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Enzymatic Hydrolysis of Fructans in the Tequila Production Process

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In contrast to the hydrolysis of reserve carbohydrates in most plant-derived alcoholic beverage processes carried out with enzymes, agave fructans in tequila production have traditionally been transformed to fermentable sugars through acid thermal hydrolysis. Experiments at the bench scale demonstrated that the extraction and hydrolysis of agave fructans can be carried out continuously using commercial inulinases in a countercurrent extraction process with shredded agave fibers. Difficulties in the temperature control of large extraction diffusers did not allow the scaling up of this procedure. Nevertheless, batch enzymatic hydrolysis of agave extracts obtained in diffusers operating at 60 and 90 °C was studied at the laboratory and industrial levels. The effects of the enzymatic process on some tequila congeners were studied, demonstrating that although a short thermal treatment is essential for the development of tequila's organoleptic characteristics, the fructan hydrolysis can be performed with enzymes without major modifications in the flavor or aroma, as determined by a plant sensory panel and corroborated by the analysis of tequila congeners.

KEYWORDS: Agave; tequila; fructans; inulinase; fructozyme

INTRODUCTION

Tequila is a Mexican alcoholic beverage produced from the fermentation and distillation of *Agave tequilana* Weber var. azul juice. The juice is extracted from the pine of this plant, which is rich in highly branched fructan polymers with complex structures combining fructose molecules linked by β (2−1) and β (2−6) bonds with a DP ranging from 3 to 29 (*1*). In the traditional tequila production process, the pines are cooked in ovens or in autoclaves to hydrolyze the fructans, liberating fructose and producing compounds that will later contribute to the aroma and flavor of tequila, as well as to soften the agave pine, facilitating the milling and extraction operations. For centuries, milling was carried out in rudimentary mills, which have since been substituted by modern milling equipment in which the cooked pines are cut and shredded to facilitate aqueous extraction of the sugars. The fructose-rich juice, derived from the chemical transformation of the agave energy reserve compounds, constitutes the basis of the tequila fermentation broth (*2*). This transformational step has been performed for decades in a traditional way with little technological input, although the physiology and chemical structure of the fructan synthesis and hydrolysis have been the subject of intensive research in a wide variety of plants, including agave (*1*, *3*−*5*). In contrast, analogous transformation processes in the beverage fermentation industry, such as the hydrolysis of starch and the extraction of fermentable sugars for beer production, have been the subject of intensive research and technological development, particularly for optimizing the activities of endogenous amylases and introducing exogenous enzymes from a wide variety of sources (*6*, *7*). It is therefore surprising that only recently has the introduction of enzymes to the tequila production process started to attract industrial interest.

In the past decade, production technology has begun to shift from traditional to modern operations as a consequence of the successful growth of the tequila industry, as demonstrated by the increase in total tequila production from 104 million liters in 1995 to 284 million liters in 2007 according to the Camara Nacional de la Industria Tequilera, the most prominent association of tequila producers. Among the modern procedures recently introduced, the extraction of sugars in "diffusers" specifically designed for the process is one of the most important (*8*). In this type of equipment, the countercurrent contact between the shredded agave (not cooked) and water is optimized, allowing for high extraction efficiency with little loss of fructan in the residual agave fibers. After extraction, chemical (thermal/acid) hydrolysis in autoclaves of fructan in solution is carried out under reaction conditions similar to those used when fructans were directly hydrolyzed in the pines before extraction. As already stated, polysaccharide hydrolysis has moved from a chemical to an enzymatic process, as the chemical process results in the

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formation of byproducts such as hydroxymethylfurfural (HMF), which is toxic for humans, and phenolic compounds from lignin, which may inhibit yeast in the alcoholic fermentation. The use of enzymes in the context of the modern tequila industry would avoid the production of toxic chemicals that result from lignin and sugar oxidation, reduce energy requirements, enhance hydrolysis efficiency, and simplify the production process, as hydrolysis and extraction could take place in a single operation.

Several hydrolytic enzymes have been reported in plants, such as 1-fructan exohydrolase (1-FEH), which degrades inulin-type fructans with β (2−1) linkages (*9*), 6-fructan exohydrolase (6-FEH), which degrades levan-type fructans with β (2−6) linkages (*4*), and 6&1-FEH, which is able to hydrolyze both inulin- and levan-type fructans (*10*). However, these enzymes are produced in low concentrations, and their purification is cumbersome. It is known that fructan exohydrolase activity is present in agave pines (*11*), but the number of enzymes and their physicochemical features are still unknown. Additionally, since the process has moved from hydrolysis in the pine to extraction and hydrolysis ex plant, an enzymatic process is possible only if it relies on microbial enzymes. Inulinases (EC 3.2.1.7) or 2,1-β-D-fructan fructanohydrolases are enzymes that catalyze the hydrolysis of β (2−1) fructan. A wide variety of microbial inulinases found in yeast, filamentous fungi, and bacteria have been reported and studied (*12*). Inulinases usually applied to the industrial hydrolysis of fructans are commercially available enzyme preparations containing exo- and endoinulinase partially purified from *Aspergillus niger*, a GRAS organism (Novozyme http://www.novozymes.com, Megazyme http://www.megazyme.com).

On the basis of the background discussed above, this paper describes efforts to introduce enzymes in the tequila production process, particularly in the extraction and hydrolysis steps. Experiments on the industrial level were carried out in "La Perseverancia", Tequila Sauza's distillery.

