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Yeast communities in a natural tequila fermentation

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Abstract

Fresh and cooked agave, *Drosophila* spp., processing equipment, agave molasses, agave extract, and fermenting must at a traditional tequila distillery (Herradura, Amatitan, Jalisco, México) were studied to gain insight on the origin of yeasts involved in a natural tequila fermentations. Five yeast communities were identified. (1) Fresh agave contained a diverse mycobiota dominated by *Clavispora lusitaniae* and an endemic species, *Metschnikowia agaveae*. (2) *Drosophila* spp. from around or inside the distillery yielded typical fruit yeasts, in particular *Hanseniaspora* spp., *Pichia kluyveri*, and *Candida krusei*. (3) *Schizosaccharomyces pombe* prevailed in molasses. (4) Cooked agave and extract had a considerable diversity of species, but included *Saccharomyces cerevisiae*. (5) Fermenting juice underwent a gradual reduction in yeast heterogeneity. *Torulaspora delbrueckii*, *Kluyveromyces marxianus*, and *Hanseniaspora* spp. progressively ceded the way to *S. cerevisiae*, *Zygosaccharomyces bailii*, *Candida milleri*, and *Brettanomyces* spp. With the exception of *Pichia membranaefaciens*, which was shared by all communities, little overlap existed. That separation was even more manifest when species were divided into distinguishable biotypes based on morphology or physiology. It is concluded that crushing equipment and must holding tanks are the main source of significant inoculum for the fermentation process. *Drosophila* species appear to serve as internal vectors. Proximity to fruit trees probably contributes to maintaining a substantial *Drosophila* community, but the yeasts found in the distillery exhibit very little similarity to those found in adjacent vegetation. Interactions involving killer toxins had no apparent direct effects on the yeast community structure.

Introduction

Tequila is a distilled beverage made from the fermented sugars of cooked *Agave tequilana* var. *azul* grown near the town of Tequila and other regions of the state of Jalisco, México. It belongs to a more general category of spirit known as mescal, which can be produced from several species of agave in different Mexican regions, in particular the state of Oaxaca. The principal carbohydrate of agave is inulin (Sánchez-Marroquín & Hope 1953), which can be hydrolysed to fermentable sugars, (mostly fructose) by heating with steam. Supplementation of tequila must with up to 49% of other sugars is allowed. Nitrogen deficiencies may be remedied by the addition of nitrogenous salts.

Myths and legends

Several misconceptions prevail regarding tequila. The most common is that it is made from cactus. Although both cacti and agaves are succulents, the Cactaceae are a family of dicots whereas the Agavaceae are monocotyledonous. A second frequently held fallacy is that authentic tequila should contain a worm. Although an agave-inhabiting grub is sometimes added to bottles of certain mescals, this custom is not the normal practice of tequila producers. Third, it is sometimes said that tequila is prepared by distillation of *pulque*, another agave-based beverage. In fact, *pulque* is fermented from sap recovered from the hollowed heart of mature, uncooked agave plants, and involves a natural microbiota that includes the bacterium *Zymomonas mobilis* in addition to other organisms. Last, to some, the name mescal may suggest that these beverages con-

tain mescaline or related psychotropic alkaloids, with the result that their intoxicating properties are significantly different from those of other spirits. In fact, such alkaloids originate from various cactus species (Nobel 1994). The special physiological reponses experienced by some may be due to the higher levels of alcohols other than ethanol in certain products, or simply the result of immoderate consumption.

Facts

The tequila industry is a remarkable amalgam of tradition and technology. Cooking equipment ranges from brick ovens sealed with bagasse to state-of-the-art autoclaves. These have progressively replaced the method still used by certain mescal producers, which consists of burying the agave hearts in a pit over hot embers. In some cases, mixed fermentations are left to occur spontaneously, and in others, cross-inoculation, seed tanks, commercial yeast cultures, or bacterial suppressants are utilized. Two-stage batch distillation in pot stills may coexist with continuous-flow processes involving rectifiers. Some products are bottled directly with no further amendments (*blanco*), and others are aged for various periods (*reposado, añejo*) in high quality oak casks that have been subjected to various degrees of charring.

