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Fermentable Sugars Production by Enzymatic Processing of Agave Leaf Juice

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The Mexican mezcal industry annually processes approximately 2.92×10^5 t of mezcal agave, generating roughly 1.46×10^5 t of agave leaves per year, which represents a potential carbon source of at least 8170 t via enzymatic processing of agave leaf juice. This carbon source is considered an attractive alternative to produce biofuels and/or chemical products since it is produced and used without adversely affecting the environment. The aim of this investigation was to determine the effect of temperature, pH, enzyme concentration, and bioreaction time on the enzymatic hydrolysis of agave leaf juice enriched in fructan to maximize the fermentable sugars production from three varieties of mezcal agave, using a low-cost commercial brand of hydrolase. This process generated a sugar-enriched juice of 80.07–136.12 g/L of reducing sugars. A Box-Behnken experimental design and a mathematical surface response analysis of the hydrolysis were used for process optimization.

Keywords: agave leaf juice, fructan, enzymatic hydrolysis, experimental design, reducing sugar production

INTRODUCTION

In the last decades, the fermentable sugars production from agro-industrial waste has been a research topic of great interest for the production of biofuels. Monomeric sugars production from agricultural waste using commercial pectinase, cellulase, and β-glucosidase have been reported.[1–3] Due to its abundance in nature, lignocellulose is considered to be a promising material for the production of alcohol fuels;[4] however, major economic disadvantages of biomass refineries include the pretreatment processing of the lignocellulose and the cost of production of the microbial enzymes required to convert the biomass cellulose into fermentable sugars.[5] Needless to say, there are other potential sources for producing fermentable sugars, such as agave leaves, which are actually considered agricultural waste in México.[6]

The Agave genus, with more than 200 species, is native to regions that range from the southern United States all the way to northern South America. There are around 150 species of native agave plants with 119 endemic species; i.e. unique in México. The maguey plant (agave) plays a very important role in the culture, history, and economy of México. Therefore, México is considered the point of origin and diversity of agave plants.[7–10]

In México, agave plants are used traditionally for making fermented beverages: tequila and mezcal. In México, the average global production of agave plants is an estimated 1798 thousand t per year,[11] from which 1506 thousand t per year are used for tequila production and 292 thousand t per year for mezcal. The tequila agave plant production was 848 thousand t in 2010, reaching a maximum production of 2191 thousand t in 2014. Furthermore, agave mezcal plant production was 398 thousand t in 2010, which has gradually decreased to 204 thousand t by the year 2015.

Similarly, to tequila, mezcal is protected by denomination of origin, a legal designation that aims to guarantee a product's authenticity based on its originating geographical region. This denomination of origin effectively limits mezcal production to certain authorized states within México and in addition the use of the following agave species: *Agave salmiana* Otto ex *Salm ssp crassispina* (Trel.) Gentry, *Agave angustifolia* Haw, *Agave esperrima* jacobi, *Agave weberi* cela, *Agave patatorum* zucc, and any other agave species which are not used as raw material to produce other beverages with denomination of origin within the same federative state.[12] In mezcal making, only part of the feedstock is converted into alcohol, rendering a substantial amount of plant material being underutilized: bagasse, leaves, and roots. In fact, the leaves, which constitute about 50 % weight of the agave plant (data obtained in this research), are discarded, and extensive processing is performed upon the stem biomass to generate a fermentable juice. Because the agave leaf has no commercial utility, it causes a serious environmental problem (since they are generally burned). At present, there are various research projects for the conversion of this agricultural waste into a value-added product: ethanol production from agave leaf juice, saccharification of the lignocellulosic residue from

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bagasse to produce ethanol, and cellulose pulp production from agave leaf bagasse.[13–19]

The main carbohydrate of agave leaf juice is fructan, which is a polymer of several fructose units and a common glucose residue.[17] Fructan, in general, is a term used for any carbohydrate in which fructosyl-fructose links constitute most of the glycosidic bonds. In agave plants, fructans are their main photosynthetic product, and are synthesized and stored in the stem. Fructans are used by plants as a source of energy and as an osmoprotector during drought and cold stress periods.[20]

Regarding the production of ethanol, yeast cells can metabolize fructose or glucose into ethanol using the pathways of fermentation. However, the agave fructans cannot be assimilated by many microorganisms without first being hydrolyzed, because of the native branched forms of agave complex fructans.[14] Enzymatic hydrolysis could be an effective strategy for producing fructose. Hydrolysis of fructans may be achieved by inulinases, which are enzymes that catalyze the hydrolysis of inulin-like fructans to produce fructose and fructooligosaccharides. Inulinases having β-fructosidase activity can be obtained from plants and microorganisms (fungi, yeast, and bacteria). Microbial inulinases can reach a yield of up to 95 % fructose by a single-step enzymatic reaction.[21] In fact, there are various reports of agave fructan hydrolysis using inulinases: Avila-Fernández et al.[22] investigated the enzymatic hydrolysis of fructans in tequila production; Corbin et al.[14] produced ethanol from agave leaf-and-steam juice using a commercial fructanase; Huitrón et al.[23] enhanced the ethanol production from raw *Agave tequilana* juice using exo-inulinases by culture of *Aspergillus niger*; and Villegas-Silva et al.[17] studied the hydrolysis of agave leaf juice and fermentation for ethanol production using a commercial brand inulinase enzyme obtained from *Aspergillus niger*.