MATERIALS AND METHODS

Extraction of Agave Fructans. Agave fructans were extracted with water from 3 kg of shredded pines, which were mixed in water for 4 h at 60 °C and 320 rpm using an agave/water ratio of 1:3 (w/v). The aqueous extract was then filtered, concentrated in a Hollow Fiber Cartridge 30000 kDa (Amikon, H1P30-20), and dried by lyophilization over 8 h in a Usifroid (SMH-IS) unit.

Quantification of Inulinase Activity. Fructozyme L (Novozymes) is a mixture of exoinulinases and endoinulinases obtained from a particular *A. niger* strain. This product achieves the purity specifications recommended by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the Food Chemicals Codex (FCC). The enzymatic activity of Fructozyme L was measured at 60 °C in 50 mM acetate buffer, pH 4.5, using 3% (w/v) agave fructan or chicory inulin as substrate at an appropriate enzyme concentration. Samples of 50 μL were withdrawn at 1 min intervals over 5 min, and the initial rate was determined following the reducing power release by the DNS method (*13*). One unit (U) of enzyme activity was defined as the amount of enzyme producing 1 μmol of fructose equivalent per minute.

Quantification of Fructans in Agave. For the quantification of water-soluble carbohydrates (WSC) in agave, 25 g of agave pines or bagasse was blended with 500 mL of water for 5 min. The suspension was treated with 750 μL of Fructozyme L at 60 °C for 30 min to hydrolyze fructans, and then it was filtered. Reducing sugars were quantified by the DNS method or by high-performance liquid chromatography (HPLC). Alternatively, 10 mL of the extract was treated with 300 μL of Fructozyme L at 60 °C over 15 min and filtered. The reducing sugar was then quantified. In the production plant, the Fehling method for the quantification of direct and total reducing sugars for alcoholic beverages was applied in accordance with Mexican regulation (NMX-V-006-NORMEX-2005). Fructan concentration in agave was calculated from the fructose obtained after hydrolysis.

Quantification of Sugars. For fructose measurement in the laboratory, samples were centrifuged and analyzed by HPLC in a Waters 510 pump equipped with a refraction index (RI) detector (Waters 410) using a carbohydrate column (Waters, 4.6 × 250 mm) at 35 °C, with an 80:20 acetonitrile/water mixture as eluent at a flow rate of 1.0 mL/min. In the distillery, analysis was performed using a Rezex RMN-Carbohydrate column (Phenomenex) at 80 °C using water as eluent at a flow rate of 0.6 mL/min. D-Fructose and inulin from chicory (Sigma) were used as standards.

Fructan Hydrolysis Essays. Enzymatic hydrolysis of fructans in the agave shredded pines was performed at different enzyme concentrations at 60 °C using a 1:1 (w/v) agave/water ratio in a batch process, in a laboratory semicontinuous system, and in an industrial diffuser. In all cases, the agave extracts were obtained under the same extraction conditions and agave/water proportions. When indicated, thermal fructan hydrolysis was carried out at 110−126 °C and 1.2 kg/cm^2 of pressure. In some experiments, 0.33 mL of concentrated sulfuric acid/L aqueous agave extract was added as is done in the traditional process.

Diffuser Operation. A few years ago, continuous diffusers were introduced into tequila factories for fructan extraction, replacing batch autoclaves. A 60 m^3 capacity industrial diffuser was fed 16 tons of agave per hour in a 1:1 (w/v) agave/water ratio with a residence time (θ) of 3.5−4 h and operated at 60 and 90 °C through direct steam injection. Around 60000 L of agave extract was obtained from each experiment and then stored in tanks for further processing.

Semicontinuous System at the Laboratory Scale. A semicontinuous extraction system was adapted at laboratory scale in order to scale down the reaction conditions used in the industrial diffuser. For this purpose, five 1 L reaction vessels each containing 200 g of agave and 200 mL of water were operated in series. The countercurrent continuous extraction flow in the industrial diffuser chambers was simulated by the manual movement of both water and filtered agave bagasse through the five vessels. Every hour, the contents of each vessel were separated and transported countercurrently to the next vessel in an operation that lasted a maximum of 3 min; as a consequence, each hour, 200 g of fresh agave entered vessel 1 while exhausted agave fibers were withdrawn from vessel 5, and while 200 mL of fresh water entered vessel 5 with the fructan aqueous extract collected from vessel 1. In theory, the system reached steady state after the fifth hour of operation, equivalent to the system residence time. The addition point (center, vessel 3; with the water feed, vessel 5; all along the diffuser, one-fifth of the enzyme added at each vessel) and the concentration of enzyme were two variables studied as described under Results. The system is illustrated in **Figure 1**.

General Sensory Descriptive Analysis. Tequila samples produced from enzyme-treated agave extracts were evaluated by an expert panel working for the plant. The evaluation derived from a previous description of samples after degustation was collected and analyzed statistically using the nonparametric Kramer's rank sum test with a significance level of 5%. This is the common method applied in the plant's quality control procedures.

