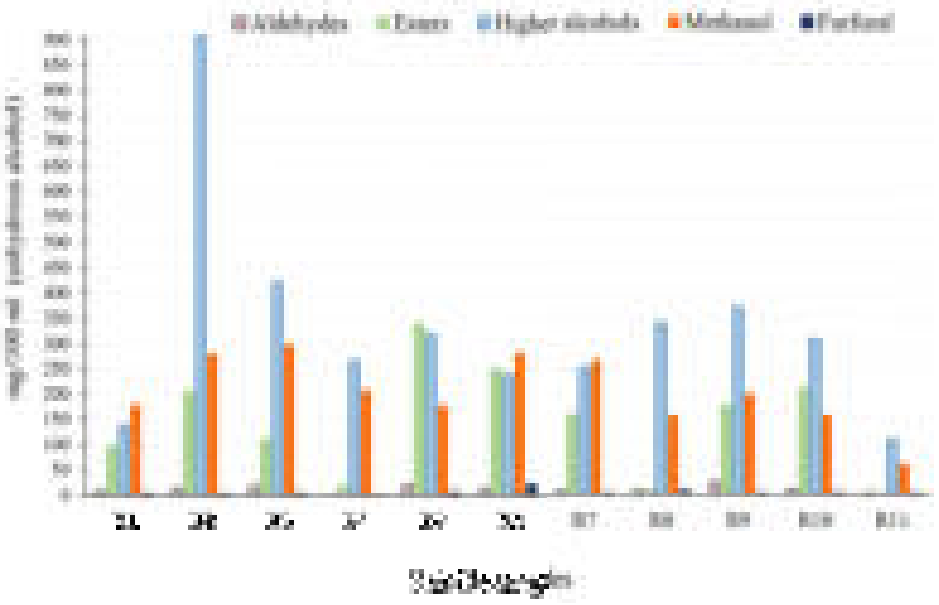

Figure. 1. Major volatile profile in different Raicillas

Individual concentration of the major volatile compounds shown significant differences and contributed to the formation of the global majority volatile profile of the Raicillas evaluated. Overall seven of the eleven Raicillas tested met the specifications for content of aldehydes, esters, higher alcohols, methanol and furfural indicated in the PROY-NOM-199-SCFI-2015 and NOM-142-SSA1/SCFI-2014. Four products exceeded the allowed limit for esters with concentrations of 205.1, 339.5, 250.5, 210.5 mg/100 ml of anhydrous alcohol for samples R2, R5, R6 y R10 respectively. Sample R2 with 908.6 ml/100 ml of anhydrous alcohol exceeded the maximum limit of higher alcohols, while samples R5, R6 y R10 were out of range for furfural with 5.9, 20.2 y 5.1 ml/100 ml of anhydrous alcohol.

Table 1. Concentration of major volatile compounds in different Raicillas.

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aRefered to alcohol/volume (20°C). bAs acetaldehyde. cMaximum and minimum values suggested in the PROY-NOM-199-SCFI-2015 for Raicilla. cMaximum values permitted by NOM-142-SSA1/SCFI-2014 for distillates.

## Minority volatile profiles

The direct injection of different Raicillas allowed the identification and quantification of 86 minority compounds (not considered in the NOM). In Table 2, the identified compounds are listed, among which, ketones, acids, miscellaneous compounds and other alcohols were the most abundant quantitativity.

Table 2. Minor volatile compounds identified en Raicilla.

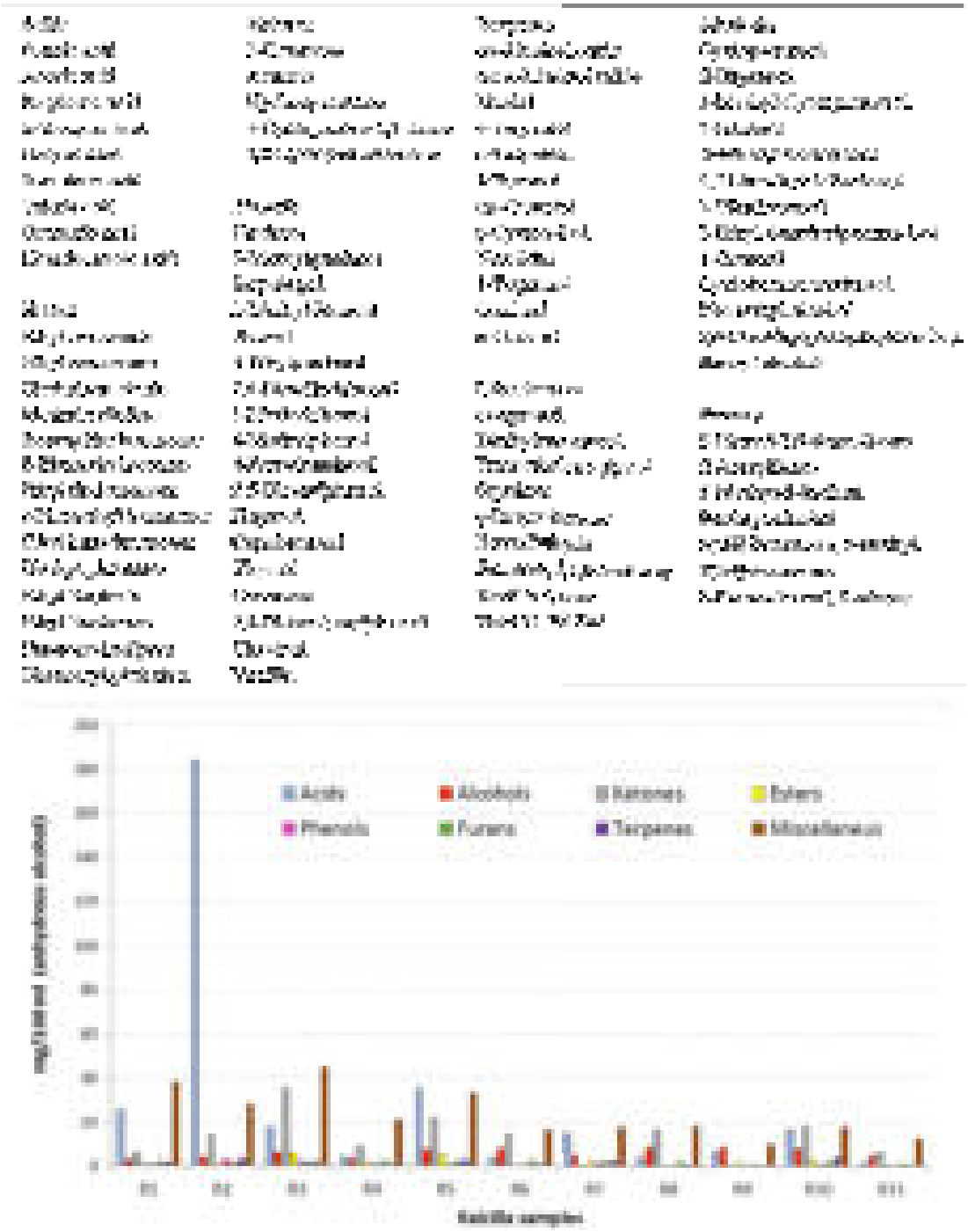


Figure. 2. Minority volatile compounds in different Raicillas

Different concentrations of the minor compounds marked the differences in their volatile profiles (Fig. 2). Esters and higher alcohols are generated during alcoholic fermentation and their concentration in spirits is influenced mainly by the microorganisms involved in the fermentation stage, as well by process conditions during elaboration. The furfural is generated mainly during the cooking stage, but its concentration change during the distillation of the fermented musts; so that the species of agave used, the type and time of cooking, and distillation conditions are determinants in the control of the concentration of this compound (Estarrón et al., 2015). Despite are few studies on the volatile majority and minority composition of this beverage; De León et al. (2008), reported concentrations of 166 mg/L, 217 mg/L and 490 mg/L for esters, methanol and higher alcohols respectively, this values are low for methanol and higher alcohols compared to those evaluated in this study. On the other hand, all the minor volatile compounds identified in this study have already been reported in previous studies on Raicilla (Arrizón et al., 2007; de León et al., 2008).

## CONCLUSION

The characterization of the volatile compounds in Raicillas showed that only 63% meets the quality specifications established by the Official Mexican Standard. The detection of significant differences in composition profiles and some physicochemical parameters outside permitted limits, suggests large differences in manufacturing processes, however it is necessary to determine the quality of this artisan agave beverage in different production regions to warranty that Raicilla products meet the law specifications.

## ACKNOWLEDGEMENTS

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THEMATIC II **Science and technology of Agave beverages and other derivatives**

# Yeasts diversity and distilleries technification of durango's mezcal.

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## ABSTRACT

Mexico is known for the production of alcoholic beverages produced by distilling the fermented juice of different species of agave, one of these beverages is the mezcal. This drink is produced mainly by artisanal process, so the fermentation is done with juice and bagasse, usually without inoculation. The fermentation process is carried out by yeast, which are responsible for the production of ethanol and other compounds that confer organoleptic characteristics to the final product.
This work analyzes the diversity of yeast during the fermentation process in two distilleries in the state of Durango, Mexico; both distilleries have different production processes, an artisanal distillery and the other one is semi-industrialized distillery.
Relevantly 34 yeasts were isolated, Where *Saccharomyces cerevisiae* were 47%, 44% *Kluyveromyces marxianus, Pichia manshurica* 3%, 3% *Torulaspora delbrueckii* and 3% *Pichia kluyveri*. Differences were observed in the distribution of *Saccharomyces* and Non-*Saccharomyces* between the two mezcal distilleries. In Lagrimas de Dolores only *Saccharomyces* obtained in the final stages of fermentation. On the other hand, artisanal distillery exhibited a high percentage of *Saccharomyces* in the early stages in April unlike june, by the end, *Saccharomyces* and *Kluyveromyces* genera were obtained.

**Key words:** yeast, fermentation, mezcal, *Saccharomyces*, Non-*Saccharomyces*.

## INTRODUCTION

Mezcal is a traditional Mexican beverage obtained from the distillation of fermented juices of cooked Agave heart (Flores-Berrios et al. 2005). Distilled beverages involve a complex of microorganisms during the fermentation, where yeasts are responsible for the production of various chemical compounds (Escalante-Minakata et al. 2008). The aim of this work was to identify and assess the

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diversity of the yeasts that occur in different fermentation stages (starting, middle and end) of must obtained from *Agave durangensis* fermentations, from two distilleries with different levels of technification.

## METHODOLOGY

Samples were taken at different times during fermentation in two mezcal distilleries: Lagrimas de Dolores located in Durango, Dgo., and Avila located in Nombre de Dios, Durango, Mexico. Both distilleries have different production processes. Avila's factory presents an artisanal process, while Lagrimas de Dolores is technified.

Samples from Avila's distillery were collected in april (NAP) and june (NAJ), while samples from Lagrimas de Dolores were taken in june (LDJ). Yeasts were isolated in YPDA. Genomic DNA was obtained by using the Wizard genomic DNA purification Kit© (PROMEGA). Identification was conducted by direct sequencing of the 26S ADNr region using the Big Dye Terminator v3.1 cycle sequencing Kit (Applied Byosistems) on an ABI 3130 sequencer.

## RESULTS AND DISCUSSION

34 yeasts were isolated, where 47% were *Saccharomyces cerevisiae*, 44% *Kluyveromyces marxianus*, 3% *Pichia manshurica*, 3% *Pichia kluyveri* and 3% *Torulaspora delbrueckii*. Differences were observed in the distribution of *Saccharomyces* and Non-*Saccharomyces* (Fig. 1) between the two mezcal distilleries. Lagrimas de Dolores exhibited a high percentage of *Saccharomyces* in the early fermentation stages, but at the end, only *Saccharomyces* was obtained. On the other hand, Avila´s factory exhibited a high percentage of *Saccharomyces* in the early stages in april (33.3%) unlike june (22.2%), by the end, *Saccharomyces* and *Kluyveromyces* genera were obtained.

Figure 1. Phylogenetic analysis of the 26S rDNA region. (Green) Beginning, (Yellow) middle and (Red) end fermentation stages. Avila (NA) and Lagrimas de Dolores (LD).

## CONCLUSION

Preliminarly we conclude that Avila's distillery showed more yeast diversity, probably because this distillery retains a more traditional process.

## ACKNOWLEDGEMENTS

We thank the financial support from Instituto Politécnico Nacional, project SIP 2016-0950 and BEIFI-IPN support to SCME.

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THEMATIC III **Biological effects of Agave fructans and other by-products**

# Automatic determination of *Kluyveromyces marxianus* fructanase enzymatic activity

**Barbosa E.[1], Arana A.[2], Pliego J.[3], Gschaedler A.[4], Arrizon J.[5], Femat R.[6], Herrera-López E.[7]***

## ABSTRACT

The aim of this work was to monitor fructanase enzymatic activity produced by a non-conventional yeast *Kluyveromyces marxianus* isolated from the mezcal process. For this purpose, a known enzymatic analytical method was adapted to a sequential injection analyzer (SIA) designed in our laboratory. Samples taken from a running bioreactor were put in a flask and were properly joined via tubing to the SIA system. Off-line fructanase determination was carried out by pumping a specific volume of free cell samples into the SIA system. When necessary, automated dilutions was performed, in function of the culture elapsed time. Substrate was added to the sample and was incubated at 50°C for 15 min. Then it was mixed with DNS reagent and heated at 90°C for 5 min, finally be cooled and read at 540 nm in a spectrophotomer. The SIA measurements were validated against the standard method by means of quantifying the amount of residual reducing sugars released from the hydrolysis of sucrose. The results indicate that SIA system measurements are as reliable as the microplate methodology. The SIA system allowed to measure the activity of a sample in a linear range of 0.01-1.4 U/ml. This methodology developed with the SIA system is a promising tool to monitoring on-line the evolution of fructanase production in a bioprocess with the purpose to consider automated control strategies.

**Key words:** *Kluyveromyces marxianus*, fructanase, monitoring, Sequential Injection Analysis, off-line.

## INTRODUCTION

In a bioprocess, the yeasts are the largest and most valuable generators of bioproducts worldwide, exceeding in production capacity and income to any other industrial microorganism (Kurtzman et al.

1, 2, 3, 4, 5, 7. Biotecnología Industrial, Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco A.C., Unidad Zapopan, Camino Arenero 1227, Col. El Bajío del Arenal, C.P. 45019 Zapopan, Jalisco, México
6. División de Matemáticas Aplicadas, IPICYT. Camino a la Presa San José 2055, Col. Lomas 4a. sección C.P. 78216. San Luis Potosí, S. L. P., México
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2011). *K. marxianus* has desirable characteristics for biotechnological applications, such as: capability to assimilate key substrates not metabolized by other yeast, such as lactose and inulin. This yeast can grow at considerable fast rates, it has thermotolerance as it has the ability to grow above 52 °C, and shows high secretory capacity of metabolites (Fonseca et al. 2008).

The fructanases are enzymes capable of hydrolyze β-fructofuranoside links of fructans and can be used to obtain fructooligosaccharides (FOS) or fructose nectars (Corona-González et al. 2014). A recent study has evaluated the fructanase and fructosyltransferase activity of non-Saccharomyces yeasts isolated from the mezcal fermentation must, where *K. marxianus* had fructanase activity (Arrizon et al. 2011).

Usually, determining enzyme activity requires taking a sample out from the bioreactor to analyze it off-line at the laboratory. The disadvantage of this approach is the extensive time needed to recover the information from the bioprocess, hindering the possibility to develop control systems. It will be of great interest to monitor enzymatic activity in real time, since from its determination of optimization and control strategies can be designed in order to guarantee the maximum yield and productivity in the bioprocess. Sequential injection analysis (SIA) systems are suitable devices to monitor biochemical key variables such as enzyme activity in a bioprocess on-line (Silvestre et al. 2011). The aim of this work was to develop an automatic method to monitor off-line, the production of a fructanase produced by a strain of *Kluyveromyces marxianus* isolated from the mezcal process using a SIA system.

## METHODOLOGY

### Microorganism and fermentation conditions

The yeast used in this study was *Kluyveromyces marxianus* SLP1 that belongs to the microorganism collection of the *Centro de Investigation y Asistencia en Tecnología y Diseño del Estado de Jalisco, A.C. (CIATEJ)*. The Samples from batch and fed-batch fermentations (chemically defined medium, 20 g/L glucose, pH 4.5) were centrifuged to separate the cell pack. Supernatant was stored at -20 °C until the samples were analyzed.

### Sequential injection analysis system description

Fructanase enzyme determination was carried out in a Sequential Injection analysis system designed at the Industrial Biotechnology laboratory of the CIATEJ. The SIA system is composed by a mini-pump for aspiration/injection, a holding coil, two multiposition valves, a reaction coil with temperature control, a flow cell with a light source and a spectrophotometer, see details in (Pliego et al. 2015).

### Calibration curve

The calibration curve used in the SIA system consisted of 5 concentrations of glucose (0.0 g/L to 2.0 g/L). Each dilution was mixed with DNS reagent and heated up at to 90 °C for 5 min, and immediately cooled with ice for 1 min. The mixing mixture was diluted 1:4-fold, and finally read at 540 nm.

### SIA system fructanase determination and validation.

The supernatant from the bioreactor samples were pumped to the SIA system and 10 g/L of sucrose with pH 5.0 were mixed with the sample and carried to the holding coil of the measuring device. The blank was obtained by mixing the sample with the DNS reagent followed by the addition of subs-

trate. The samples and blank were treated following the same procedure performed in the calibration curve. Samples were diluted in a ratio 1:4, and read at 540 nm. The SIA system measurements were compared against the standard methodology used to quantifying enzyme activity in microplate (Arrizon et al. 2011), our method does not use phenol. From this analysis a curve for each sample was obtained.

## RESULTS AND DISCUSSION

The developed methodology for the calibration curve showed that the response generated by the SIA system was proportional to the glucose concentration of the enzymatic reaction. Fig. 1A shows the summary of curves generated by the sequential analysis system for diverse runs of the calibration curve. It is noteworthy that the method is reproducible. Linear regression with areas under the curve shows an adjustment of 99.7% (Fig. 1B).

