AGAVE JUICE

Fermentation and Chemical Composition Studies of Some Species

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Several species of Mexican agave are used in fermentative processes. The yeast *Saccharomyces carbajali* is considered the principal alcohol producer from "aguamiel." Two *Lactobacilli* species are believed to be responsible for lactic acid production and two species of *Leuconostoc* cause the actual viscosity found in the final product. Some pulque yeasts are used in the production of dry yeast. The alcoholic beverage tequila is obtained by the distillation of the fermented juice of the *Agave tequilana*. Very small amounts of readily fermentable sugar were found in the eight species studied, and the presence of starch was not proved. The dominant polysaccharide was found to be inulin, and the dominant fermentable monosaccharide, fructose. The polysaccharides contained in these agaves were hydrolyzed by autoclaving. From the distillates obtained in experimental fermentations it was found that the strain of yeast determines the quality of the tequila. Because of the very small nitrogen content, it was necessary to add ammonium salts to the substrate in order to achieve a strong fermentation. The amino acids in the fermentable substrate did not seem to be responsible for the large quantities of higher alcohols found in the tequila.

PULQUE IS THE PRODUCT of the fermentation of the juice, "aguamiel," obtained from various species of agave and mainly from *A. atrovirens* and *A. americana*. The juice is extracted from these plants when they are 8 to 10 years old—i.e., when they have reached full maturity. The main sugars found in 8-year-old plants, "magueyes," are sucrose, glucose, and small amounts of gums and mannitol. Fermentation takes place spontaneously as a result of the growth of the microorganisms naturally present in the juice, or of pitching the unfermented juice with the lees of previously fermented must.

Del Rio (*1*) and Sánchez-Marroquín (*8*) have reviewed the microbiological and chemical studies of aguamiel and pulque. The work of other investigators throws some light on the vitamin and amino acid content of pulque.

The present paper refers to more recent studies, some already published in Mexican publications and others not yet published.

Pulque

Biochemical Activity of Microorganisms

Sánchez - Marroquín *et al.* (*5–17*) have studied the following microorganisms found in pulse: homofermentative and heterofermentative *Lactobacillus* species, which carry on the lactic acid fermentation; *Leuconostoc mesenteroides* and *L. dextranicum*, which are mainly responsible for the viscosity of the beverage; *Saccharomyces carbajali*, an alcoholic fermenter; and finally, *Pseudomonas lindneri*, which also produces ethanol.

Homofermentative *Lactobacillus* sp. In the isolation of these microorganisms, media enriched with some growth factors were used and all *Lactobacillus* strains were found to be microaerobic. The optimum temperature was 28° C., and the optimum pH was 6.6 to 6.9.

Isolated cultures, appearing as streptobacilli, have been found to be capable of fermenting pentoses, but they are unable to ferment disaccharides. They do not modify milk, nor assimilate nitrates, nor do they produce indol or catalase.

During the lactic fermentation of the juice aguamiel enriched with glucose, this *Lactobacillus* assimilated 53% of the sugar present, yielding 141% of lactic acid, expressed as fixed acidity.

The optimum sugar concentration for their growth was found to be 10%.

In some experiments using hydrolyzed aguamiel, when enriched with some source of growth factors or phosphates, the lactic acid production was increased and higher yields resulted than from those obtained when *L. delbrueckii* and *L. leichmanii* were used under similar conditions. The bacterial metabolism for glucose (Table I) yields 96% of lactic acid, when this *Lactobacillus* sp. is grown in synthetic media. The amounts of carbon dioxide evolved were so small that they were considered as having been produced by cellular respiration. The fermentation balance in Snell's glucose medium by one of the heterofermentative cultures is shown in Table I. When a medium containing hydrolyzed commercial sucrose was fermented, 10.88 gram % of the sugar was utilized and 26.80 gram % of lactic produced. *Lactobacillus plantarum*, when grown under similar conditions, almost doubled the above lactic acid figure. The homofermentative *Lactobacillus* sp. grew well in a medium containing 10% sugar-cane molasses and yeast extract, utilizing 8.81 gram % of the sugars present with a yield of 16.25 gram % of lactic acid, while *L. plantarum* under the same conditions yielded 31.29 gram % of lactic acid. If corn-steep liquor was added to the medium instead of yeast extract, the amount of lactic acid produced by *Lactobacillus* sp. was 25 gram %, that produced by *L. plantarum* being 29 gram %. The production was slightly increased when the molasses concentration was raised to 20%.