Congeneric Compound Analyses. Several compounds with known importance for the organoleptic properties of tequila grouped in five chemical classes (*14*, *15*) were analyzed: [fusel oil (FO)] acetaldehyde, ethyl acetate, methanol, 1-propanol, isobutanol, and isoamyl alcohol; [terpenoids (TERP)] α-terpineol, farnesol, linalool, linalool oxide (1), linalool oxide (2), β-citronellol, 4-terpineol, and damascenone; [alcohols and esters (A&E)] 2-phenylethyl acetate, 2-phenylethanol, 1-butanol, 3-methyl acetate, 2-methylpropyl acetate, ethyl butanoate, hexadecanol, ethyl propanoate, and farnesyl acetate; [cyclic oxygenated compounds (CO)] 1-(2-furanyl)ethanone, cyclopentanone, 2,5-dimethylfurane, 2-furanmethanol acetate, furfural, 2-methyltetrahydrothiophen-3-one, and 3-methylcyclopentanone' and [free fatty acids and fatty acid ethyl esters (FFA&EE)] C8:0, C10:0, C12:0, C14:0, C16:0, EE8:0, EE10:0, EE12:0, EE14:0, EE16:1, and EE16:0, where C*n*:*m* and EE*n*:*m* indicate the number of carbon atoms (*n*) and double bonds (*m*) for acids (C) and ethyl esters (EE).

Gas Chromatography (GC)−Mass Spectrometry (MS). All analyses were performed using an Agilent Technologies 6890N GC and a 5973N MS detector. A ZB-5 M (30 m × 0.32 mm i.d.), 0.25 μm film thickness (Zebron Phenomenex), column was used. The gas chromatographic conditions were as follows: initial oven temperature at 40 °C for

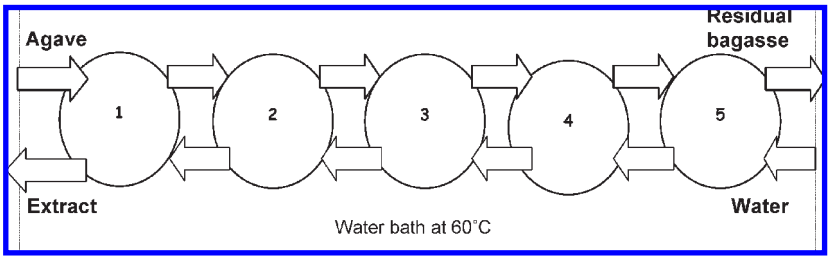

Figure 1. Simulated diffuser containing five 1 L agitated vessels (1−5) immersed in a water bath. Agave and water are moved in countercurrent as described under Materials and Methods.

1.0 min, then programmed to increase from 40 to 210 at 5 °C/min, then increased from 210 to 280 at 10 °C/min. The injector temperature was 250 °C, in splitless mode (1.0 min). The MS was operated in scan mode (m/z 50−550), ionization potential was 70 eV; ionization current was at 350 μA, and the ion source and transfer line temperatures were at 230 and 280 °C, respectively. Identification was conducted by comparison of the retention times with those of standards (when available) or with spectral data obtained from NIST libraries. Quantitative analysis was done on chromatographic peak areas by calibration with standards (Sigma-Aldrich). A&E and CO concentrations were estimated by comparison of peak areas with that of 2-phenylethanol, as were damascenone and linalool oxides with the linalool peak area.

Sample Preparation. FO and TERP were analyzed by a headspace (HS) solid phase microextraction (SPME) and GC-MS procedure (*14*). One milliliter of the tequila sample, diluted with 4 mL of saturated NaCl solution, was HS-SPME extracted with stirring at 1200 rpm. FO extraction was carried out with a Carbowax−divinylbenzene fiber (Supelco) at 65 °C for 5 min, whereas for TERP, a polydimethylsiloxane−divinylbenzene fiber (Supelco) was used at 25 °C for 30 min. The fiber was allowed to remain in the inlet for 10 min, thereby preparing the fiber for the next sample.

For A&E and CO analyses, a general extraction procedure was used. In a separatory funnel, 30 mL of tequila was diluted 1:10 with cold (4 °C) distilled water and extracted with 10 mL of dichlomethane. To remove the acids, the organic layer was washed three times with 2 mL of cold saturated sodium bicarbonate solution and five times with 2 mL of cold water. The extract was dried over anhydrous sodium sulfate, and 1 μL was injected into the GC-MS.

FFA&EE were extracted from tequila and FFA silylated to enhance their GC properties. Ten milliliters of tequila and 10 mL of saturated NaCl were thoroughly mixed in a 25 mL volumetric flask and extracted in a Vortex for 2 min with 2 mL of hexane. NaCl solution was carefully added until the organic layer reached the flask's narrow neck and removed with a Pasteur pipet. Hexane extract was dried over anhydrous sodium sulfate, decanted to a vial with screw cap, and derivatized with 250 μL of a 2:2:1 BSTFA/HMDS/pyridine mixture (Regis Technologies) at 80 °C for 1 h. When reaction products reached room temperature, 1 μL was injected into the GC-MS system.

RESULTS AND DISCUSSION

Enzyme Activity and Quantification of Total Sugars in Agave. The enzymatic activity of the inulinase preparation (Fructozyme L) was measured using both agave fructan and chicory inulin according to the manufacturer's recommendation, and it was found that the commercial products used in these experiments respectively contained 2400 and 2500 U/mL of inulinase activity under the reported assay conditions. Chicory inulin is a lineal polymer containing β (2−1) linkages, whereas agave fructan is a branched polymer containing a high proportion of β (2−6) linkages (*1*), making the hydrolysis process more difficult and therefore explaining the difference in reaction rates (*15*). Exoinulinases in the inulinase blend may act upon β (2−6) linkages, but at lower reaction rates.

