MINIREVIEW

Yeasts associated with the production of Mexican alcoholic nondistilled and distilled *Agave* beverages

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Received 29 February 2008; revised 23 June 2008; accepted 14 July 2008.
First published online 28 August 2008.

DOI:10.1111/j.1567-1364.2008.00430.x

Editor: Patrizia Romano

Keywords
Agave; alcoholic and distilled beverages; pulque; tequila; mezcal; yeasts.

Abstract

The great variety of agaves and their multiple uses have played an important role in the cultural identification of Mexico. They have been exploited in many ways for over 10 000 years, and one of these applications is the production of alcoholic nondistilled and distilled beverages. Most of the production processes of these Mexican beverages involve a complex fermentation in which bacteria (mainly lactic and acetic acid) and yeasts (non-*Saccharomyces* and *Saccharomyces*) are present in stable mixed populations, or succeeding one another, and have a significant impact on the sensorial characteristics and nutritive value of the final product. This minireview focuses on several nondistilled and distilled *Agave* beverages, their production area, the *Agave* species used in their elaboration, the functional microbiota involved in the fermentation process, their fermentation products (when known), the biochemical changes of these unique fermentations, and their impact on the quality and sensorial characteristics of the product.

Introduction

In Mexico, traditional alcoholic beverages have had great relevance in the daily life of indigenous communities. Since pre-Hispanic times, the Mesoamerican civilizations fermented a variety of native plants for the production of alcoholic beverages and their consumption played an important role in their religion, ritual, divination, and curing for millennia (Bruman, 2000).

Since ancient times, agaves or magueyes (*Agave*) have been among the most important and widely used plants in Mexico. These are succulent plants of great biological, ecological, and economic importance, and have been sustaining the establishment and development of different human groups for centuries. They belong to the family *Agavaceae*, which is endemic to America, and are often misidentified with cacti, growing in the same environment. Agaves contain a high concentration of fructans as reserve substances, and have only one reproductive event in their life (García-Mendoza, 1995, 1998).

In Mexico, during ancient times, the integral use of these plants (for food, alcoholic beverage production, water substitute, clothing, and herbal medicine) was of such importance that the survival and cultural development of indigenous civilizations could not be explained without its existence (García-Mendoza, 1995, 1998).

Today, different *Agave* species are exploited for the production of alcoholic nondistilled and distilled beverages with national and international recognition. In Mexico, pulque, a pre-Hispanic, nondistilled beverage, is the most traditional and represents a Mexican icon (Loyola-Montemayor, 1956; Gonçalves de Lima, 1990). Traditionally, it is obtained by spontaneous fermentation of the sap or aguamiel of different *Agave* species (Sánchez-Marroquín, 1967; Gonçalves de Lima, 1978, 1990; Steinkraus, 1997; Ramírez *et al.*, 2004). At present, pulque is also produced on a small industrial scale through a pure mixed starter culture industrialized process to control the quality and safety of the product, which is canned and exported as a novel Mexican product (Del Razo, 2004; Ramírez *et al.*, 2004).

Nowadays, distilled drinks are more popular. They are produced by distillation of the fermented must of several *Agave* species, and are generically known by the name mezcal. For the commercial production of the different

types of mezcal (mezcal, bacanora, raicilla), several Agave species are used in Mexico (Table 1). The elaboration process has five main steps: removal of the leaves, leaving the stem and the leaf bases, known as head or pine; cooking of the pines; extraction of the must; fermentation (spontaneous or induced); distillation; and sometimes maturation. Tequila differs from other mezcal in that it is produced only from *Agave tequilana* Weber var. *azul* (Cedeño, 2003).

Table 1. Mexican alcoholic nondistilled and distilled agave beverages

Beverage	Type of beverage	*Agave* species	Substrate	States of production	Functional microbiota	Fermentation products	References
Pulque	Nondistilled	*A. atrovirens* *A. mapisaga* *A. salmiana*	Sap	México Tlaxcala Hidalgo Querétaro México DF Puebla Morelos San Luis Potosí	In spontaneous fermentation homo and heterofermentative LAB (*Lactobacillus* spp., *L. brevis*, *L. plantarum*, *Leuconostoc mesenteroides* ssp. *dextranicum*, *L. mesenteroides* ssp. *mesenteroides*), *Zymomonas mobilis* ssp. *mobilis*, non-*Saccharomyces* (*Candida* spp., *C. parapsilosis, Clavispora lusitaniae*, *Hanseniaspora uvarum, Kluyveromyces marxianus, Kluyveromyces lactis, Pichia membranifaciens, Pichia* spp., *Torulaspora delbrueckii*) and *Saccharomyces* (*S. bayanus*, *S. cerevisiae, S. paradoxus*) yeasts. In industrialized process a mixed culture starter (*Lactobacillus* spp., *Z. mobilis* ssp. *mobilis* and *S. cerevisiae*) with or without *Leuconostoc* species	Ethanol, organic acids, dextrans, vitamins, amino acids, fusel oil, esters, aldehydes	Lappe & Ulloa (1993), Steinkraus (1997), Ramírez *et al*. (2004)
Mezcal	Distilled	*A. tequilana* var. *azul*	Must	Jalisco, regions of the states of Nayarit, Michoacán Tamaulipas Guanajuato	In spontaneous fermentation LAB, non-*Saccharomyces* (*Candida* spp., *C. magnolia*, *Hanseniaspora guilliermondii, H. uvarum, H. vinae, K. marxianus, P. membranifaciens, T. delbrueckii*) and *Saccharomyces* (*S. cerevisiae*) yeasts. In industrialized process select *S. cerevisiae* strains cultures	Ethanol, organic acids, fusel oil, esters, terpenes aldehydes, furans, ketones, nitrogenous compounds	Lachance (1995), Cedeño (2003), López (1999), Mancilla-Margali & López (2002)
Tequila	Distilled	*A. salmiana*	Must	San Luis Potosí	LAB, non-*Saccharomyces* (*C. lusitaniae*, *K. marxianus, Pichia fermentans*) and *Saccharomyces* (*S. cerevisiae*) yeasts	Ethanol, organic acids, fusel oil, esters	De León-Rodríguez *et al*. (2006)
Mezcal	Distilled	*A. cupreata* *A. inaequidens*	Must	Michoacán	Nondetermined	Ethanol, organic acids, fusel oil, esters	Gschaedler *et al*. (2008)
Mezcal	Distilled	*A. angustifolia* *A. americana* var. *oaxacensis* *A. karwinskii* *A. marmorata* *A. potatorum* *A. rhodacantha*	Must	Oaxaca	Non-*Saccharomyces* (*Candida* spp., *Hanseniaspora* spp.) and *Saccharomyces* (*S. cerevisiae*) yeasts	Ethanol, organic acids, fusel oil, esters, terpenes aldehydes	Andrade Meneses & Ruiz Terán (2004)
Mezcal	Distilled	*A. duranguensis*	Must	Durango	Non-*Saccharomyces* (*K. marxianus*) and *Saccharomyces* (*S. cerevisiae*) yeasts	Ethanol	Cisneros *et al*. (1997)

Table 1. Continued.

Beverage	Type of beverage	*Agave* species	Substrate	States of production	Functional microbiota	Fermentation products	References
Mezcal	Distilled	*A. fourcroydes*	Must	Yucatán	Non-*Saccharomyces* (*Candida parapsilosis, Clavispora lusitaniae*, *Debaryomyces hansenii, Kluyveromyces marxianus, Pichia caribbica, P. guilliermondii*, *T. delbrueckii*) and *Saccharomyces* (*S. cerevisiae*) yeasts	Ethanol	Lappe *et al*. (2004)
Raicilla	Distilled	*A. angustifolia* *A. inaequidens* *A. maximiliana*	Must	Jalisco	Non-*Saccharomyces* (*C. lusitaniae*, *K. marxianus*) and *Saccharomyces* (*S. cerevisiae*) yeasts	Ethanol, organic acids, fusel oil, esters, aldehydes, terpenes	Arrizon *et al*. (2007)
Bacanora	Distilled	*A. angustifolia*	Must	Sonora	Nondetermined	Ethanol, organic acids, esters, aldehydes, ketones, terpenes, hydrocarbons	Vallejo-Córdoba *et al*. (2005)

Most of the production processes of the Mexican alcoholic and distilled *Agave* beverages involve a complex fermentation in which bacteria (lactic and acetic acid) and yeasts (non-*Saccharomyces* and *Saccharomyces*) are present in stable mixed populations, or succeeding one another. This microbiota is responsible for the production of several chemical and volatile compounds that confer the particular characteristics to the final product (Sánchez-Marroquín, 1967; Gonçalves de Lima, 1990; Steinkraus, 1997; Ramírez *et al*., 2004). Table 1 shows several alcoholic nondistilled and distilled *Agave* beverages, their production area, the *Agave* species used in their elaboration, and the functional microbiota involved in the fermentation process.