The lactic acid obtained by fermentation of aguamiel by this pulque bacteria was inactive, as determined by the water of crystallization and the specific rotation of the zinc lactate obtained "ex professo" by fermentation of hydrolyzed aguamiel inoculated with the same *Lactobacillus*.

Heterofermentative *Lactobacillus* sp. The metabolic products formed by this heterofermentative species grown under the same conditions as the homofermentative species were lactic acid, ethanol, and carbon dioxide in some

Table I. Fermentation Balance in 100 Ml. of Snell's Glucose Medium

(Homofermentative Cultures, *Lactobacillus* sp. C-35)

Compounds	Mg.	Mg., %	Millimoles	Carbon, Millimoles	Oxid. Value	Red. Value	Carbon Recovery, %
Utilized glucose	135	100	0.75	4.5	0	0	0.96
CO_2	1.72	1.28	0.05	0.05	0	0	
Lactic acid	129.60	96.00	1 44	4.32	0	0	
Recovery	129.60	96.00	1.44	4.32	0	0	
						Error, %	4.0

Heterofermentative Cultures, *Lactobacillus* sp. N-22

Utilized glucose	385	100	2.12	12.72	0	0	0.95
CO_2	24	6.31	0.54	0.54	2	0	
Ethanol	160	41.5	3.47	6.94	0	−2	
Lactic acid	183.6	47.6	2.04	6.12	0	0	
Recovery	367.6	95.5	6.05	13.6	2	−2	
						Error, %	4.46

cultures, and lactic acid, acetic acid, and carbon dioxide in other cultures.

Lactobacillus, either species, homofermentative and heterofermentative, showed antigenic reactions similar to *L. plantarum* and *L. brevis*, but differed quantitatively in antigenic constitution, while the *Lactobacillus* sp. from pulque showed the same serological characteristics among themselves. The cross-agglutination reactions among the isolated cultures stressed the possibility of the presence of an antigen common to all of these cultures—i.e., an antigenic constitution qualitatively similar but quantitatively different.

***Leuconostoc* sp.** Some cultures seemed to be related to *Leuconostoc dextranicum*, except for the fact that they do not ferment lactose and do not reduce litmus milk. Some other cultures showed characteristics similar to *L. mesenteroides;* however, the former does not ferment mannitol.

The production of dextrans in aguamiel by the isolated cultures of *Leuconostoc*, and the production of a sirup of high viscosity, were studied. Appreciable quantities of dextrans were obtained from aguamiel with a sucrose content varying from 4.6 to 7.5 gram %, after incubation for 24 hours at 23° C. The sirup thus obtained by fermentation had the following characteristics: specific gravity 1.103; refractive index 1.371; specific rotation of the hydrolyzate, 1.6°; viscosity at 17° C. 419 centipoises; dry extract 28.3%; total nitrogen 0.06%; direct reducing sugars 4.1 gram % (expressed as glucose); and total reducing sugars 10.2 gram % as glucose. The cultures were adaptable to sugar concentrations up to 70° Brix, the resulting sirup having a viscosity of 2500 centipoises at 17° C.

Serological studies with the cultures isolated corroborated their relation to *L. dextranicum* and *L. mesenteroides*.

The cultures which did not ferment lactose showed metabolic similarities to *L. dextranicum*, and those which fermented lactose also fermented glucose and arabinose. From the glucose consumed, the pulque cultures gave the following results (expressed as grams per 100):

Lactic acid	46.5 to 52.5
Ethanol	28.3 to 35.7
Volatile acid	3.4 to 6.8
CO_2	3 to 19.3

Saccharomyces carbajali. This is the most important yeast in the production of pulque. Metabolic studies of *S. carbajali* show a fermentation yield of 84.7 to 90.2% glucose utilization (Table II). This yeast can ferment molasses, especially if 1% of ammonium sulfate is added, and will also ferment rice mash, corn mash, and other starchy materials if previously hydrolyzed. The yields obtained were from 79.2 to 89.6% depending on the substrate used, the highest yield being derived from corn mash substrate (Table III).

The results obtained demonstrate that this yeast ferments glucose stereoisomers and sucrose directly. It is capable of fermenting one third of the raffinose molecule—that is, it ferments fructose but not melibiose.

The pH value at which the fermentation is most active is 4.5, at which value pulque is derived at the onset of alcoholic fermentation. The aguamiel has a slightly alkaline reaction, a pH of 7.4, probably due to the phosphate content; hence bacteria develop first.