WSC of different agave plant samples were extracted and hydrolyzed according to the enzymatic method as described under Materials and Methods to quantify total sugars in the agave pines. Total sugars in the range of 129−174 g of fructose equivalents/kg of pine fresh weight were found in the pines used for this project (12.9−17.4% w/w).

Effect of the Particle Size on the Enzymatic Extraction−Hydrolysis Process. The agave was shredded, and the resulting particles were separated into three main size categories of around 0.5, 5, and 100 cm^3 on average. Extraction and hydrolysis reactions were performed in the conditions already described and the released sugars monitored as shown in **Figure 2**. As expected, it was found that sugar extraction and hydrolysis rates are dependent on particle size, and although there is a difference in the extraction rates for 0.5 and 5 cm^3 average volume particles, after a few minutes, all of the available fructose was extracted in both cases. Nevertheless, larger particles (100 cm^3) not only require longer times, but also result in lower yields, as can be observed in **Figure 2**. In conclusion, 5 cm^3 is a recommended upper size limit in the pine shredding step to avoid diffusion limitations in the extraction−hydrolysis process.

To increase the fructose yield or the sugar extraction rate, additional enzymatic activities besides inulinases were introduced in the extraction−hydrolysis processes. This included cellulolitic and hemicellulolitic complexes (Celluclast, Olivex) active against macrocomponents of the cell wall. However, no significant advantage in the production of reducing sugars was observed.

Simultaneous Enzymatic Reaction and Extraction Process. The introduction of enzymes in the extraction−hydrolysis process of agave pine fructans can take place at different levels depending on the facilities and flexibility of the processing plant. In industries already extracting fructans in diffusers, the simplest approach is to add enzymes to the agave extract after the diffuser in the settling tanks while the extract is still hot and before thermal treatment. For this type of application, the effect of enzyme concentration was analyzed using as substrate an agave extract provided by the distillery obtained by aqueous extraction in a diffuser installed in the plant. Enzyme concentrations in the assays were varied from 0.1 to 20 mL/L of extract, which are equivalent to 240−48000 U/ L or to 0.017−3.4% (v/w) (mL of Fructozyme L/100 g of agave), as reported in **Figure 3**, where the evolution of the hydrolysis reaction is shown. It may be observed that above 0.05% v/w of enzyme, 50% of fructans are hydrolyzed after 15 min.

In all cases, a drop in the hydrolysis rate is observed after 30 min of reaction as fructan concentration decreases. Total hydrolysis is achieved after 30 min when an enzyme concentration of 3.4% v/w is used, but it takes 3.5 h if the enzyme concentration is reduced to 1.7% v/w. Much longer reaction times are required to complete the hydrolysis when enzyme concentrations lower than 0.85% are used.

An alternative approach for the enzymatic hydrolysis is possible if the enzyme is incorporated into the extraction process. In this particular case, the extraction from shredded agave was studied in laboratory experiments in 1000 mL reaction vessels as a function of time and enzyme concentration. As shown in **Figure 4**,

it was found that when the enzyme is used at a concentration of 0.5% v/w, < 1 h is required to extract and hydrolyze 50% of the total sugars, but if the enzyme concentration is decreased to < 0.1% v/w, > 3 h is required. In these experiments complete hydrolysis and extraction were observed after 4 h using 3% v/w of enzyme. The difference found in enzyme requirements and reaction times in the extraction−hydrolysis process (**Figure 4**) as compared to those reported in **Figure 3** is clearly the consequence of diffusion. Therefore, to have reaction kinetics similar to those found in soluble fructan hydrolysis (**Figure 3**), the optimization of particle size, mixing, and contact conditions is required. The optimum enzyme concentration combines a suitable extraction and reaction time at a minimum of enzyme cost. Considering an enzyme dose between 0.05 and 0.1% (v/w), at a cost of U.S. $28/L of enzyme and a process yield of 3−4 kg of agave required to produce 1 L of tequila (100% agave), the increase in tequila production cost would be between U.S. $0.04 and $0.11/L of tequila. It is also possible to construct a process in which a partial enzymatic hydrolysis is followed by a thermal treatment; this could reduce processing times and would allow 100% conversion with higher productivity and lower energy requirements.

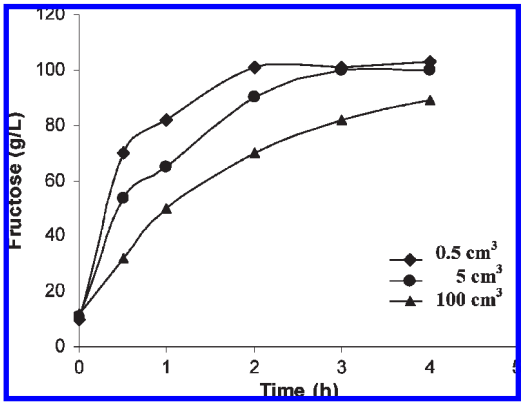

Figure 2. Effect of agave fiber particle size on the enzymatic fructan extraction−hydrolysis: size A (0.5 cm^3), size B (5 cm^3), and size C (100 cm^3). Reaction conditions: 60 °C, agave/water ratio 1:1 w/v (wet basis), 1% (v/w) Fructozyme L.