In this minireview, the characteristics, history, and process of elaboration of several Mexican *Agave* alcoholic beverages are discussed briefly. Special attention is focused on the fermentation process and essential microorganisms involved, their fermentation products (when known), the biochemical changes of these unique fermentations, and their impact on the quality and sensorial characteristics of the product.

Mexican alcoholic *Agave* beverage: pulque

Pulque is probably the oldest and most traditional Mexican alcoholic beverage. It is a milky white, viscous, slightly acidic, and alcoholic beverage ($< 6\%$) produced by the fermentation of the sap that is extracted from several *Agave* species, mainly *Agave salmiana*, *Agave atrovirens*, and *Agave mapisaga*. They grow in semi-arid and temperate zones, in poor soils, with scarce and irregular precipitations (Sánchez-Marroquín, 1967; Gonçalves de Lima, 1990; García-Mendoza, 1998).

Owing to its great historical, religious, social, medical, and economical importance, pulque is the most widely studied beverage from different points of view (Gonçalves de Lima, 1990).

In ancient Mexico, pulque had a dominating presence in the daily life of the indigenous populations, as well as a decisive influence on religious and war rituals. This has been illustrated in pre-Hispanic and post-Hispanic codices (richly illustrated hieroglyphic texts made of fig-bark paper folded like an accordion) (Gonçalves de Lima, 1978).

The *Aztecs* distinguished different pulque types: metoctli or *Agave* wine, iztacoctli or white wine, and teoctli or ceremonial or god wine, and the name poliuhquioctli was given to the decomposed wine with an unpleasant flavor and odor. From this word, pulque was derived and used by the Spaniards to designate the freshly produced beverage (Gonçalves de Lima, 1990; Lappe & Ulloa, 1993). When the Aztec empire fell, pulque lost its religious importance, but retained its relevance as a nutritional supplement and water substitute. The beverage became profane and its consumption was introduced to the cities, mainly to Mexico City (Gobierno del Estado de Hidalgo y Museo Nacional de Culturas Populares, 1988).

Currently, pulque persists as a typically popular beverage, and it is only sold in pulquerías. Its consumption varies with the age and type of consumer, as well as with the occasion. For the low-income population, pulque is still the preferred stimulating drink. It also forms an important part of their daily diet, because of its contribution of vitamins and essential amino acids (Steinkraus, 1997; Ramírez *et al.*, 2004).

Nowadays, a re-evaluation of the beverage can be observed. Pulque is promoted in restaurants and gastronomic festivals in an attempt to recover old traditions and it is becoming popular among young people.

Traditional pulque production

Pulque is produced from the sweet, yellowish, slightly cloudy sap, extracted from 8- to 10-year-old mature agaves, that are about to produce their inflorescence, which, once productive, lasts only from 90 to 120 days, with a total production of 200–1000 L per plant.

The first step in pulque production is the elimination of the floral bud, leaving a cavity or cajete in the center of the *Agave* stem (Fig. 1a). An aging period follows, allowing the maturation of the central leaves, and increasing the sap sugar content (7–14% w/v) (Loyola-Montemayor, 1956; García-Mendoza, 1998). The healed cavity is scraped to open the vessels, allowing the sap to flow, which accumulates in the cavity. The sap is removed by oral suction through a dried gourd (*Lagenaria siceraria*) called an acocote (Fig. 1b), and is carried to the tinacales where the fermentation takes place in open 700-L capacity containers (Fig. 1d). The fermentation occurs spontaneously or can also be induced by adding a small portion of a sap-fermented starter (semilla).

The starter is made with 10–15 L of the best-quality sap, which is poured into a closed vessel, for a first natural fermentation. After several days, depending on the temperature, seasonal changes, and other uncontrolled factors, the alcoholic fermentation ends and the acetic fermentation begins. A thick floating layer is formed, indicating that the starter is ready for a second fermentation (Fig. 1d) (Loyola-Montemayor, 1956; Gobierno del Estado de Hidalgo y Museo Nacional de Culturas Populares, 1988). Another batch (600–900 L) of best-quality sap is then mixed with the starter (1–3% v/v) in a first tank (head fermenter) and fermented until the limiting sugar content controls the microbial growth. A batch of 300–450 L of supernatant pulque (pie de cuba) is removed, poured into a second tank (tail fermenter), and used as inoculum (50% v/v) to ferment a batch of regular sap (SECOFI, 1972a; Ramírez *et al.*, 2004). After several days of fermentation in the second fermentor, pulque finally reaches a specific alcoholic degree and viscosity and acquires the sensorial characteristics of the final product; at this point, it must be barreled and commercialized (Fig. 1e) before acidification and putrefaction begins. Pulque is considered a rapid consumption product and its shelf-life is 1–3 days (Loyola-Montemayor, 1956).

These fed-batch tanks provide a rapid low-cost technique for settling the microbial inoculum in both fermenters, so that when pulque is withdrawn semi-continuously, the pulque biomass is retained for further sap fermentations (Ramírez *et al.*, 2004).

In the market, besides white pulque, cured pulque can also be found, which is prepared with white pulque added with macerated fruits, cereals, grains, nuts, and vegetables (Steinkraus, 1997).

Characteristics and types of sap and pulque

Agave sap is a white, yellowish, lightly cloudy, thick, sweet, fresh-flavored, and lightly acid liquid (Fig. 1c). It contains water, sugars (glucose, fructose, and sucrose), proteins, gums, and mineral salts as the most important components (Table 2) (Loyola-Montemayor, 1956; Ortiz-Basurto *et al.*, 2008).

The Mexican regulation NMX-V-022 (SECOFI, 1972a) distinguishes two types of sap. Type I refers to the one of best quality (cleanest and with the highest sugar content) and Type II includes all other types of sap.

The sap constitutes a favorable medium for the proliferation of numerous species of microorganisms that induce its spontaneous fermentation; this begins when the liquid accumulates in the cavity and is strongly activated when the liquid mixes with the starter in the fermentation vats.

The Mexican regulation NMX-V-037 (SECOFI, 1972b) recognizes two types of pulque: Type I includes pulque de semilla (starter) and pulque pie de cuba and Type II refers to commercial pulque.

Pulque starter is prepared to increase the natural microbiota that determines the correct fermentation in the elaboration of the beverage. Pulque pie de cuba is prepared in the first fermentor tank with Type I sap and inoculated with pulque starter, and is used to establish the optimum biochemical equilibrium between the fermentable substrates and the basic microorganisms involved in fermentation; it is the base of the commercial production (Loyola-Montemayor, 1956; Ramírez *et al.*, 2004). The characteristics of both types of pulque are shown in Table 2.

Agave sap fermentation

Fermentation is carried out over several days, depending on the sap quality, pulque starter maturity, microbiota present in both substrates, season, and temperature changes. The degree of fermentation is adequate when a specific alcoholic

Fig. 1. Pulque elaboration process. (a) Scrapping of a central cavity in the *Agave* stem where the sap accumulates. (b) Oral suction of the sap with an acocote or dried gourd. (c) Sap. (d) Open fermentation containers. (e) Traditional pulque in plastic recipient. (f) Canned natural pulque.

degree and viscosity are reached (Loyola-Montemayor, 1956; Sánchez-Marroquín, 1977; Steinkraus, 1997).

The sap, rich in carbohydrates (8–10%), mineral salts, and growth factors, constitutes a favorable medium for the proliferation of numerous microorganisms that appear on the walls of the cavity or derive from the scraping or extraction utensils, from dust, or from insects (*Drosophila* spp.) that introduce a heterogeneous population of microorganisms that quickly adapt to the new ecological niche. Through the action of these microorganisms and those provided by the starter, a series of consecutive fermentations (lactic, alcoholic, viscous, acetic, and putrid) take place (Loyola-Montemayor, 1956; Sánchez-Marroquín, 1977; Gonçalves de Lima, 1990).

The microorganisms present in the sap and pulque are classified into two main groups, bacteria and yeasts, although fungi (*Aspergillus*, *Mucor*, and *Penicillium* species) may also be present. In both sap and fresh or young pulque, there is a predominance of bacteria ($8.0–15.0 \times 10^8$ cells mL^{-1}) over yeasts ($3–6 \times 10^6$ cells mL^{-1}), due to the neutral initial pH (7–7.4) of the substrates. The yeast population increases gradually during fermentation, predominating ($2.5–3.0 \times 10^8$ cells mL^{-1}) over bacteria ($1.0–2.0 \times 10^8$ cells mL^{-1}) (Ruiz Oronoz, 1953; Loyola-Montemayor, 1956). This is probably due to the acidity (pH 4.5) during the fermentation, which favors the development of yeasts.