The yeast community of a natural tequila fermentation

The premium product called Tequila Herradura is prepared uniquely from agave, using plants harvested at full maturity, up to 12 years of age. Cooking and distillation follow mostly traditional methods. No effort is made to interfere with the implantation of a natural microbiota to initiate fermentation. Various hypotheses have been formulated locally about the origin, composition, and importance of the yeasts. First, the possibility existed that agave plants in the field harbour an indigenous microbial community that is carried to the distillery at harvest. In addition, the Herradura estate encompasses sizeable plantations of fruit trees, leading to the possibility that yeasts originate from the neighbouring vegetation. Alternatively, the milling and crushing equipment present near the fermentations vessels could be a major source of yeasts. Although this equipment is maintained to the highest standards of cleanliness, harsh cleaning agents are not used, and it is reasonable to assume that a low but significant degree of surface contamination exists.

Harvested plants in the field or at the distillery, as well as sections of the factory where agave pulp, fibre, or juice are accumulated all teem with *Drosophila* species. The possible involvement of these flies as yeast vectors, repositories, or both, could not be discounted. As a last possibility, although no conscious effort is made to propagate yeast in a designated seed tank, it is conceivable that the juice collecting vessels could act as major sources of inoculum. This study was undertaken to resolve some of these questions.

Materials and methods

Study site

The Tequila Herradura estate is located in Amatitan, Jalisco, México. The distillery is bordered by an immense agave plantation. Mature plants are harvested as needed. The leaves are trimmed off (with a special tool called *jima*) in the field and the hearts (*piñas*) are carried to a reception area near the ovens. Usually the same day, the hearts are cooked in steam for 24 h, and allowed to cool for an equivalent period. During cooking, a syrup referred to as honey (*miel*) or molasses is collected from the oven floor and stored in an open tank before being added to must, usually the same day. The cooked hearts are transferred to a mill to be ground and repeatedly crushed and sparged with water to extract soluble sugars. The resulting extract reaches an underground reservoir from which it can be pumped into one of a dozen open fermentors ranging in size from 25,000 to 50,000 L approximately. The separately stored syrup is mixed with agave juice into the fermentors at a rate of ca. 10% of the must volume. The fibrous solids (bagasse) are piled outside the building and disposed of the same day.

Sampling and identification

Samples were taken over a 7 day period in February 1992. The various materials examined and the number of samples obtained from each are listed in Table 1. Not included were a few samples of decaying fruits collected in the estate garden, and which did not give rise to yeast colonies. Agave or fruit solids were dissected with a sterile spatula and diluted ca. ten-fold in sterile distilled water. Juice, molasses, or fermenting must were collected in sterile vials. A loopful (ca. 10 μL) of sample was streak-inoculated onto YM agar acidified to pH 3.7 with HCl. *Drosophila* were caught

Table 1. List of materials sampled.

Substrate type		Number of samples	
		Yeasts present	Yeasts absent
Agave rots		22	5
Fruit			8
Drosophila spp.	Outdoor	6	
	Indoor	13	1
Agave molasses		6	
Mill and crusher		8	
Agave extract		2	
Active fermentation	Early	21	
	Intermediate	11	
	Advanced	7	

with a net and transferred to sterile shell vials. Individual flies were allowed to reside in 60 mm Petri dishes containing acidified YM agar for about 1 hour and then released. Plates were examined at suitable intervals. For each colony type, the approximate number of colonies was recorded and a representative was picked for identification. The abundance values reported in Table 2 are based on these estimates, with the three categories 1 (1 to 50 colonies), 2 (51–500 colonies, and 3 (over 501 colonies). Standard methods (van der Walt & Yarrow 1984) were followed for species determination. Growth tests were performed by replica plating with a multipoint inoculator (Lachance 1987). Bacteria were isolated by streak-inoculation of 5% glucose, 0.5% yeast extract, 0.5% calcium carbonate agar containing 100 mgL^{-1} cycloheximide. Identification of bacterial genera followed the methods given in Bergey's manual (Buchanan & Gibbons 1974).

Killer reactions

Production of and sensitivity to killer toxins was determined by the method of Woods & Bevan (1968). All isolates were screened using *Candida glabrata* Y55 as target. Positive strains were tested further against representatives of every major species isolated in this study. For this purpose, the putative killers were spot-inoculated heavily by replica plating, and the target strains were spray-inoculated by vaporization with sterilized spray bottles.