Finally, the best approach when including enzymes in the tequila process would be to perform the extraction and hydrolysis processes simultaneously in the contact unit (diffuser) followed (or not) by a complementary thermal treatment. This will reduce processing time and energy consumption while avoiding sugar losses through oxidation.

Bench-Scale Experiments Simulating Diffuser Operation. The efficiency of simultaneous extraction and hydrolysis was

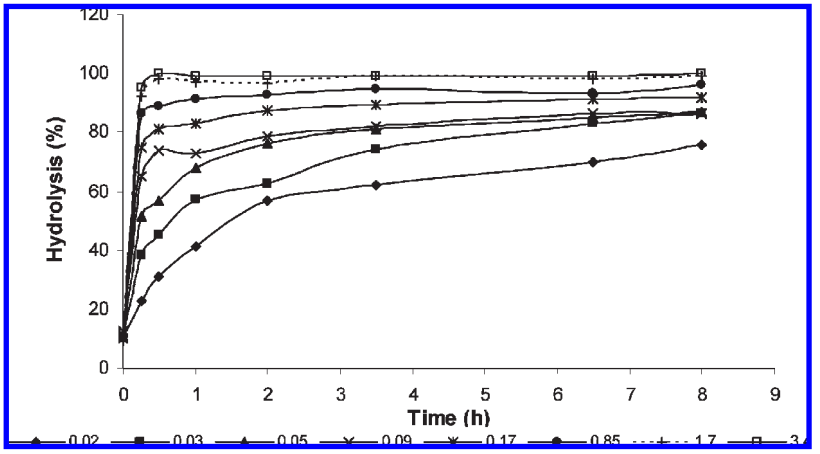

Figure 3. Effect of enzyme activity on fructan hydrolysis in reactions performed at 60 °C using an aqueous agave extract obtained from an industrial diffuser operating at an agave/water ratio of 1:1 w/v. Enzyme concentration is referred to the agave weight from which the extract is obtained. Final fructose concentration (100% hydrolysis) is 97.5 g/L.

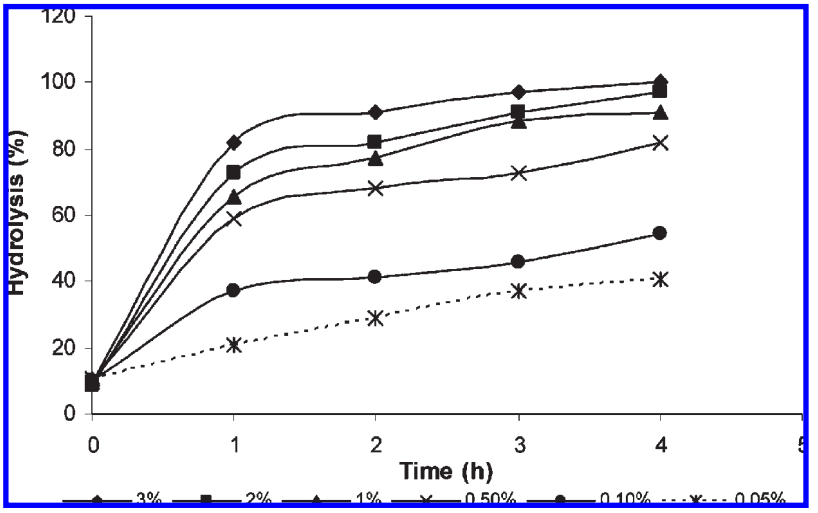

Figure 4. Effect of enzyme activity on reaction rate in reactions performed at 60 °C using agave particles of 5 cm^3 on average on batch process and an agave/water ratio of 1:1 w/v. Enzyme concentration is referred to the agave weight from which the extract is obtained. Final fructose concentration (100% hydrolysis) is 110 g/L.

Table 1. Comparison of the Efficiencies of Enzymatic Hydrolysis When the Enzyme Was Added at Different Points of the Simulated Diffuser[a]

enzyme concentration [% (v/w)]	addition place	fructose (g/L)	hydrolysis efficiency (%)
0.085	vessel 3 in Figure **1**	89	58
0.17	vessel 3 in Figure **1**	78	61
0.17	vessel 5 in Figure **1**	70	62
0.17	all along the diffuser	77	50
control at 60 °C without enzyme		5	1
control at 90 °C without enzyme		9	8

[a] Reaction conditions: residence time, 5 h; temperature, 60°C; water/agave ratio, 1:1 (v/w).

evaluated at laboratory scale in experiments simulating a semicontinuous process as illustrated in **Figure 1** (see Materials and Methods for a detailed description of the experiment). As enzymes have to be continuously added to the extraction process, the location of the enzyme feed should be carefully selected. For this purpose, three experiments were designed in which the conversion was analyzed after a steady state was reached in the system through an hourly addition of the enzyme dose (a) in the middle of the system, (b) with the agave feed, or (c) distributed in the five vessels. These results are summarized in **Table 1**, where it is shown that the worst performance was observed when the enzyme is added throughout the extraction system. No differences were found when the enzyme was added with the agave feed or in the middle of the extractor. It is important to point out that most of the enzyme remains in solution (results not shown) with only a small proportion absorbed by the agave fibers. Therefore, after the extraction system, the hydrolysis may continue in the extract during storage and before thermal treatment or fermentation.