In the present study, fermentable sugars production achieved from agave leaf juice was researched using different mezcal agave varieties (salmiana, crassispina, and americana) and processing conditions (temperature, pH, enzyme concentration, and bioconversion time). For this, the temperature levels for the experiments were selected in manner that the energy requirements were minimal (30–40 °C). A four factor, three-level Box-Behnken experimental design combining response surface modelling (RSM) and quadratic programming (QP) was employed for maximizing the production of reducing sugars. In addition, the hydrolysis kinetics of fructans uptake and reducing sugars production were evaluated, which follow a first-order kinetic behaviour. This kinetic analysis can be of valuable utility in the process design of the bioconversion from fructans to reducing sugars; it is due to allow optimization of the bioconversion time.

MATERIALS AND METHODS

Collection of Agave Plants

Agave leaves were collected at Ejidos Molino de San José and Emiliano Zapata, a municipality of San Felipe, Guanajuato, México (21° 28′ 51″ N, 101° 12′ 49″ W). At the time of harvest, the 8 year-old plants of three agave varieties: salmiana (*Agave salmiana* ssp salmiana), crassispina (*Agave salmiana* ssp crassispina), and americana (*Agave Americana* L. ssp americana), were separated into leaves, roots, and stems. The leaves were immediately transported to the Technological Institute of Celaya in Celaya, Guanajuato, México.

Extraction of Agave Leaf Juice

After cutting the leaves from the plant, the thorns are removed and the agave leaves reduced in size using a generic grinder: a mill was used to extract the juice from the agave leaves for each variety. The agave leaf juice was filtered through a Whatman 40 filter to eliminate fibres, and stored at 4 °C. This extraction process can yield up to 0.7 L of agave juice/kg of agave leaf. For the conservation of this juice, we use sodium benzoate, as a preservative (0.1 g/L), at this stage.

Enzymatic Material

For this study a commercial brand of fructan hydrolase enzyme was used (ENMEX, producer of food and industrial grade enzymes in México). This hydrolase is a consortium of inulinases and sucrases (invertases) obtained from *Aspergillus niger*. Specifically, constituted of endo-inulinase (EC 3.2.1.7), exo-inulinase (EC 3.2.1.80) and sucrase (EC 3.2.1.26). ENMEX's characterization indicated an inulinase activity of about 1367 U/g.

Determination of Reducing and Total Sugars

The samples were filtered through 0.45 μm membrane filters to remove the suspended solids. Total sugar content was estimated by the phenol-sulphuric acid method[24] and using a sucrose calibration curve obtained by reading the absorbance of the samples at 490 nm (Perkin-Elmer Lambda 25 UV/Vis, USA). The reducing sugars were determined by the DNS method.[25] This colorimetric method involves the oxidation of the aldehyde or ketone functional groups and the 3, 5-dinitrosalicylic acid (DNS) is reduced to 3-amino, 5-nitrosalicylic acid under alkaline conditions. The reducing sugars concentrations were determined using a glucose calibration curve and reading the absorbance of the samples at 545 nm (Perkin-Elmer Lambda 25 UV/Vis, USA). In all cases, the experiments performed and carried out at room temperature in triplicate and the mean values were calculated. Finally, the fructan content in the agave juice was determined as the difference between the total carbohydrates and the reducing sugars.

Enzymatic Hydrolysis

Enzymatic hydrolysis of agave leaf juice was carried out in a 500 mL baffled Erlenmeyer flask containing 250 mL of reaction materials (agave leaf juice and enzymatic extract) on a rotatory shaker (SI 600R, Lab Companion, Korea) at 200 rpm and constant temperature. Scaling up experiments were carried out in a Stirred Tank Bioreactor (Applikon, Schiedam, The Netherlands; 7 L) with a 2 L working volume, under the following conditions: the optimized parameters of temperature and pH obtained for each agave variety, and a stirring speed of 200 rpm.

Experimental Design

A 3^4 Box-Behnken experimental design[26] was employed. The levels of the independent variables studied are as follows: temperature (30, 35, and 40 °C), pH (4.0, 4.5, and 5.0), ratio of enzyme volume/juice volume (0.0001, 0.0002, and 0.0003 L/L (0.01, 0.02, and 0.03 v/v %)), and hydrolysis time (5, 10, and 15 h) while the response variable used was the reducing sugars production in agave leaf juice for each agave variety (salmiana, crassispina and americana). Tables 1–2 show the experimental matrix and performance and the mean values were calculated.