As the microbial sap transformation begins, the resulting chemical changes propitiate the development of different microorganisms, as follows: (1) lactic acid-producing bacteria (*Leuconostoc* and homo- and heterofermentative *Lactobacillus* species), (2) yeasts (non-*Saccharomyces* and *Saccharomyces* species) and *Zymomonas mobilis* ssp. *mobilis* which transform sugars into alcohol and other products, (3) dextrans-producing bacteria (*Leuconostoc* spp.), (4) acetic

Table 2. Mexican official specifications of agave sap and pulque

	Agave sap*			Pulque†			
	Type 1		Type 2	Type 1		Type 2	
Specifications	Min	Max	At least	Min	Max	Min	Max
pH	6.6	7.5	4.5	> 3.7	4.2	3.5	4.0
Density (Baumé degrees)	5	7	4.5	32	345	25	–
Refractive index (Immersion at 20°)	59	100	27	1.339	1.3406	1.3365	1.3380
Total solids (g 100 mL^{-1})	13	17	7	–	–	–	–
Total reducing sugars [(glucose) g 100 mL^{-1}]	8	12	6	0.10	0.80	0.20	0.50
Direct reducing sugars [(glucose) g 100 mL^{-1}]	2	3	3	–	–	–	–
Gums [(glucose) g 100 mL^{-1}]	2	6	0.20	–	–	–	–
Proteins (g 100 mL^{-1})	0.3	0.6	0.1	–	–	–	–
Ashes (g 100 mL^{-1})	0.3	0.43	0.18	–	–	–	–
Not greater than							
Acidity [(lactic acid) mg 100 mL^{-1}]	0.90	1.03	4.00	400	750	400	700
Alcohol degree in % per volume	–	–	–	6.0	9.0	4.0	6.0

Source: *NMX-V-022 (SECOFI, 1972a).
†NMX-V-037 (SECOFI, 1972b).

acid-producing bacteria (*Acetobacter* spp.), and (5) putrefactive microorganisms.

Microbial studies on pulque

Microbial studies on pulque have been undertaken since the end of the 19th century. Currently, the microbiota identified from pulque is: Bacteria: *Acetobacter aceti, A. aceti* ssp. *xylinus, Bacillus simplex, Bacillus subtilis, Cellulomonas* sp., *Escherichia* sp., *Kokuria rosea, Lactobacillus* spp., *Lactobacillus delbrueckii, Lactobacillus vermiforme, Leuconostoc* spp., *Leuconostoc mesenteroides* ssp. *dextranicum, L. mesenteroides* ssp. *mesenteroides, Macrococcus caseolyticus, Micrococcus luteus, Sarcina* spp., *Zymomonas mobilis* ssp. *mobilis* (Herrera, 1953; Gonçalves de Lima, 1990), and Yeasts: *Cryptococcus* spp., *Candida parapsilosis, Clavispora lusitaniae, Debaryomyces carsonii, Hanseniaspora uvarum, Kluyveromyces lactis, Kluyveromyces marxianus, Geotrichum candidum, Pichia* spp. *Pichia guilliermondii, Pichia membranifaciens, Rhodotorula* sp., *Rhodotorula mucilaginosa, Saccharomyces bayanus, Saccharomyces cerevisiae, Saccharomyces pastorianus,* and *Torulaspora delbrueckii* (Ruiz Oronoz, 1953; Lappe & Ulloa, 1993).

These species have frequently been isolated from pulque of the central plateau of Mexico, and it has been verified that a degree of uniformity exists in the distribution of the microorganisms of the beverage, with variants according to the production regions.

In recent years, new microbiological studies on pulque have been carried out. In the search for yeast strains for use in biotechnological applications, Estrada-Godina *et al*. (2001) determined the incidence of killer and killer-resistant (neutral) yeasts among *Agave* sap and pulque isolates. Three killer strains from *Agave* sap (*K. marxianus*) and pulque (*K. lactis* and *P. membranifaciens*) were identified. Also, a neutral *S. cerevisiae* strain with remarkable ethanol tolerance was isolated, which has been used in the industrial production of red and white wine and to develop a pulque-like beverage (P. Lappe, pers. commun.).

Escalante *et al*. (2004) determined the bacterial diversity in pulque by 16S rRNA gene sequences. They detected the species *Lactobacillus* strain ASF360, *Lactobacillus acidophilus*, *Lactobacillus kefir*, *Lactobacillus acetotolerans*, *Lactobacillus hilgardii*, *Lactobacillus plantarum, Leuconostoc pseudomesenteroides*, *Microbacterium arborescens, Flavobacterium johnsoniae, Acetobacter pomorium, Gluconobacter oxydans*, and *Hafnia alvei* for the first time in pulque, and indicated the possible presence of new species. They observed the dominance of *Lactobacillus* species (80.97%), although acetic acid bacteria were also present.

Essential fermentative microorganisms and main chemical changes during fermentation

Leuconostoc species (*L. mesenteroides* ssp. *mesenteroides* and *L. mesenteroides* ssp. *dextranicum*) played a vital role through the production of polysaccharides for the development of pulque viscosity (Sánchez-Marroquín & Hope, 1953; Steinkraus, 1997; Ramírez *et al*., 2004). In the initial stages of the fermentation, they increased the sap acidity and evacuated oxygen by the production of CO_2. Once the pH decreased (7.4–6.5), these bacteria presented a slower development; at the same time, homo- and heterofermentative *Lactobacillus* spp. developed, producing more lactic acid,

and their higher growth rate at pH 4.0–6.0 guaranteed their dominance over *Leuconostoc* spp. (Swings & De Ley, 1977; Steinkraus, 1997; Ramírez *et al*., 2004).

As a result of lactic acid bacteria (LAB) fermentation, the total solids (Brix), the direct reducing sugars (glucose, fructose), and the sucrose diminished; they were used in the synthesis of lactic acid, ethanol, and dextrans, while other chemical compounds present in the sap were used for the generation of secondary fermentation compounds (fatty acids and their esters, organic acids, and higher alcohols) that acted as important flavor congeners in the final product, as reported in wine and whisky (Fleet, 1997; Passoth *et al*., 2007). These microorganisms also inhibit the growth of pathogenic bacteria, through organic acid and bacteriocin production, and create the environment for the growth of ethanol-producing species: *Z. mobilis* ssp. *mobilis* and yeasts (Sánchez-Marroquín, 1977; Steinkraus, 1997; Ramírez *et al*., 2004). *Zymomonas mobilis* ssp. *mobilis* plays a key role in the fermentation of pulque, and also produces some lactic acid, acetylmethylcarbinol, gums, acetate, acetone, glycerol, acetaldehyde, and some acetic acid from glucose and fructose (Swings & De Ley, 1977; Gonçalves de Lima, 1990; Steinkraus, 1997; Ramírez *et al*., 2004).

Saccharomyces (*S. cerevisiae, S. bayanus, and S. paradoxus*) and non-*Saccharomyces* (*Candida* spp., *C. parapsilosis, C. lusitaniae, K. marxianus, K. lactis, H. uvarum, Pichia* spp., *P. gulliermondii, and T. delbrueckii*) yeasts were essential fermentative microorganisms that produced ethanol from glucose, fructose, and sucrose, or synthesized both nutritive (amino acids and vitamins) and flavor-volatile compounds that influenced the quality and aromatic profile of the beverage (Sánchez-Marroquín & Hope, 1953; Sánchez-Marroquín, 1967; Steinkraus, 1997; Ramírez *et al*., 2004). At the early stages of fermentation non-*Saccharomyces* yeasts developed. As the ethanol concentration increased, they were replaced by primary ethanol-producing yeasts, *S. cerevisiae* and several *K. marxianus* ethanol-tolerant strains; these dominated until the end of fermentation when pulque had 7% of ethanol (P. Lappe & M. Herrera, pers. commun.).

Some of these yeast species are involved in pulque nutritional bioimprovement with essential amino acids, proteins, and vitamins of microbial origin (Massieu *et al*., 1959; Sánchez-Marroquín, 1967; Steinkraus, 1997).

Although many microbial studies of pulque have been carried out over the last 100 years, the complex microbiota of the beverage has not been well defined. In order to establish the microbial communities involved in the complex pulque fermentation, molecular ecological studies, including non-culture-dependent approaches and PCR-based-molecular techniques, are being performed. These studies will lead to the identification of microbial groups not detected previously in pulque, extending the knowledge of the genetic diversity of this microbial community.