Enzymatic Extraction−Hydrolysis in an Industrial Diffuser. As agave extraction takes place in industry at 90 °C and the enzymatic reaction requires a temperature reduction to 60 °C, at which most of the microbial inulinases work, sugar extraction was evaluated at 60 °C in the industrial diffuser. During several hours of operation, no significant difference was found in sugars extracted in the 60 m^3 diffuser when the temperature was reduced to 60 °C, around 99.5% of extraction yield, as compared to the usual extraction efficiency found at 90 °C (98.4%). This indicates that temperature can be reduced without affecting sugar extraction yield. It was later shown by plant experiments in which both extracts were subjected to the traditional tequila production process (thermal hydrolysis, fermentation and distillation) that no differences exist in tequila produced from agave extracts at the two temperatures. This conclusion was reached by the test panel that systematically evaluates each tequila batch in the plant as part of the process control protocol. It is also a conclusion derived from the correlation coefficient given in **Table 3** when congeneric compounds of T_{60} and TC are compared. Unfortunately, when the enzyme was incorporated to the industrial diffuser with the agave feed, the enzyme was inactivated. In effect, as the water/agave mixture is heated by direct steam injection in the diffuser chambers, the enzyme in solution is subjected to short contact with water vapor at deactivating conditions. In conclusion, although feasible, as demonstrated at low scale, the simultaneous extraction and reaction process requires a redesigning of diffusers to include heating systems suitable for the enzymatic processes. Alternatively, the use of thermostable enzymes would allow a less sophisticated temperature control.

Tequila Produced with Enzymatically Hydrolyzed Fructan and No Thermal Treatment. The role of the thermal treatment on the production of tequila color and organoleptic properties has been described (*16*). However, the simplest alternative process after the extraction and the enzymatic hydrolysis of fructans would be to perform the fermentation of the fructose-rich agave extract without further treatment (heat treatment). To explore this alternative, an experiment was performed with 15000 L of agave extract obtained at 60 °C from the diffuser and hydrolyzed by the addition of Fructozyme L (0.05% v/v), which is equivalent to a dose of 0.085% (v/w) in terms of agave weight. Usually, the agave extracts are stored from 12 to 24 h prior to fermentation; in this particular experiment, the extract remained stored for 20 h before fermentation of the formulated broth, but no thermal treatment was applied. Due to the inulinase activity, during this time residual fructans were hydrolyzed. The fermented broth was distilled and the product analyzed both by gas chromatography and by the plant sensory panel. In this particular case, the fermentation was delayed for 14 h and the total yeast number reached only 46×10^6 in 18 h, whereas in the traditional fermentation process 114×10^6 yeast cells are produced in 10 h and the fermentation starts after the third hour. Although there are several factors that may be responsible for this delay in the fermentation, no further attempts to study the fermentation were made, considering the unfavorable flavor properties of the tequila produced from the nonthermally treated extracts (TE), as will be described later.

Tequila Produced with Enzymatically Hydrolyzed Fructan Followed by a Thermal Treatment. Two 60000 L hydrolysis experiments were performed in tanks in which the aqueous agave extract is usually accumulated before the thermal treatment in autoclaves. The enzyme (0.05% v/v) was added to the agave extract obtained at 60 °C from the diffuser. The enzymatic reactions proceeded without temperature control and with eventual agitation through air bubbling, as these facilities were not available in the plant. After 2 h of reaction, both experiments resulted in less conversion (60 and 50%) than the expected 75%, equivalent to 62 and 53 g/L of fructose. Samples were taken at different points of the tank. The partially hydrolyzed aqueous agave extracts were transferred to 30000 L autoclaves (four experiments). By the time the autoclaves were ready for operation, an additional hour had elapsed, increasing fructan conversion by an additional 20%, so that thermal treatment began with a fructan concentration of 21 g/L, which decreased to 5 g/L. Once the autoclave temperature reached 100 °C (after the second hour), the residual fructans were rapidly hydrolyzed.

At this point, the effect of sulfuric acid addition (0.33 mL/L aqueous agave extract) was also evaluated. In this case, the thermal treatment began with a fructan concentration of 23 g/L and decreased to 4.3 g/L, but no differences in extraction efficiency were found between experiments performed with or without sulfuric acid. It was concluded that the addition of acid is not required to hydrolyze fructans and that process optimization to increase productivity requires an efficient heat transfer operation in the autoclaves, as the effective hydrolysis of residual fructans takes place once 100 °C is reached in the autoclaves.

It was also found that to avoid excessive heating, residual fructan can be hydrolyzed by a final enzyme addition to the fermentation must, as with the addition of 0.0075% v/v to the fermentation broth, no fructan remains after 2 h of fermentation.

To evaluate the feasibility of the enzymatic process for tequila production, fermentation of 15000 and 30000 L of the already