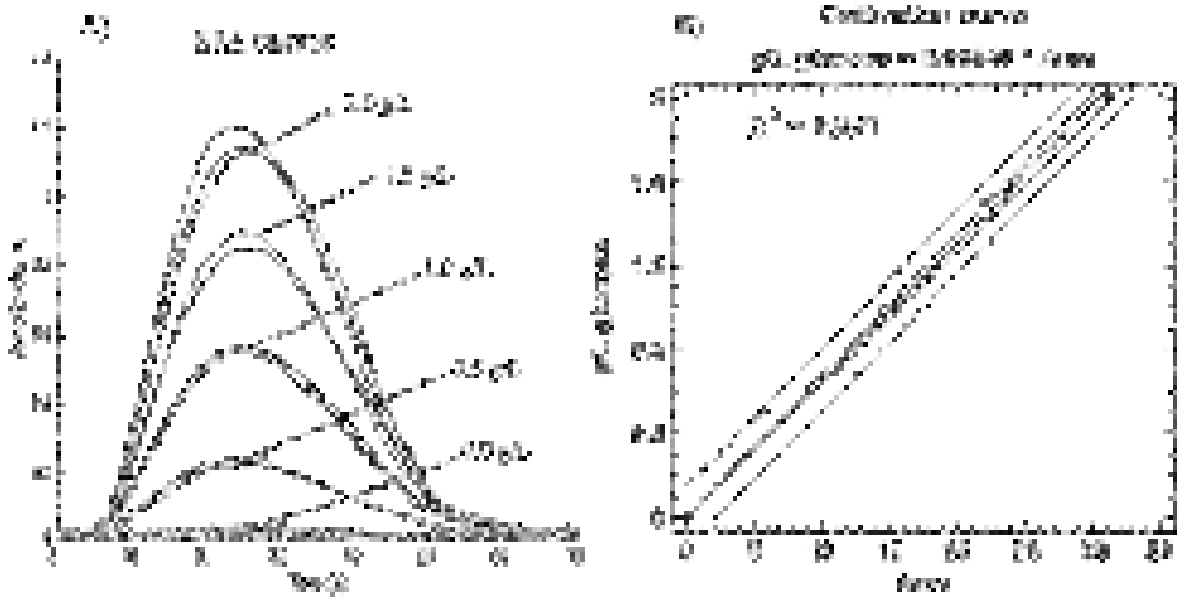

Figure 1. A) Response curves obtained from the processed samples in the SIA system and the calibration curve. B) linear regression with areas of the calibration curve.

Table 1 compares the enzymatic activity values obtained from microplate with the SIA system. It can be observed not significant differences (below 1%) between the microplate with the SIA system at high enzymatic activity obtained from the fed-batch cultures, this samples required dilution of 1:100 to fit the calibration curve. However, at low enzymatic activity values obtained from the batch cultures the dissimilarities appeared between the SIA and the microplate measurements since the standard deviation varied from 14% to 20%. This variability could be caused by an inadequate mixing due to the presence and/or generation of air bubbles during processing and measurement; therefore, the SIA performance is still under study to improve its reliability at low activities. The SIA system allowed the measurement of the enzymatic activity of a sample in a linear range of 0.01-1.4 U/ml. In a next stage, on-line fructanase activity determinations will be performed with the SIA system.

Table 1. Comparison of enzyme activity microplate vs SIA

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## CONCLUSION

The results obtained let us show that the fructanase activity measurement through the SIA system is as effective as the measurement in microplate, although its performance still needs to be improved to increase reliability at low enzymatic values. The methodology developed with the SIA system is a promising tool for on-line monitoring the evolution of fructanase production in a bioprocess, with potential use for optimization and control purposes.

## ACKNOWLEDGEMENTS

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THEMATIC III **Biological effects of Agave fructans and other by-products**

# Characterization of polysaccharides extracts of *A. durangensis* pineapple agave.

**Valencia-del Toro G.[1*], Aguilar-Doroteo J.[2], Aguilar-Doroteo L.[3], Torres-Ricario R.[4], Garín-Aguilar M. E.[5]**

## ABSTRACT

The *Agave durangensis* is an important complex specie of agave in Mexico because represents an economic and cultural source for Durango City. The agave is used principally for mezcal production in our country. The fructooligosaccharides are necessary in such production. At date, there are few reports about type and amount of polysaccharides of A. *durangensis*. We used fresh pineapple from A. *durangensis* of nine years of age, this sample was cut in 1 $cm^3$ pieces of and two processes were developed. On one hand, fragments were dehydrated and grinded, the resulting flour was extracted with water, the aqueous extract was dried out (in Spray dryer) and the polysaccharides were precipitated with absolute ethanol. On the other hand, the pineapple juice was obtained and was mixed with absolute ethanol and then polysaccharides were collected. The extract yield obtained in spray dryer was 33% while the extract from maceration was 7%, but the IR spectra of polysaccharides extracts obtained of spray dryer extract had additional peaks with respect to spectrum of polysaccharides obtained from pineapple juice. The use of spray dryer to obtain polysaccharides extracts of agave allowed increase in yield. Therefore is important to perform the purification from the obtained extract.

**Key words:** *Agave durangensis*, Frutooligosaccharides, IR spectra, Spray dryer, polysaccharides.

## INTRODUCTION

Members of the Agavaceae family are species rich in carbohydrates, which are, enable to produce fructans, this polysaccharides are reserve material and osmoprotectans against hydric stress. In different Agave species, the fructans are synthesized and stored in the stems. The fructans have different structure and molecular weights depended of specie and region of study. The comparison of fructan linkage analysis between *A. tequilana, Agave potatorum, Agave cantala, Agave fourcroydes*

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*and Agave angustifolia* showed differences in the proportions of glucose polymerized, as well as in the proportions of β(2-6) and β(2-1) linkages of the principal chains (Mancilla-Margalli and Lopez, 2006).

Different studies have been realized on *Agave* fructans (i.e. characterization of their molecular structure, thermal and enzymatic hydrolysis, characterization their physiological mechanisms). However, reports regarding the factors of the extraction process and the possible interactions among them are limited (Flores et al. 2015). Recently, the attention of researchers has focused on the *Agave* fructans extraction and several methods for fructans extraction have been proposed, among which hot-water treatment (Laurenzo et al. 1999) and maceration in distilled water extraction to obtain the agave juice are used. In both methods is required the polysaccharides precipitation with ethanol. The aim of this research was to obtain and characterize the polysaccharides extracts from *Agave durangensis* pineapple.

## METHODOLOGY

### Polysaccharides extraction process

A fresh pineapple from *A. durangensis* of nine years of age and harvested in the State of Durango, México was used, this sample was cut in 1 $cm^3$ pieces and two processes were developed. On one hand fragments were dehydrated (Mellado and López, 2012) in an oven at 60 °C, once dry the material was grinded in a blender and pass through a 60-mesh sieve, the resulting flour was extracted four times with water at 90 °C during 4 h. The aqueous extract was dried out (in Spray dryer) and the polysaccharides were precipitated with absolute ethanol. On the other hand, the pineapple juice was obtained; the pieces of pineapple agave were added with distilled water in 1:0.7 proportion weight: volume, after this mix was crushed in a blender for 3 minutes, the macerated pulp was maintained at 60-70 °C for 15-20 minutes, the obtained juice was mixed with absolute ethanol and the polysaccharides were collected (Mancilla and López, 2006). The polysaccharides extraction yield was determined according to the following equation: Extraction Yield (%) = (polysaccharides weight/wet raw material weight) × 100%.

### Characterization of Polysaccharides of *A. durangensis*

The polysaccharides content in the extracts was determined as the difference between the total carbohydrates and reducing sugars. The reducing sugars were quantified via the DNS (Miller, 1959) method using D-glucose as the standard and total carbohydrates were determined by phenol-sulphuric acid method (Dubois et al. 1956). The polysaccharides content was measured with the difference between total carbohydrate and reducing sugars. The average chain length, as an index of degree of polymerization (DP), was calculated according to (Paseephol et al. 2007): degree of polymerization (DP) = Total amount of carbohydrate/Total amount of reducing sugar.

Infrared spectrums of polysaccharides were recorded in the solid state using a Fourier Transform InfraRed spectroscopy (FTIR) in Perkin-Elmer 16F PC Spectrophotometer, between 550 and 4000 $cm^{-1}$. The qualitative identification of polysaccharides was performed by thin-layer chromatography (TLC). Phase ascension was composed of a mixture of solvents: butanol–propanol–water (7:5:4:2). After drying the plate, the spots were visualized by spraying aniline–diphenylamine–phosphoric acid–acetone (1:1:5:50), followed by drying at 80 °C for 10 min (Mellado and López, 2012).

## RESULTS AND DISCUSSION

The aqueous extract yield obtained in spray dryer was 42% and the polysaccharides extract was of 33% while the extract from maceration was 7%. The agave polysaccharides obtained via spray dyer exhibited a DP of 49.5. Of the total sample obtained, 143.6 g $kg^{-1}$ corresponded to total carbohydrates and 2.9 g $kg^{-1}$ were reducing sugars, whereas the polysaccharides obtained via maceration of extracts exhibited a DP of 6.7, and the sample was composed of 281.6 g $kg^{-1}$ carbohydrates and 46.6 g $kg^{-1}$ reducing sugars. DP and concentration of fructans are highly dependent on the environmental conditions: temperature, light, nutrients in the soil, among others (Flores-Girón et al. 2015). The FTIR spectra of the polysaccharides extracted by different methods are shown in Fig. 1. The spectra are basically similar to the previously analyzed spectra of other examined systems (Cai et al. 2008; Pourfarzad et al. 2015; Zhao et al. 2011). In the IR area of about 3,200 $cm^{-1}$, there is a wide intense band. This band can be assigned to the O-H stretching vibrations of CH-OH groups from a fructo-furanose unit. Some weak absorption peaks of about 2,888 – 2,977 $cm^{-1}$ for C-H stretching vibrations, were observed in the spectra. The relatively strong absorption peak at around 1,581 $cm^{-1}$ and 1,583 $cm^{-1}$ reflected the absorption of the C=O group which was part of glycosides. This is a characteristic of monomers fructose and glucose (Zhao et al. 2011). Other peaks, between 1,020 and 1,050 $cm^{-1}$ are resulted from C-O bond and C-C bond, the peak at about 1,395 $cm^{-1}$ represents the angular deformation of C-H (CH3 group). In addition, a series of common spectra at about 1,269 and 1,455 $cm^{-1}$ appeared in the spectra of fructans, and they were related to bending vibrations and internal deformations of the methylene CH2–OH group from the fructose ring (Pourfarzad et al. 2015).

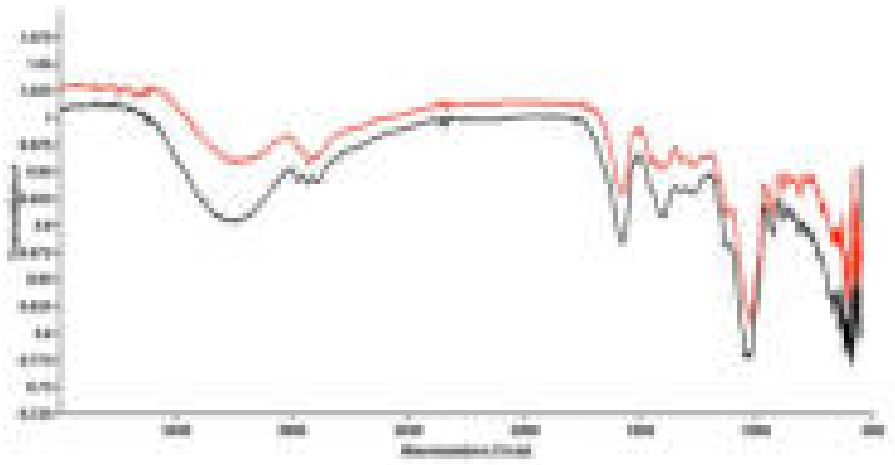

Figure 1. IR spectra of polysaccharides extracts of *A. durangensis* obtained from agave juice **(a)** and dry agave **(b)**.

The TLC profile of polysaccharides extracts of *Agave durangensis* (Fig. 2) shown retention factors (Rf values) and spot colors similar. In both methods of extraction were obtained the same carbohydrates and this also was observed in the IR spectra.

Figure 2. TLC of polysaccharides extracts of *Agave durangensis* Obtained from agave juice (F1) and dry agave (M1, M2).

## CONCLUSION

The use of spray dryer to obtain polysaccharides extracts of agave allowed increase in yield. Therefore is important to perform the purification from the obtained extract.

## ACKNOWLEDGEMENTS

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THEMATIC III **Biological effects of Agave fructans and other by-products**

# Effect of agave fructans addition on technological properties of white bread.

**Camacho-Ruíz R.M.[1], Torres-Pedraza C.[2], Alvarado-Osuna C[3]***

## ABSTRACT

Agave fructans are dietary fiber, which has been shown to have prebiotic activity and improve mineral absorption. Besides offering health benefits when fructas are added to white bread, they also improve their technological properties as texture, volume and color. Inulin type fructans have been extensively studied in bakery; however, there are few studies of agave fructans. The aim of this work was to study the impact of agave fructans addition on technological properties of white bread. White bread formulations containing different proportions of chicory or agave fructans (including 10% or 18%) were developed. White bread without fructan addition was used as control. After baking process, technical properties of breads were analyzed: color, specific volume and texture (hardness, elasticity, cohesiveness, chewiness, resilience). Breads added that contain 18% of agave fructans (Faa18) developed an intense brown color than other breads. In all formulations containing fructans, either, chicory or agave, an important reduction in the specific volume was observed. Hardness, increase depending of the percentage of fructan added, while elasticity was decreased. It is important to note that agave fructans had less negative impact than chicory fructans. However, cohesiveness and chewiness were highly affected when agave fructans were added, mainly in 18% addition. Agave fructans in bakery have interesting features and brownish breads can be obtained, on the other hand, chewiness was increased. We recommend addition of no more than 10% of fructan, because higher concentrations produce a decrement in the cohesiveness, resulting in crumbling breads.

**Key words:** agave fructans, white bread, technological properties, dietary fiber, bakery.

## INTRODUCTION

Fructans are fructose polymers linked with β bonds between their units; it could be linear or branched depending on their source. Inulin type fructans are linear polymers composed of fructose units linked by β(2-1) fructose-fructose glycosidic bonds. Agave fructans are branched polymers with fructose units

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linked by β(2-1) and β(2-6) fructose-fructose glycosidic bonds, and could have an internal or external glucose unit (Lopez et al. 2003). Fructans are considered dietary fiber or prebiotics because human digestive enzymes are not able to hydrolyze them. Fructans can be fermented by colonic microbiota promoting digestive health through short chain fatty acids production, improving the balance between healthy and unhealthy bacteria in the colon (Gibson et al. 2004, Ramnani et al. 2015, Marquez et al. 2013). Besides the digestive health benefits, fructans can also improve the technological properties of food. The benefits that fructans provide to food products depend on the characteristics of the polysaccharide as chemical structure, solubility or degree of polymerization. Branched fructans are more soluble than lineal fructans. Higher degree of polymerization fructans are used as fat replacement, stabilizer, thickener, improving flavor and texture (Roberfroid, 2005; Madrigal and Sangronis, 2007). In the other hand lower degree of polymerization fructans are used as sugar substitute, humectant, improving palatability and favoring Maillard reactions. Inuline type fructans have been extensively used in bakery with a strong impact in dough developing time, rheological characteristics, volume, color, moisture and texture of bread (Hager et al., 2011; Meyer and Peters, 2009). On the other hand, it has been observed that fructan addition on bread formulations decreases cooking times maintaining the aromatic quality (Poinot et al., 2010). Nevertheless there are few research exploring agave branched fructans incorporation into bakery products. This research aims to contribute to this knowledge.

## METHODOLOGY

### Bread formulations and cooking

White bread formulations containing different proportions (10% or 18%) of chicory (*GR* and *HP* from Beneo) or agave (*Olifructine*® from Nutriagaves) fructans were developed. *GR* and *Olifructine*® are raw fructans, while *HP* are mainly long chain fructans. In all formulations, flour was replaced by fructans. White bread without fructan addition was used as control. The same method of preparation was used in all formulations, mixing 20 min, dwell time 30 min and cooking 30 min at 180°C.

### Technological properties

*Color*. The color of the crust and the crumb of different breads was determined using a colorimeter (Color Quest XE, Hunter Lab, USA), which measures the color on flat surfaces. The measuring principle is based on recording the intensity of light absorbed by the black color and reflected by the white color, and the decomposition of light in the red, green, yellow and blue (L*a*b*).
*Specific volume*. The bread volume was determined using the displacement method of millet seed.
*Texture*. The texture analysis was conducted with TPA (texture profile analysis). For which slices of 10 mm thickness were cut with a strain of 50%, preventing the fracture. The transverse arm speed was 5 seconds. The sample was compressed twice to resemble two bites. Area under different curves was determined to evaluate attributes: hardness, elasticity, cohesiveness, chewiness, resilience.

## RESULTS AND DISCUSSION

The effect of fructan addition on color of crumb and crust bread was measured through L parameter in colorimeter, showed a soft effect when 10% of fructans were added. Only GR fructan in crust and chicory ingredients in crumb showed less darkness (L parameter). In contrast, 18% of agave fructan addition (Faa) developed an intense brown color in crust compared with control bread. Bread crumbs added with agave fructans had similar behavior than control, while breads containing chicory

fructans were darker (Fig. 1). Volume is linked to density and softness of bread crumb, fermentation period and interaction of ingredients with gluten. In all formulations containing fructans, both chicory and agave, significant reduction in the specific volume was observed (Fig. 2). The effect of fructan introduction on the texture profile is shown in Table 1. Addition of 18% of fructan caused bad quality bread, crumbly and hard; but in the case of agave fructans the parameter measurement were not possible to do. Resilience showed no differences between control, agave fructan and HP. In the bread added with 10% of fructans, hardness increase only in breads added with chicory ingredients, but breads with agave fructans remain soft like in bread control. Elasticity and cohesiveness decrease in all breads added with fructan ingredients compared with control.

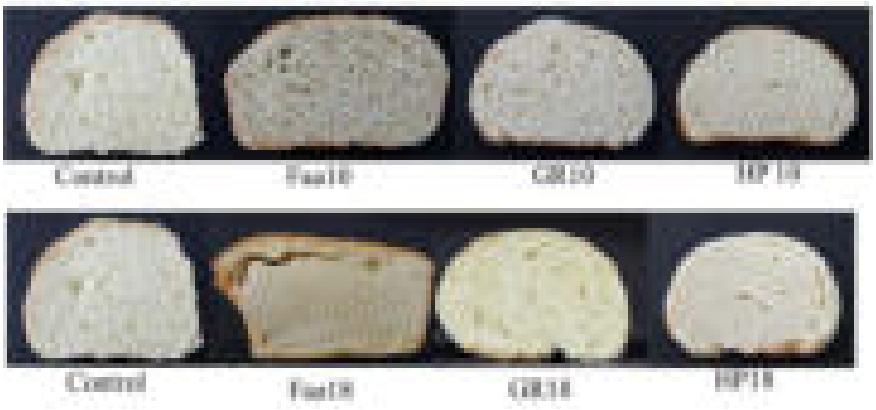


**Figure 1.** Appearance of baked breads. Faa: agave fructans (Olifructine®), GR: Chicory fructans GR, HP: Chicory fructans HP.