Statistical Analysis and Optimization

The experimental data was fit to a second-order polynomial equation taking into account each dependent variable which is

Table 1. Independent variables values used at different levels of Box–Behnken experimental design

		Levels		
Independent variable	Symbol	−1	0	1
Temperature (°C)	x_1	30	35	40
pH	x_2	4.0	4.5	5.0
Ratio of enzyme volume/juice volume (L/L)	x_3	0.0001	0.0002	0.0003
Hydrolysis time (h)	x_4	5	10	15

given below:

$$y = \beta_0 + \sum_{i=1}^{4}\beta_i x_i + \sum_{i=1}^{4}\beta_{ii}x_i^2 + \sum_{i=1}^{3}\sum_{j=i+1}^{4}\beta_{ij}x_i x_j + e_i \quad (1)$$

where y is the response variable (reducing sugars concentration) and the x_i's represent the original independent variables (x_1, temperature in °C; x_2, pH; x_3, relation of enzyme volume/juice volume in L/L; x_4, treatment time in h). Therefore β_0 is the value of fitted response at the centre point of design, and β_i, β_{ii}, and β_{ij} are the linear, quadratic and cross product or interaction regression coefficients, respectively, and e_i is the error. The model permitted evaluation of linear, quadratic and interactive terms of the independent variables on the dependent variable. Determination and regression coefficients were estimated using STATISTICA software (version 12; Stat Soft, Inc.). In addition, an analysis of variance (ANOVA) was conducted to guarantee the significance of the obtained model, and a probability value of $p < 0.05$ was considered statistically significant. The different interactions were obtained using the response surface and contour plots of any two independent variables, while keeping the value of the third and fourth variables constant at the central level. Such three-dimensional surfaces could give accurate geometrical representation and provide useful information of the behaviour of the system within the experimental design. The

Table 2. Experimental design matrix for response surface analysis in terms of coded and actual values of optimization parameters

	Temperature (°C)		pH		Ratio of enzyme volume/juice volume (L/L)		Hydrolysis time (h)	
Statistical order	Coded values	Actual values	Coded values	Actual values	Coded values	Actual values	Coded values	Actual values
1	−1	30	−1	4.0	0	0.0002	0	10
2	+1	40	−1	4.0	0	0.0002	0	10
3	−1	30	+1	5.0	0	0.0002	0	10
4	+1	40	+1	5.0	0	0.0002	0	10
5	0	35	0	4.5	−1	0.0001	−1	5
6	0	35	0	4.5	+1	0.0003	−1	5
7	0	35	0	4.5	−1	0.0001	+1	15
8	0	35	0	4.5	+1	0.0003	+1	15
9	0	35	0	4.5	0	0.0002	0	10
10	−1	30	0	4.5	0	0.0002	−1	5
11	+1	40	0	4.5	0	0.0002	−1	5
12	−1	30	0	4.5	0	0.0002	+1	15
13	+1	40	0	4.5	0	0.0002	+1	15
14	0	35	−1	4.0	−1	0.0001	0	10
15	0	35	+1	5.0	−1	0.0001	0	10
16	0	35	−1	4.0	+1	0.0003	0	10
17	0	35	+1	5.0	+1	0.0003	0	10
18	0	35	0	4.5	0	0.0002	0	10
19	−1	30	0	4.5	−1	0.0001	0	10
20	+1	40	0	4.5	−1	0.0001	0	10
21	−1	30	0	4.5	+1	0.0003	0	10
22	+1	40	0	4.5	+1	0.0003	0	10
23	0	35	−1	4.0	0	0.0002	−1	5
24	0	35	+1	5.0	0	0.0002	−1	5
25	0	35	−1	4.0	0	0.0002	+1	15
26	0	35	+1	5.0	0	0.0002	+1	15
27	0	35	0	4.5	0	0.0002	0	10
28	0	35	0	4.5	0	0.0002	0	10
29	0	35	0	4.5	0	0.0002	0	10
30	0	35	0	4.5	0	0.0002	0	10

Temperature (°C) = x_1, pH = x_2, ratio of enzyme volume/juice volume (L/L) =x_3, hydrolysis time (h) = x_4.
The coded values are dimensionless parameters.

Table 3. Characterization of agave leaf per sections: base, middle, and tip

	Mass fraction (g/g)			Juice yield L of juice/kg of biomass (kg/L)			Reducing sugars content (g/L)			Fructans content (g/L)		
Leaf part	s	c	a	s	c	a	s	c	A	s	c	a
Base	0.575	0.592	0.499	0.793	0.724	0.775	26.1	22.8	19.7	86.4	53.9	39.8
Middle	0.310	0.284	0.310	0.731	0.682	0.662	20.4	19.7	16.1	33.6	18.0	17.2
Tip	0.114	0.124	0.191	0.183	0.115	0.124	16.5	14.3	12.6	0.5	0.0	0.6

s = salmiana, c = crassispina, a = americana.

optimization of the hydrolysis process was addressed by finding the levels of independent variables (temperature, pH, relation of enzyme volume/juice volume and hydrolysis time), which would give maximum reducing sugars production. The optimum values of the selected variables were obtained by solving the regression equation proposed. To verify the prediction model, three experimental units were performed, under optimal conditions using an Erlenmeyer flask for each agave variety.

RESULTS AND DISCUSSION

Characterization of Agave Leaves

As a first step in this research experiment, the agave leaves were divided into three equal parts with the same length (base, middle, and tip), subsequently each leaf part was analyzed using the following parameters: mass fraction, juice yield, reducing sugars concentration, and fructan concentration: those results are reported in Table 3.