Ethanol tolerance

Growth in the presence of ethanol was assessed by replica plating onto YM agar supplemented with 8% glucose. The autoclaved medium was cooled to 45–50° C and warm ethanol (6 to 12% v/v in 1% increments) was added immediately before pouring. The plates were sealed with parafilm soon after setting of the agar, and again after inoculation. Final readings were taken 9 days after inoculation.

Results

Yeast identification

The species recovered from the various habitats sampled are listed in Table 2. To keep Table 2 to a manageable size, a number of taxa isolated at low frequencies were pooled under common names. The isolates reported as *Rhodotorula* spp. were not purified for identification. Reported as *Candida* species were seven different yeasts recovered from agave. Of these, one was clearly identified as *Candida oleophila*, three exhibited various degrees of resemblance to *Candida silvicola*, one keyed imperfectly to *Candida valvidiana*, and two had novel combinations of characters which made it impossible to identify them. Two filamentous species of basidiomycetous affinity were designated as *Trichosporon* spp. Amongst the 11 isolates recovered from substrates in the distillery and reported as *Candida* species, four keyed imperfectly to *Candida rugosa*, four were slow growing organisms forming chains of large ovoid cells and a pigment similar to pulcherrimin, and three had different combinations of characters that did not match any known descriptions.

The agave isolates designated as *Sporopachydermia*-like exhibited a superficial resemblance to that genus in their physiological traits, but could not be identified adequately. The isolate identified as *Pichia amethionina* differed from the standard description by its lack of growth on ethyl acetate.

The isolates identified as *Kluyveromyces marxianus* (Table 2) could be assigned to five biotypes on the basis of presence or shape of ascospores and utilization of β-glucosides. Similarly, the isolates identified as *Torulaspora delbrueckii* belonged to four categories differentiated by utilization of nitrite, lysine, or cadeverine. Three biotypes of *P. membranaefaciens* were recovered. The majority were asporogenous and

Table 2. List of yeasts recovered from samples listed in Table 1. An abundance score (1 to 3, see Materials and methods) is given, preceded by the number of positive samples in the case of multiple isolations.

Designation	Sample	Agave rots	*Drosophila* species		Agave molasses	Cooked agave	Agave extract	Fermentation		
			Indoor	Outdoor				Early	Intermediate	Advanced
Prototheca zopfii		2 × 2, 3 × 1				1				
Rhodotorula spp.		2, 1, 1, 1				1				
Clavispora lusitaniae		2 × 3, 6 × 1		2						
Metschnikowia agaveae		3,3 × 2,2 × 1								
Candida guilliermondii		2,3 × 1					1	3,5 × 2, 4 × 1	3,2 × 2	
Sporopachydermia-like		3,2 × 2						2, 1	2, 1	
Candida spp.		2,6 × 1					1	2 × 2, 2 × 1	2 × 3,2	3 × 3,2,1
Pichia amethionina-like		1					1	3,2 × 2, 2 × 1		
Debaryomyces hansenii		1						8 × 2, 6 × 1	2 × 2, 2 × 1	
Geotrichum penicillatum		1						2 × 1		
Trichosporon spp.		1	1				2 × 1			
Arthroascus javanensis			1				3,2 × 2,1	8 × 3, 8 × 2, 2 × 1	11 × 3,2,1	2 × 3, 2 × 2
Pichia kluyveri			4 × 1			2,2 × 1	1			
Candida boidinii						1				
Schizosaccharomyces pombe					1					
Candida intermedia		1			2 × 3, 2 × 2					
Candida krusei						2 × 1				
Brettanomyces anomalus		3,2,2 × 1	1			3 × 1	1			
Kluyveromyces marxianus		3,2,3 × 1	2 × 1		6 × 1	2,4 × 1				
Pichia membranaefaciens		3,2,4 × 1	1		5 × 1	1				
Torulaspora delbrueckii			2 × 1		3 × 1					
Hanseniaspora spp.			2,3 × 1		2 × 2, 8 × 1					
Candida spp.					1					
Saccharomyces cerevisiae					1	1				
Pichia anomala										
Issatchenkia/*Pichia* sp.										
Saccharomycodes ludwigii										
Zygosaccharomyces baillii										
Candida milleri										
Brettanomyces bruxellensis										
Zygosaccharomyces rouxii										