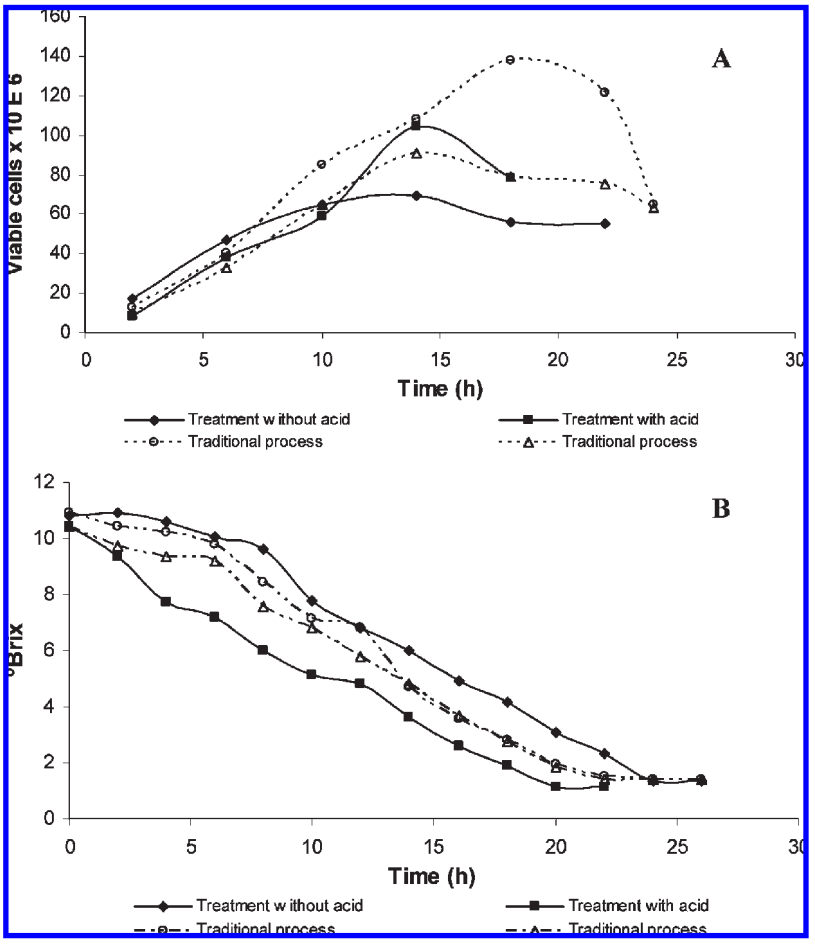

Figure 5. Fermentation behavior of musts after enzymatic/thermal treatment with and without acid as compared to the traditional process: growth cell (**A**); carbohydrate consumption (**B**).

described enzyme hydrolyzed extracts was carried out. These results are shown in **Figure 5**, where cell growth and carbohydrate consumption (°Brix) are shown. It was found that the overall performance of the fermentation is very similar to that observed in musts prepared following the traditional process. Afterward, the fermented broths were distilled to produce tequila following the usual process conditions, both with and without acid addition (TETA and TET).

Preliminary Sensory Descriptive Analysis. The sensory evaluation of tequila obtained from enzyme-treated agave extracts followed by a short thermal treatment (TET and TETA) demonstrated no significant differences in terms of tequila bouquet (scent, aroma) and tequila taste when compared to tequila prepared according to the standard method. However, with regard to the bouquet, it was concluded by the expert panel that the enzymatic tequila (TET and TETA) had herbaceous notes, a lack of cleanliness in the bouquet and aftertaste, and a slightly fruity aroma; this evaluation was reinforced with the volatiles analysis. Although some of these organoleptic characteristics were also observed in standard tequila produced by the traditional process (TC), they were more evident in the enzymatic tequila, which was produced with a shorter thermal treatment.

The tequila produced without the thermal treatment (TE) was rejected by the panel due to its strong herbaceous note, allowing us to conclude that the thermal treatment is required to eliminate compounds related to yeast inhibition (a fermentation delay) and compounds related to the herbaceous flavor and thus to avoid undesirable notes in the product; the thermal treatment is also required to eliminate methanol and to produce flavor-related compounds. Otherwise, the general conclusion reached by the panel was that the enzymatic products (TET and TETA) are similar to the traditional tequila (TC).

Congeneric Compounds Analysis. Besides water and ethanol, its major components, tequila contains a complex chemical composition with well over 200 congeners (*17*, *18*). Identities and concentrations of these compounds account for the subtle differences in the organoleptic properties of the spirits. Attempts to find a simple combination of compounds responsible for the characteristic flavor and aroma of tequila have not been successful (*17*). It has also been pointed out that the generation of Maillard compounds as a result of heating in the cooking of agave plays an important role (*16*). It seems more likely that the final and overall properties of tequila are the result of a complex production process combined with the chemical composition of agave. However, as tequila results from the transformation of a natural product, and as only alcohol and fusel oil concentration are used as the criteria used to control the distillation process, the concentration of congeners varies widely between batches. Their relative composition affects their global organoleptic properties, whereas their individual concentrations correspond to the intensity of the sensory experience they provide. Selected compounds were analyzed to evaluate the chemical composition changes caused by acid and thermal treatments that might explain the differences pointed out by the sensory descriptive analysis (**Table 2**). GC-MS analyses were performed with procedures described elsewhere in this paper. Total concentrations of selected compound groups for TC ranged in the following intervals (mg L^{-1}): FO, 1500−2000; FFA&EE, 100−150; A&E, 200−250; CO, 20−50; and TERP, 1−3. The traditional tequila (TC)

Table 2. Relative Areas of Selected Compounds in Tequila Produced from Traditional Process (TC), Normalized to 100% within Each Group