Pulque industrialization

Attempts to obtain a stable, hygienic, best-quality, bottled product with a long shelf-life were initiated at the beginning of the 20th century, but it was not until the 1960s that the first pilot plant was established to produce pulque with pasteurized sap (8°Brix, pH 6.0–7.0) inoculated with 5–10% v/v of a mixed starter (homofermentative *Lactobacillus* spp., *Z. mobilis* ssp. *mobilis*, *Leuconostoc* sp., and *S. cerevisiae*). Also, three industrial production facilities were developed where 50 000 L of industrialized pulque was produced daily. Owing to technical difficulties in the preservation of the quality of the final product and its bottling, these plants are no longer in operation (Sánchez-Marroquín, 1967, 1977; Steinkraus, 1997; Ramírez *et al*., 2004).

Del Razo (2004) developed a technique to ferment pasteurized sap with LAB, *Z. mobilis* ssp. *mobilis*, and *S. cerevisiae* starters, and to can pasteurized natural and flavored pulque (Fig. 1f). This industry produces 50 000–200 000 pulque cans per month, which are exported. At present, in the pulque-producing states, other small-scale production facilities exist that offer six new brands of this beverage, which is a good indication of the re-evaluation that pulque experiences.

In summary, the new pulque industrial processes are focused on improving the nutritional value of the product, as well as its hygienic quality, and yield a high shelf-life.

Chemical and nutritional studies on pulque

Since the second half of the 19th century, several researchers have studied the chemical composition of traditional and industrialized pulque.

Table 3 gives the chemical and physico-chemical characteristics of sap, traditional pulque (several studies average), pasteurized sap, pilot plant sap, and industrialized sap. The main changes observed during the fermentation of natural or pasteurized sap are: decrease in total solids (°Brix) and pH, increase in total and volatile acidity, and a decrease in the sugar content, which are used in different metabolic pathways for the production of ethanol, lactic acid, dextrans, and other compounds (Sánchez-Marroquín, 1977; Steinkraus, 1997; Ramírez *et al*., 2004).

Ortiz-Basurto *et al*. (2008) determined the main chemical characteristics of *A. mapisaga* sap during the harvest period. They detected an average dry matter of 11.5%, composed mainly (75%) of sugars (being mostly glucose and fructose, followed by fructo-oligosaccharides with prebiotic properties, and sucrose), protein (3%), and ashes (3.3%).

Regarding the presence of volatile compounds in pulque, only the work of Sánchez-Marroquín (1977) exists. He

determined the presence of higher alcohols and esters in traditional and pilot plant pulque. The most abundant compound was ethyl acetate (121 μg mL^{-1}), followed by isoamylic alcohol (76 μg mL^{-1}), *n*-propyl alcohol (13 μg mL^{-1}), isobutyl alcohol (22 μg mL^{-1}), and active amyl alcohol (22 μg mL^{-1}). These compounds are products of the LAB, *Zymomonas*, and yeast metabolism, but it remains unknown which species are responsible for each synthesis.

The nutritional value of pulque relates to its content of vitamins (B complex and vitamin C, mainly synthesized by yeasts), essential amino acids (lysine and tryptophane), and bioavailable iron (Cravioto *et al*., 1951; Loyola-Montemayor, 1956; Sánchez-Marroquín, 1967; Steinkraus, 1997; Backstrand *et al*., 2002; Ramírez *et al*., 2004). Table 4 presents the essential compounds in *Agave* sap and the different types of pulque. The concentration of amino acids is higher in traditional than in pilot plant and industrialized pulque. The vitamins presented a similar concentration in both types; because yeast populations increased at the end of the fermentation, it is possible that these microorganisms were responsible for the synthesis of the essential compounds. The greater the yeast population in pulque, the higher the nutritional value of the beverage (Gonçalves de Lima, 1990).

Recently, Ortiz-Basurto *et al*. (2008) detected free amino acids (all essential amino acids except methionine) and four neurotransmitter amino acids with excitatory (glutamine, aspartic acid/asparagine, γ-aminobutyric acid) or inhibitory (glycine) activity in *A. mapisaga* sap, whose stability has not been determined after the sap fermentation (Ramírez *et al*., 2004).

Owing to the presence of these compounds, pulque represents an important part of the daily diet of the low-income population as it supplies several important nutritional compounds, which are lacking in corn diets.

Currently, it is thought that pulque may have prebiotic activity, due to its partially fragmented fructan content (Ortiz-Basurto *et al*., 2008) and probiotic activity because of the presence of LAB and yeasts (Steinkraus, 1997).

The characteristics of this ancient beverage are of such value that techniques must be developed that will allow its nutritional properties to be conserved while providing the low-income consumers with the benefit of a safe product of homogenous quality.

Mexican alcoholic distilled spirits: tequila, mezcal, bacanora, and raicilla

In Mexico, tequila, mezcal, bacanora, and raicilla are specific names of distilled beverages obtained from *Agave* species (Table 1).

Table 3. Amino acid and vitamin content in agave sap, traditional, pilot plant and industrialized pulque

	Pulque types				
Essential compounds	Agave sap*	Traditional pulque†	Traditional pulque‡	Pilot plant pulque‡	Industrialized pulque§
Amino acids (mg 100 mL^{-1})					
Lysine		16.2	3.0	7.5	12.0
Tryptophane		2.7	2.5	9.0	6.0
Hystidine		4.7	4.0	1.0	–
Phenylalanine		11.2	6.5	7.5	–
Leucine		10.5	4.0	6.0	–
Threonine		6.4	1.5	5.0	–
Methionine		0.7	3.0	5.0	–
Valine		6.6	2.5	7.5	–
Arginine		10.9	2.5	3.2	–
Tyrosine			3.0	7.5	
Vitamins (mg 100 mL^{-1})					
Thiamine	0.06	0.02	0.02	0.02	0.02
Riboflavin	0.02	0.025	0.02	0.03	0.03
Niacin	0.45	0.32	0.28	0.37	0.35
Pyridoxine	–	–	0.02	0.03	–
Biotine	–	–	0.02	0.02	–
Ascorbic acid	9.0	5.6	–	–	5.1

Source: *Cravioto *et al*. (1951).
†Massieu *et al*. (1949, 1959) and Loyola-Montemayor (1956).
‡Sánchez-Marroquín (1977).
§Del Razo (2004).

Table 4. Concentrations (mg L^{-1} of must) of volatile compounds in tequila (A) and raicilla (B) musts fermented with yeast strains isolated from pulque, mezcal and raicilla

	Yeast strains isolated from *Agave* beverages							
	Pulque		Mezcal		Raicilla		Raicilla	
	Clavispora lusitaniae		*Kluyveromyces marxianus*		*Kluyveromyces marxianus*		*Kluyveromyces marxianus*	
	Strain 22		Strain 216		Strain 80		Strain158	
Compound	A	B	A	B	A	B	A	B
2-Phenyl ethanol	0	0	3.1	1.8	1.1	2.5	2.5	4.7
Acetaldehyde	0	0	3.3	0	4.2	8.6	4.1	14.0
Ethyl acetate	5.51	9.5	150.3	0	45.1	20.3	63.9	23.4
2-Methyl-propanol	0	0	47.5	0.6	0	84.1	20.3	84.6
Amyl alcohols	0	0	53.8	0.9	0	95.7	57.8	105.5
1-Propanol	0.18	0	33.0	0.4	0.18	48.5	49.3	43.5

Source: Arrizon *et al*. (2007).

Tequila is the distilled beverage classically associated with México and various works have been published describing its microbiology (Lachance, 1995; Arrizon & Gschaedler, 2002; Cedeño, 2003; Faria *et al*., 2003). The word tequila derives from the Nahuatl tequillan, from tequitl = tribute, tequiotl = work or employment, and tlan = place. Nowadays, tequila is produced exclusively in the territory of appellation of origin; production is subject to the Mexican regulation (SECOFI, 2005), and is carried out on an industrial scale, with an annual production of 242.6 million liters in 2006.

The word mezcal derives from the Nahuatl metl = maguey or *Agave*, and from ixcahui = to roast, meaning roasted *Agave*; the word mezcal is the generic name of all the *Agave*-distilled beverages. This word also refers to a spirit with denomination of origin whose elaboration process is subject to the Mexican regulation NOM-070I-1994 (SECOFI, 1994). The processes of elaboration of the different types of mezcal are more traditional, made in small distilleries with lower production levels (6 million liters in 2006) in comparison with the tequila industry.

Characteristics of the raw material

The principal characteristics of the different agaves used as raw material (Table 1) are the presence of fructans (highly branched fructans and neo-fructans) (Mancilla-Margalli & Lopez, 2006), which are used as a reserve substance by the plant; the presence of some chemical compounds (terpenes) contributes to the flavor and taste of the final product. In some cases, the agaves are cultivated (Oaxaca or Zacatecas) or the wild plants are harvested directly (San Luis Potosi, Durango, Guerrero). Propagation is carried out by vegetative means. *Agave* plants are harvested between 8 and 12 years of age, depending on the species and cultivation conditions. Harvesting is carried out in an artisan way: the complete plant is cut, the leaves are removed, leaving the stem, and the bases of the leaves, known as the head or the pine (Fig. 2a and e), are transported to the distilleries and cut into halves to facilitate handling and uniform cooking (A. Gschaedler, pers. commun.).