formed a large-celled pseudomycelium. Three strains recovered in *Drosophila* (indoors) and in the crushing equipment sporulated abundantly and produced killer toxins; two of these assimilated sorbose. Strains reported as *Hanseniaspora* species were members of either *Hanseniaspora guilliermondii, Hanseniaspora vineae*, or their anamorphs. They could not be assigned unequivocally to particular species because of variation in their assimilation of 2-ketogluconic acid (2 kg), their growth at 37° C, or their resistance to cycloheximide. Strains of *Saccharomyces cerevisiae* could be assigned to a number of biotypes, based on their responses to various tests. Most noteworthy is the fact that approximately one quarter of the strains did not utilize maltose. Among maltose utilizers, a few strains exhibited strong flocculation characteristics, and one produced a killer toxin. Last, the majority of strains identified as *Zygosaccharomyces bailii* were unable to utilize lysine as sole nitrogen source and grew well on sorbose as sole carbon source; a few strains were more typical.

Identification of communities

(1) Agave

It is evident in Table 2 that the yeast community of agave is distinct from the others. Agave plants undergo small infections at the base of their leaves. These are seen in harvested agave hearts as bright red to purple areas that often release an odour typical of fermenting succulents, and in some cases a clear smell of acetic fermentation. The dominant yeasts in these rots were *Clavispora lusitaniae* and the recently described (Lachance 1993) *Metschnikowia agaveae*. In addition to various other species (Table 2), agave rots yielded a few isolates of *Brettanomyces anomalus*, which would account for the acetic smell, and a significant number of a *K. marxianus* biotype different from those present in distillery samples (the agave isolates were the only ones that produced reniform ascospores). The asporogenous biotype of *P. membranaefaciens* was the only yeast that appeared to be truly shared with other habitats, including the fermentations.

(2) Drosophila

The *Drosophila* species captured out of doors were recovered in proximity to fermenting bananas (2 specimens) and near a heap of bagasse accumulating outside the distillery building (4 specimens) for the time of the milling operation. *P. kluyveri* (killer biotype) and rather typical isolates of *H. guilliermondii* (2 kg^+) were the dominant species. Samples of materials that could serve as yeast and *Drosophila* habitats were collected from the estate gardens. These included a eucalyptus exudate, several samples of gummy exudates of mango trees, frass from an unidentified tree, and a decaying fruit of *zapote prieto*. None of these contained ascomycetous yeasts.

The indoor *Drosophila* samples were swept in proximity to cooked agave, the molasses reservoirs, the milling equipment, and the juice collection reservoir. Some of the yeast species in these flies were the same as those found in outdoors specimens, although a higher species overlap existed with the communities found in the materials where flies were collected. Most of the *Hanseniaspora* biotypes recovered from flies captured indoors were similar to those found in fermentations, but differed from those obtained from flies captured in the garden or in other locations outside the building. These strains either failed to grow at 37° C (*H. vineae*?) or did not assimilate 2-ketogluconate (atypical *H. guilliermondii*?). An analogous situation applies, perhaps less strikingly, to biotypes of *Pichia* spp., *K. marxianus*, and *T. delbrueckii*. It should be noted that very few flies were observed in the immediate vicinity of the fermentation vessels themselves. The nearly pure carbon dioxide blanket present at the top of active fermentations would no doubt be lethal to approaching flies.

(3) Agave molasses

The yeast biota of cooked agave molasses is dominated by *Schizosaccharomyces pombe* and lower numbers of *K. marxianus*. These and other minor species were similar to those found in some indoor flies that probably serve as a reservoir in this case.

(4) Crushing equipment and fresh juice

Yeasts from cooked agave were pooled with those from scrapings from the various components of the mill and crushers, because these represent essentially the same material. Yeast composition and diversity of these samples were very similar, being characterized by a vast array of yeast species. A few of these species were unique, and several were killers. Among shared species, two biotypes of *T. delbrueckii* and one biotype of *Hanseniaspora* (*H. vineae*?) were also found in *Drosophila* species captured indoors and in the earlier stages of fermentations. Also present in these samples were strains of *Saccharomyces cerevisiae* representing

the three biotypes found in active fermentations, plus a killer biotype. The two samples of freshly extracted agave juice exhibited much resemblance in yeast composition to crusher materials, but also shared species with early fermentations.