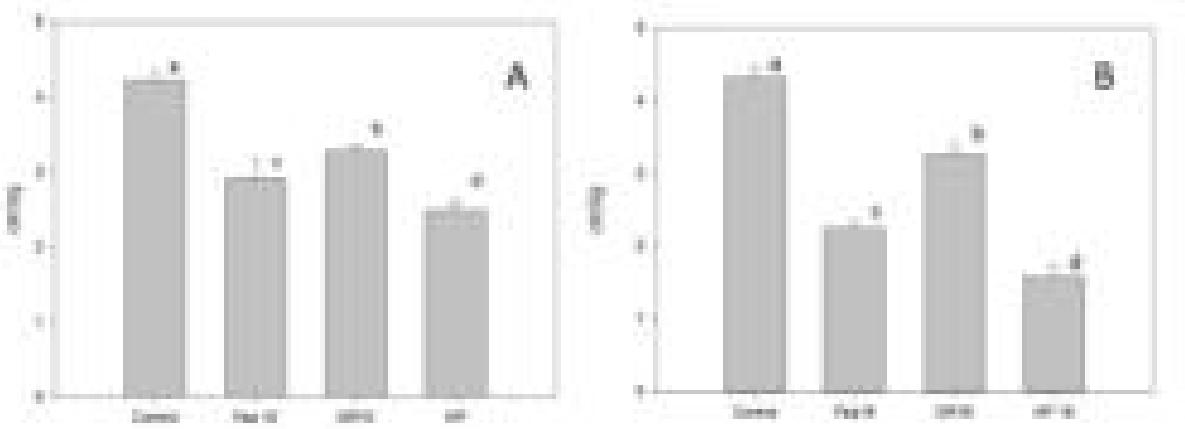
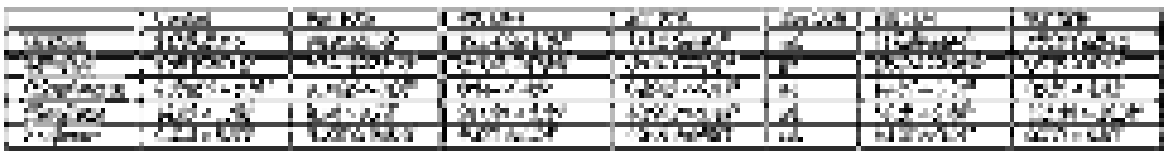



**Figure 2.** Specific volume of baked bread. A: (10% fructans), B: (18% fructans). Faa: agave fructans (Olifructine®), GR: Chicory fructans GR, HP: Chicory fructans HP.

**Table 1.** Texture profile of white breads with agave fructans (Faa) and inulin (GR, HP).



Means within columns that share different letter are statistically significant ($p < 0.05$).

## CONCLUSION

In order to find some bakery product matched with functional food, a couple of formulations with high concentration of fructans were evaluated on white bread. More than 10% of ingredient addition is not recommended because of their negative impact on technological properties. The addition of 10% of frutans from different origin showed increase of hardness, had no effect on resilience and reduce elasticity and cohesiveness. Agave ingredients added at 10% showed no bad impact in hardness and resulted in acceptable volume and quality of bread.

## ACKNOWLEDGEMENTS

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THEMATIC III **Biological effects of Agave fructans and other by-products**

# Effect of fructans on quality parameters of dark chocolate

**Domínguez-Murillo A.C.[1]*, Urías-Silvas J.E.[2]**

## ABSTRACT

The high caloric value of traditional chocolate formulations leads consumers who seek a more balanced diet to avoid it. The first modification of chocolate recipes for nutritional ends was the elimination of sucrose. The rise in diet-related illness has led consumers to take a greater interest in the ingredients used in the manufacture of many sugar-free products. Agave fructans and inulin are functional food ingredient with potential prebiotic activity. The aim of the present study was to obtain a prebiotic dark chocolate and the effects of agave fructans and inulin in the rheological, textural and melting properties were examined and analyzed. The use of agave fructans had statistically significant effects ( $p<0.05$ ) on the rheological properties of the samples, especially in viscosity, showing the highest value, due a strongest inter-particle force. Otherwise, no statistically significant differences were found between samples in the mechanical and molten properties, so it can be deduce that the substitution of sucrose by inulin or agave fructans produces no change in the crystallinity.

**Key words:** Chocolate, agave fructans, inulin, rheology.

## INTRODUCTION

Dark chocolate can be described as a suspension of nonfat particles (sugar and cocoa solids), that shows a non-Newtonian behavior (Beckett, 2008). Foods that contain low-calorie sugar and fat replacers are popular due to a desire for dietary caloric reduction. Depending on the type of chocolate, sucrose constitutes more than 40–50% of solids dispersed in fat and thus, its functional properties including sweetness, stability, particle size distribution, mouthfeel (texture), and its impact on rheological properties of the product are important for chocolate products (Belščak-Cvitanović et al., 2015), therefore low-sugar chocolate with prebiotic properties have been formulated with several different sugar replacements as inulin, polydextrose, and maltodextrin, however, an important point to consider is their functionality in a variety of sugar and/or fat-containing products to obtain

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products with similar quality parameters (Konar et al., 2014).

The aim of this study was to explore the relationship between rheological, textural and melting properties of dark chocolate as influenced by the utilization of agave fructans as sugar replacements and compared with chicory inulin.

## METHODOLOGY

Dark chocolate samples were prepared in a refining-conching machine with 2 rolls and manual tempering. Experimental samples were taken after the refining-conching step. The samples were identified by the letters AF, IN or SA representing agave fructans, inulin or sucrose, respectively.
Rheological properties.

The rheological properties of the chocolate samples were carried out in a controlled stress–strain rheometer, (AR-1000, TA-Instruments, USA) using bob and cup geometry. Each chocolate sample was incubated at 50°C for 75 min, melted, transferred to the rheometer and pre-sheared at a rate of 5.00 $s^{-1}$ for 10 min at 40°C in the rheometer. The shear rate started at 5.0–60 $s^{-1}$ within 120 s, and 50 measurements were taken. The data from the measurements were applied to the Casson model, and related rheological parameters, such as yield stress and viscosity were determined (Konar et al. 2014).

### Melting properties.

Melting properties were carried out in a differential scanning calorimeter (DSC Q200, TA-Instruments, USA) and was calibrated using indium and octa-decane, using an aluminium pan as reference, adopting the modified method reported by Afoakwa et al. (2008). Samples (15-20 mg) were loaded into 40µl capacity pans. Pans were heated at 10°C/min from 15 to 200°C in a N2 stream. Onset temperature ($T_{onset}$), end temperature ($T_{end}$) and enthalpy of melting ($\Delta H$) were calculated for each peak presents in the thermogram obtained. Each sample was analyzed in triplicate and mean values and standard deviations are reported.

### Mechanical properties.

The mechanical properties of the chocolate, such as hardness, were measured using a TA-TXPlus Texture Analyser (Stable Micro Systems, UK) according to the modified method of Konar et al. (2014). A penetration test were performed using a uniaxially cone ($\alpha$ = 40°) at the center of the rectangular piece of chocolate (1.87 x 2.7 cm) at room temperature, to a penetration rate of 1.6 mm/s with a 60% of compression of the original thickness. The results for the hardness (N) are expressed as the mean value of six replicates.

## RESULTS AND DISCUSSION

Chocolate mass exhibited a non-Newtonian behavior, and is observed in fig. 1, in which the apparent viscosity decreases increasing the shear rate, which proves the pseudoplastic or shear thinning nature of chocolate. This behavior can be attributed to the breakdown of the inner structure dispersions; in fact, the increase of shear rate causes the drop in the apparent viscosity of the molecules orientating along the flow lines (Glicerina et al., 2015). Viscosity relates to pumping characteristics, filling of rough surfaces, coating properties and sensory character of body, and is defined as the amount of

energy necessary to maintain a non-Newtonian fluid in movement, while the shear stress is the energy required to start flow, and is related to the inter-particle interaction forces at rest, and both rheological parameters, are given in Table 1. Formulation AF, which differs from SA (control) only in the substitution of sucrose with agave fructans (Table 1), had the highest viscosity. Also this may be result of a more effective crystallization and that increases the inter-particle forces in chocolate containing agave fructans, which requires a greater force to maintain the flow. Viscosity ranged from 2.93 to 3.44 Pa.s and the yield stress from 3.30 to 4.60 Pa, which agrees with the standards for traditional dark chocolate (2-4 Pa.s and 4-32 Pa, respectively) (Beckett 2008).

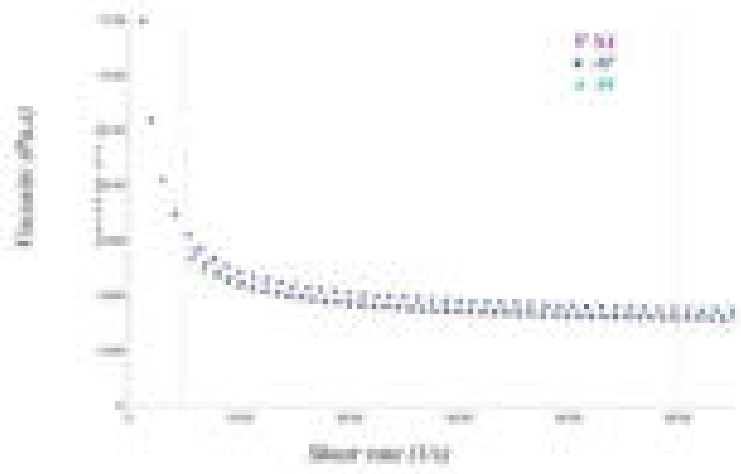

Figure 1. Rheological behavior of dark chocolate samples.

Table 1. Rheological and mechanical properties of dark chocolate.

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Otherwise no statistically significant differences ($p$<0.05) were found between samples in the mechanical properties (Table 1), however the chocolate sample that exhibits the highest hardness was the inulin containing formulation, while the lowest was the one with fructans.

The molten behavior of the dark chocolate samples is presented in fig. 2, also the results of the characterization of the molten properties are presented in the Table 2. No statistically significant differences ($p$<0.05) were found between samples in the molten properties, then deducing that the crystalline form achieved in tempering begins to melt at the same temperature, and therefore the substitution of sucrose by inulin or agave fructans produces no change in the crystallinity and melting properties.

Table 2. Molten properties of dark chocolate.

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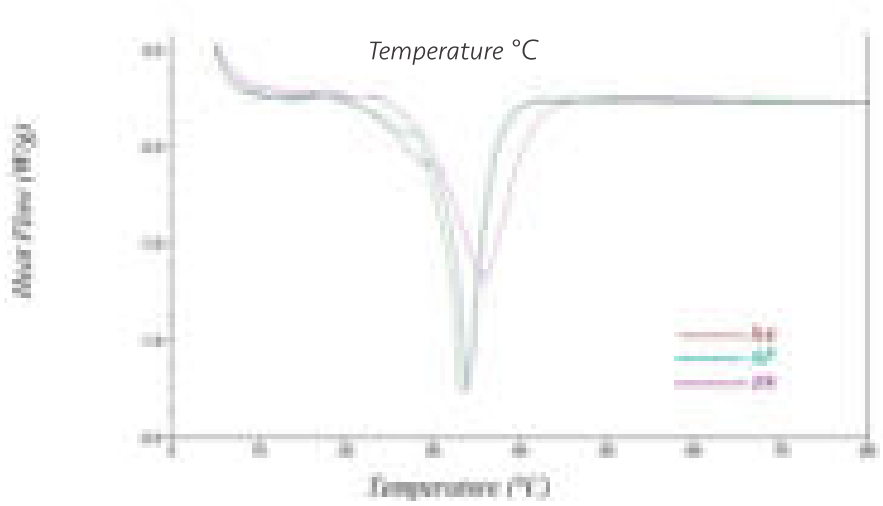


Figure 2. Melting behavior of dark chocolate samples.

## CONCLUSION

With increasing consumer interest in functional foodstuffs and compounds, development studies have been accelerated in this area. However, it is important that the products that are being developed are consistent with the standard products with respect to other quality parameters in addition to their functional features from the point of view of consumers' acceptance level. Therefore, investigating the variations in these parameters with the inclusion of agave fructans as a prebiotic substance could have strategic significance. Chocolate with agave fructans shows the highest viscosity due a strongest inter-particle force, but it was found no significant differences in the mechanical and molten properties, so it can be deduce that the substitution of sucrose by inulin or agave fructans produces no change in the crystallinity or thermal properties.

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THEMATIC III **Biological effects of Agave fructans and other by-products**

# Evolution of sugars from *Agave tequilana* Weber during cooking by using infrared spectroscopy

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## ABSTRACT

In this work, the transformation of fructans to fructose induced by cooking process in samples of cooked agave juice was monitored using FTIR-ATR spectroscopy. The uncooked juice showed a FTIR spectrum with typical groups related to the fructan molecule. The FTIR spectra of the cooked agave juice changed in function of the cooking times, bands related to the partial and total transformations from fructans to fructose. FTIR spectrum of the cooking sample at 48 h presented vibrational groups related only to fructose. In the tequila industry, FTIR-ATR spectroscopy could be an efficient and fast tool for controlling the cooking agave juice process.

**Key words:** agave, fructans, cooking, infrared, FTIR-ATR.

## INTRODUCTION

The elaboration of distilled beverages, involve the fermentation of sugars, principally fructose. However, the sugars content in agave raw are not fermentable, for this reason, it needs a hydrolysis process, commonly by cooking. Agave plants are collected in the field and later peeled. Then, agave heads are cooked in traditional ovens or modern autoclaves during a time period. Next, the cooked agave heads are crushed to extract the juice. Subsequently, these juices are fermented to obtain ethanol. The principal components of uncooked agave's head are water and fructans. The fructans are formed by fructose molecules united by β (2→1) and β (2→6) links and in minor proportion chains of glucose and saccharose (López et al. 2003).

In this work, the transformation of agave juice during the stage of cooking was observed by using the technique of Fourier Transform Infrared (FTIR)-Attenuated Total Reflectance (ATR) spectroscopy.

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FTIR-ATR analysis are fast and needs a small amount of sample (Lohumi et al. 2015). This technique is widely used for the qualitative and quantitative analysis of components in organic samples that uses information from the functional groups of the molecules involved (Stuart, 2004). These groups are obtained from the interaction of the infrared beam with the sample placed on the ATR accessory (Karoui et al. 2010). The aim of this study was to analyze the spectral changes of cooked agave juice samples using FTIR-ATR spectroscopy.

## METHODOLOGY

Agave head samples were cooked in an autoclave during 6, 12, 24, 36 and 48 h at 95°C. The cooked juice was extracted by milling. Spectra from all samples were obtained using a spectrometer CARY 360 (Agilent, USA) equipped with an ATR accessory. Samples were placed on diamond / ZnSe crystal plate of 18 mm diameter and scanned from 4000 to 650 $cm^{-1}$, 20 scans with resolution of 4 $cm^{-1}$ at room temperature (~20ºC). Spectra of the samples were collected and rationed against a background of water and presented in absorbance units versus wavenumber ($cm^{-1}$). Each sample was run in duplicate. Data manipulations were carried out via resolution-pro software (Agilent, USA).
Fructose content was measured in the agave juice samples by high performance liquid chromatography (HPLC).

## RESULTS AND DISCUSSION

Agave juices have two major components: fructans and water. Fructans are a diverse group of polysaccharides that contains two or more β-linked fructose. Fig. 1 shows the infrared spectrum of uncooked agave juice between 4000 and 750 $cm^{-1}$. The spectral region between 1200 and 800 $cm^{-1}$ can be attributed only to signals from the fructan molecule (Grube et al. 2002). The region between 3100 y 2800 $cm^{-1}$ exhibits peaks related to C-H groups. The spectrum show around 3200 $cm^{-1}$ a peak associated with O-H group. Fig. 1 (a) shows only the infrared spectrum in the region from 1300 to 700 $cm^{-1}$. In this region can be observed mainly peaks associated with fructans. At 1025 $cm^{-1}$ appears a high peak, which can be associated to C-C and C-O groups. The peaks around of 1135 and 930 $cm^{-1}$ are attributed to the complex vibration of the C-O-C group and ring vibrational modes in the composition of cycle structure.

Fig. 2 shows the infrared spectra of agave juice in function of the cooking times. In this figure can be observed clearly the gradual disappearance of the peaks originally located in the infrared spectrum of the uncooked juice (rich in fructans) (Fig. 2 (a)). For example, the band located at around 1025 $cm^{-1}$ decrease drastically.

In the spectrum of the cooked sample at 48 h can be observed the total transformation of the fructans to fructose (Figure 2 (f)). The peaks located at 1135, 1025 and 930 $cm^{-1}$ in the fructans spectrum (Figure 2 (a)) practically disappeared. Fructose molecule spectrum presents a principal peak approximately at 1060 $cm^{-1}$, which is related to C-C and C-O groups. In the same figure the peaks located in 835 y 775 $cm^{-1}$ are also related to the fructose molecule (Grube et al. 2002).

Fig. 3 shows the fructose content in cooked agave juice samples. In this figure can be observed that fructose concentration increase in function of the cooking time. These results are consistent with the characteristic of the absorption peaks shown in the obtained infrared spectra (Fig. 2).

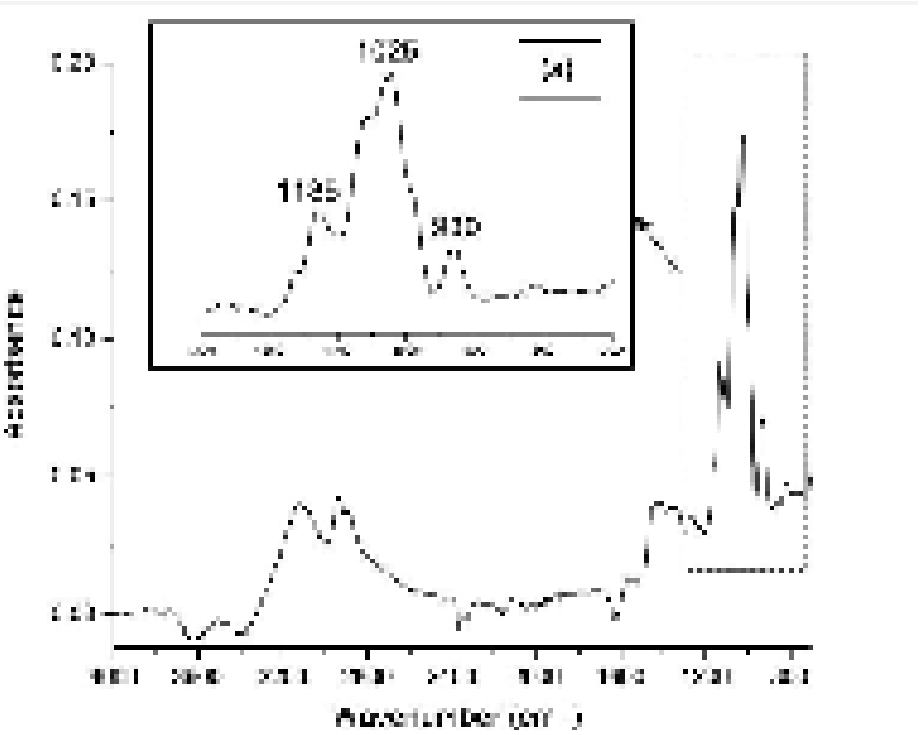


Figure 1. Infrared spectrum of uncooked agave juice. The expanded region (1300-700 $cm^{-1}$) shows the most representative area related to agave fructans.