In all agave varieties studied, it is possible to observe that most of the biomass is found in the base and middle sections of the agave leaf (approx. 0.80 g/g), which contain most of the juice (0.7–0.8 L of juice per kg of biomass). Interestingly, there is a reducing sugars profile along the agave leaf, reducing sugars concentration decreased significantly from the leaf base to the leaf tip. This is likely due to the fact that when glucose is synthesized via photosynthesis, it is immediately metabolized as an energy source, and any surplus amounts are isomerized into fructose, or along with the latter converted into sucrose, which is then used as a transport sugar, and it is translocated from leaves through the

Table 4. Comparison between the reducing sugars production obtained experimentally (g/L) and the reducing sugars production predicted (g/L) by a second order polynomial model

	Salmiana		Crassispina		Americana	
Statistical order	Experiment	Prediction	Experiment	Prediction	Experiment	Prediction
1	105.85 ± 2.29	103.27	69.56 ± 1.02	71.64	64.02 ± 1.76	63.17
2	125.33 ± 1.55	127.45	84.09 ± 1.58	85.40	71.55 ± 0.78	70.65
3	103.57 ± 1.32	99.15	65.73 ± 1.56	66.37	56.47 ± 0.72	57.27
4	118.02 ± 2.24	118.31	81.93 ± 0.12	81.80	67.21 ± 0.89	67.97
5	68.42 ± 2.24	73.00	50.20 ± 2.74	55.02	39.01 ± 0.94	39.42
6	109.51 ± 1.83	109.74	72.82 ± 0.81	73.22	60.05 ± 1.82	59.42
7	112.48 ± 0.90	109.96	77.85 ± 0.64	79.41	60.62 ± 1.11	61.15
8	129.27 ± 1.79	122.40	87.13 ± 0.19	84.27	72.59 ± 1.93	72.09
9	110.84 ± 2.97	110.66	77.33 ± 0.97	77.50	61.06 ± 1.15	61.66
10	94.3 ± 2.47	87.42	59.39 ± 0.04	56.80	49.49 ± 0.74	49.69
11	116.71 ± 3.03	112.10	77.92 ± 0.41	74.86	60.69 ± 2.28	58.04
12	113.14 ± 0.99	115.24	76.14 ± 0.86	77.99	63.82 ± 0.95	66.16
13	129.53 ± 2.43	133.90	87.73 ± 1.16	89.12	76.5 ± 1.71	75.98
14	99.34 ± 2.14	95.52	73.23 ± 0.83	70.86	54.64 ± 1.05	54.49
15	91.14 ± 2.94	87.20	66.59 ± 1.51	66.79	50.65 ± 1.14	50.67
16	116.99 ± 2.04	118.42	84.16 ± 0.23	82.76	70.76 ± 1.81	70.43
17	112.17 ± 2.22	113.48	76.79 ± 0.68	77.96	65.83 ± 0.73	65.67
18	110.5 ± 1.69	110.66	77.75 ± 1.85	77.50	61.42 ± 1.30	61.66
19	83.33 ± 1.20	89.68	63.12 ± 1.33	60.41	52.85 ± 2.57	51.00
20	117.52 ± 1.62	116.90	78.32 ±0.84	76.85	56.98 ± 1.27	58.04
21	114.38 ± 2.39	119.82	73.05 ±1.91	73.79	65.06 ± 0.64	64.42
22	137.46 ± 1.55	135.93	84.57 ± 1.02	86.54	73.29 ± 1.11	75.56
23	89.66 ± 1.76	92.04	68.20 ± 0.31	69.00	52.32 ± 1.80	54.57
24	79.30 ± 1.40	83.62	63.38 ± 1.39	63.05	48.86 ± 0.84	49.28
25	114.56 ± 1.59	115.06	85.60 ± 1.54	85.20	70.77 ± 1.44	70.77
26	107.77 ± 0.73	110.22	83.82 ± 0.83	82.29	69.31 ± 0.84	67.49
27	110.79 ± 1.95	110.66	77.43 ± 1.00	77.50	60.97 ± 1.43	61.66
28	110.26 ± 2.48	110.66	77.1 ± 1.85	77.50	62.71 ± 2.27	61.66
29	110.52 ± 1.62	110.66	77.22 ± 2.30	77.50	61.64 ± 1.01	61.66
30	111.03 ± 1.04	110.66	78.11 ± 1.89	77.50	62.15 ± 1.79	61.66

phloem into the stem for the biosynthesis of fructans. Regarding the fructan content in raw juice, fructan presence was only significantly detected in the base and middle sections of agave leaf; and most significantly in the leaf base. In fact, it is in the stem and the attached leaf base that agave plants store huge quantities of fructans and other nonstructural carbohydrates.[27] With respect to the agave varieties, these results indicate that the leaf juice of all agave varieties studied here are a potential source of reducing sugars. This potential source of reducing sugars can be ranked from highest to lowest in the following order: salmiana, crassispina, and americana. Certainly, fructan content differs among Agave species and varieties and it changes over the lifetime of the plant.[28]

Statistical Analysis for Reducing Sugars Production

With respect to the reducing sugars production, a comparison between the experimental data and the predicted data obtained by the second order polynomial model are shown in Table 4 and Figure 1 for each agave variety studied. The regression equation obtained indicated an r^2 value of 0.9487, 0.9606, and 0.9819 for the agave varieties salmiana, crassispina, and americana, respectively. In this sense, the quadratic model describes properly the behaviour of experimental data.