Traditional production process of *Agave* spirits

The process begins with the cooking of the *Agave* pines. In the traditional process, this step is generally carried out in rustic ovens (hole in the ground, filled with stones, heated with wood, and covered with earth) (Fig. 2b). The cooking process has three main objectives: (1) hydrolysis of fructans into simple sugars (fructose, glucose, sucrose) easily fermented by yeasts; (2) to facilitate the milling operation and the extraction of sugars, because during cooking, the pines acquire a soft texture; and (3) generation of some important chemical compounds involved in the sensorial characteristics of the final product, and some of these are produced by caramelization and Maillard reaction (Cedeño, 2003).

The cooked *Agave* is then milled with wood or steel mallets, and the juice is collected in a canoa a hollow-log fermentation container. The most common method consists of a rudimentary mill or tahona (a circular stone about 1.5 m in diameter rotating in a circular pit where the cooked *Agave* is placed) (Fig. 2c). In the traditional process, two kinds of fermentations are carried out: one with *Agave* juice only and another with the complete *Agave* plant including the juice, pulp, and fiber.

Fermentation tanks are diverse and usually have a capacity of 500–10 000 L. The most rustic tanks are round holes carved directly in the ground. In Oaxaca and Guerrero, the most common tank is a 1000-L wooden vat. In San Luis

Fig. 2. Mezcal traditional and tequila industrial elaboration processes. (a) *Agave* pine of *Agave* sp. (b) Rustic ground oven. (c) Stone mill or tahona. (d) Clay pot stills. (e) Pines of *Agave tequilana* Weber var. *azul*. (f) Extraction of *Agave* juice in industrial mill. (g) Stainless steel industrial fermentation tanks. (h) Detail of the fermenting must.

Potosí, the tanks are stone, rectangular recipients with a variable capacity. The fermentation process is carried out spontaneously by the microbiota present in the must (nitrogen compounds, salts, and water can be added) and lasts for several days depending on the temperature, region, and weather conditions. The fermented must is distilled twice in clay (Phillipine-type clay pot stills) (Fig. 2d) or metal stills.

In the traditional processes, both fermentation and distillation are performed without specific control. This

process is followed in the production of mezcal, bacanora, and raicilla.

Mezcal is produced in the region of denomination of origin, which includes the states of Durango, Guerrero, Oaxaca, San Luis Potosi, Zacatecas, and some regions of Guanajuato and Tamaulipas; the process is subject to the Mexican regulation NOM-070-1994 (SECOFI, 1994). Two categories of mezcal are recognized: Mezcal type I (Mezcal 100% *Agave*), obtained exclusively from *Agave* sugars, and Mezcal type II (Mezcal 80% *Agave*), produced using 80% of *Agave* sugars and 20% from other sugar sources. In each category, there are three types, as in tequila: mezcal joven without maturation, mezcal reposado (aged), and mezcal añejo (extra aged). Bacanora is another *Agave* spirit with government recognition and appellation of origin. Four types of bacanora are recognized: blanco (silver), joven or oro (gold), reposado (aged), and añejo (extra aged) with the same description as the different tequila types. Outside these areas with appellation of origin, other *Agave* spirits are produced. Raicilla is elaborated in the western part of Jalisco with different *Agave* species (Table 1).

Production process of tequila

Tequila is obtained exclusively from distillation of *A. tequilana* var. *azul*-fermented must (Fig. 2e); it is produced in the state of Jalisco and some regions of the states of Michoacán, Guanajuato, Nayarit, and Tamaulipas. The process is subject to the Mexican regulation NOM-006-2005 (SECOFI, 2005), which recognizes two categories: 'Tequila 100%', obtained exclusively from *A. tequilana* var. *azul* sugars, and 'Tequila 51%' or 'Tequila', produced using 51% of *Agave* sugars and 49% from other sugar sources. In each category, there are five types: tequila blanco (silver) without maturation; tequila joven u oro (gold) containing permitted additives and colors (caramel color); tequila reposado (aged) matured at least 2 months in white oak barrels, which is the most popular kind of tequila; tequila añejo (extra aged); and tequila extra añejo (ultra aged), matured from 1 to 3 years, respectively, in white oak barrels (Cedeño, 2003; Gschaedler Mathis *et al.*, 2004)

The industrial production of tequila includes the five stages of the mezcal traditional process. Each step is precisely controlled to increase the efficiency of the process and the production. In some distilleries, cooking is still carried out in brick ovens heated by steam injection, and in others steel autoclaves are used. The cooked *Agave* is milled to extract the sweet must containing a high concentration of fructose; the mills used in the tequila industry combine water extraction and milling (Fig. 2f). In recent years, a diffusion process has been developed using hot water to extract fructans or fructose from crude crushed *Agave* or *Agave* fibers.

Most distilleries use stainless-steel fermentation tanks (2000–120 000 L) (Fig. 2g). The fermentation wort of 'Tequila 100%' is formulated with *Agave* must, with an initial sugar concentration of 4–10% v/v, depending on the amount of water added during milling. For 'Tequila', other sugars are added to obtain an initial sugar concentration of 8–16%, depending on the yeast strain sugar tolerance. Wort formulation is based on the composition of raw materials and nutritional requirements for yeast growth. It is left to ferment spontaneously or selected yeast strains are used (Fig. 2h). Some companies maintain this kind of natural fermentation, as in the traditional process, because the great diversity of microorganisms produces more compounds contributing to the flavor and bouquet of tequila, despite the lower ethanol production. In other distilleries, the wort is inoculated with commercial fresh baker yeast or dry yeast (prepared for wine, beer, or rum production). Another practice is to use autochthonous yeast strains isolated from a natural fermentation. When an inoculum is used, it is scaled up with continuous aeration to produce 5–10% of the final must volume (Cedeño, 2003; Gschaedler Mathis *et al.*, 2004).

Distillation involves the separation and concentration of alcohol from the fermented wort. Tequila is obtained after two consecutive differential distillations in copper or stainless-steel stills. Some distilleries use rectification columns to improve the efficiency of this step and to achieve better control of the final product (Prado-Ramírez *et al.*, 2005). Finally, tequila is matured in different ways, depending on the type of tequila to be obtained. The regulation specifies that the maturation tanks must be made of oak or holm oak wood with a capacity of 600 L. Before bottling, tequila is filtered through cellulose filter pads or polypropylene cartridges. In the case of Tequila 100% the product has to be bottled in the region of denomination of origin; tequila can be exported in bulk outside Mexico.

Yeast in the fermentation process

In the few works dealing with the characterization of the mycobiota involved in the fermentation process of the different *Agave* spirits, the role played by non-*Saccharomyces* and *Saccharomyces* yeasts has been determined. In tequila, Lachance (1995) reported the characterization of yeast communities present in a tequila distillery, where a natural fermentation was carried out. Fresh *Agave* contained *C. lusitaniae* and *Metschnikowia agaves* as dominant yeasts and *K. marxianus* (biotypes different from the distillery strains) and *P. membranifaciens* as secondary yeasts. Cooked *Agave*, fresh must, and crushing equipment had a considerable diversity of species (*Candida* spp., *Candida intermedia*, *Hanseniaspora vineae*, and *P. membranifaciens*) including three *S. cerevisiae* biotypes and *T. delbrueckii* as dominant

yeasts. During fermentation a succession of species was observed. In earlier fermentation stages *Dekkera bruxellensis*, *Hanseniaspora guilliermondii, Hanseniaspora vinae*, *K. marxianus*, *P. membranifaciens*, and *T. delbrueckii* were present as secondary yeasts and *S. cerevisiae* as the dominant species. As fermentation progressed, the heterogeneity of species diminished, and at the end of the fermentation the maltose-positive nonflocculent *S. cerevisiae* biotype became dominant.

Gschaedler Mathis *et al.* (2004) reported the isolation of *Candida magnoliae*, *H. vineae*, *H. uvarum, Issatchenkia occidentalis*, *K. lactis*, and seven *S. cerevisae* strains, with six different karyotype profiles, from 13 tequila distilleries.