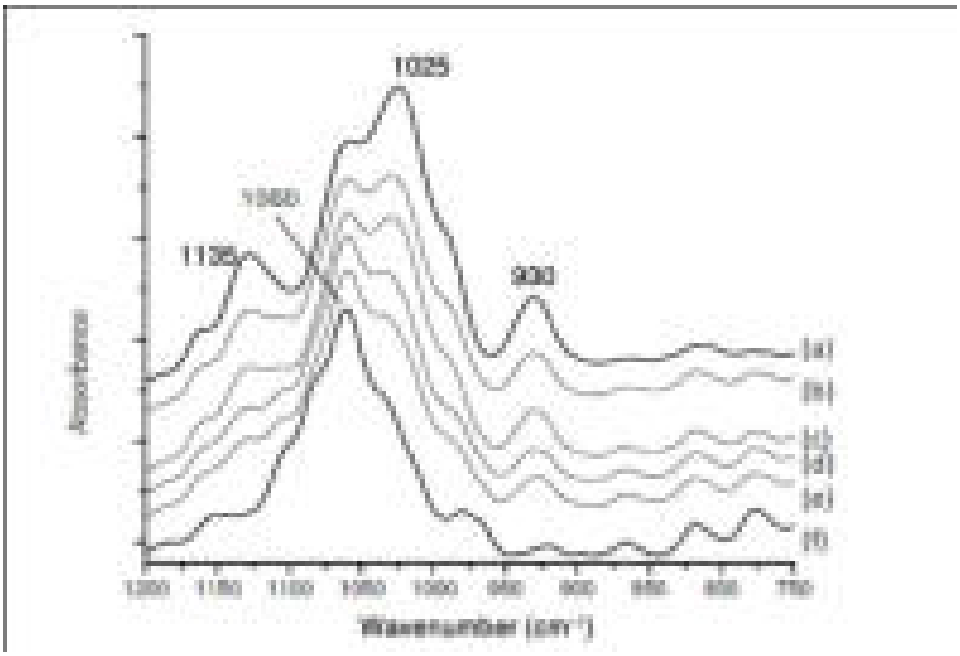


Figure 2. Infrared spectra evolution of cooked agave juice samples. **Figure 2 (a),** uncooked agave juice.

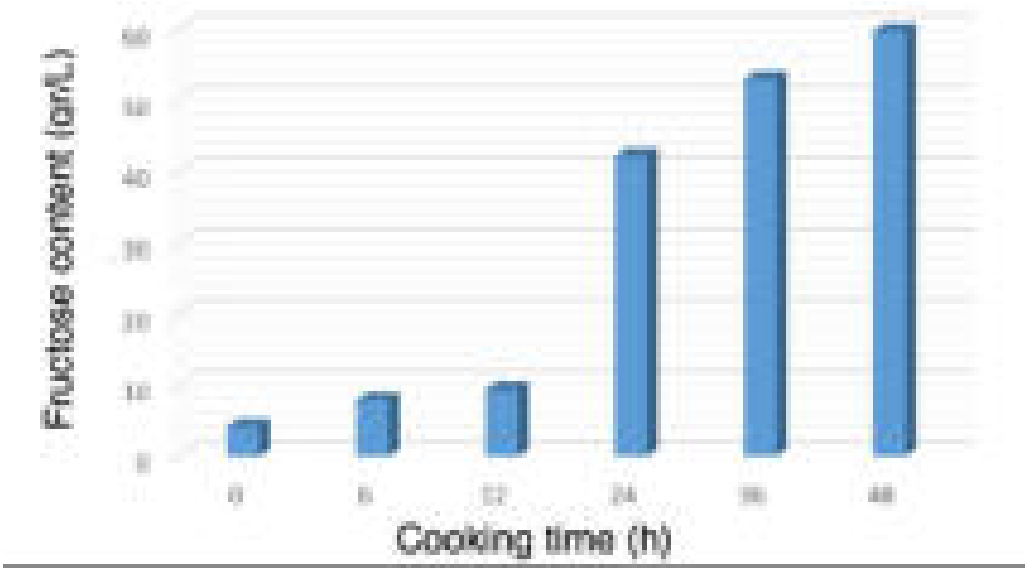


Figure 3. Fructose content in cooked agave juice samples obtained by HPLC

## CONCLUSION

In this work, by using FTIR-ATR spectroscopy the partial or total transformation of fructans to fructose in cooked agave juice samples at different times were observed. The complete transformation of fructans to fructose was observed in cooked agave juice sample after 48 h of cooking at 95°C. FTIR-ATR spectroscopy could be an efficient, flexible and fast tool to control of the cooking process of agave juices.

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THEMATIC III **Biological effects of Agave fructans and other by-products**

# *In silico* analysis of predicted fructanase from a filamentous fungus for agave fructans hydrolysis

**Ordaz E.[1], Camacho-Ruíz R.M.[2], Mateos-Díaz J.C.[3], Rodríguez J.A.[4]***

## ABSTRACT

Agave fructans are water-soluble polysaccharides and they are a rich source of sugars to obtain fermented beverages, functional sweeteners and/or fructose syrups. However, their branched structure limits its hydrolysis with enzymes from several sources. Nevertheless, filamentous fungi (*e.g. A. niger*) are potential sources of exo-inulinases to produce fructose syrups from Agave fructans. In this work, a predicted fructanase from *A. terreus* (ATPF) which, was compared with an exo-inulinase from *A. awamori* (AAEI) through *in silico* docking with three substrates: sucrose, bifurcose and neofructosylnystose. When ATFP was docked with bifurcose and neofructosylnystose (which represents a more challenging hydrolysis such as agave fructans), these were closer by approximately 1 Å from the main catalytic residues in comparison with AAEI, suggesting that ATPF could easily hydrolyze both substrates and therefore agave fructans; however, a biochemical characterization of ATPF is needed to verify these findings.

**Key words:** *Agave fructans, hydrolysis, fructanases, inulinases, filamentous fungi.*

## INTRODUCTION

Fructans are water-soluble fructose polysaccharides that resist gastrointestinal human digestion. They are commonly found in angiosperms, fungi and bacteria (Hendry, 1993). Depending on structural features such as initial trisaccharide and glycosidic bonds, fructans can be classified into, inulins, neo-inulins, levans, neo-levans and neo-fructans. Neo-fructans (Fig. 1), also known as agavins, mainly found in *Agave* spp., are the most complex poly-fructans due to their highly branched structure (Salinas et al., 2016).

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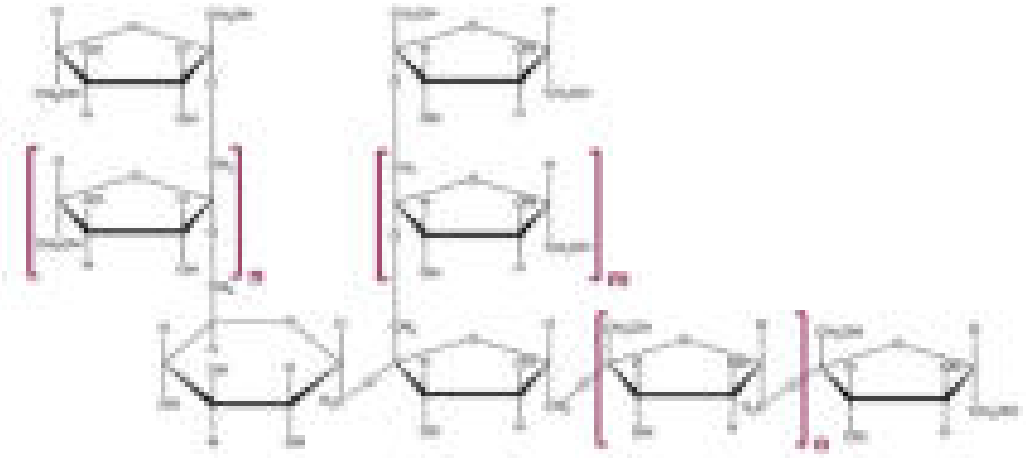

Figure 1. General structure of neo-fructans.

Most fructans can be easily hydrolyzed to obtain fermented beverages, functional sweeteners or fructose syrups. However, in the case of neo-fructans such as agave fructans, their branched structure limits its enzymatic hydrolysis. Because of this, finding endo- and exo-fructanases capable of hydrolyzing Agave fructans has become a goal for researchers and the industry. Exo-inulinases from *A. niger* have been successfully used as effective enzymes for exo-hydrolysis of agave fructans (Huitrón et al., 2013), positioning the filamentous fungi as potential sources of such enzymes.

The 3D structure of *A. niger* exo-inulinase (ANEI) has not been reported yet; however, the 3D structure of an exo-inulinase from *A. awamori* (AAEI) with a 92.3% sequence identity with ANEI has been reported (Nagem et al., 2004). This means that this structure could be used as template for *in silico* analysis to find new potential biocatalysts for enzymatic hydrolysis of agave fructans. The aim of this work was to demonstrate the potential application of a predicted fructanase from *A. terreus* (ATPF) through *in silico* docking in comparison with an exo-inulinase from *A. awamori*.

## METHODOLOGY

Amino acid sequence of ATPF (XP_001216776) was taken from *A. terreus* NIH2624 genome. The protein domains and potential catalytic amino acids were determined using the PROSITE Database of protein domains. ATPF 3D structure was modeled using Swiss-model server (Arnold et al., 2006; Peitsch et al., 1995) based on the known X-ray structure of *A. awamori* exo-inulinase (PDB ID: 1Y9G). *In silico* docking analysis was performed by using AutoDock Vina (Trott and Olson, 2010) and visualized with Pymol Molecular Graphics System. Substrate molecules were built with Avogadro and ChemOffice software.

## RESULTS AND DISCUSSION

ATPF (499 residues) contains the motifs corresponding to the GH32 family (Table 1). These motifs include two aspartate residue and a glutamate residue which are known as the 'catalytic triad' (Lammens et al., 2009). This enzyme has a 39% of sequence identity with AAEI, therefore, it was

possible to use this structure as a template to build the ATPF model. *In silico* docking analysis of both enzymes (Fig. 2) was performed with three substrates: sucrose which is an easily hydrolyzable substrate for these enzymes, bifurcose and neofructosylnystose which represent a more challenging hydrolysis (such as agave fructans).

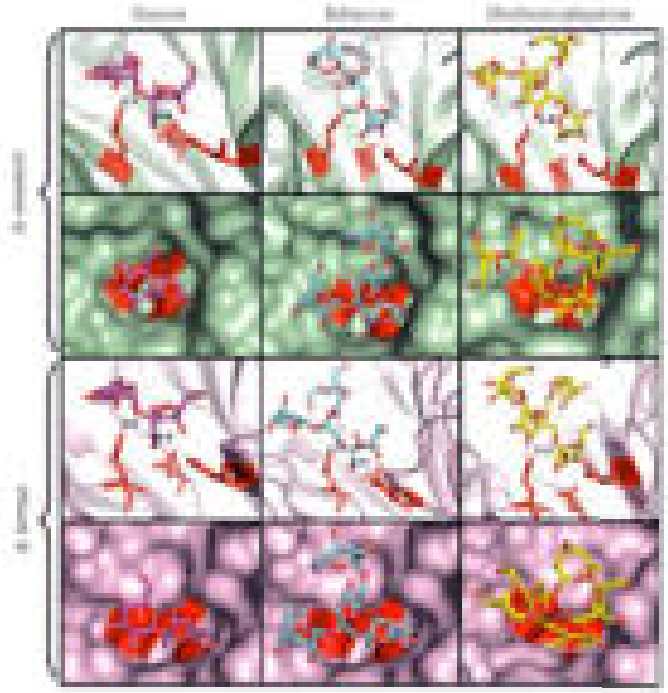

Figure 2. AAEI and ATPF dockings with sucrose (pink), Bifurcose (blue) and Neofructosylnystose (yellow) as substrates. Distance in Å from the catalytic residues and the glycosidic bond are shown in black numbers.

For AAEI and ATPF, the fructosil residue of the sucrose molecule was correctly introduced in their active site cavity with similar coupling energy, suggesting that this substrate can be easily hydrolyzed by both enzymes. When bifurcose and neofructosylnystose were docked with the AAEI, the substrates were farther apart by approximately 1 Å from the main catalytic residues in comparison with ATPF. These results suggest that ATPF could easily hydrolyze bifurcose and neofructosylnystose and therefore agave fructans, in spite of their branched structure. These variations may affect the biochemical properties of the enzyme, such as reaction velocity; however, a biochemical characterization of ATPF is needed to verify these findings.

**Table 1.** Conserved motifs of GH32 family. The active site residues are shown in bold.

## CONCLUSION

In silico docking analysis of predicted fructanase from A. terreus suggests this enzyme could be a potential candidate for the exo-hydrolysis of bifurcose, neofructosylnystose and therefore of agave fructans, however these results must be validated at a biochemical level.

## ACKNOWLEDGEMENTS

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THEMATIC III **Biological effects of Agave fructans and other by-products**

# New dietary supplement from *Agave fourcroydes* in broiler rabbits

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## ABSTRACT

This study was conducted to determine the effects of *Agave fourcroydes* powder as a dietary supplement on the growth performance, gut morphology, serum concentration of IgG and the hematology parameters of broiler rabbits. A total of 32 rabbits [New Zealand × Californian] were weaned at 35 days. They were randomly selected for two dietary treatments (eight repetitions per treatment), which consisted of a basal diet and a basal diet supplemented with 1.5 % dried-stem powder of *A. fourcroydes*. On day 60 from the initiation of treatment, gut histomorphology (duodenum and cecum), serum concentration of IgG and hematology parameters were all measured. The results showed that *A. fourcroydes* powder supplementation improved (P<0.05) the average daily feed intake, Average daily gain and final body weight. Correspondingly, this treatment increased (P<0.05) the muscle and mucosa thickness, height and width of villi. However, duodenum crypts depth was lower (P<0.05) when rabbits were fed with this natural product, compared with the basal diet treatment. Results also indicated that the *A. fourcroydes* powder increased (P<0.05) the serum concentration of IgG, but did not change the hematology parameters. This data indicates that *A. fourcroydes* powder, as a supplement, had beneficial effects on increasing the growth performance and serum concentration of IgG, as well as improving the gut morphology without affecting the hematology parameters in broiler rabbits.

**Key words:** *Agave fourcroydes*, dietary supplementation, rabbit, growth performance, gut integrity.

## INTRODUCTION

Natural products are considered better alternatives to Antibiotic Growth Promoters (AGP), from the point of view of bio-safety and low residue (Martínez et al., 2013). The *Agave* genus, part of the

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Agavaceae family, is native to Mexico. They have been cultivated since the pre-Columbian era for the production of textile fibers, alcohol, molasses, pulp and fodder, as well as for erosion control and soil conservation (García et al., 2015). Specifically, the stem of the *Agave fourcroydes* has a high oligosaccharide content, which forms a poly-disperse mixture (García et al., 2015). In addition, our previous studies have demonstrated the presence of flavonoids, anthocyanins, saponins, coumarins, reducing sugars and tannins in this plant material (Iser, 2016). On the other hand, the dried-stem powder of *Agave tequilana*, which are rich in fructans type inulin, is used as a supplement in the diets of swine, this, enhance immunity, microbiology and intestinal morphology, as well as promote the growth performance and meat quality (Sánchez et al., 2015). There are many chemical benefits of *Agave spp*. for animal feed. However, there have been few studies done on the dietary use of dried-stem powder of *A. fourcroydes* in animal nutrition; especially on the diet of rabbits. Thus, the objective of the current study was to evaluate the effects of dried-stem powder of *Agave fourcroydes* on growth performance, gut morphology, serum concentration of IgG.

## METHODOLOGY

### Animal, housing, treatment and growth performance

A total of 32 rabbits [New Zealand × Californian], that were weaned at 35 days with an initial BW of 768 to 769g, were randomly selected to two dietary treatments. The dietary treatments consisted of a basal diet (BD) and a basal diet supplemented with 1.5% dried-stem powder of *A. fourcroydes*. Feed and water were freely available during the entire experimental period, which lasted for 60 days. The temperature was kept at 22(±2) °C, and relative humidity was maintained between 60 and 65%. BD was prepared according to the nutritional requirements of broiler rabbits (de Blas and Mateos, 2010). The dried-stem powder of *A. fourcroydes* was kindly provided for the study by the Study Center of Animal Production, Faculty of Veterinary Medicine, University of Granma, Cuba. Rabbits were weighed (BW) on days 35 and 95. Feed intake (g/rabbit/day) was measured daily. Average daily gain (ADG), average daily feed intake (ADFI), feed/gain ratio (F/G) and viability were calculated for the period of 1 day to 60 days during the trial. At the end of the experiment, rabbits (one rabbit/pen) were killed to sample gut tissues.

### The analysis of gut morphology

The gastrointestinal tract (GIT) was divided into two segments; duodenum and cecum. Approximately 5 cm of intestinal tissue was cleaved, removed and fixed at 10% formalin in PBS, at 4°C for the histomorphological analysis. After dewaxing, hydrating and staining the tissues with Hematoxylin-Eosin, the thickness of muscle and mucous membrane, the width and depth of the crypts, as well as the height and width of villi of the duodenum and cecum were determined by using an Axiostar microscope (Carl Zeiss, Oberkochen, Germany) connected to a computer with Opti-AnalySIS Basic and soft imaging system software. Images to 500x and 100x were obtained (Jiang et al., 2012). Then, the villus height/crypt depth ratio was calculated.

### Determination of hematology parameters and serum concentration of IgG

Blood samples were collected from the jugular vein of eight rabbits, one rabbit per pen per treatment, on the day of euthanasian at 95 days old. Leukocytes, hemoglobin, hematocrit, total proteins, erythrocytes and platelets were analysed. The Concentration of the Mean Corpuscular Hemoglobin (CMCH), the Mean Corpuscular Hemoglobin (MCH) and the Mean Corpuscular Volume (MCV) were determined by the following formulas: CMCH=Hb (g/100 ml) x100/ (Ht (%); MCH:

(Hgb*10)/leukocytes; MCV: Ht (%)*10)/ (RBCs (millions/mm$^3$). The serum concentration of IgG was determined using an analyser kit with γ-calculating instrument GC-300 (Beijing, China).

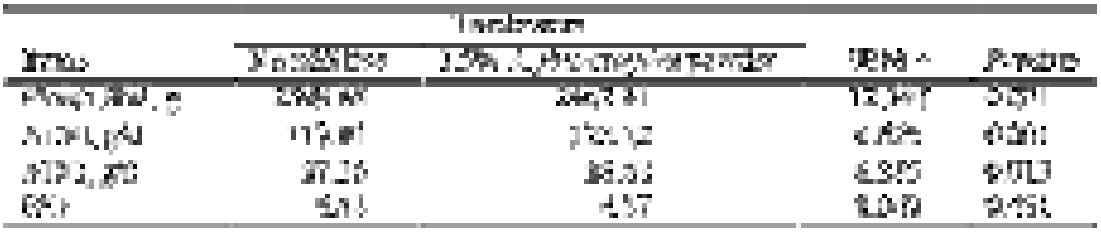
### Statistical analysis

Results are expressed as mean ± SEM. The statistical analysis was performed by unpaired t-test using SPSS 13.0 (SPSS Inc., Chicago, IL, USA). P values \0.05 were taken to indicate significance.

## RESULTS AND DISCUSSION

All rabbits were healthy and grew well throughout the entire experimental period of 60 days. *A. fourcroydes* powder supplementation improved ($P$<0.05) the final BW, ADG and ADFI compared with BD (Table 1). However, F/G did not show significant differences ($P$>0.05) among treatments.

**Table 1.** Effects of dietary supplementation of *Agave fourcroydes* powder on growth performance of broiler rabbits (95 days old).