(5) Fermentations

Although yeasts from the various stages of fermentation exhibited a certain amount of overlap, a simple examination of Table 2 will make it obvious that a certain succession takes place as the fermentation proceeds. In most cases, a vigorous activity was evident very early in the process, sometimes before a vessel was even filled completely. By 5 days, the effervescence had ceased, frequently giving way to the formation of a dull pellicle. The three categories used in Table 2 consist of fermentations of up to 2 days, 2 to 4 days, and 5 or more days. It is worthwhile to state that a high fermentation turnover is not a goal at Tequila Herradura.

The earlier fermentation samples contained a rich mixture of species, including 3 biotypes of *T. delbrueckii*, 3 of *Hanseniaspora* species, 3 of *Brettanomyces* species, and 3 (or more) biotypes of *S. cerevisiae*. Many samples also contained *Z. bailii* and *Candida milleri*, in addition to a few other species. *Z. bailii* was more abundant in fermentations conducted in smaller tanks, and its presence was betrayed by extensive foaming at the surface of the fermenting must. As fermentations progressed, the species diversity was reduced considerably, as the maltose-positive, nonflocculent biotype of *S. cerevisiae* became dominant. The surface film of older fermentations invariably contained an abundance of *P. membranaefaciens*, although this species was found, in lesser numbers, at all stages of the process.

Fermentation samples contained relatively large numbers of acid-producing bacteria. A few representatives were subjected to a preliminary characterization, which showed that early phases of the fermentation were dominated by the lactic acid bacteria *Leuconostoc* spp., and *Lactobacillus* spp. Older fermentations that had formed a film or a froth layer contained significant numbers of *Acetobacter aceti*.

Killer toxins

As shown in Table 3, only one strain of *S. cerevisiae*, isolated from crusher scrapings, exhibited killer activity. Moreover, the only strain sensitive to that killing reaction was *C. glabrata* Y55, which is used in this laboratory for killer screening because of its sensitivity to many different toxins. A known commercial strain capable of producing the K1 toxin was also tested (Table 4). Neither strain had any effect on tequila isolates. Among yeasts isolated in actual fermentations, *T. delbrueckii* was the only species that exhibited killer activity. The broadest killer activity was observed in *Pichia anomala*, of which only one isolate was obtained. All strains of *K. marxianus* tested were sensitive to at least one killer toxin.

Ethanol resistance

Ethanol resistance ranged from less than 6% up to 12%, as shown in Table 5. In addition, preliminary experiments indicated that *Brettanomyces bruxellensis* may exhibit higher ethanol resistance than *B. anomalus*, although the replica plating method made it difficult to evaluate the results systematically due to the slow growth of these yeasts. Three strains of *P. kluyveri* and one strain of *P. membranaefaciens* (dominant biotype) exhibited growth in the presence of up to 7% ethanol.

Discussion

The tequila 'ecosystem' comprises 5 principal yeast communities that exhibit various degrees of species overlap (Table 6). The yeasts of agave rots are clearly distinct from others, and no evidence would support the hypothesis that important yeasts are carried from the field. Major components of that community, *C. lusitaniae*, *M. agaveae*, and *Candida guilliermondii*, share the common characteristics of being moderately fermentative and of utilizing a relatively large number of carbon compounds. In particular, all three grow well on hexadecane, suggesting the possibility that agave waxes and other lipids may be important factors in shaping the composition of this microflora. In addition, *C. guilliermondii* and *K. marxianus* hydrolyse inulin, the major carbohydrate of agave.

P. kluyveri, *K. marxianus*, and *Hanseniaspora* species are not at all unusual in *Drosophila* that feed on fruit. The fact that the last two species were also found in early fermentations could have been indicative of a connection between shared fly habitats. However, the actual overlap between flies collected indoors and outdoors is low when biotypes are taken into account. In addition, the higher similarity between the yeasts

Table 3. Killer yeasts isolated in this study and their sources.

Killer yeast species	N killers/N tested	Source of killer strains	N sensitive species[1]
S. cerevisiae	1/46	Crusher	1
P. membranaefaciens	3/28	*Drosophila* spp., crusher	5
P. anomala	1/1	Crusher	9
T. delbrueckii	4/17	Agave juice, young fermentation	7
P. kluyveri	4/8	*Drosophila* spp.	2

[1] Includes *Candida glabrata* Y55. Nineteen species (72 representative strains) isolated in this work were tested.