In mezcal from Oaxaca, Andrade Meneses & Ruiz Terán (2004) isolated *Candida*, *Hanseniaspora*, *Rhodothorula*, and *S. cerevisiae* species. From a natural fermentation of *Agave fourcroydes* must, Lappe *et al.* (2004) reported a great diversity of yeasts (*Candida* spp., *C. parapsilosis, C. lusitaniae, Debaryomyces hansenii, K. marxianus, Ogataea siamensis, Pichia angusta*, *Pichia caribbica, P. guilliermondii, Rhodotorula mucilaginosa*, *Rhodotorula* spp., and *T. delbrueckii*) and a population of 3.9×10^5 cells mL^{-1} at the beginning of the fermentation, which increased to 1.3×10^8 cells mL^{-1} after 24 h. Fermented must underwent a dramatic reduction in yeast heterogeneity and the population diminished to 1.4×10^7 cells mL^{-1} after 48 h, with *K. marxianus* and *S. cerevisiae* being the predominant species. Escalante-Minakata *et al.* (2008) identified yeast and bacteria present in *A. salmiana* fermentations, where the microbial diversity was dominated by LAB and *Z. mobilis* ssp. *mobilis*. Regarding yeast species, only *C. lusitaniae*, *K. marxianus*, and *Pichia fermentans* were identified. In these few papers published on the mezcal mycobiota, it appears that non-*Saccharomyces* yeasts and LAB play an important role in the initial fermentation stages and influence the generation of volatile compounds involved in the aromatic profile of the final product (Escalante-Minakata *et al.*, 2008; A. Gschaedler, pers. commun.).

Flores Berrios *et al.* (2005) used amplified fragment length polymorphism (AFLP) to detect DNA polymorphism, genotype identification, and genetic diversity between *S. cerevisiae, Candida* spp., and *Hanseniaspora* spp. strains isolated from different *Agave* species, sotol (*Dasylirion* spp.), and grape musts. A direct correlation between the genetic profile, origin, and fermentation process was found particularly in *Agave* must strains.

Little information is available on the evolution of yeast populations during the fermentative process. In the case of tequila, the population of *S. cerevisiae* reached $1.8–2.0 \times 10^8$ cells mL^{-1} after 7 h of cultivation, when the inoculum was developed under optimal conditions (sugar concentration between 50 and 80 g L^{-1}, continuous aeration, temperature of 30 °C, and addition of a nitrogen source). During fermentation, with an initial population of $2.0–2.5 \times 10^7$ cells mL^{-1} and an initial concentration of sugar 140 g L^{-1}, the fermentative process took 24 h; the yeast population reached $1.1–1.2 \times 10^8$ cells mL^{-1}, with an alcohol production between 50 and 60 g L^{-1}. The yeast population remained high throughout the process (A. Gschaedler, pers. commun.). In mezcal from Oaxaca, the native yeast population, mainly non-*Saccharomyces*, reached $1.5–4.0 \times 10^7$ cells mL^{-1} and declined during the fermentative process. This reduction could be associated with the lower alcohol tolerance of these kinds of yeasts or some nutritional limitation; also, 50 g L^{-1} of ethanol was obtained after 5–8 days of fermentation with an initial 150 g L^{-1} of sugar concentration.

De León-Rodríguez *et al.* (2008) optimized the fermentation conditions for the production of *A. salmiana* mezcal with the native microbiota. The highest ethanol production (37.7 g L^{-1}) was obtained in must with 105 g L^{-1} of sugars, and 1 g L^{-1} of ammonium sulfate, fermented 15 h at 28 °C. At the end of the fermentation the biomass (yeasts and bacteria) concentration reached 1.04 g L^{-1}.

Arrizon *et al.* (2006) compared the behavior of yeasts of different origins during fermentation of *A. tequilana* var. *azul* and grape musts. In comparison with *Agave* yeasts (*C. magnoliae*, *Issatchenkia orientalis*, *H. uvarum*, and *S. cerevisiae*) grape yeasts (*H. uvarum* and *S. cerevisiae*) exhibited a reduced fermentation performance in *Agave* musts with a high sugar concentration, while both groups of yeasts showed similar fermentation behavior in grape must. The presence of toxic compounds like furfural and vanillin and the high concentration of fructose in the *Agave* most could explain the poor fermentation performance of the wine yeasts. Fiore *et al.* (2005) demonstrated that non-*Saccharomyces Agave* yeast strains (*Candida krusei*, *C. magnoliae*, and *H. vineae*) possess a high sulfite and ethanol (10–12%) tolerance in controlled fermentations under laboratory conditions. These experimental results on ethanol tolerance contradict what was found in the traditional mezcal process where different yeast strains and different fermentation conditions prevail; however, it highlights an important characteristic that must be studied more thoroughly.

Volatile compounds produced during the fermentation

The raw material volatile compounds play an important role in the development of the sensorial characteristics of the final product. In *A. angustifolia*, *A. salmiana*, and *A. tequilana*, Peña-Álvarez *et al.* (2004) reported the same fatty acid profile, and different terpenes were identified in each species. Of these terpenes, geraniol, linalool, limonene, and 4-terpineol were also detected in the final product. Arrizon

et al. (2007) determined the volatile compounds in tequila and raicilla. Sixteen terpenes were found in both beverages (β-Myrcene, isocineole, linalool, 1-terpineol, 4-terpineol, citronellol, nerol, geraniol, and nerolidol) and 15 more were detected only in raicilla (α-pinene, camphene, limonene, γ-terpinene, ρ-cymene, mycenol, neryl acetate, geranyl acetate, and α-eudesmol). Terpenes are important aromatic compounds that, in low concentrations, confer distinctive characteristics to the final product. Some terpenes are present in the raw material as complex polysaccharide (cellulose and hemicellulose) compounds, and may be released by the action of microbial enzymes like β-glucosidase, β-cellobiosidase, and β-xylosidase. A screening for the presence of β-glucosidase and β-xylosidase has been performed on six yeasts (three *S. cerevisiae strains*: *C. magnoliae*, *C. krusei*, and *H. vineae*) isolated from *Agave* musts. *Saccharomyces* strains produce β-xylosidase and the non-*Saccharomyces* produce both enzymes. The highest activity of the β-glucosidase was obtained in the *C. magnoliae* isolate (Fiore *et al*., 2005). Hence, the yeasts present in the fermentation process could play an important role in the release of terpenes. Moreover, each strain seems to have different enzymatic activities and so the aromatic profile of the beverage could be different depending on the mycobiota involved in the fermentation process.

Several works have been published on the volatile compounds present in tequila (Benn & Peppard, 1996; López, 1999; López & Dufour, 2001; Faria *et al*., 2003; Escalona Buendía *et al*., 2004). Escalona Buendía *et al*. (2004) reported 237 volatile compounds in white tequila samples.

Estarrón (1997) determined, in five tequila samples, the concentration of the most important compounds controlled by the Mexican regulation. Ethanol presented the highest concentration, followed by higher alcohols, methanol, ethyl acetate, and acetaldehyde. They also reported 24 different esters, the most abundant being ethyl decanoate, ethyl dodecanoate, ethyl lactate, and ethyl octanoate, whose concentration varied in the products analyzed. Detailed studies on the relation of the microorganisms present in the fermentation with the generation of esters are still missing.

Arrizon *et al*. (2007) determined the production of 2-phenylethyl alcohol, 2-methyl-propanol, amyl alcohols and 1-propanol, acetaldehyde, and ethyl acetate on tequila, and raicilla musts by different yeast strains isolated from pulque, raicilla, and mezcal. Table 4 shows the different volatile compound concentrations. The *C. lusitaniae* strain isolated from pulque did not ferment any of the must tested. The two strains of *K. marxianus* from raicilla produced more volatile compounds in raicilla than in tequila must, and the *K. marxianus* strain from mezcal fermented the tequila must actively and the raicilla must poorly. In both musts, the compound concentrations were quite different; however, on comparing the concentration of each alcohol with the total higher alcohol concentration, it was similar in both musts. A different behavior was observed with the production of ethyl acetate, which was higher in tequila than in raicilla must. This study highlights the importance of the yeast strains and its origin in the generation of volatile compounds and the relationship of the yeast with the musts.

Another important higher alcohol detected in tequila was phenyl ethyl alcohol. This alcohol was produced in *A. tequilana* must by yeast strains isolated from *Agave* must fermentation but not by grape yeast strain (Arrizon *et al*., 2006).

Pinal *et al*. (1997) demonstrated that the most important factor influencing the production of isoamyl alcohol and isobutanol was the yeast strain used as inoculum. It was found that a native *S. cerevisiae* strain isolated from tequila must produced a higher amount of isoamyl and isobutyl alcohol than when compared with baker yeast. The carbon/nitrogen (C/N) ratio also has a significant influence on higher alcohol production. In tequila musts, it was found that when the C/N ratio was small, the production of higher alcohols was low (19 mg L^{-1} in baker yeast and 30 mg L^{-1} for native strain), and when this ratio was high, a higher alcohol production was observed (27 and 64 mg L^{-1} isoamyl alcohol, respectively).