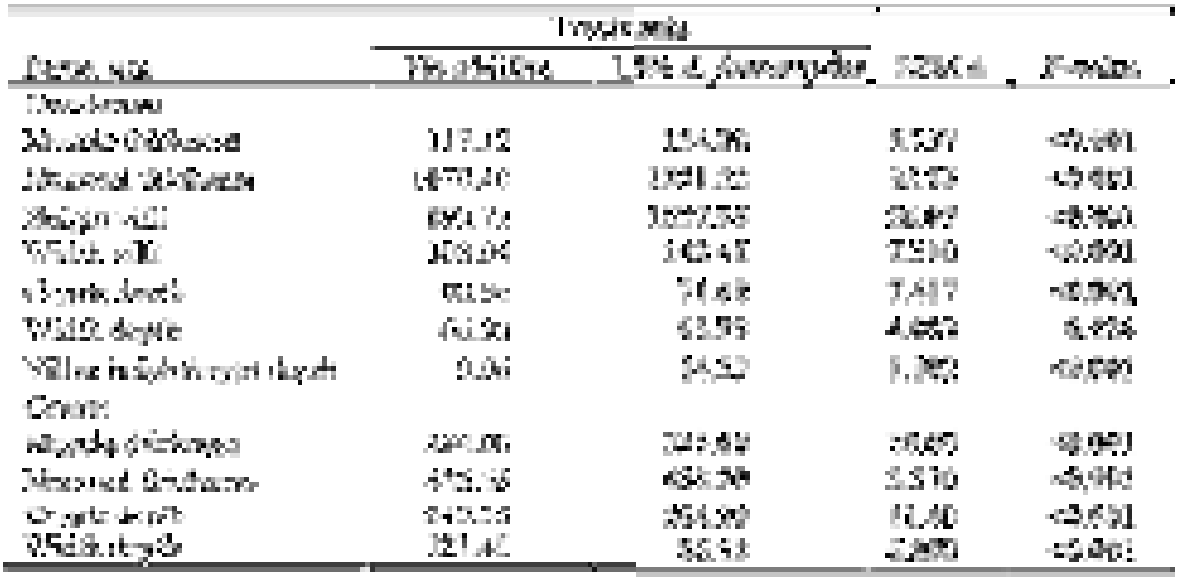


The experiment lasted for 60 days; n=16. BW: Body Weigh, ADFI: Average Daily Feed Intake, ADG: Average Daily Gain, F/G: Feed/Gain ratio.

Fructans, which are recognized as one kind of oligosaccharides presented in the *A. fourcroydes* can be metabolized by microorganisms in the large intestine, which is beneficial for the synthesis of short-chain fatty acids. Thus, *A. fourcroydes* is capable of positively affecting the growth performance by increasing the production of short-chain fatty acids (Falcão-e-Cunha et al., 2007). In rabbits, fructans were reported to increase the population of lactic acid bacteria, particularly *Lactobacillus* spp. and *Bifidobacterium* spp., which provokes a competitive exclusion against pathogenic bacteria in the GIT, as well as a beneficial influence on body weight (Falcão-e-Cunha et al., 2007). Also, this natural product has a high presence of flavonoids, a polyphenolic compound with the ability to inhibit the production of nitric oxide (NO), interleukin-6 (IL-6) and prostaglandin E2 (PGE2) in LPS-induced macrophage cells, which ensures improvement in the absorption of nutrients, subsequently increasing the body weight (Han et al., 2014). Table 2 illustrates the data from the analysis of the gut morphology of broiler rabbits at 95 days old. In the duodenum and cecum, the *Agave fourcroydes* powder increased ($P$<0.05) the muscle and mucosa thickness compared with BD, as well as improve the ($P$<0.05) height and width of villi. However, the duodenum crypts depth of A. fourcroydes-treated group was lower ($P$<0.05) than that of BD group (table 2). Meanwhile, the width and depth of cecum did not show significant differences ($P$>0.05) amongst the treatments. Supplementation of *A. fourcroydes* increases the thickness of muscle and mucosa in broiler rabbits, and subsequently improves intestinal health (Jiang et al., 2012). The improvement of an intestinal barrier function, though modulating the intestinal microbial community diversity, is mostly beneficial for the

animal's health. Our results are consistent with these findings, as it was observed that *A. fourcroydes* powder supplementation (1.5 %) increased ($P<0.05$) the height and width of the villi, due to suitable intestinal conditions. These conditions include higher proliferation of lactic acid bacteria, decrease of cecum pH (Iser, 2016), as well as thickening of the intestinal mucosa, suggesting that there may indeed be an association between the intestinal health status and the absorptive capacity.

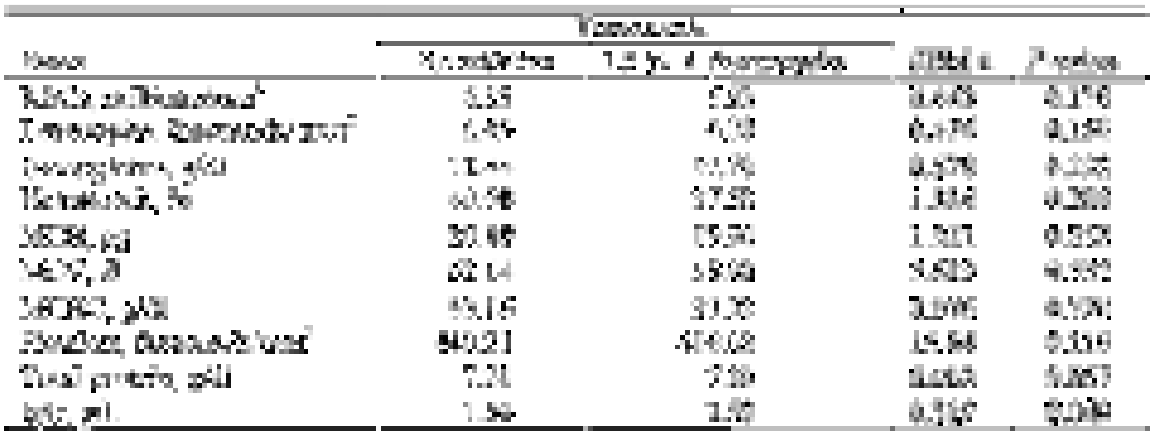
Table 2. Effects of dietary supplementation of *Agave fourcroydes* powder on gut morphology of broiler rabbits.



The experiment lasted 60 days; n=8.

Furthermore, rabbits from the BD group had a higher width and depth of crypts than those from the *A. fourcroydes* group. It is reported that the migration of specialised cells to the villi, especially with the decrease of villus height, would elevate the depth of crypts (Jiang et al., 2012). *A. fourcroydes* powder did not influence ($P>0.05$) the hematology parameters of broiler rabbits according to Table 3. In addition, the serum concentration of IgG was higher ($P<0.05$) when rabbits were fed with the *A. fourcroydes* powder as feed additives compared with the BD group. After the 60-day treatment, it was found that *A. fourcroydes* powder, with high fructan concentrations and secondary metabolites, did not cause adverse symptoms or diminish the defenses (white blood cells) in rabbits. The amount of Serum antibody is an indicator of humoral immunity in all mammals. The rabbits can generate abundant antibodies or proteins and promote the proliferation of B lymphocytes to defend against any parasitic or pathogenic infections. Curiously, IgG represents about 80% of the immunoglobulins in serum, which participated in humoral immunity against bacteria and pathogens (Gong et al., 2014). Thus, *A. fourcroydes* powder supplementation may improve antitoxic and antibacterial immune responses through the elevation of serum concentration ($P<0.05$) of IgG in rabbits. Several studies have shown that certain functional foods can improve the phagocytic activity of the intestinal leukocytes, as well as promote the proliferation of leukocytes B and secretion of immunoglobulins A and G. Other studies on feed additives in non-ruminants have also found similar results when animals were fed with foods rich in secondary metabolites (Gong et al., 2014).

Table 3. Effects of dietary supplementation of *Agave fourcroydes* powder on hematology parameters and concentration of IgG of broiler rabbits.



The experiment lasted for 60 days; n=8.
MCH: Mean Corpuscular Hemoglobin; MCV: Mean Corpuscular Volume; MCHC: Mean Corpuscular Hemoglobin concentration.

## CONCLUSION

In summary, feeding with 1.5% of *A. fourcroydes* powder improved the growth performance, as well as the serum concentration of IgG and gut morphology in broiler rabbits. Supplementation of this product did not affect the hematology parameters, suggesting that it can be used safely as a food additive at a dose of 1.5% for broiler rabbits.

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THEMATIC III **Biological effects of Agave fructans and other by-products**

# Yield and composition of *Agave salmiana* Otto ex Salm-Dick y *A. tequilana* F.A.C. Weber fructans

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## ABSTRACT

Production, local use and export of *Agave tequilana* F.A.C. Weber (AT) fructans has recently grown significantly. However, their classification as GRAS substance is still in progress by the FDA of USA. So, still needs much to standardize its raw material to control variation in yields, composition and purity. Therefore, the aim of this study was to compare the yield and composition of fructans extracted from stems individuals in full physiological maturity, "quiotillos" for *Agave salmiana* Otto ex Salm-Dick (AS) and "novillos" AT. Each individual was sectioned into leaf bases and stem and measured its dry matter, and the fructans concentrate (FC) of each stem was extracted and humidity carbohydrate composition and ash were mesured. In both species the proportion of FC recovered in the primary, secondary and tertiary juices was similar ($p < 0.05$); quaternary only was higher in AT. The total average yield of FC of a stem of AS is almost a third of AT, although its concentration in the stem dry matter was similar in both species ($p < 0.05$). In both species, more than 80% of FC is composed mainly by fructans of higher than five polymerization degrees. The ash content was higher in AS ($p < 0.05$) than AT, and are mainly composed of Ca, in the form of oxalate crystals type raphides insoluble in water. These crystals are larger ($p < 0.05$) in AS than AT, but similar in thickness ($p < 0.05$). These results are relevant to scaling, design and process optimization in industrial level.

**Key words:** *Agave salmiana, A. tequilana, fructans*, composition, yield, raphides.

## INTRODUCTION

Production, local use and export of fructans of tequila maguey (*Agave tequilana* F.A.C. Weber) (AT) has recently grown tremendously (Godinez *et al.* 2016b) and together with syrup production derived from them can help reduce cyclical swings in this maguey market. For the production of fructan erroneously the head or pineapple maguey (formed by the stem and the base of the leaves)

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is still using as raw material, instead of taking only the stem where fructans are stored (Aguirre *et al*., 2001). In addition, by incorporating, the bases of the leaves extracts obtained are contaminated with various compounds (chlorophylls, waxes, saponins and sugars) that have to be cleared, and the harvest of this maguey usually is based on the vague chronological age. Fructans of this maguey cataloging as GRAS substances is in progress by FDA of USA (Anonymous, 2015). However, there is still need to standardize the raw material, to control the variation in yield, composition and purity of these fructans, which depends on the physiological maturity of the harvested plants and the plant structure used as raw material for this purpose. For *Agave salmiana* Otto ex Salm-Dick (AS), with potential in this respect for its largest natural distribution in the country (Aguirre *et al*., 2001) we have advanced the standardization of its raw material, using only the stem, instead of the traditional head or pineapple and plants in full physiological maturity (Godinez *et al*., 2016b). This stage is known in the field as maguey "quiotillo" (i.e little flowering stalk) for the case of AS, and maguey "novillo" (i.e. steer) for AT. These stages of physiological maturity are recognized in the field by the thinning of the bud and that the tips of its last pencas to deploy are below the apex of the already deployed; that is, this is before the appearance of flower stalk stage involving consumption of stored fructans (Aguirre *et al*., 2001). Thus, the objective of this study was to compare the yields and composition of the fructan extracted from stems of quiotillos or novillos individuals of these two species.

## METHODOLOGY

Five heads of AS were selected at quiotillo stage in Charcas, San Luis Potosi, and five AT at novillo stage AT in the municipality of Arandas, Jalisco, prepared in the usual way in both cases. They were weighed and sectioned in stem and leaf bases; each section was weighed and processed separately; a subsample of each stem and leaf bases was taken and dried at 60 ° C / 48 h (Shel-Lab FX-14, USA) to constant weight (Ohaus 5120, USA) for measure its moisture. For the extraction of fructans another subsample of each stem was taken and fructans were extracted according to the methodology that we have developed (Godinez *et al*., 2016b). The yield was calculated according to the amount of fructans concentrate (FC) obtained, and referred to the dry and fresh weight of each subsample. Carbohydrate composition by HPLC (HP-1100 series Agilent Technologies, Germany) was assessed with the methodology of Zuleta and Sambucetti (2001) modified. The ash content was obtained by incineration in muffle (Sartorius BP221S, Germany) and composition of macro elements by ICP-OES (Thermo Scientific iCAP 7000 Series, USA) and micro elements by ICP-MS (Therrno Scientific X - Series 2, USA) at the Institute of Geology of the UASLP. The average size of raphides (crystals of calcium oxalate) present in the stem raw juices was obtained from photomicrographs taken with a camera and an optical microscope (Leica DM2000, USA), and with the help of software ImageJ 1.50 and a reference scale. The mean values, and standard deviation (SD) were calculated and compared using a t-test.

## RESULTS AND DISCUSSION

In both species the proportion of FC obtained from primary, secondary and tertiary juices (PJ, SJ and TJ) of the extraction process was similar, except for the quaternary (QJ), which was higher ($p < 0.05$) in AS than AT (Table 1); so, with two washes of bagasse (SJ and TJ) most FC is extracted in both species. The average yield of FC of a stem of AS is almost a third of AT, although its concentration in the stem dry matter was similar in both species (Table 2). In both species, without differences ($p < 0.05$),

the FC contains mainly fructans higher than five polymerization degrees, and similar 1-kestosa and sucrose content, but the content of nystose, glucose and fructose was different ($p<0.05$) (Table 3). Also, according to Mancilla and López (2006), the content of fructans in AT dry basis is about 73%, and little more than 15% the amount of fructose, glucose and sucrose.

Table 1. Average ratio (%) of FC recovered during the extraction process

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Table 2. Average weight (kg) of fresh (SFW) and dry (SDW) stem of *A. salmiana* and *A. tequilana*, ratio (%) pineapple/stem fresh (RFS) and dry (RDS), and FC total (TFC) and relative (RFC) in the dry matter of the stem.

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These values contrast with 87.0% and 4.7%, respectively, found in this work for the same species, which is probably due to differences in physiological ripeness, extraction method and the structure used as raw material. Instead for AS we confirmed previous results by Godinez *et al*. (2016).

Table 3. Composition (%) of FC of *Agave salmiana* and *A. tequilana*

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The FC ash content of AS was higher (5.16%) than AT (1.77%) and is mainly composed of Ca, K, S, Mg, Na, Si, P and Sr (75.8, 9.4, 7.6, 2.2, 1.7, 1.2, 0.9 and 0.4%, respectively) and the ashes of AT of Ca, S, K, Mg, P, Si, Na, Sr, Ba and Zn (51.9, 15.0, 10.6, 9.1, 4.4, 4.1, 1.9 , 0.6, 0.6 and 0.6%, respectively). Calcium is as crystals of calcium oxalate (raphides), whose length was greater (315.12 ± 32.93µm) in AS than

in AT (192.57 ± 29.82µm), but were similar in thickness (6.08 ± 1.33 and 4.56 ± 1.01 µm, respectively). These raphides type III (hex section), insoluble in water, are present naturally in species of *Agave* (Raman *et al*., 2014.); their presence in the raw juices of maguey stem is little known, although being insoluble in water and resistant to high temperatures, it is likely to persist until the distillation of maguey spirits. In both species raphides tips are about 1 micron in diameter, which facilitates puncture and death of yeast by contact of saponins or other enzymes of the maguey. The larger size of the raphides and saponins content of AS may explain the greater pungent effect of their raw "guishes" on skin (Salinas *et al*., 2001), and the inhibition of alcoholic fermentation (Zamora *et a*l., 2010). However, these insoluble in water raphides can be separated mechanically (León, 2000) by centrifugation of raw or cooked maguey juices.

## CONCLUSION

Knowledge of each of the components of the raw material used for the extraction of fructans and for the production of maguey spirits is necessary as background to support scaling, design and optimization of processes at industrial.

## ACKNOWLEDGEMENTS

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THEMATIC IV **Industrial processing of Agave wastes and subproducts**

# Agave fiber (*A. salmiana*). Proposal for industrial Eco utility

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## ABSTRACT

In Mexico, only 45% of the weight of *Agave salmiana* is used for industrial purposes (pineapple to pulque-mezcal and pulpa); the remains are generally disposed in open areas, leading to serious environmental problems. In the southeastern of Guanajuato, this specie is widely distributed and it is mostly used as ornament due to the lack of another practical application. The objective of this study, was to investigate the physical and chemical characteristics of the structural fiber of adult leaves from the agave and, therefore, offer alternatives for industrial applications. Some of these properties include mechanical strength, preset chemical attack and water and oil retention.

**Key words:** Hard fiber, structural fiber, maguey, agave, *Agave salmiana*

## INTRODUCTION

Mexico is very biodiverse, containing one of the most fibrous vegetables, the agave. However, its exploitation is limited to the production of tequila and mezcal drinks. Only 45% of the weight of *Agave salmiana* (pineapples to pulque-mezcal and pulpa) is used for industrial purposes; the remains are generally disposed in open areas, leading to serious environmental problems (Silva and Caballero, 2004; Parra-Negrete *et al*., 2010). Agavacea family produces an average of at 300 leaves by plant. *A. salmiana*, is one of them, presenting the widest morphological variability and the greatest diversity of environments. After taking advantage of the agave pineapple to produce "pulque", the remanent of the plant (leaves) is not further used.

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## METHODOLOGY

The adult leaves were harvested and peeled by mechanical friction (Maiti, 1995). An instrument formed by two wooden beams in an 45° angle were used as the basis, while another one was used to do mechanical friction on the vegetal material, this is shown in fig. 1. After, the fiber is dried at room temperature.

Figure 1. Agave´s fiber (*Agave salmiana*). Methodology for obtention fiber by mechanical friction.

About the mechanical characterization, rupture-weight was tested (NMX-A-069-1990) on fiber twisted manually, using 3 mm of caliber. Other adicional tests to the fibers, were employed cleaning agents used in the textile industry, they were exposed to chemical agents such as NaCl, $CH_3COOH$ and NaOH at three different concentrations during 20h at 25°C. Finally, the capabilities for water and oil retention were evaluated, with methodology of dietary fiber.

## RESULTS AND DISCUSSION

The methodology for obtention of agave´s fiber by mechanical friction, produces a final product, if desired. It has one straightening good, as appears in fig. 2.

Figure 2. Agave´s fiber (*Agave salmiana*). Final product no cleaning in "jarcias" form.

The fibers without treatment, and according with the specifications for henequen agave provided by the company CORDEMEX©, showed a low tensile strength with a value of 3.752 ± 0.79 kg per 3 mm jarcia. However, this can be improved by wetting or lubricating the fibers and a greater braided, which increase the lineal density. Chemical degradation assay showed that the agave samples are sensitive to NaOH (2.5, 5.0 and 10.0%); Fig. 3A shows the appearance before and fig. 3B hydrolyzate appearance after treatment. However, presented high resistance to NaCl at all concentrations tested (20 and 40%), see fig. 3D and 3E. Chávez & Domine (2013), which explained that chemical degradation, occurs in the ligning-carbohydrates bonds, and thus causing the permanent loss of mechanical properties. Finally, the liquid absorption assays for water and oil retention showed results of 813.1± 37.92% and 69.23 ± 13.69% respectively, like the highly demanded fiber from bamboo.