The highest yield in sugar assimilation was found to be obtained when the yeast was grown on substrates containing 21.6% sugar, even though this figure does not correspond to the highest yield in ethanol which is obtained at a sugar concentration of 18.5%. As far as the industrial process is concerned, it is necessary to take into consideration the fact that higher sugar concentrations involve longer fermentation times. The optimum growth temperature was found to be 28° C.

The products of fermentation follow, quantitatively and qualitatively, the first scheme of fermentation of Neuberg.

It has been shown that yields of fermentation are increased by the addition of copper sulfate, magnesium sulfate, sodium cyanide, sodium sulfide, or sodium sulfite. Copper sulfate (at a concentration of 1 to 2000) appeared to give the highest yields, but the fermentation time was longer. The stimulating effect of copper sulfate appeared to be effective only when added to the starter, and not when added to the main fermentation.

Saccharomyces carbajali associated with *Endomycopsis fibuliger* (an amylase producer) was added to study fermentative properties on some starchy materials. With malted-wheat meal 84.6% of the sugar was utilized and 87.9% of the starch was assimilated. *Endomycopsis fibuliger*, fermenting alone, yielded 40.9 and 72.6%, respectively.

Pseudomonas lindneri. Gonçalves Lima *et al.* (*2*), working at the authors' laboratory with cultures isolated by them, obtained appreciable amounts of ethanol when *Pseudomonas lindneri* was grown on glucose (Table IV), thus corroborating the results obtained by Kluyver, Schreder, *et al.*

The cultures studied were active in fermenting glucose, fructose, and sucrose, and produced gums. Their colonies were slimy and very different from the normal shapes described by Lindner and Kluyver, especially when grown on beer wort containing 2% sucrose and an excess of calcium carbonate.

Table II. Chemical Analysis of 18% Glucose Broth Fermented with *S. Carbajali*

Compounds	Grams Per 100 Ml. of Fermented Medium
Initial reducing substances	18.1
Final reducing substances	1.5
Sugars utilized	16.6
Theoretical maximum ethanol yield	8.48
Ethanol produced	7.19
Yield	84.78
Higher alcohols	Traces
Acetic acid	0.38
Succinic acid	0.14
Glycerol	0.42
CO_2	7.12
Carbon recovery, %	90.23
O/R balance	0.995

Control of Pulque Fermentation by Starters (*7*) After the three fundamental microorganisms found in aguamiel and pulque had been studied, the possibilities for their use in pure mixed culture fermentation for commercial production were examined. The starter was composed of *Saccharomyces carbajali*, homofermentative *Lactobacillus* sp., and the two *Leuconostoc* species.

The optimum conditions for a mixed culture fermentation of the sterile agua-

Table III. Fermentation by *S. Carbajali*

Expt.	Residual Sugars, G. %	Utilized Sugars, G. %	Theoretical Maximum Amount of Ethanol	Ethanol After 24 hours	Ethanol After 96 hours
			Rice Mash		
1	1.10	16.14	8.24	6.41	6.96
2	2.04	15.20	7.76	5.96	6.34
3	1.73	15.51	7.92	6.41	7.12
Initial reducing sugars, %			17.24		
Yield, %			79.19		
			Corn Mash		
1	1.41	14.97	7.67	5.21	6.49
2	1.26	15.12	7.72	5.06	6.73
3	0.15	16.23	8.29	5.88	6.80
Initial reducing sugars, %			16.38		
Yield, %			84.03		

Table IV. Glucose Fermentation of Schreder's Medium plus 1% Yeast Extract, by *P. Lindneri*

Ethanol	CO_2	Lactic Acid	Succinic Acid	Acetic Acid	Total
46.3–48.3	44.1–48.8	0.3–0.5	0.14–0.15	0.44	91.3–94.2

miel were established as follows: pH 5.0. inoculum 5%, and temperature 28° C.

Successive inoculation with bacteria, followed by yeast, was found to give higher yields of the desirable products, as compared with the simultaneous inoculation of the bacteria and yeast. The results obtained by the use of pure mixed cultures and the data pertaining to the sterile, unfermented aguamiel are given in Table V.

It seems to be possible to produce a good hygienic beverage by using pure cultures in the commercial production of pulque.