De León-Rodríguez *et al*. (2006) analyzed several commercial mezcal samples produced from *A. salmiana*: white, rested, aged, and white and rested with worm. The addition of the worm is primarily a commercial ploy, and there is no technical reason for, or any improvement in the sensorial characteristics from, the worm inside the bottles of some mezcal brands (Cedeño, 2003). De León-Rodríguez *et al*. (2006) identified 37 compounds, and nine were classified as major compounds (Table 5); ethanol was the most abundant compound, followed by methanol and n-propanol and other higher alcohols (2-butanol, 2-methyl-propanol, 2-methyl-1-butanol, and 3-methyl-1-butanol). During pine cooking, methanol is directly produced from pectin and lignin, which are present in the cell wall. The other compounds are synthesized during the fermentation process as result of sugar biotransformation or by the catabolism of amino acids. Significant differences were observed in the concentrations of the compounds that could be explained by the presence of a different microbiota (which is not controlled) or by different fermentation conditions during the production of the samples analyzed. In the same study, a high concentration of acetic acid was also detected, which could be due to a high contamination of the fermentative musts with acetic acid bacteria.

In mezcal obtained from *A. angustifolia*, López & Guevara-Yáñez (2001) reported the same compounds; furfural and 5-methyl-furfuraldehyde were also detected as major compounds. It is clear that in the case of mezcal, detailed

Table 5. Concentrations (mg L^{-1}) of the major compounds found in different samples of mezcal from San Luis Potosi

	Type of mezcal				
Compound	White ($n = 4$)	White with worm* ($n = 3$)	Aged ($n = 3$)	Aged with worm* ($n = 3$)	Extra aged ($n = 3$)
Ethyl acetate	182 ± 11	103 ± 6	150 ± 12	113 ± 5	107 ± 10
Methanol	816 ± 72	703 ± 89	891 ± 46	881 ± 38	834 ± 76
Ethanol†	42 ± 1	39 ± 2	39 ± 2	42 ± 0	42 ± 5
2-Butanol	61 ± 17	54 ± 19	66 ± 20	56 ± 6	ND
n-Propanol	700 ± 108	728 ± 18	738 ± 87	615 ± 62	388 ± 26
2-Methyl-propanol	17 ± 4	ND	ND	37 ± 4	48 ± 7
2/3-Methyl-1-butanol	30 ± 7	26 ± 10	117 ± 15	17 ± 4	98 ± 19
Ethyl-2-hydroxypropanoate	105 ± 13	101 ± 4	207 ± 27	109 ± 17	192 ± 32
Acetic acid	169 ± 51	224 ± 48	843 ± 64	133 ± 5	219 ± 9
High alcohol	809 ± 122	808 ± 32		876 ± 52	533 ± 51

Source: De León-Rodríguez *et al*. (2006).

*The addition of the worm is a commercial ploy. There is no technical reason for, or any improvement in the sensorial characteristics from, the worm inside the bottles of some mezcal brands.

†Ethanol concentration in % v/v. ND, not detectable.

studies on the relation of the microorganisms present in the fermentation with the generation of volatile compounds should be carried out.

Conclusion and perspectives

The great variety of agaves and their multiple uses have played an important role in the cultural identification of Mexico. They have been exploited in many ways for over 10 000 years, and one of these applications is the production of alcoholic and distilled beverages. Until today, the microbiota that participates in the fermentation and its biochemical role in this process remain largely unknown; therefore, it is essential to carry out more studies on the traditional processes that are still in use because they are the source of important microbial consortia that could disappear with the introduction of new technologies.

A detailed phenotypical and genotypical characterization of the microbiota must be carried out in order to conserve this specific biodiversity and subsequently evaluate its potential as starter cultures and in the production of different chemical compounds of biotechnological importance.

Acknowledgements

The authors thank B.Sc. Concepción León Cano.

References

Andrade Meneses OE & Ruiz Terán F (2004) Study of yeast populations in a mezcal fermentation. Poster PF 17, p. 157. 11th International Congress on Yeasts. Yeasts in Science and Technology, The quest for Sustainable Development. Rio de Janeiro, Brazil.

Arrizon J & Gschaedler A (2002) Increasing fermentation efficiency at high sugars concentrations by supplementing an additional source of nitrogen during the exponential phase of the tequila fermentation process. *Can J Microbiol* **48**: 965–970.

Arrizon J, Fiore C, Acosta G, Romano P & Gschaedler A (2006) Fermentation behavior and volatile compounds production by agave and grape must yeasts in high sugar *Agave tequilana* and grape must fermentations. *Antonie van Leeuwenhoek* **8**: 181–189.

Arrizon J, Arizaga JJ, Hernández RE, Estarrón M & Gschaedler A (2007) Production of volatile compounds in tequila and raicilla musts by different yeasts isolated from Mexican agave beverages. *Hispanic Foods Chemistry and Flavor* (Tunick MH & González de Mejía E, eds), pp. 167–177. American Chemical Society Symposium Series 946, Washington, DC.

Backstrand JR, Allen LH, Black AK, Mata M & Pelto G (2002) Diet and iron status of non pregnant women in rural central Mexico. *Am J Clin Nutr* **76**: 156–164.

Benn SM & Peppard TL (1996) Characterization of tequila flavor by instrumental and sensory analysis. *J Agric Food Chem* **44**: 557–566.

Bruman HJ (2000) *Alcohol in Ancient Mexico*. The University of Utah Press, Salt Lake City, Utah, UT.

Cedeño M (2003) Tequila production from agave: historical influences and contemporary processes. *The Alcohol Textbook*, 4th edn (Jacques KA, Lyons TP & Kelsall DR, eds), pp. 223–245. Nottingham University Press, Nottingham, UK.

Cisneros ME, Llovera L & Lappe P (1997) *Identificación de las levaduras aisladas de tesgüino, sotol y mezcal*, pp. 157. VI Congreso Nacional de Micología, Tapachula, Chiapas, México.

Cravioto RO, Massieu HG, Guzmán J & Calvo de la Torre J (1951) Composición de alimentos mexicanos. *Ciencia México* **11**: 129–156.

De León-Rodríguez A, González-Hernández L, Barba de la Rosa A, Escalante-Minakata P & López M (2006) Characterization

of volatile compounds of mezcal, an ethnic alcoholic beverage obtained from *Agave salmiana*. *J Agric Food Chem* **54**: 1337–1341.

De León-Rodríguez A, Escalante-Minakata P, Barba de la Rosa A & Blaschek HP (2008) Optimization of fermentation conditions for the production of the mezcal from *Agave salmiana* using response surface methodology. *Chem Eng Process* **47**: 76–82.

Del Razo R (2004) Valor nutritivo del pulque. Available from www.pulqueazteca.com.

Escalante A, Rodríguez ME, Martínez A, López-Munguía A, Bolivar F & Gosset G (2004) Characterization of bacterial diversity in *Pulque*, a traditional Mexican alcoholic fermented beverage, as determined by 16S rDNA analysis. *FEMS Microbiol Letters* **235**: 273–279.

Escalante-Minakata P, Blaschek HP, Barba de la Rosa AP, Santos L & De León-Rodríguez A (2008) Identification of yeast and bacteria involved in the mezcal fermentation of *Agave salmiana*. *Lett Appl Microbiol* **46**: 629–638.

Escalona Buendía HB, Villanueva Rodríguez SJ, López Ramírez JE, González Herrera RM, Martin del Campo Barba T, Estarrón Espinosa M, Cosio Ramírez R & Cantor Solórzano E (2004) Calidad del tequila como producto terminado: normatividad, composición volátil y la imagen sensorial. *Ciencia y Tecnología del Tequila, Avances y Perspectivas* (Centro de Investigación y Asistencia Tecnológica y Diseño del Estado de Jalisco, ed), pp. 171–256. CIATEJ, Guadalajara, Mexico.

Estarrón M (1997) Identificación de los principales compuestos volátiles que caracterizan al tequila 100% de agave. MSc Thesis, University of Guadalajara, Mexico.

Estrada-Godina AR, Cruz-Guerrero AE, Lappe P, Ulloa M, García Garibay M & Gómez Ruiz L (2001) Isolation and identification of killer yeasts from *Agave* sap (aguamiel) and pulque. *World J Microbiol Biotech* **17**: 557–560.

Faria JB, Loyola E, López MG & Dufour JP (2003) Cachaça, pisco and tequila. *Fermented Beverages Production* (Lea AGH & Piggott JR, eds), pp. 335–364. Kluwer Academic/Plenum Publishers, New York.

Fiore C, Arrizon J, Gschaedler A, Flores J & Romano P (2005) Comparison between yeasts from grape and agave musts from traits of technological interest. *World J Microbiol Biotechnol* **21**: 1141–1147.