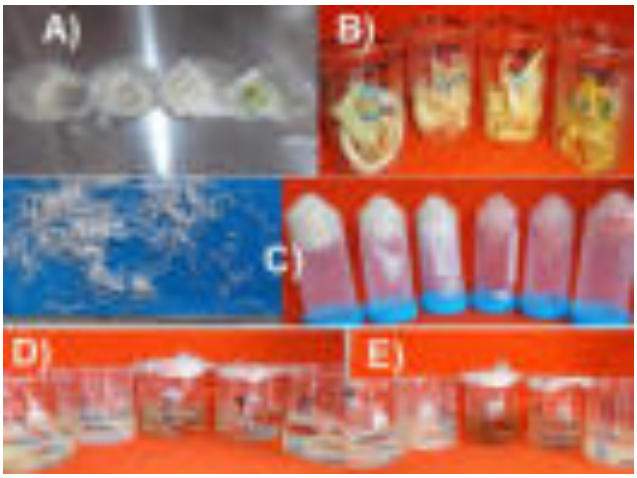


Figure 3. Chemistry and physical tests in the fiber (Agave salmiana). A) y B) Fiber before and after attack at different concentrations of NaOH, C) Appearance and granulometry (0.42-1.42mm) of the fiber employed in the determinations. Test to determine the water retention capacity and retention capacity of organic substances (edible oil), D) Fiber before and E) After to attack by salt (NaCl) and acid (HCl) at differents concentrations.

## CONCLUSION

The high liquid retention capabilities of the fiber from adults leaves of *A. salmiana*, allowed to suggest its application in eco-products as diapers, dietary fiber or as automotive oil filters.

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THEMATIC IV Industrial processing of Agave wastes and subproducts

# Evaluation of tequila vinasses as a substrate for the fermentative production of *Trichoderma harzianum* inhibitors against the phytopathogen *Phytophthora infestans*

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## ABSTRACT

Tequila vinasses are produced during tequila distillation, they contain essential nutrients that may be used for the formulation of economic culture media and nonetheless tequila vinasses are toxic for several microorganisms. Vinasses from different tequila production processes, such as masonry oven, autoclave and diffuser were used to formulate a culture media which was compared with the Weidling medium (WM) for the production of *Trichoderma harzianum* inhibitors against the phytopathogen *Phytophthora infestans*.

Reformulated fermented and unfermented vinasses showed more than 3.5 times inhibition of *P. infestans* growth. Interestingly, fermented and unfermented vinasses showed a higher inhibition than the fermented WM, suggesting that tequila vinasses could be an interesting substrate for the fermentative production of *Trichoderma harzianum* inhibitors against the phytopathogen *Phytophthora infestans*.

**Keywords:** Tequila Vinasses, *Trichoderma harzianum*, Phytopathogen, *Phytophthora infestans*.

## INTRODUCTION

Vinasses are produced during ethanol distillation, and they are also known as must, thin stillage, distillery wastewater and distillery slop (Kuusisto, 2013). These kind of wastes are dark brown colored because of the presence of melonoidins, have a low pH, between 3 to 5, and a high biochemical oxygen demand (BOD) and chemical oxygen demand (COD), ranging from 35,000 to 50,000 mg and 100,000 to 150,000 mg, respectively. On the other hand, vinasses contain many essential nutrients

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which may be used for the formulation of culture media for the growth of microorganisms, however vinasses are toxic and recalcitrant for many of them (Robles González et al. 2012).

When such agroindustrial wastes are not correctly treated they can be very hazardous when disposed to the environment, because they are a source of possible diseases which can damage humans, animals and other natural resources (Saval, 2012). For example in the Tequila industry, each liter of Tequila produced, generates 1.4 kg of bagasse and 10–12 l of vinasses (López-López et al. 2010). Previous research revealed the efficacy of biofumigation for the control of phytopathogenic fungi and nematodes, using sugar beet, sugar cane and wine vinasses, (Santos et al. 2008). In another study, the actinobacterium *Streptomyces sp*. MC1 was very effective for the vinasses treatment; this strain was able to grow at very high vinasse concentrations (until 50% v/v) and remove over 50% of the biodegradable organic matter in only 4 days (Colin et al. 2016). This works suggest an alternative use of raw vinasses as a substrate for the production of high value metabolites of biotechnological interest, such as bioemulsifiers as well as the possibility of growing microorganisms in loud wastewater concentrations.

*Trichoderma harzianum* is the most commonly used fungus for many agricultural applications, including biological control of plant diseases. This microorganism has been largely studied because its well-known produced antifungal secondary metabolites as well as other signaling molecules that positively affect the metabolism of the host plant, protecting it against pathogenic micro- and macro-organisms (Vinale et al. 2013). For example harzianic acid is a *Trichoderma* secondary metabolite which shows antifungal and plant growth promotion activities. In our research group, the weidling medium has been successfully used for grow and production of *T. harzianum* secondary metabolites, however low cost agrowaste based culture media are needed in order to be able to scale and transfer a feasible technology. Thus the aim of this work was to evaluate vinasses from the tequila industry as a possible substrate for the fermentative production of *Trichoderma harzianum* inhibitors against the phytopathogen *Phytophthora infestans*.

## METHODOLOGY

### Microbial strain

*Trichoderma harzianum* was maintained on potato dextrose agar (PDA; SIGMA) slants at room temperature and subcultured bimonthly. This strain has shown an effective biocontrol agent of several soilborne plant diseases, including *Phytophthora infestans*.

### Vinasses characterization

Tequila vinasses from different processes such as masonry oven, autoclave and diffuser were used. Each tequila vinasse was took immediately after discharge and frozen at -20°C until use. Vinasses were filtered and centrifuged to lower non soluble solids. The nutrients as N, Ca, Mg, Mn, K, Cu, Zn, Fe, Ni, P, sugars and pH were measured every 24h.

### Fermentation

The tequila vinasses medium was prepared by adding nutrients as necessary. Fermentations were performed in 250 mL Erlenmeyer flasks. The pH of the medium was adjusted to 4.0 before autoclave sterilization (121°C/15 min). Flasks were inoculated with 1 mL of a conidial suspension containing $10^6$ conidia/mL. The cultures were incubated on a rotary shaker (120 rpm) at 28°C during 7 days. Three

flasks were sampled each day. Glucose consumption was measured using DNS (3,5-Dinitrosalicylic acid) at 540 nm (Miller, 1959). Biomass was spectrophotometrically estimated by measuring the optical density at 600nm. Secondary metabolites were obtained after clarification by centrifugation of a 7 day fermented vinasse culture. The fermented weidling medium using identical culture conditions was used as control.

### Phytopathogen inhibition

*Phytophthora infestans* was cultured at 20°C in Petri dishes with PGA (PGA: potato glucose agar; absolute control) containing fermented and unfermented tequila vinasses and weidling media (positive control). Growth was measured every 24 hours during 7 days.

## RESULTS AND DISCUSSION

Tequila vinasses from different processes such as masonry oven, autoclave and diffuser, were inoculated with *Trichoderma harzianum*, and the potential of the corresponding fermented vinasses as growth inhibitors of the phytopathogen *Phytophthora infestans* was compared. All tequila vinasses were good substrates for *T. harzianum* growth, but the tequila vinasses obtained from the oven 1 showed the best performance. While the weidling medium showed the worst performance for *T. harzianum* growth as shown in fig. 1, however this medium is one of the most used for growing *T. harzianum* and producing secondary metabolites. All media tested needed almost 4 days to promote the maximum fungal growth.

When the unfermented and fermented tequila vinasses (f) were used to evaluate the *Phytophthora infestans* growth inhibition, a higher inhibition was observed using tequila unfermented vinasses (Fig. 2). These results can be probably explained by the high content of toxic compounds in non-treated vinasses such as furans and saponins, thata are well known microorganism inhibitors (España-Gamboa et al., 2011). Otherwise, all tequila vinasses exhibited a better P*hytophthora infestans* growth inhibition capacity than the weidling fermented medium. F-Oven 1 and F-Diffuser were the most interesting fermented tequila vinasses in terms of *P. infestans* growth inhibition (table 1).

In 2008 Santos et al. observed the biocide effect of three agroindustrial byproducts; sugar beet, sugar cane and wine vinasses. They mentioned from *in vitro* assays showed that wine vinasse has 100% capacity to suppress fungal growth at concentrations between 5% to 7% for *Fusarium oxysporum, Sclerotinia sclerotiorum, Pythium aphanidermatum* and *Phytophthora parasitica* in melonis; and was necessary 10% to 15% for *F. oxysporum* f.sp. in radicis-cucumerinum (Santos et al. 2008). In the present work, it is confirmed that the vinasses has a potential as substrate for the phytopathogen inhibitor production and the best results using unfermented vinasses achieving until full inhibition.

## CONCLUSION

Tequila stillage can be used to grow microorganisms of industrial interest. Diffuser vinasses and oven 1were better substrates for growing *Trichoderma harzanium*. Both fermented and unfermented tequila vinasses showed greater *P. infestans* growth inhibition than the fermented weidling medium. Tequila vinasses are an interesting substrate for the fermentative production of *Trichoderma harzianum* inhibitors against the phytopathogen *Phytophthora infestans*.

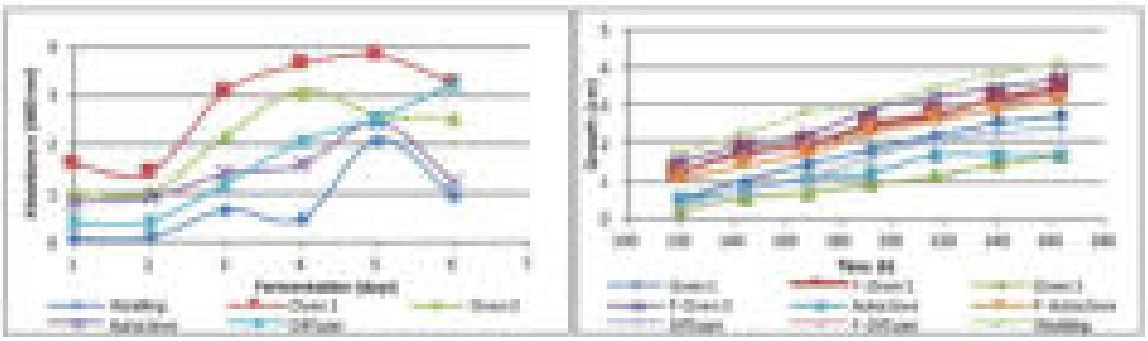
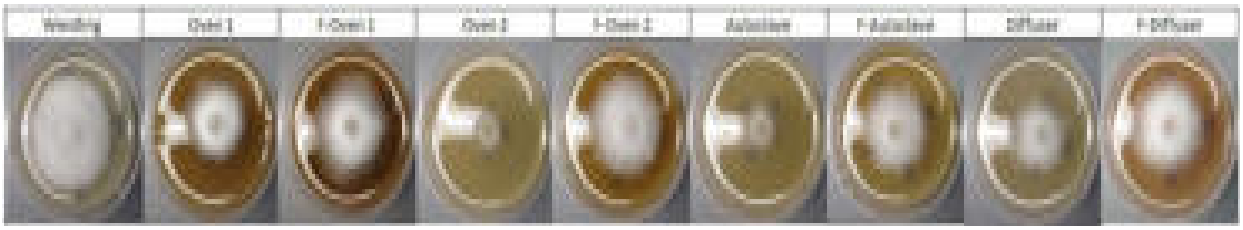


Figure 1A. *Trichoderma harzianum* growth in tequila vinasses. B. Inhibition comparison of *Phytophthora infestans* (F=Fermented).

Table 1. *Phytophthora infestans* growth in PGA medium with fermented and unfermented vinasses after 264 hours.



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THEMATIC IV **Industrial processing of Agave wastes and subproducts**

# Identification of volatile compounds found in Tequila vinasses

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## ABSTRACT

Samples of vinasse (stillage) obtained from two different Tequila production processes were analyzed to identify the presence of some volatile compounds considered as inhibitory compounds for dark fermentative hydrogen production. Volatile compounds were extracted with a liquid-liquid extraction method using different solvents. The extracts were analyzed by gas chromatography coupled to a mass-selective detector. A total of approximately 110 compounds ($p<0.05$) were identified in the extracts obtained with a mixture of pentane-dichloromethane 3:1 (v/v). Principal component analysis (PCA) made it possible to define two groups belonging to different production processes. Cooking (C) and raw (NC) agave stems processes were separated mostly by PC1. The presence of some reported inhibitory compounds such as furans (furfural, 5-hydroxymethylfufural), organic acids (butyric acid, propionic acid) and phenols (guaiacol, 2,6-dimethylphenol) were found in the vinasses from agave stem cooking process. Therefore, tequila vinasses obtained from processes in which the agave stem is not cooked would be more viable as a substrate for biohydrogen production, due to the reduced presence of potential inhibitory compounds.

**Key words:** Agave, stillage, furans, phenols, furfural, organic acids.

## INTRODUCTION

Tequila is one of the most consumed alcoholic beverages worldwide. It is obtained from the fermentation of hydrolysable sugars from the stems of *Agave tequilana* Weber var. Azul (Bautista-Justo *et al*. 2001; López-López et al. 2010). Distilleries for tequila production use similar processes, with relatively few modifications in the hydrolysis process (Salgado, 2012).

Sugars from raw Agave plant require a hydrolysis, in order to transform complex sugars (fructans)

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to fermentable sugars (fructose and glucose), which can be metabolized by yeast for alcoholic fermentation (Valenzuela, 2003). Currently there are different processes used to accomplish sugar hydrolysis.

The traditional process consists in placing the raw agave stems in brick ovens or autoclaves for stem injection (0.5-1.4 kg/cm$^2$) at 80-120 °C for approximately 48 hours or 8-10 hours, respectively (Martínez and Pérez, 2008; Peréz *et al*. 2015). Once the agave stems are cooked, they are placed on conveyer belts to a mill with rotatory knives where they are shredded and washed under pressure with potable water to dissolve the sugars. Finally, the agave stems are introduced to a press where the agave juice is extracted (Valenzuela-Zapata, 1995; Salgado, 2012).

Nowadays another way to obtain agave juice directly from raw agave stems is being implemented due to tequila's process optimization (Salgado, 2012). The juice is obtained either by expeller pressing or lixiviation extraction (solid-liquid extraction) through the physical phenomenom known as diffusion (using diffuser equipment), collecting fructans before hydrolysis, which is done subsequently (Pérez *et al*. 2015).

The processes for tequila production described previously might impact the distillery wastewater (i.e. tequila vinasses) composition. Tequila vinasses (TV) are characterized for having low pH, high temperature, high BOD, dissolved salts and a brownish color due to the content of polyphenols and melanoidins (España-Gamboa *et al*. 2012; Buitrón *et al*. 2014[a]). Actually TV are considered as feasible feedstock for the dark fermentative biohydrogen production (Buitrón et al. 2014[b]). However, the presence of some volatile compounds such as furans, organic acids and phenols, which are considered as inhibitory compounds for the dark fermentative process, could be an issue for this process (Zumar *et al*. 2016; Lin *et al*. 2015).

Currently there is no reported evidence that proves the presence of such volatile compounds in TV, nonetheless previous studies held in tequila, agave juice and sugar cane (Prado-Jaramillo *et al*. 2015; Mancilla-Margalli and López, 2002; Awad *et al*. 2015), respectively, showed their presence. Therefore, it is expected to find some volatile compounds reported as inhibitors in TV according to literature, which could be a handicap for TV utilization.

Thus, the aim of this work was to evaluate the use of three solvents in a liquid-liquid extraction (LLE) method in order to identify the presence of some volatile compounds considered as inhibitory compounds for biohydrogen production by dark fermentative process. TV samples obtained from two different production processes were used.

## METHODOLOGY

### Samples

Tequila vinasses were sampled from five distilleries located at Tequila, Jalisco. Two of them came from the raw agave process (NC) and the rest were collected from agave stem cooking process (C). A 20 L sample of each vinasse was kept at 4° C until further utilization.

### Volatile compounds extractions

Volatile compounds were extracted by the liquid-liquid method proposed by Prado-Jaramillo *et al.*

(2015) using different solvents as penthane/dichloromethane 3:1 (v/v) (Prado-Jaramillo *et al*. 2015), hexane/dichloromethane 1:4 (v/v) (Awad *et al*. 2015) and dichloromethane (Mancilla-Margalli and López, 2002). All solvents (HPLC grade) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Previously 40 ml of tequila vinasse were centrifuged at 10 000 rpm for 10 minutes and filtered by 0.45 µm membrane. Afterwards the sample was settled in a centrifuge tube, to which 10 ml of the solvents previous described were added. Subsequently the samples were shaken for 5 minutes and centrifuged for 5 min at 5 000 rpm and 10 °C. The organic phase was separated, dried with anhydrous $Na_2SO_4$ (Fermont, Mexico) and conserved at -21° C in amber flasks before concentration. The extracts were concentrated to a final volume of 1.5 ml with a nitrogen gas flow. For their posterior analysis the extracts were placed in suitable vials and preserved at -21° C until the chromatographic analysis was performed.

### Chromatographic analysis

Concentrated extracts were analyzed in a gas chromatograph 6890N Network system (Agilent Technology, USA), coupled to a selective mass inert detector 5975 (Agilent Technology, USA). Compound separations were performed using a HP-FFAP of 25 m x 0.32 mm (i.d) capillary column, coated with a 0.52 µm film (Agilent Technologies, USA). Helium was used as carrier gas, using a 2 ml/min flow with an initial temperature of 40° C at 5 min. Followed by a temperature program of 20° C/min to 100° C for 1 min, followed by a second rate of 3° C/min to a final temperature of 230° C for 40 min. The injector and detector temperature were 220 and 240° C, respectively (Mancilla-Margalli and López, 2002).

### Volatile compounds identification by GC-MS

Unknown volatile compounds identification was performed through an abundant ion comparison with NIST library. Comparison of the mass spectra with the database library was performed with a similarity percentage of 85-90%.

### Statistical Analyses

The CG-MS chromatograms obtained were integrated and the peak areas were documented for each compound identified. Statistical analyses were performed with STATGRAPHICS CENTURION XVI software (StatPoint Technologies, USA). An analysis of variance (ANOVA) was performed to evidence the compounds showing significant differences between solvents. Once the best solvent for volatile compounds extraction was chosen, the compounds that matched the reported in previous works were submitted to a principal component analysis (PCA) seeking to highlight the hydrolysis process.

## RESULTS AND DISCUSSION

Significant differences in the volatile compound profiles were observed between the solvents used in the LLE method. The chromatograms obtained with the blend of pentane/dichloromethane showed significantly higher number of compounds identified (about 110 volatile compounds, $p<0.05$) belonging to different chemical groups as alcohols, furans, acids, aldehydes, ketones, pyrans, among others. Only around 44% of them have been reported in previous studies with different samples (Mancilla-Margalli and López, 2002; Prado-Jaramillo *et al*. 2015; Fagier *et al*. 2015).