Supplementary Tables 1–3 show the results for the second order of the response surface model in the form of analysis of variance (ANOVA) for all agave varieties studied. Fischer's, F-test and *p*-values demonstrate significance for the regression model. The ANOVA table indicates the overall significant effect of linear, square, and interaction terms on enzyme activity at 5 % level of significance ($p < 0.05$ and $F_{calculated} > F_{table}$). The regression summary (Supplementary Tables 1–3) indicates that the effect of all the process variables was significant at a 5 % level (*p*-value less than 0.05) for all agave varieties studied. Additionally, the significance of the factors for reducing sugars production is in the following order: hydrolysis time > enzyme concentration > temperature > pH, for the variety salmiana; hydrolysis time > temperature > enzyme concentration > pH, for the variety crassispina; and hydrolysis time > enzyme concentration > temperature > pH, for the variety americana. The effect of the quadratic term of the various process variables is also significant except for the terms: enzyme concentration in variety salmiana; temperature and pH in crassispina variety; and pH in variety americana. In addition, some interaction terms for the various process variables are not significant; except, the interaction term of 'temperature and enzyme' concentration for all agave varieties, 'temperature and time' for crassispina, and 'temperature and concentration' for the varieties salmiana and americana.

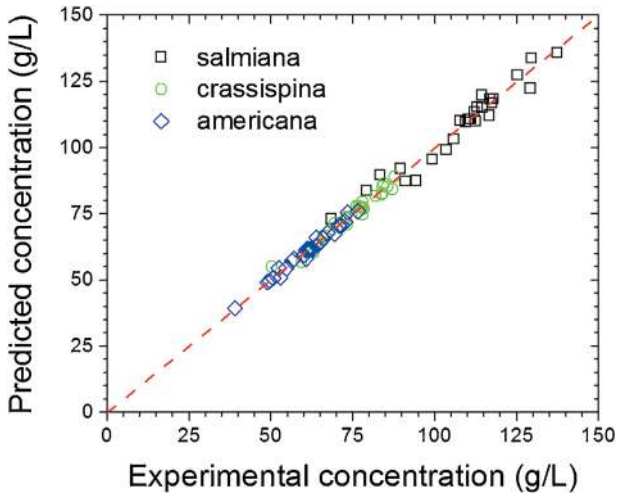

Figure 1. Comparison between the experimental reducing sugars production and the predicted reducing sugars production from the agave leaf juice of three different varieties.

Equations of the fitted model (based on the original variables) after neglecting the effect of non-significant terms are as below.

For the variety salmiana:

$$RSC = -12.5 \cdot T + 193.8 \cdot pH + 4.322 \times 10^5 \cdot C + 9.532 \cdot t + 0.266 \cdot T^2 - 21.09 \cdot pH^2 - 0.206 \cdot t^2 - 5.558 \times 10^3 \cdot T \cdot C - 1.215 \times 10^4 \cdot C \cdot t \quad (2)$$

For the variety crassispina:

$$RSC = 3.717 \cdot T + 3.015 \times 10^5 \cdot C + 5.861 \cdot t - 2.408 \times 10^8 \cdot C^2 - 8.461 \times 10^{-2} \cdot t^2 - 6.940 \times 10^{-2} \cdot T \cdot t - 6.67 \times 10^3 \cdot C \cdot t \quad (3)$$

And for the variety americana:

$$RSC = 238.3 - 8.153 \cdot T - 37.48 \cdot pH + 1.494 \times 10^5 \cdot C + 2.579 \cdot t + 0.1 \cdot T^2 - 1.929 \times 10^8 \cdot C^2 - 6.861 \times 10^{-2} \cdot t^2 + 2.047 \times 10^3 \cdot T \cdot C - 4.537 \times 10^3 \cdot C \cdot t \quad (4)$$

where *RSC* is the reducing sugars concentration in g/L; *T* is the hydrolysis temperature in °C; *C* is the ratio of enzyme volume/juice volume in L/L; and *t* is the time of the hydrolysis in h. Notice that the models expressed by Equations (2–4) are not coded. Also, Supplementary Table 4 shows all coefficients of the quadratic models.

Figure 2 exhibits the effect of temperature and enzyme concentration on the reducing sugars production, and it is found that the increase of both temperature and enzyme concentration enhances very significantly the reducing sugars production; concluding, under the conditions of study, that the maximum reducing sugars production is achieved at the highest levels of temperature and enzyme concentration; for this case, 40 °C and 0.0003 L/L (0.03 v/v %). This behaviour is found for all agave varieties studied.