Table 4. Killing reactions among yeast species isolated in this study. The number of target strains tested is indicated in parentheses. Total numbers of positive killer/sensitive interactions are given.

Target species	Killer species giving a response				
	S. cerevisiae[1]	*P. membranaefaciens*	*P. anomala*	*T. delbrueckii*	*P. kluyveri*
C. glabrata Y55 (1)	2	3	1	4	1
K. marxianus (13)			8	28	1
S. cerevisiae (18)			4	4	
P. membranaefaciens (4)				3	
C. milleri (4)				4	
C. krusei (1)		1		4	
T. delbrueckii (6)			3		
C. rugosa (1)			1		
M. agaveae (2)			1		
B. bruxellensis (3)			1		
B. anomalus (6)		2	3		
C. lusitaniae (2)		1			
Hanseniaspora spp. (3)					
Z. bailii (2)					
P. kluyveri (2)					
Candida sp. (1)					
C. intermedia (1)					

[1] Includes K1 commercial strain.

of indoor flies and those from cooked agave, juice, or molasses suggests that *Drosophila* species are probably important in maintaining a communication between these habitats within the distillery building. The supposition that the neighbouring vegetation affords a constant supply of yeast inoculum does not receive a clear substantiation, which is not to say that these plants could not serve as an occasional alternate habitat for the flies themselves.

The data presented above suggest that young fermentations build their yeast community from yeasts found in agave molasses, cooked agave, and freshly extracted agave juice. The must reservoirs and the crushing equipment may serve as important repositories of yeasts which end up playing a major role in the fermentation, and indoor *Drosophila* may serve some function as vectors during the process. The non-flocculent, maltose-assimilating biotype of *S. cerevisiae* becomes the climax organism, outnumbering any others in mature fermentations, in spite of the fact that it was not recovered from *Drosophila*. It is presumed that this organism was selected, over time, for certain growth advantages. Although impeccable conditions of hygiene are maintained through extensive washing of equipment with water only, juice reservoirs and the pipes linking them to fermentors may contain a fair number of that yeast. As fresh agave extract accumulates in reservoirs, the juice comes in contact directly or indirectly with an inoculum. New contributions from yeasts in the external environment only seem to

Table 5. Ethanol tolerance of yeasts from tequila fermentations. The number of strains able to grow in the presence of each maximum ethanol concentration is given.

Species/biotype	Maximum ethanol concentration (% v/v)							
	<6	6	7	8	9	10	11	12
S. cerevisiae mal$^+$				1		5	26	3
S. cerevisiae mal$^-$						2	13	
S. ludwigii							2	
C. milleri					2	12		
Z. bailii			1		11	1		
T. delbrueckii		2	5	2				
K. marxianus			12					
H. vineae			3					
H. guilliermondii 2 kg$^+$		14						
H. guilliermondii 2 kg$^-$	1	6						

Table 6. Yeast communities identified in tequila fermentation and related habitats.

Habitat	Dominant yeasts	Secondary yeasts
Fresh agave	*C. lusitaniae*	*P. membranaefaciens*
	M. agaveae	*K. marxianus*
Sporopachydermia-like		
		B. anomalus
Drosophila	*H. guilliermondii*	*K. marxianus*
		P. kluyveri
		P. membranaefaciens
		C. krusei
		H. vineae
Agave molasses	*S. pombe*	
	K. marxianus	
Cooked agave, must,	*S. cerevisiae*	*H. vineae*
crushing equipment	*T. delbrueckii*	*Candida* sp. (pulcherrimin)
		P. membranaefaciens
		C. intermedia
Fermentation	*S. cerevisiae*	*B. bruxellensis*
	Z. bailii	*H. guilliermondii*
	C. milleri	*H. vineae*
	B. anomalus	*P. membranaefaciens*
		T. delbrueckii
		K. marxianus

have a minor impact beyond that point. It was reported that after closure of the distillery for a significant (but unspecified) amount of time, the first fermentation began spontaneously within a very short time. In such a case, it is possible that *Drosophila* normally associated with the distillery resided in the neighbouring vegetation to return along with an adequate yeast inoculum upon resumption of operations.