Fleet G (1997) The wine. *Food Microbiology. Fundamentals and Frontier* (Doyle M, Beuchat L & Montville T, eds), pp. 699–721. American Society for Microbiology, Washington, DC.

Flores Berrios EP, Alba González JF, Arrizon Gaviño JP, Romano P, Capece A & Gschaedler Mathis A (2005) The uses of AFLP for detecting DNA polymorphism, genotype identification and genetic diversity between yeasts isolated from Mexican agave-distilled beverages and from grape musts. *Lett Appl Microbiol* **41**: 147–152.

García-Mendoza A (1995) El maguey una planta maravillosa. *En torno al pulque y al maguey* (Cofradía en Apoyo de la Mayora Mexicana AC, ed), pp. 38–42. Grupo Editorial Siquisiri, Mexico.

García-Mendoza A (1998) *Con sabor a maguey: Guía de la colección nacional de Agaváceas y Nolináceas del Jardín Botánico*. Instituto de Biología, Jardín Botánico, Universidad Nacional Autónoma de México, Mexico.

Gobierno del Estado de Hidalgo y Museo Nacional de Culturas Populares (1988) *El maguey: árbol de las maravillas*. Instituto Nacional Indigenista, Secretaría de Educación Pública y Dirección General de Culturas Populares, Mexico.

Gonçalves de Lima O (1978) *El maguey y el pulque en los códices mexicanos*. Fondo de Cultura Económica, Mexico.

Gonçalves de Lima O (1990) *Pulque, balché y pajauarú*. Fondo de Cultura Económica, Mexico.

Gschaedler A, Gallardo J & Villanueva S (2008) El proceso de elaboración del mezcal en el Estado de Michoacán. *La producción de mezcal en el Estado de Michoacán* (Gobierno del Estado Michoacán, ed), pp. 72–92. Michoacán, Mexico.

Gschaedler Mathis A, Ramírez Cordova J, Diaz Montaño D, Herrera López H, Arellano Plaza M, Arrizon Gaviño J & Pinal Zuazo L (2004) Fermentación: etapa clave en la elaboración del tequila. *Ciencia y Tecnología del Tequila, Avances y Perspectivas* (Centro de Investigación y Asistencia Tecnológica y Diseño del Estado de Jalisco, ed), pp. 32–120. CIATEJ, Guadalajara, Mexico.

Herrera T (1953) *Trabajos que se han hecho en México sobre bacterias de líquidos fermentados. Memoria del Congreso Científico Mexicano*, pp. 36–53. IV Centenario de la Universidad de México. Universidad Nacional Autónoma de México, Mexico.

Lachance MA (1995) Yeast communities in a natural tequila fermentation. *Antonie van Leeuwenhoek* **68**: 151–160.

Lappe P & Ulloa M (1993) Microbiología del pulque. *Alimentos fermentados indígenas de México* (Wacher MC & Lappe P, eds), pp. 75–80. Universidad Nacional Autónoma de México, Mexico.

Lappe P, Ulloa M, Arce Rocha G, Cáceres Farfán M, Tapia Tussel R, Pérez Brito D & Larque A (2004) Isolation and identification of the mycobiota present in *Agave fourcroydes*. Poster PE 11, p. 175, 11th International Congress on Yeasts. Yeasts in Science and Technology, The Quest for Sustainable Development. Rio de Janeiro, Brazil.

López MG (1999) Tequila aroma. *Flavor Chemistry of Ethnic Foods* (Shahidi F & Ho CT, eds), pp. 211–217. Kluwer Academic/Plenum Publishers, New York.

López MG & Dufour JP (2001) Tequila: charm analysis of blanco, reposado, and añejo tequilas. *Gas Chromatography-Olfactometry: The State of the Art* (Leland JV, Schreirer P, Buettner A & Acree TE, eds), pp. 62–72. American Chemical Society Symposium Series 782, Washington, DC.

López MG & Guevara-Yáñez SC (2001) Authenticity of three Mexican alcoholic beverages by SPME-GC-MS. Paper 10-3. Annual Meeting of Institute of Food Technologists, New Orleans, LA.

Loyola-Montemayor E (1956) *La industria del pulque*. Departamento de Investigaciones Industriales. Banco de México SA, Mexico.

Mancanilla-Margalli N & López MG (2002) Generation of maillard compounds from inulin during the thermal processing of *Agave tequilana* weber var. *azul. J Agric Food Chem* **50**: 806–812.

Mancilla-Margalli N & Lopez M (2006) Water-soluble carbohydrates and fructan structure patterns from *Agave* and *Dasylirion* species. *J Agri Food Chem* **54**: 7832–7839.

Massieu HG, Guzmán J, Cravioto RO & Calvo J (1949) Determination of some essential amino acids in several uncooked Mexican foodstuffs. *J Nutr* **38**: 293–304.

Massieu HG, Cravioto RO, Guzmán G & Olivera BH (1959) Contribución adicional al estudio de la composición de los alimentos mexicanos. *Ciencia, México* **19**: 53–66.

Ortiz-Basurto RI, Pourcelly G, Doco T, Williams P, Dornier M & Belleville MP (2008) Analysis of the main components of the aguamiel produced by the maguey pulquero (*Agave mapisaga*) throughout the harvest period. *J Agri Food Chem* **56**: 3682–3687.

Passoth V, Blomqvist J & Schnurer J (2007) *Dekkera brouxellensis* and *Lactobacillus vini* form a stable ethanol-producing consortium in a commercial alcohol production process. *Appl Environ Microbiol* **73**: 4354–4356.

Peña-Álvarez A, Díaz L, Medina A, Labastida C, Capella S & Vera LE (2004) Characterization of three *Agave* species by gas chromatography and solid-phase microextraction-gas chromatography-mass spectrometry. *J Chromat A* **1027**: 131–136.

Pinal L, Cedeño M, Gutierrez H & Alvarez-Jacobs J (1997) Fermentation parameters influencing higher alcohol production in the tequila process. *Biotechnol Lett* **19**: 45–47.

Prado-Ramírez R, Gonzáles-Alvarez V, Pelayo-Ortiz C, Casillas N, Estarrón M & Gómez-Hernández H (2005) The role of distillation on the quality of tequila. *Int J Food Sci Technol* **40**: 701–708.

Ramírez JF, Sánchez-Marroquín A, Álvarez MM & Valyasebi R (2004) Industrialization of Mexican pulque. *Industrialization of Indigenous Fermented Foods*, 2nd edn (Steinkraus K, ed), pp. 547–586. Marcel Deckker, New York.

Ruiz Oronoz M (1953) *Estudios en México sobre levaduras. Memoria del Congreso Científico Mexicano*, pp. 127–149. IV Centenario de la Universidad de México. Universidad Nacional Autónoma de México, Mexico.

Sánchez-Marroquín A (1967) Estudio sobre la microbiología del pulque. XX. Proceso industrial para la elaboración técnica de la bebida. *Rev Lat-Am Microbiol Parasitol* **9**: 87–90.

Sánchez-Marroquín A (1977) *Mexican Pulque- a Fermented Drink from Agave Juice*. Symposium on Indigenous Fermented Foods, Bangkok, Thailand.

Sánchez-Marroquín A & Hope PH (1953) Agave juice fermentation and chemical composition studies of some species. *J Agric Food Chem* **1**: 246–249.

SECOFI (1972a) *NMX-V-022-1972*. Hidromiel. Normas Mexicanas, Dirección General de Normas. Diario Oficial Septiembre 26, Mexico.

SECOFI (1972b) *NMX-V-037-SECOFI-1972*. Pulque manejado a granel. Norma Oficial Mexicana Dirección General de Normas. Diario Oficial Septiembre 26, Mexico.

SECOFI (1994) *NOM-070-SCFI-1994*. Bebidas Alcohólicas-Mezcal, Especificaciones. Norma Oficial Mexicana Diario Oficial 17 de Agosto, Mexico.

SECOFI (2005) *NOM-006-SCFI-2005*. Bebidas Alcohólicas–Tequila, Especificaciones. Norma Oficial Mexicana Diario Oficial 6 de Enero, Mexico.

Steinkraus K (1997) Mexican pulque. *Handbook of Indigenous Fermented Foods*, 2nd edn (Steinkraus K, ed), pp. 389–397. Marcel Deckker Inc., New York.

Swings SJ & De Ley J (1977) The biology of *Zymomonas*. *Bacteriol Rev* **41**: 1–46.

Vallejo-Córdoba B, González-Córdoba AF & Estrada-Montoya MC (2005) Latest advances in the characterization of Mexican distilled agave beverages: tequila, mezcal and bacanora. AGFD-113 229th ACS meeting, San Diego, CA.