The catalytic activity of enzymes has long been understood in terms of the Michaelis-Menten mechanism: a substrate S binds reversibly with an enzyme E to form an enzyme-substrate complex ES, whose decomposition forms the products P and regenerates the original enzyme E:[29]

$$S + E \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} SE \xrightarrow{k_2} P + E \quad (5)$$

So, the rate of products' formation is dependent on the substrate concentration, which can be expressed by:

$$\frac{dP}{dt} = \frac{k_2 E_t S}{\left(\frac{k_{-1}+k_2}{k_1}\right) + S} \quad (6)$$

where E_t is the total enzyme concentration. Usually the terms $k_2 E_t$ and $(k_{-1} + k_2)/k_1$ are expressed as the maximum rate ($V_{max} = k_2 E_t$), and the substrate affinity constant

($K_m = (k_{-1} + k_2)/k_1$). In accordance with Equation (6), it is possible to establish that $dP/dt \propto E_t$, which is consistent with the experimental observed behaviour in this study: when the enzyme concentration is increased, the reducing sugars production rate is also increased. Sometimes, the affinity between the enzyme and the substrate is so high that it is possible to consider $K_m >> S$, which allows the simplification of the Michaelis-Menten equation to a first-order kinetic model (which occurs in this study and is demonstrated in the next section):

$$\frac{dP}{dt} = kS \tag{7}$$

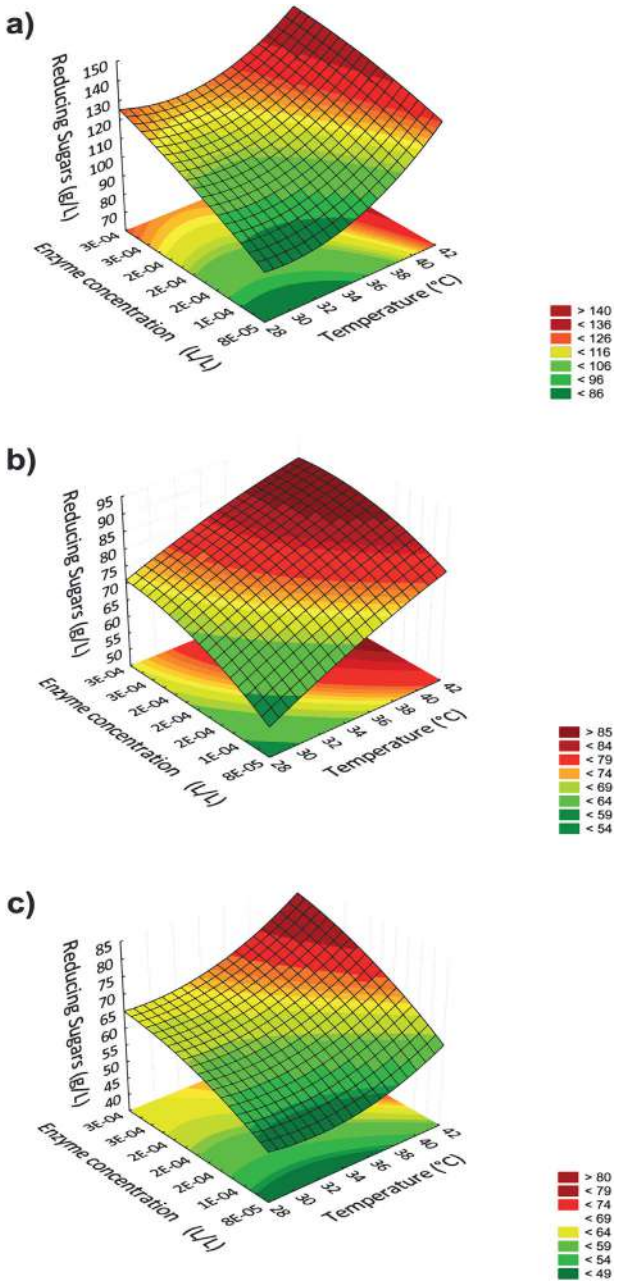

Figure 2. Effect of temperature and enzyme concentration on reducing sugars production, at pH of 4.5 and hydrolysis time of 10 h, for three agave varieties: (a) salmiana; (b) crassispina; and (c) americana.

where $k = V_{\max}/K_m = k_1 k_2/(k_{-1} + k_2)$ is the specific rate constant. In general, the parameters k_i obey Arrhenius' law; i.e. $k_i = A_i \exp(-E_i/RT)$; in this case, A_i is the pre-exponential factor and E_i is the activation energy. Therefore, Equation (7) we expressed in terms of temperature as follows:

$$\frac{dP}{dt} = \frac{A_1 A_2 e^{-(E_1+E_2)/RT}}{A_{-1} e^{-E_{-1}/RT} + A_2 e^{-E_2/RT}} E_t S \tag{8}$$

Mathematically, in Equation (8), the product formation rate exhibits a maximum value, which is established by the difference between the activation energy of the forward reaction ($SE \xrightarrow{k_2} P + E$) and the reverse reaction ($SE \xrightarrow{k_{-1}} S + E$). Therefore, an optimal temperature (T_{opt}) can obtained by solving this equation:

$$\frac{d}{dT}\left(\frac{dP}{dt}\right) = 0 \tag{9}$$

which is,

$$T_{opt} = \frac{(E_2 - E_{-1})}{R \ln\left[\frac{A_2}{A_{-1}} \frac{E_1}{E_1 + (E_2 - E_{-1})}\right]} \tag{10}$$

So when temperature is lower than optimal temperature, the forward reaction rate of $SE \xrightarrow{k_2} P + E$ increases more rapidly than the reverse reaction rate of $SE \xrightarrow{k_{-1}} S + E$, until the reaction temperature reaches the optimal temperature. In the case of the commercial enzyme used in this study, Segura-Cerda[30] reported an optimal temperature of 45 °C for the fructan hydrolysis from the mezcal agave leaf. Therefore, the experimental data of reducing sugars production is consistent with the expected behaviour in the temperature range of 30–40 °C; i.e. when the temperature is increased it is followed by an increase of the reducing sugar production rate, because the temperature used in this study is less than the optimal temperature of hydrolysis.