Factors which may affect the fermentation community

Evidence exists that killer toxins may play an important role in shaping the yeast community of certain natural large-scale fermentations (Young 1987). This is of special interest in this case. Assuming that the community composition is a major determinant of the quality of the final product, one should be concerned with the possibility that killer yeasts from the environment could threaten the stability of the existing mycobiota. This would be particularly important in deciding whether a commercially grown yeast isolated from the natural community should be used as an inoculant in cases where the process fails to begin spontaneously. In fact, the only conspicuous killer species found in significant numbers in early fermentations was *T. delbrueckii*. This species is rapidly overcome by others as the fermentation process unfolds, indicating that killer toxins may not offer any significant advantages in this case. The broadest killer activity spectrum was observed in *P. anomala*, of which only one isolate was obtained. All strains of *K. marxianus* tested were sensitive to at least one killer toxin, which could have accounted for their low abundance in fermentations, but the yeasts producing those toxins do not dominate fermentations. From the above, one should conclude that killer factors are not a major determinant of the tequila fermentation community. This would not discount the possibility that the *S. cerevisiae* biotype composition has shaped itself, over time, to exclude strains that are sensitive to killer factors. In that case, the present balance of biotypes would be the final result of earlier killer interactions. The second conclusion is that a potential displacement of *S. cerevisiae* by killer strains in the present community appears unlikely.

Fermentative growth rates are likely to be the pivotal element affecting succession in the tequila fermentation community. These in turn must be influenced by availability of nutrients and growth factors, physical-chemical conditions, and toxicity of ethanol and other elements of the must. The initial density of the must is typically ca. 14° Brix (data from Herradura records), approximately 90% of which is accounted for by fructose (Sánchez-Marroquín & Hope 1953). Typical agave tissue is poor in nitrogen (ca. 0.02%), although the material used at Herradura, because of its low cultivation density and late harvest, may exhibit higher values. All the same, nitrogen is likely to be limiting in the must. The final ethanol concentration usually is less than 6° Gay-Lussac. Agave juice also contains waxes and other compounds which may be inhibitory to certain yeasts. Many of these factors and their effects are poorly understood, but it is common practice in the tequila industry (but not at Herradura) to enrich the must with nitrogenous salts.

The higher ethanol resistance of *S. cerevisiae* compared to several other species would account well for the observed succession. It is less clear whether the lower numbers of *H. guilliermondii* compared to *H. vineae*, the replacement of *B. anomalus* by *B. bruxellensis*, or the dominance of maltose-positive biotypes of *S. cerevisiae* over others can be attributed to the slight differences in ethanol tolerance within groups of otherwise similar yeasts. The presence of large numbers of *Brettanomyces* spp. in mature fermentations may be affected by their high tolerance to ethanol, as suggested in the literature for other fermentations (Subden 1990). Overall, very little doubt remains that the progressive increase in ethanol concentration is probably the most important factor shaping the yeast community of fermenting tequila.

Although this may not be apparent in the data as presented in the results section, the distribution of yeasts in different fermentation samples was not entirely independent of such factors as the fermentation vessel itself. Very large numbers of *P. membranaefaciens* were a characteristic of samples taken from older fermentations that had developed surface films, a phenomenon which was more likely to take place in the smaller (25,000 L) vessels. The smaller fermentors were located at a lower level in the distillery and were connected to the juice reservoir by less well drained pipes, which may be of significance. These tanks were also more likely to undergo substantial foaming, which was linked with a high abundance of *C. milleri* and *Z. bailii*. Although *Zygosaccharomyces* species are known to occur in wine and other fermentations, they are often isolated as spoilage agents of foods preserved with high sugar, sulfite, benzoate, or sorbate, because of their high tolerance to these inhibitors. Their flocculation characteristics render them particularly tenacious, and they tend to build up in plumbing and fermentation equipment (Subden 1990).

In conclusion, the exact origin of the yeasts responsible for the fermentation of agave sugars in the production of natural tequila escapes a complete explanation. However, it is quite clear that the most important yeast in the process, *S. cerevisiae*, is not brought in from the field or from neighbouring vegetation, but rather perpetuates itself in the distillery, possibly with

an involvement from *Drosophila* species that forage on cooked agave during the milling process. Most of the several yeast species that reach each new batch of must are rapidly eliminated or at least outgrown by *S. cerevisiae*. Killer toxins do not appear to play a major role in maintaining the existing community, but the ability to grow rapidly in the presence of increasing concentrations of ethanol is likely to be a central factor.

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