FO	%	TERP	%	A&E	%	CO	%	FFA&EE	%
acetaldehyde	1	α-terpineol	52	2-phenylethyl acetate	36.3	ethanone, 1-(2-furanyl)-	51.2	C8:0	16
ethyl acetate	8	farnesol	12	2-phenylethanol	32.2	cyclopentanone	15.7	C10:0	30
methanol	35	linalool	10	1-butanol, 3-methyl acetate	26.6	2,5-dimethylfurane	10.9	C12:0	14
1-propanol	7	linalool oxide (1)	10	2-methylpropyl acetate	2.2	2-furanmethanol, acetate	8.3	C14:0	1
isobutanol	9	linalool oxide (2)	9	butanoic acid, ethyl ester	1.1	furfural	4.8	C16:0	3
isoamyl alcohol	40	β-citronellol	3	hexadecanol	0.7	2-methyltetrahydrothiophen-3-one	4.6	EE8:0	3
		4-terpineol	2	propanoic acid, ethyl ester	0.6	3-methylcyclopentanone	4.5	EE10:0	12
		damascenone	2	farnesyl acetate	0.3			EE12:0	14
								EE14:0	2
								EE16:1	2
								EE16:0	3
sum	100	sum	100	sum	100	sum	100	sum	100
C^a (mg L^{-1})	1668	C^a (mg L^{-1})	1.8	C^a (mg L^{-1})	224	C^a (mg L^{-1})	28	C^a (mg L^{-1})	121

[a] Mean values (n = 5).

Table 3. Linear Correlation Matrices for Selected Compounds within Each Group[a]

		TC	TE	T_{60}	TET	TETA
FO	TC	1				
$r_{0.01}$ = 0.917	TET	0.983			1	
	TETA	0.991			0.995	1
TERP	TC	1				
$r_{0.1}$ = 0.669	TE	**0.084**	1			
	T_{60}	0.827	**0.389**	1		
	TET	0.677	**0.534**	0.966	1	
	TETA	**0.433**	**0.422**	0.854	0.927	1
A&E	TC	1				
$r_{0.001}$ = 0.925	TE	0.993	1			
	T_{60}	0.974	0.945	1		
CO	TC	1				
$r_{0.001}$ = 0.951	TE	0.953	1			
	T_{60}	0.983	0.963	1		
FFA&FAEE	TC	1				
$r_{0.001}$ = 0.847	TE	0.989	1			
	T_{60}	0.985	0.979	1		
	TET	0.963	0.992	0.972	1	
	TETA	0.959	0.987	0.962	0.997	1

[a] Indicated r_α values correspond to the critical values where correlation can be rejected at a $1 - \alpha$ significance level for n (number of compounds in the group) data. Boldface values indicate no correlation.

typical relative composition (n = 5) for these chemical groups is also given in **Table 2**.

FO concentration is increased by a factor of 1.8 in the diffuser compared to the classical autoclave extraction process, demonstrating the higher efficiency obtained through this extraction procedure. Temperature and acid treatments result in an additional increase in concentration of FO in the range of 30−60%. Methanol concentration increases, whereas acetaldehyde, ethyl acetate, and other esters decrease. Concentrations for these compounds are regulated by the Mexican government (NOM-006-SCFI-2005), but even if they are high, they meet the regulations. Temperature treatment also affects concentrations in other chemical groups. Comparison of TC with TE shows that without temperature treatment TERP is higher by a 6-fold factor, whereas for A&E and CO a 5-fold decrease was observed, and there is a 1.6 reduction in FFA&EE concentration. Besides the same 1.6 reduction in FFA&EE, there are no important differences in concentrations of TERP, A&E, and CO between TC and T_{60}. Even if there are differences in concentrations of chemical groups and some individual compounds, in general, the global profiles are quite similar as shown by the linear correlation coefficients.

Linear correlation coefficients (r) are a simple and straightforward way to estimate composition profile differences between similar chemical matrices. Relative chromatographic areas normalized to 100% within each chemical group (**Table 2**) were used for correlation analyses. Correlation matrices (CM) are shown in **Table 3**, where critical r values for the specified significance level ($1 - \alpha$) are also indicated. Correlations between samples for the studied chemical groups are significant in all cases except for TERP in TE. Closer inspection of TERP data shows a 4-fold increase in the proportions of linalool and 4-terpineol in TE as compared to TC and a similar decrease in the proportions of α-terpineol and β-*trans*-farnesol.

As a general conclusion, the sensory analysis and the chemical composition demonstrated significant differences in the tequila when agave fructans were exclusively hydrolyzed by enzymes without thermal treatment (TE) prior to fermentation. However, tequilas produced by enzymatic hydrolysis followed by a short thermal treatment (TET and TETA) were different neither based on sensory analysis nor in terms of their chemical composition. The use of enzyme allows a more efficient process as extraction and hydrolysis can take place simultaneously, increasing the overall process efficiency and productivity.

ABBREVIATIONS USED

CO, cyclic oxygenated compounds; CM, correlation matrices; DNS, dinitrosalicylic acid; EE, ethyl esters; FCC, Food Chemicals Codex; FEH, fructan exohydrolase; FFA&EE, fatty acids and fatty acids ethyl esters; FO, fusel oil; GC, gas chromatography; HMF, hydroxymethylfurfural; HPLC, high-performance liquid chromatography; JECFA, Joint FAO/WHO Expert Committee on Food Additives; MS, mass spectrometry; RI, refraction index; TC, tequila produced from traditional process; TE, tequila produced from the nonthermally treated extracts; T_{60}, tequila produced from agave extracted to 60 °C; TET, tequila produced from agave extracted to 60 °C without acid addition; TETA, tequila produced from agave extracted to 60 °C with acid addition; TERP, terpenoids; U, unit of enzymatic activity; WSC, water-soluble carbohydrates.

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