The pH plays a key role in the hydrolysis process. During hydrolysis, fructans monomers or oligosaccharides are produced; this release begins with the addition of a proton (H^+) to the glycosidic oxygen, thereby breaking the glycosidic bond and permitting the formation of a carbocation (C^+) cyclic; subsequently an electron pair is donated by a water molecule enabling the formation of two molecules (monomers or oligosaccharides).[31] Therefore, we investigated the effect of the pH and enzyme concentration on the reducing sugars production and it is shown in Figure 3. It was found that the effect of enzyme concentration on the reducing sugars production is remarkable, and is more significant than the pH. In addition, it is noted that the response surface's curvature as a function of pH remains the same, regardless of the enzyme concentration value. In fact, in Equations (2–4) it is possible to observe that the reducing sugars production does not depend on the interaction of the pH-enzyme concentration. This is due to the enzyme concentration being so low that the hydronium ion equilibrium is not affected by its variation.

Both temperature and pH are the main factors that define the biocatalytic activity of an enzyme; in Figure 4, the effect of the temperature and the pH on the reducing sugars production is shown. Although it is widely known that the temperature influences the chemical-physical equilibrium; the variation of temperature used in this study is so low that the hydronium ion equilibrium is not affected significantly. So that in Equations (2–4) it is possible to observe that the reducing sugars production does not

depend on the temperature-pH interaction. Nevertheless, the effect of temperature is more significant than pH for all agave varieties studied. This may be due to the experiments being limited to a narrow range of pH (4.0–5.0). In fact, ANOVA revealed that the pH has the lowest statistical influence on the reducing sugars production. In Figure 4, it is possible to observe that there are a few significant differences in the selected pH range of the reducing sugars production. In general, we can confirm that the highest level of temperature maximizes the production of reducing sugars under the conditions of this study.

Figure 5 shows the effect of temperature and hydrolysis time on reducing sugars production; and it is found that both variables contribute significantly to the hydrolysis process, hydrolysis time being the most import factor for reducing sugars production. In fact, for all agave varieties, the coefficient of the independent variable time is positive, and it is the more significant with respect to the other coefficients (see coded model coefficients in Supplementary Table 4). Additionally, Figure 6 shows the effect of enzyme concentration and hydrolysis time on reducing sugars production, in which the behaviour of the enzyme concentration and time effect on the reducing sugars production is similar to the temperature and time effect, that is to say the reducing sugars production increases with increasing both variables. However, it is found that the temperature-reducing sugars production curves are concave upward (except for the variety crassispina) and the enzyme concentration-reducing sugars production curves are concave down (for all the varieties). It is possibly due to the strong exponential dependence of hydrolysis process on the temperature