Such compounds were analyzed by a PCA, as it can be observed on the positive side of the principal

component 1, compounds such as tetradecane, acetoin, dibuthyl phthalate are grouped together showing a higher relationship with the raw agave process. Otherwise in the negative side, the presence of inhibitory compounds (furfural, hydroxymethylfurfural, propionic acid, etc.) was found, presenting a direct relationship with agave stem cooking process (Fig. 1).

According to the PCA, it can be inferred that the hydrolysis developed by cooking the agave stems by autoclave, shows a significant impact in generation of acids, furans and phenols, which are considered as inhibitory compounds for dark fermentative process (Quéméneur *et al*. 2012).

Fructans are insoluble in water at room temperature (25 °C), but are soluble at >50 °C. Reason why, a lixiviation process and hydrolysis is required to obtain fermentable sugars. When the cooking process is carried out, sugars are subjected to a series of complex reactions, among the principal reactions performed, caramelization, Maillard and oxidation-dehydration reactions stand out (Pérez *et al*. 2015).

These reactions are influenced by several factors, such as temperatures and time of cooking process (Waleckx *et al*. 2008). In the research performed with agave juice by Mancilla-Margalli and López (2002), the generation of furfural, 5-hydroxymethylfufural among others increased continuously throughout the agave cooking process.

Therefore, the vinasses from an agave stem cooking process showed a different volatile compound profile than those vinasses from the raw agave process. In the first, the presence of furans, organic acids and phenols is important.

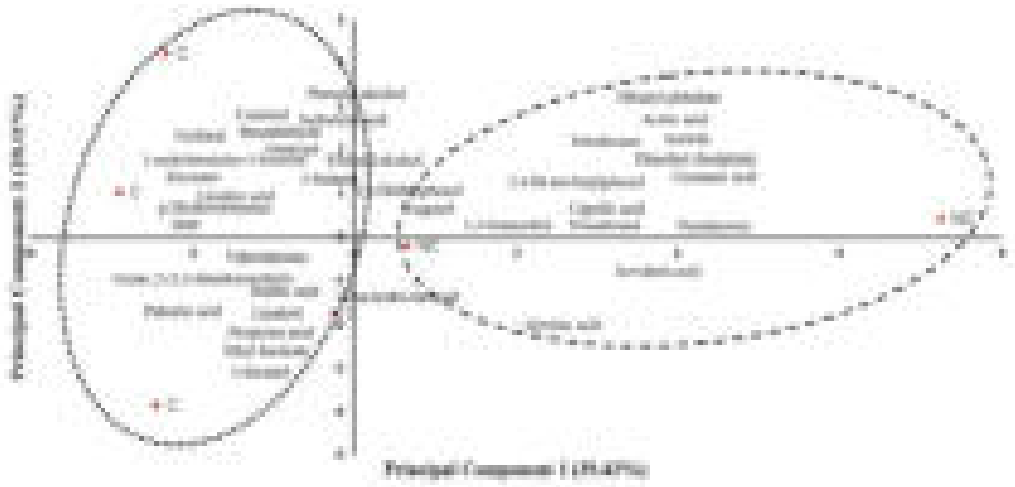

Figure 1. Volatile compounds principal component analysis (PCA) for scores (●) and loadings in tequila vinasses extracts by hydrolysis process.

## CONCLUSION

Tequila vinasses obtained from hydrolysis processes in which the agave stem is not cooked would be more viable as a substrate for biohydrogen production, due to the reduced presence of potential inhibitory compounds, compared to the vinasses obtained through the traditional tequila production process.

## ACKNOWLEDGEMENTS

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THEMATIC V **Social and ethnobotanics aspects**

# Analysis of the social, economic and productive characteristics of Agave-Mezcal producing municipalities in Mexico using a logistic regression model

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## ABSTRACT

In Mexico, indigenous rural Agave-Mezcal producing communities reflect high levels of marginalization presenting a contrast due to the fact that_they maintain high potential of increased productivity becasue of weather and environmental conditions governing cultivation. Municipalities having indigenous populations are more likely to engage in these activities; however, the probability of being an Agave-Mezcal producing municipality is 8.6% ($p < 0.05$) when compared with families in rural municipalities having the PROSPERA program ranging from 1,500≤2,150 holders with those of areas only having between 200≤850. Through the logistic regression analysis from the program Stata®, it was possible to show that Agave-Mezcal producing municipalities correlate with indigenous populations susceptible to social deprivation. Furthermore they were characterized as having less members of the "PROSPERA" program and living mostly in rural areas; it is relevant to note that for each additional percentage unit of occupants with dirt-floor housing, Agave-Mezcal producing municipalities increased by 27.5%, *ceteris paribus*. These results show the necesity to form a national public policy aimed at strengthening Agave-Mezcal production in most rural municipalities having desigation of origin (keeping in mind that the states of Oaxaca, Michoacan and Guerrero are the main producers of Mezcal in the country). In this sense, the importance of promoting the coordinated participation between institutions of social, agricultural and economic development with a perspective outlook is considered highly important. Furthermore, it is necessary to focus public budget policies on technology and innovation in rural areas of Agave-Mezcal production.

**Key words:** Agave-Mezcal, marginalization, technology, logistic regression model.

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## INTRODUCTION

Prospection defined as "the process that is used systematically to glimpse at long term, the future of science; technology; economy and society, in order to identify strategic areas of research and emerging generic technologies that will likely bring economic and social benefits "(Martin, B, 2001) is made necessary to provide for economic; political and social problems, including the use of and / or exploitation of food and technologies in emerging economies where resources are increasingly essential to development. According to the diagnosis of the National Congress of Public Policies for Field Labor held by the Mexican Senate of the Republic (2013), Mexico is considered the biggest spender with the lowest results due to the misapplication of public policy. Also here we note a contradiction which mandates the law for sustainable rural development (LDRS: ley de desarrollo rural sustentable) where the agricultural budget is concentrated in areas with greater development in infrastructure, contrasting that of the most marginalized zones. From this perspective, it is essential to develop comprehensive and holistic studies (Lopez et al. 2014); it is also necessary to encourage technological innovation, research and training in this sector (Garcia and Macias, 2010).

In 2014, the production of Agave-Mezcal accounted for approximately 2.79% of the national value corresponding to the total of all agricultural products in the country (SIAP-SAGARPA, 2015). The states of Jalisco, Guanajuato, Oaxaca, Michoacán and Guerrero accumulated 82.11% of the total value of production, as well as 75.91% of total domestic production with a utilized sown area of 131,434.56 hectares generating direct and indirect employment. According to the Tariff Information System Via the Internet (SIAVI) from the Secretary of Economy (2015), tequila exports totaled 916.9 million dollars from January to September 2015, 80.22% coming from the United States.

## METHODOLOGY

Escobar et al., (2012) notes that, "the estimation of the logistic regression model was performed by the method of maximum likelihood. This method estimates the values of parameters "b" and the regression that is most likely to have generated the values of the dependent variable of the sample, that is, if the assumptions of the model are true". How can odds ratios be interpreted? The values of the odds ratio express how much the ratio of occurrence varies based on the event of change within the independent variables.

Information obtained from INEGI, CONAPO, CONEVAL and SIAP-SAGARPA databases.

### Logistic model

$$\left(\text{[illegible]}\right) = \frac{1}{1+e^{-(\beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_5 X_5 + \beta_6 X_6 + \beta_7 X_7 + \beta_8 X_8 + \beta_9 X_9 + \beta_{10} X_{10} + \beta_{11} X_{11} + \beta_{12} X_{12} + \beta_{13} X_{13} + \varepsilon)}}$$

$$\text{[illegible]} = \left(\frac{\Pr(y=1)}{\Pr(y=0)} \Big/ \frac{\Pr(y=1)}{1-\Pr(y=0)}\right) = e^{\beta_1 \text{[illegible]}}$$

After studying a set of variables we resolved to use those shown in table 1 considering them to be relevant to the subject matter of the investigation. The logistic regression model seeks to interpret the regression coefficients and the predictive probability (LONG and FREESE, 2014). The binary dependent variables establish Agave-Mezcal producing municipalities as (value 1) versus those of non-production as (value 0).

Table 1. Socio-economic variables of the municipalities producing agave in Mexico

| Variable | Code |
|---|---|
| Type of Agave-Mezcal | TASE |
| PROMEKA holders (Mar-Apr 2015 | PPT |
| Categories: 1to500; 501to1500; 1501to2500; 2501to3500; 3501to4500 | |
| Sown area of Agave (Ha) | SSE |
| Indigenous municipalities | MUI |
| Medical staff in municipalities | PSIM |
| Percentage of occupants with dirt floor housing | OVT |
| Total population | POT |
| Percentage of population in towns with less than 5,000 inhabitants | PM5 |
| Percentage of population aged 15 years or more without complete primary education | PSF |
| Percentage of occupants in houses without running water | POA |
| Percentage of occupants in houses without electricity | PVE |
| Municipalities with indigenous presence | MPI |
| Gini coefficient | GNI |

Source: Prepared based on data from CONAPO, SIAP-SAGARPA, CONEVAL (2015).

The purpose of introducing economic, social and Agave-Mezcal production variables is to identify and analyze the characteristics of the producing municipalities that are suspected to explain the relative frequency that agave producers resemble in different regions of the country. It is worth noting that soil, climate and production conditions will be the study of further research. Also, it is important to point out that diverse qualitative and quantitative variables, from the Municipal Strategic Planning Index (IPE) of the National Institute for Federalism and Municipal Development (INAFED), have been explored (Marginalization indexes (IMG) of the National Population Council (CONAPO)). However, these were not statistically significant in the logistic regression model.

## RESULTS AND DISCUSSION

The Hosmer-Lemeshow test (HL) was performed in order to determine the goodness of fit of the logistic regression model, 10 groups were generated obtaining HL chi2 (8) = 12.18, with 552 observations and Prob> chi2 = 0.1435. Table 3 shows the predictive power and discrimination of the model. Sensitivity generates a test to correctly identify the mezcal producing municipalities. According to Martinez Sanchez and Faulín (2006) this corresponds to the probability that a unit of study is classified by the prediction model. In this case the classified + if predicted Pr (D)> = 0.5. It can be observed that the positive predicted responses are 133 (municipalities) of which 123 are properly

classified with a positive observed response of (y = 1); while 10 units are classified incorrectly with a negative observed response of (y = 0). Of the 419 municipalities specified with negative responses only 44 are correctly classified, 375 are classified incorrectly. In short, the whole model correctly classifies 90.22% of the observations (Table 2).

Table 2. Logistic model for Mezcal producing municipalities

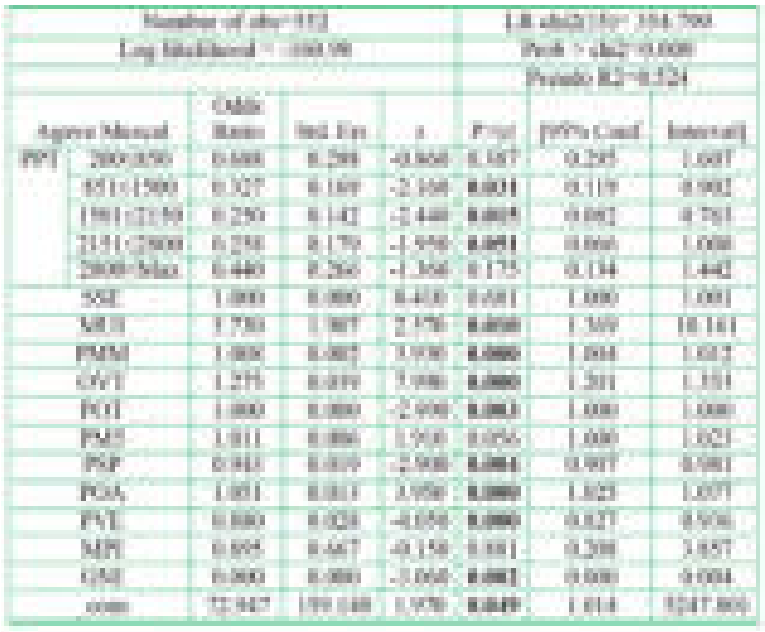
| | ---- True ---- | | |
|---|---|---|---|
| Classified | D | ~D | Total |
| + | 123 | 10 | 133 |
| - | 44 | 375 | 419 |
| Total | 167 | 385 | 552 |
| Classified + if predicted Pr(D) | >= .5 | | |
| True D defined as Agave-Mezcal producing municipality | != 0 | | |
| Sensitivity | | Pr( +\| D) | 73.65% |
| Specificity | | Pr( -\|~D) | 97.40% |
| Positive predictive value | | Pr( D\| +) | 92.48% |
| Negative predictive value | | Pr(~D\| -) | 89.50% |
| False + rate for true ~D | | Pr( +\|~D) | 2.60% |
| False - rate for true D | | Pr( -\| D) | 26.35% |
| False + rate for classified + | | Pr(~D\| +) | 7.52% |
| False - rate for classified - | | Pr( D\| -) | 10.50% |
| Correctly classified | | | 90.22% |

Source: Data based on logistic regression model.

The estimated coefficients appear below in table 3. The results of the fit model are considered adequate, due to the fact that the Pseudo R2 test is 52.43% with a probability of (p = 0.000). In this case, it can be said that the ratio of the 552 agave producing municipalities are statistically significant in eleven socio-economic variables of the model; the confidence level is 95% (p <= 0.05). Most covariates maintain statistical significance in the contrast of related hypotheses with the dependent variable (Agave- Mezcal producing municipality).It is worth noting that the PPT (200≤850, 2800≤Max), SSE, PM5 and MPI variables reflect higher values in hypothesis testing (not statistically significant).

The odds ratio represents the frequency of the respected event in conjunction with the frequency of its non-occurrence (Escobar, Fernandez and Bernardi, 2012); however, it does not reveal the magnitude of change in the probability of outcome. It can thus be said that the Agave-Mezcal producing municipalities having a level of PROSPERA members between 851≤1500 compared with 0≤200 decreases the odds ratio to 0.327; while the OR of localities having between 2151≤2800 holders is reduced to 0.25, conserving the other variables as constant. Indeed, 67% of the Agave –Mezcal producing municipalities have less than 1000 beneficiaries per municipality. For each additional unit of indigenous municipalities the OR of Agave-Mezcal producing municipalities experiences an increase of 3.73 while the percentage of occupants in dirt floor housing: OVT (ocupantes en vivienda con piso de tierra) is increased by 1.27.

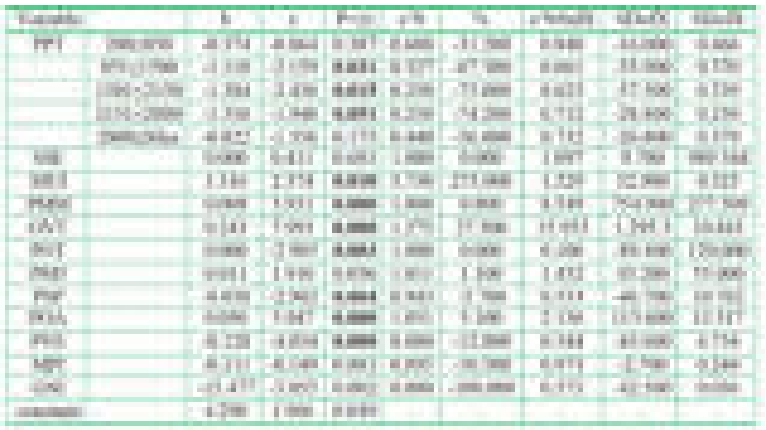
Table 3. Results of logistic regression model



Note: 4 failures and 3 successes completely determined.
Fuente: elaboración propia con datos del SIAP-SAGARPA (2015), CONAPO (2015).

The first three columns of table 4 show the standard logit coefficients (b), z value and probability (P> | z |). Column "e ^ bStdX" expresses the change in reason for an increase of the independent variable in a standard deviation. It is evident that for each additional unit of Agave producing indigenous municipalities, there is a probable frequency increase of 273% of being an Agave-Mezcal producer; the other variables remain constant. Also, for each additional percentage unit of occupants in dirt floor housing, the quotient percentage of Agave-Mezcal producing municipalities is increased by 27.5%, ceteris paribus. Furthermore, when a standard deviation of the percentage of occupants in homes without running water increases, the odds ratio of Agave-Mezcal producing municipalities is increased by 113.60%; the other variables remain constant (p <0.01).

**Table 4.** Probabilistic properties of the logistic regression model



Source: Unique elaboration based on results of logistic regression model.

b = raw coefficient
z = z-score for test of b=0
P>|z| = p-value for z-test
e^b = exp(b) = factor change in odds for unit increase in X
e^bStdX = exp(b*SD of X) = change in odds for SD increase in X
SDofX = standard deviation of X
% = percent change in odds for unit increase in X
%StdX = percent change in odds for SD increase in X

Table 5 shows the average marginal predictions over probabilities based on the logistic regression model for Agave-Mezcal producing municipalities according to the percentage of population aged 15 years or older with incomplete primary education (PSP) and indigenous municipalities (MUI). On average, the PSP shows that with a standard deviation increase of one, approximately 0.5% increase the likelihood of having an Agave-Mezcal producing municipality. In addition, with a one unit increase of indigenous municipalities, the effect on the probability of being an Agave-Mezcal producing municipality is 14.3%. On average, municipalities with PROSPERA members between 1500≤2150 compared with those of 200≤850 decrease the probability of being an Agave-Mezcal producing municipality by 8.6% ($p < 0.05$). The sum of members between 2150≤2800 compared with those of 0≤200 decreases the probability of being an Agave-Mezcal producing municipality by 12.3% ($p < 0.05$). This evidence demonstrates that the fundamental problem facing the Agave-Mezcal sector is located in rural locations that incorporate higher levels of marginalization.

Table 5. Marginal average predictions of PSP, PVP and MUI.

| Variable | | | Change | From | To | p-value |
|---|---|---|---|---|---|---|
| PPI | | | | | | |
| 850<1500 | vs | 0≤200 | -0.109 | 0.374 | 0.268 | 0.035 |
| 1500≤2150 | vs | 0≤200 | -0.123 | 0.374 | 0.49 | 0.014 |
| 2150≤2800 | vs | 0≤200 | -0.123 | 0.374 | 0.251 | 0.036 |
| 2800<Más | vs | 0≤200 | -0.081 | 0.374 | 0.293 | 0.167 |
| 850<1500 | vs | 200≤850 | -0.086 | 0.359 | 0.268 | 0.086 |
| 1500≤2150 | vs | 200≤850 | -0.086 | 0.359 | 0.246 | 0.038 |

| PSP | Change | From | To | p-value |
|---|---|---|---|---|
| +1 | -0.085 | 0.303 | 0.597 | 0.003 |
| +SD | - .049 | 0.303 | 0.253 | 0.001 |
| Marginal | 0.085 | .e | .e | 0.001 |
| MUI | Change | From | To | p-value |
| +1 | 0.143 | 0.303 | 0.445 | 0.028 |
| +SD | 0.040 | 0.303 | 0.342 | 0.014 |
| Marginal | 0.115 | .e | .e | 0.009 |

Source: Unique elaboration based on results of logistic regression model.