Table V. Average Chemical Determinations of Unfermented Substrate and Final Product Obtained by Mixed Pure Culture Fermentation

Determinations	Substrate Sterile Aguamiel	Final Product
Brix	11.0	6.0
Sp. gravity	1.042	0.978
pH	7.0	4.6
Refractive index at 20° C.	...	1.338
Total acidity in lactic acid	0.018	0.348
Direct reducing sugars, in glucose	2.40	0.06
Total reducing sugars, in glucose	10.00	0.48
Sucrose	7.6	0.42
Gums in glucose	0.60	0.33
Crude proteins	0.17	0.17
Dry residue	15.29	2.88
Ash	0.31	0.29
Ethanol, °GL at 20° C.	0.00	5.43
Higher alcohols in amylic acid	...	0.51
Volatile acidity in acetic acid	...	0.02

Dry Yeast from Pulque Yeasts (7) Four species of pulque yeasts, *Torulopsis aquamellis*, *Saccharomyces carbajali*, *T. hydromelitis*, and *Pichia barragani*, were compared with *Torulopsis utilis*. Sugar-cane molasses was used as the main source of carbohydrate in the experiments. All species showed fairly good growing conditions. A study of the chemical composition of each yeast was undertaken, with the following results: *Torulopsis hydromelitis* and *S. carbajali* gave a similar protein content but grew more slowly than *T. utilis* (Table VI).

Bacterial Growth Factors Because pulque is a popular beverage in certain regions of Mexico, its food value is of interest. The content of certain growth factors (especially vitamins) in aguamiel and pulque was investigated by microbiological methods and the results obtained are summarized in Table VII.

In addition to pantothenic acid, biotin, thiamine, *p*-aminobenzoic acid, and pyridoxine in pulque, other investigators have reported the presence of riboflavin, nicotinic acid, niacin, ascorbic acid. and several amino acids, thus attributing to this beverage a rather high nutritive value.

Tequila

Tequila is a liquid obtained from another maguey plant, *Agave tequilana*. When the plant is from 8 to 10 years old, the central portion is removed, cooked, mashed, and fermented. This fermented mash is then distilled and the final product has an alcoholic content of from 40 to 50% (*4*).

Hope and Novoa (*3*) have analyzed the central portion of 8- and 9-year-old tequila-producing plants (maguey) of eight different species, with the following results: fibrous matter, 11.0 to 11.5%; ash, 2.4 to 4.0%. The readily fermentable sugars were small, 1.0 to 1.5%, and the presence of starch was not demonstrated. Gums are present in the pentose forms, and aldoses were found by the hypoiodite method. Ketoses were present and identified by the Selliwanoff reagent. Because of the very small protein nitrogen content, 0.02 to 0.03%, it was necessary to add ammonium salts to the substrate in order to achieve active fermentation.

Inulin was the polysaccharide found in these agaves, which hydrolyzes into the fermentable sugars. Inulin was identified in microtome sections and identified by characteristic sphoero crystals, and by hydrolysis which gave a

Table VI. Determination of 5 Growth Factors Present in Pulque

(Average obtained in 24 different samples. Results given in micrograms per 100 ml. of pulque biotin given in millimicrograms per 100 ml. of pulque)

Growth Factors	Average	Maximum	Minimum
Panthothenic acid	65.17	86.17	55.70
Biotin	19.60	32.24	11.43
Thiamine	30.74	37.12	22.60
p-Aminobenzoic acid	21.64	28.72	15.36
Pyridoxine	22.98	33.48	16.20

Table VII. Comparative Study of Four Aguamiel Yeasts with *Torulopsis Utilis*

Yeast	Average Yield, %	Comparison of Growth, %	Average Protein, %	Comparison of Protein, %	Average Phosphorus, %	Comparison of P, %	Average Ash, %	Comparison of Ash, %	NH_4 Consumed, Mg.
T. utilis	41.02	100.0	49.40	100.0	1.85	100.0	8.10	100	468
T. hidromelitis	47.40	108.0	48.25	97.5	1.46	79.0	4.60	57.0	430
T. aquamellis	18.50	45.1	44.35	90.5	1.47	79.5	4.30	53.0	487
P. barragani	42.70	102.0	42.85	86.5	1.54	83.2	7.40	91.5	350
S. carbajali	41.50	100.6	46.35	93.8	1.61	87.6	2.20	27.3	436

levorotatory solution. The fructose, obtained by the hydrolysis of inulin, was identified and determined by using both the Munsen and Walker and the hypoiodite methods (Table VIII).

The total reducing sugar content of the maguey juice, after acid hydrolysis, varies from 16 to 27%, according to the variety of the plant. The fructose content varies from 13 to 25%; and the aldoses from 1 to 4%. These results (Table IX) confirmed the presence of inulin as the principal monosaccharide in the agave plant.