## CONCLUSION

When a municipality in Mexico is considered indigenous, there is a 14% probability that it is engaged in the production of Agave-Mezcal. However, when said municipality is comprised of PROSPERA holders having between 1500≤2150 members compared with those of 200≤850, the probability of being an agave-mezcal producer decreases by 8.6% ($p < 0.05$). Indigenous-rural Agave-Mezcal producing communities reflect higher levels of marginalization in Mexico. Therefore, it is necessary to create a national and / or multi-regional strategy where assigned institutions coordinately participate in regional, economic and agricultural development aimed at rural agave producing locations in Mexico. This intervention should not be homogeneous nor come from above, but should be case by case, adhering to the needs and characteristics of said localities. In general, prior knowledge of an area is needed before attempting to implement public policies solely focused on economic growth rather than sustainability and/or diversity.

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THEMATIC V Social and ethnobotanics aspects

# Municipal socioeconomic characteristics of Agave-Mezcal in Mexico

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## ABSTRACT

The Maguey-Agave production system is an important source of regional development for small producers located in 552 municipalities of the 2,457 that exist in the country of Mexico; in this context, the involved producers share ethnic, cultural, social, economic, environmental and productive aspects. The objective of this research is to analyze the characteristics and socioeconomic conditions among Agave-Mezcal producing municipalities, with a multifactorial variable approach on planting, marginalization, local planning and poverty. This information was obtained through domestic sources from the Instituto Nacional de Estadística y Geografía (CONAPO), Consejo Nacional de Evaluación de la Política de Desarrollo Social (CONEVAL), Comisión Nacional para el Desarrollo de los Pueblos Indígenas (CDI) y Sistema de Información Agroalimentaria y Pesquera de la Secretaría de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación (SIAP-SAGARPA). In general, prior knowledge of an area is needed before attempting to implement public policies solely focused on economic growth rather than sustainability or in respect to diversity. With this being said, territories need to improve design, planning and process of public policy focused on economic growth in order to improve social conditions.

**Key words:** Agave-Mezcal, Marginalization, poverty, public policies.

## INTRODUCTION

Presently the production of Agave-Mezcal is experiencing structural problems involving not only productive, competitive and technological innovation aspects but also those of marginalization, poverty, null regional development, lack of access to market values and asymmetries in the approach to the knowledge of economy. According to information from the National Commission

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for the Development of Indigenous Peoples: CDI (Comisión Nacional para el Desarrollo de los Pueblos Indígenas), of the 552 municipalities producing Tequila, Mezcal, Bacanora and Maguey, 65 are classified as indigenous and 448 with a sparse indigenous presence. This means that there is a close relationship between agave producers and poor conditions presented by the population in these municipalities. Regarding the 167 Agave-Mezcal producing municipalities nationwide, 62.87% (105) have dispersed indigenous populations, 7.18% (12) maintain an indigenous presence in the population and 29.94% (50) are predominately indigenous (CDI, 2015). In this scenario it becomes essential to rethink strategies on strengthening local capacities by involving stakeholders (producers, entrepreneurs, and academia) and encourage scientific, technological and innovational research in the sector to improve social development and competitiveness.

In Mexico, Agave-Maguey production was concentrated, in 2011, in 552 municipalities that were classified into four categories: Bacanora (5 municipalities, representing 0.91% of total), Maguey (77, 13.95%), Mezcal (167, 30.25%), Tequila (297, 53.80%) and unclassified (6, 1.09%) (SIAP-SAGARPA, 2015). According to the SIAP-SAGARPA information system (2015) , in 2014, the planted area of Agave-Mezcal was mainly concentrated in 167 municipalities located in 12 states (Baja California Sur with 3 hectares constituting 0.01% of the national total), Colima (46.5 ha, 0.22%), Durango (235 ha, 1.12%), Guanajuato (7,200 ha, 34.42%) Guerrero (1663.50 ha, 7.95%), Jalisco (765.66 ha, 3.66%), Michoacán (1,971 ha, 9.42%), Nayarit (2 ha, 0.01%), Oaxaca (8587.39 ha, 41.05%), Puebla (202 ha, 0.97%), San Luis Potosi (34 ha, 0.16%) and Sonora (210 ha, 1.00 %).

When we relate the variables of the National Population Council (CONAPOa) with databases from 2010 and from those of SIAP-SAGARPA (2015), there are 45 municipalities with very high marginalization indexes (IMG - Índice de Marginación), 56 with high IMG, 256 with average, 132 low and 63 with very low . Most Agave producers are located in rural areas with high levels of marginalization; however, this constitutes an alternative livelihood for many families. In particular, the primary Agave-Mezcal production sector undergoes a difficult social, economic and ecological agricultural position. From an official perspective, it is to be considered that "if there were public policies, the situation of sustainability would improve, that is, there would be no exploitation or mismanagement", in other words, when the state is present through politics, the indigenous population cease to be exploited and mismanagement diminishes. However, it is important to study why the indigenous population, who are considered to be the most sustainable type of persons, began to have such practices with adverse effects in regards to ecological terms. Sometimes the non-presence of the state is more beneficial; this problem is generally focused on how the agribusiness chain is articulated and in turn puts the market into a context of increased poverty, vulnerability and insecurity. The Mezcal Product System notes that in the absence of public policies targeted on reversing the damage caused by overuse and mismanagement in the utilization of Agave species for agro industrial and artisan productive use, agave inventories have become more expensive (communication staff, November 12, 2015). In applying this approach to the Agave-Mezcal production system and in establishing starting points, it appears that production undergoes structural problems of marginalization, poverty, null strategic planning, market failures on arrival, and unequal access to information among other variables; all of which involve not only technological innovation and productive aspects but also ones of socio-economic and agro-ecological descent (Garcia et al., 2010).

## METHODOLOGY

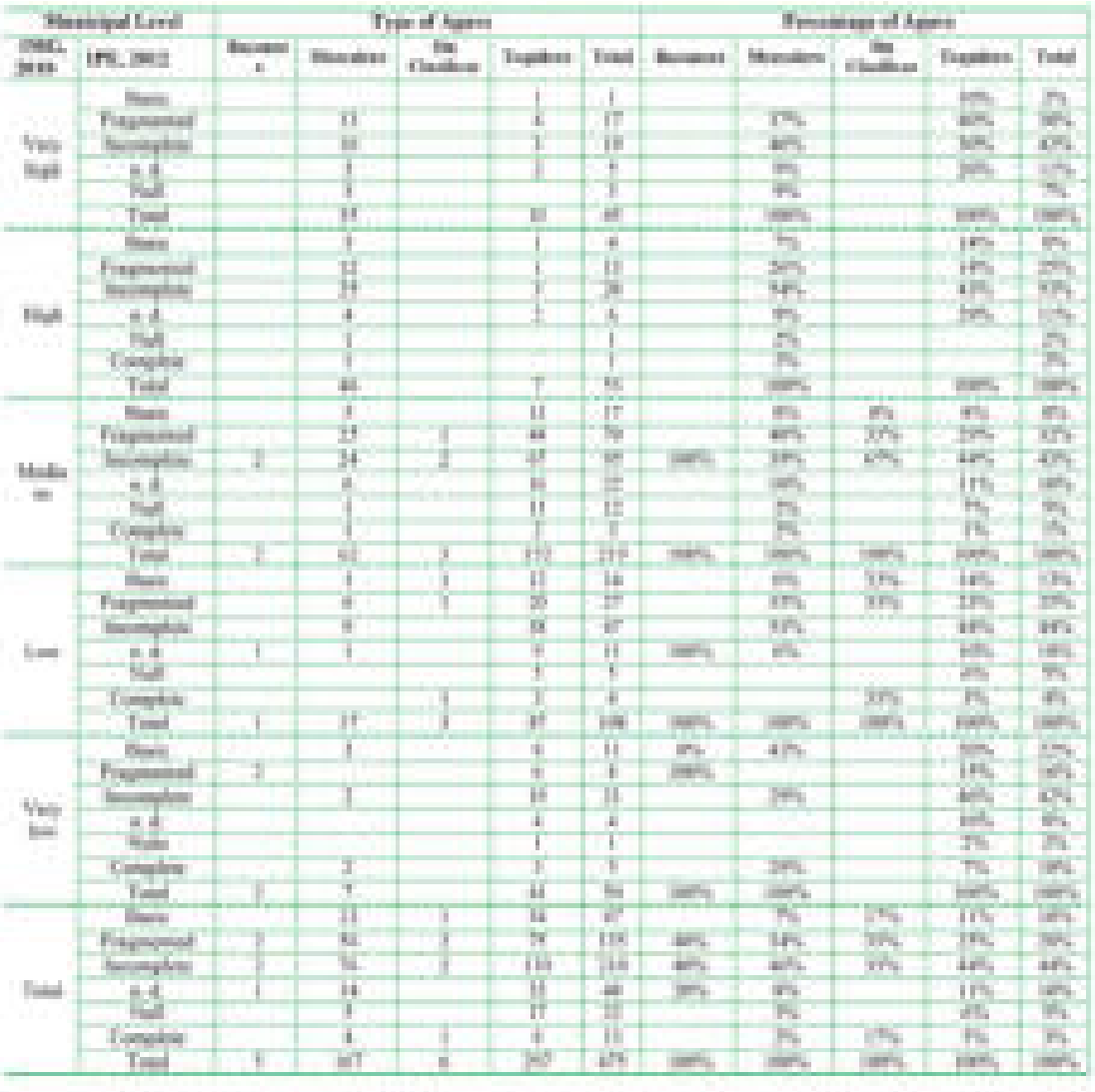
Agave-Mezcal production chain incorporates cultural and social aspects that relate to a set of socio-economic variables and those of production. In this regard we seek to analyze the production and utilization from a multifactorial approach that includes planting variables, municipal marginalization and poverty to relate the justification of the study among Agave-Mezcal producing municipalities. This information was obtained from various national sources such as: INEGI, CONAPO, CONEVAL and SIAP-SAGARPA.

## RESULTS AND DISCUSSION

Local agricultural development of municipalities is based on planning that is developed toward its interior. The Strategic Planning Index: IPE (Índice de Planeación Estratégica), compiled by the National Institute for Federalism and Municipal Development (INAFED) of the Secretary of the Interior (SEGOB, 2015) seeks to measure municipality conditions through local planning management enhancements and is classified as a Full, Basic, Incomplete, Fragmented and Null. In this regard, in 2012, the IPE of the 76 Agave-Mezcal producing municipalities resulted incomplete, 56 fragmented, 12 basic, 5 null, 4 full and 14 provided no information. The most representative states with this local issue were Oaxaca and Guerrero. In this first case, there are 67 municipalities with a scattered indigenous presence in the population, 3 with an indigenous presence and 48 municipalities which are predominately indigenous. In Guerrero there are 11 municipalities, 5 of which have a dispersed indigenous presence and 3 with an indigenous presence (CDI, 2015). This reality generates a need to transform an area's social conditions by means of better proposals, decisions and effective and efficient public policies.

Table 1 shows the relationship of strategic planning variables and those of municipal assessment with the rates of marginalization of the Agave producing municipalities (not considering maguey). There is evidence that 82% of Mezcal producing municipalities (29 municipalities) with fragmented and incomplete strategic planning levels have very high percentages of poverty, and 70% (7) correspond to those dedicated to the production of Agave-Tequila having an equal index of fragmented and incomplete planning. Municipalities with high poverty dedicated to Agave-Mezcal production account for 46 and have a municipal strategic planning characteristic that is also found to have fragmented and incomplete levels (80%). In this sense prospecting becomes one of the most important strategic elements for the integrated use of food; its technological and commercial resources thereby favorably aiding in the combat and elimination of social problems (marginalization) that plague many rural areas. For this, in the practice of prospection we are able to identify the mode of operation of the environment and thus be competent to generate conclusions and recommendations for the authorities to adopt an adequate solution (Malte et al., 2015).

**Table 1.** Characteristics of Agave producing municipalities in Mexico.



Source: Unique elaboration based on data from CONAPO, SAGARPA, SEGOB.

The need to implement better strategies and sectorial planning programs is not atypical in agave producing municipalities, which have low and medium marginalization rates given that their characteristics are very similar governing lack of planning. This reality demonstrates the need to implement public policies in order to raise the levels of local planning for more effective and efficient public services as well as to generate better development plans for the agricultural sector in order to impulse development.

## CONCLUSION

The production of Agave-Mezcal presents opportunities for growth and regional development due to the diversity and potential that most territories and climates have where it has been produced. It is essential that the basic administrative units (municipalities) also endorse their commitment to the development of Agave-Mezcal production in their localities given that the analysis shows

precarious planning in Mezcal producing territories. It is necessary to strengthen the link between municipalities and Agave producing associations (Bacanora, Tequila, Maguey Mezcal) with research institutions, technological development and national and international innovation in order to strengthen capacities and encourage the development and implementation of integrated solutions sustained by the knowledge derived from scientific and technological activities focused on said sector.

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THEMATIC V **Social and ethnobotanics aspects**

# Appellations of origin as a developmental strategy for the designation of mezcal origins

**Gallardo-Valdez J.** [1] *

## ABSTRACT

In Mexico, the term Designation of Origin (DO), is associated with the name of a geographical region of the country used to describe a product that originates there. The quality or characteristics are exclusively due to the geographical environment, understood in its natural and human factors (Cámara de Diputados, 2016), this being the case for Mexico as it is mega-diverse in both biotic resources and cultural aspects with a high natural resource potential. Despite this, to date it has 15 Appellations of Origin (Instituto Mexicano de la Propiedad Industrial, 2016), 7 of which correspond to fruit and vegetables, 3 to crafts and 5 to alcoholic beverages, three of which are protected distillates of agave: Tequila, Mezcal and Bacanora. This is a very small number compared to some European countries like Spain with 62, and France with 300 for this type of product (InfoDrinks, 2016). Some experts attribute this phenomenon to the lack of vision and commitment from state governments to manage resources or promote local products even though some of them could function as a trigger for the development not only of primary activities, but also, tourist and gastronomic activities as well. This could benefit hundreds of farmers and their families, particularly considering the case of Mezcal Denomination of Origin (DOM) due to its links that make up the Maguey-Mescal production chain.

**Key words:** Mezcal, Rural Development, Sustainability.

## INTRODUCTION

One of the goals outlined in the National Development Plan 2013-2018 (Gobierno de la República, 2013-2018), whose main objective called "México Prospero" is to lead Mexico to its fullest potential, is aimed at increasing productivity and democratizing the Mexican economy through actions to consolidate macroeconomic stability, promote efficient use of productive resources, strengthen the field of business and establish sectoral and regional policies to promote development. In this context, agencies like the Secretary of Agriculture, Livestock, Fisheries and Food (SAGARPA),

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the Secretary of Tourism, the National Forestry Commission (CONAFOR), among others have contributed to strengthen all the links of the Maguey-Mezcal chain crossed from various sectoral programs in support with the producers. The Regulatory Council of Mescal's 2015 report shows important figures in the growth of production and consumption of this liquor that is mostly is made by artisanal producers, showing that consumption of mezcal has gone from being a fashion trend, which sees a flattering and promising future scenario in economic terms, to demonstrating that the benefits generated by this phenomenon are coming to rural producers, who for decades had been the least benefited from the policies and programs promoted by various government agencies (Consejo Regulador del Mezcal-CRM, 2016). Currently, the mezcal industry is valued at just over one thousand 200 million pesos and more than 8000 families depend on it. It is estimated that the production of mezcal generates a total of 5,000 direct jobs and more than 25 thousand indirect jobs nationwide (CRM, 2016), benefiting many rural communities, contributing to the entrenchment of the population slowing migration, strengthening identity, culture and traditions surrounding this iconic liquid.

Given this scenario, the production of mezcal is making an employment alternative and economic development for many communities as well as raising the social and economic level of the population engaged in the exploitation of raw materials, production, marketing and distribution. Several states have turned their gaze to the DOM considering it as an alternative to promote development.

## METHODOLOGY

Based on the provisions in the Industrial Property Law, the Law on Sustainable Rural Development (Cámara de Diputados, 2016), the National Development Plan 2013-2016 (Gobierno de la República, 2013-2016), the resolution which granted the protection provided for the designation of mezcal origin (Diario Oficial de la Federación, 1994) and the Mexican Official Standard NOM-070-SCFI-1994 (Instituto Mexicano de la Propiedad Industrial-IMPI, 1994), some of the states that were excluded in the declaration of protection of the designation of mezcal origin issued in November 1994, have asked the IMPI to demonstrate that they meet the requirements established by the Law of Industrial Property by preparing technical studies showing that there is not only the potential of raw material constituted in natural populations (wild) and induced cultures of various species of agave, but tradition and craft techniques used in the production of mezcal involving social and cultural aspects that strengthen the identity and traditions of the people allowing for more competitive development options and better pay.

## RESULTS AND DISCUSSION

From 2001 to present, the territory covered by the DOM has had five changes promoted by the state governments of Tamaulipas, Michoacán, Guanajuato and Puebla, on the grounds that the owner of the DOM is the Mexican state, and that there is a legal interest from the states of the federation in calling for incorporation in just right. With the growth in domestic and international demand for this liquor, it is understandable that in the near future new states like Morelos, Mexico State and Aguascalientes, who have openly expressed interest, will become incorporated. This due to that most rural communities have potential raw material and process knowledge thusly making it an employment and economic development alternative (Fig. 1).

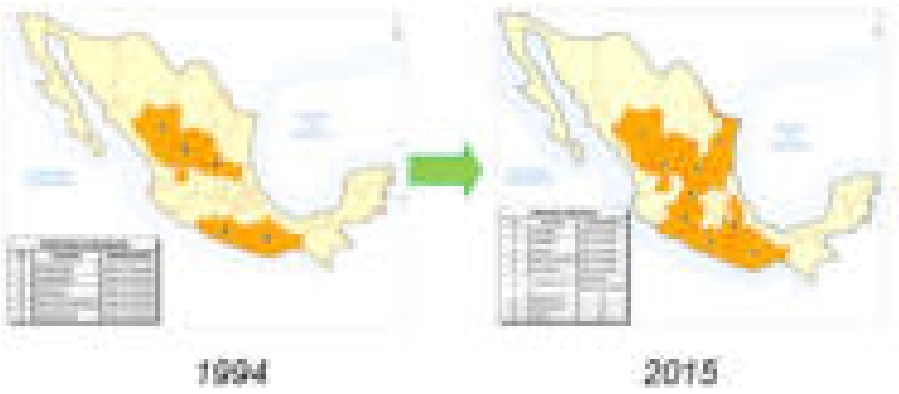


Figure 1. Addition of new territories to the DOM 1994-2015. Source: Prepared based on information published in the Government Official Gazette.

## CONCLUSION

With the addition of new territories to the DOM, Agave-Mescal production has been removed from the informality of being an activity that for years has been conducted clandestinely in various states of the country, becoming a productive option that benefits thousands of farmers and their families who make up the Agave-Mescal production chain in hundreds of rural communities. The development of mescal represents not only an economic option, but also aligns policies and social and sectoral programs implemented by the federal government, such as combating poverty, contributing to the entrenchment of the population in their communities, slowing the migration in the absence of alternative economic development, thus the identity of mezcal is strengthened, being recognized as a craft product originating from a specific region.

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