Influence of Cooking When the plants were cooked at pressures of 10 and 15 pounds per square inch for 3 and 4 hours, the differences in juice extracted were small: 50.7% juice with a specific gravity of 1.108 and 25% reducing sugars in the first case; and 52.7% juice with a specific gravity of 1.107 and 24.6% of reducing sugars in the second experiment. The pH in both cases ranged from 4.2 to 4.6.

After having been cooked, the juice was diluted to 10° Brix, 0.2 to 1.0% ammonium sulfate was added, and this was pitched with yeast (Table X). Using autochthonous *Saccharomyces* sp. and pure *S. cerevisiae* and *S. cerevisiae* var. *ellipsoideus* starters, it was found from the distillates obtained that the strain of yeast used in the process was a determining factor in the low impurity content of the product. These results are summarized in Table XI.

The nitrogen content of the substrate is very small; hence ammonium salts have to be added during fermentation. The influence of this addition to the formation of higher alcohols has not been very clearly demonstrated.

Table VIII. Composition of Main Stem of Agave Plants

(Results expressed in parts per hundred)

Common Name	H_2O	Crude Fiber	Inulin	Total Reducing Sugars	Protein Nitrogen	Ash	pH
Carpintero	70	11	15.4	1.03	0.021	3.9	5.5
Pata de mula	63	12	19.8	1.00	0.019	2.9	5.0
Bermejo	65	12.5	18.1	1.06	0.022	2.5	5.0
Azul	62	11.8	20.1	1.03	0.024	2.5	5.5
Zopilochino	70	12	14.3	1.03	0.023	2.7	4.5
Sihuin	65	12.5	17.5	1.09	0.021	2.5	4.5
Chato	68	12.5	15.6	1.23	0.020	2.4	5.0
Azul 2	60	11	24.1	1.50	0.020	2.7	4.5

Table IX. Sugars in Hydrolyzed Agave Juice

(Results expressed in parts per hundred)

Common Name	Total Reducing Sugars	Fructose	Aldoses	Total Sugars, % Fructose	Total Sugars, % Aldoses
Carpintero	17.2	15.9	1.3	92.5	7.5
Pata de mula	22.0	18.8	3.2	85.5	14.5
Bermejo	20.0	17.8	2.2	89.0	11.0
Azul	22.3	23.0	1.3	93.5	6.5
Zopilochino	16	13.7	2.2	86.5	13.5
Sihuin	19.5	15.6	3.9	80.0	20.0
Chato	17.2	15.1	2.1	88.0	12.0
Azul 2	26.8	25.5	1.3	95.2	4.8

Table X. Composition of Pressed Agave Juice Medium

Sample	A	B	C_1	C_2	C_3	D_1	D_2	D_3
pH	4.0	4.5	...	4.3	...	...	4.2	...
Total reducing sugars, %	10.0	9.3	...	9.8	...	...	9.5	...
Ammonium sulfate added, g./liter	1.0	0.6	0.2	0.5	0.8[a]	...	0.9	...

[a] 0.2 g./l. of diammonium phosphate also added.
All samples diluted to 10° Brix; temperature kept constant during fermentation at 28° C.

Table XI. Analysis of Fermented Agave Juice Distillates

(Mg. per 100 ml. of anhydrous alcohol)

Determinations	Autochthonous Yeast, Saccharomyces sp.					S. cerevisiae	S. cerevisiae var. ellipsoideus	Autochthonous Saccharomyces sp.
Sp. gr., 20°/20° C.	0.928	0.939	0.937	0.938	0.927	0.936	0.936	0.934
Alcohol, %	53.0	47.5	48.7	48.1	53.6	48.7	49.0	50.0
Total acids	12.6	12.6	18.6	14.2	10.6	7.0	13.0	19.0
Volatile acids	4.3	6.3	8.1	5.9	5.2	3.6	10.4	4.8
Fixed acids	8.4	6.3	10.5	8.3	5.4	3.4	2.6	14.2
Esters	37.3	30.5	53.2	36.3	42.4	36.0	12.5	61.0
Furfural	13.2	8.5	2.0	4.2	8.9	1.5	4.0	1.5
Aldehydes	0.9	0.6	0.4	0.8	0.4	0.6	0.9	0.3
Higher alcohols	216.0	291.0	283.0	342.0	160.0	123.0	75.0	290.00
Nonalcoholic coefficient	280.0	243.0	357.0	397.0	322.0	168	105.0	372.0

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