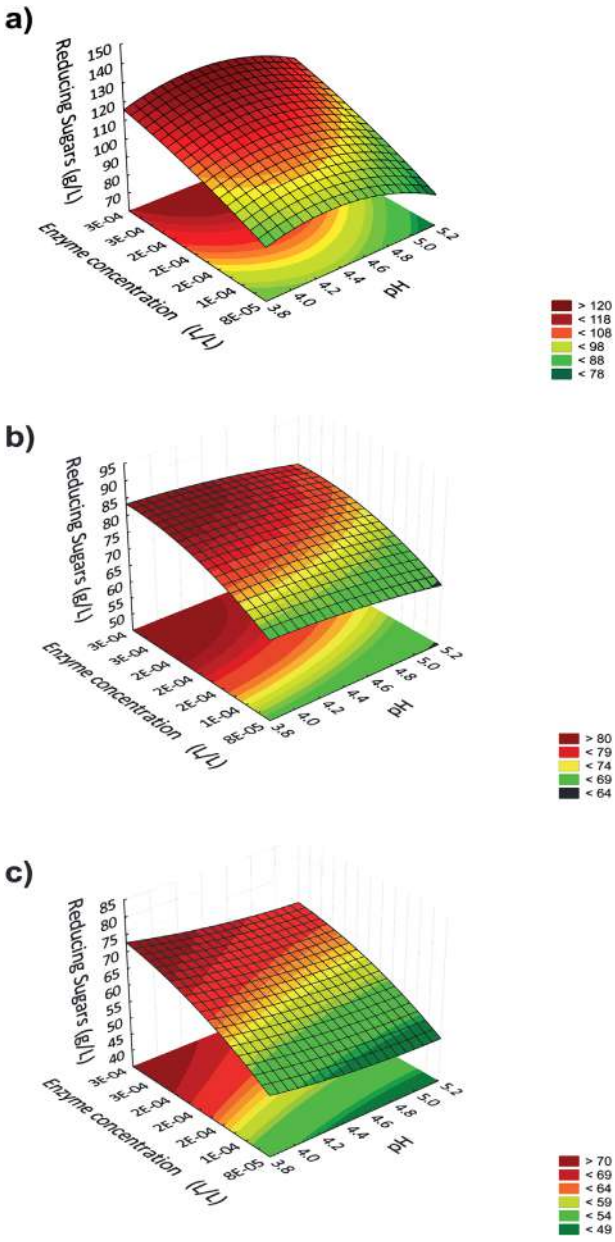

Figure 3. Effect of pH and enzyme concentration on reducing sugars production, at temperature of 35 °C and hydrolysis time of 10 h, for three agave varieties: (a) salmiana; (b) crassispina; and (c) americana.

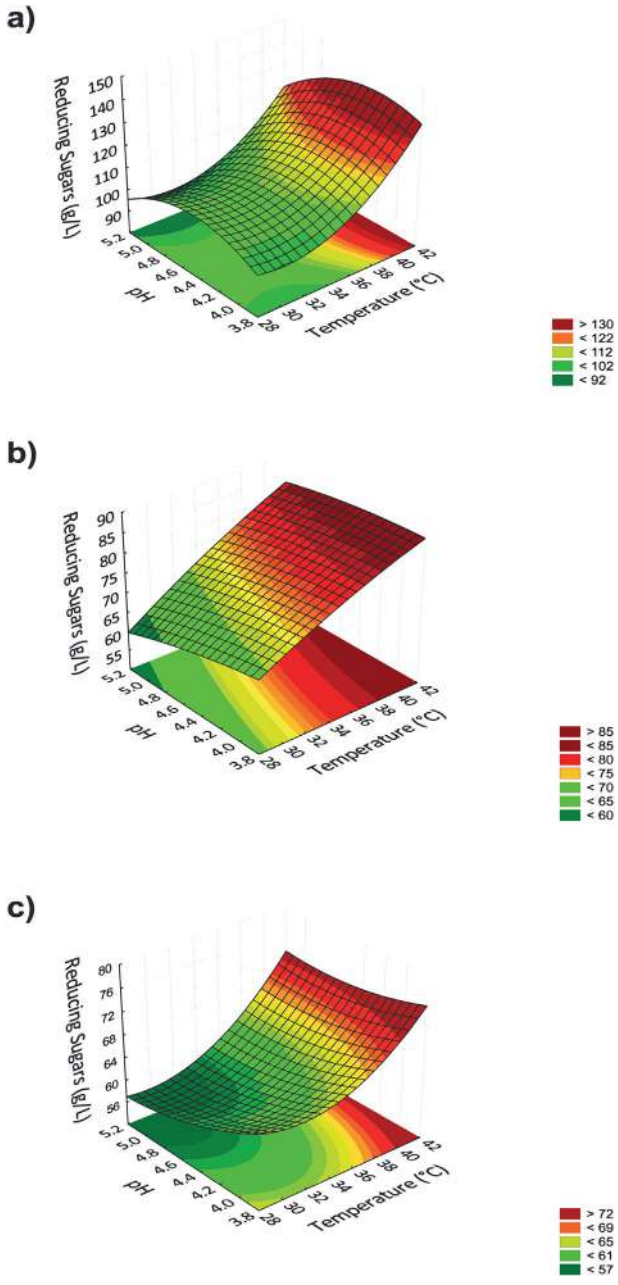

Figure 4. Effect of temperature and pH on reducing sugars production, at enzyme concentration of 0.0002 L/L (0.02 v/v %) and hydrolysis time of 10 h, for three agave varieties: (a) salmiana; (b) crassispina; and (c) americana.

at temperature range studied, and a saturation effect occurred at high levels of enzyme activity.

Notice that, prior to this study, the behaviour expected for the reducing sugars production (as function of enzyme concentration and temperature) has a monotonic increase, because the enzymatic reaction is irreversible, and the temperatures used in this study were less than the optimal temperature of hydrolysis, which is consistent with the linear coefficients of the models coded for all agave varieties (see Supplementary Table 4); that is, all the coefficients of the independent variables enzyme concentration and temperature are positive, and highly significant with respect to the other coefficients. In this sense, the quadratic model describes properly the physics of the enzymatic process. With respect to the pH effect on the reducing sugars production, it is significantly low, which is probably due to the experiments being performed over a narrow range of pH (4.0–5.0). Thus, a deeper examination is suggested to evaluate the effect of pH on the enzymatic hydrolysis of fructans.

In general, increasing the temperature and the enzyme concentration could decrease the hydrolysis time; undoubtedly, it may increase dramatically the processing cost, because it causes a higher energy and enzyme consumption. On the other hand, a high hydrolysis time leads to a higher energy demand in the mixing operation, and requires the use of larger stirred tank bioreactors. Although the use of the enzyme could reduce by its immobilization, it would imply a longer hydrolysis time. This

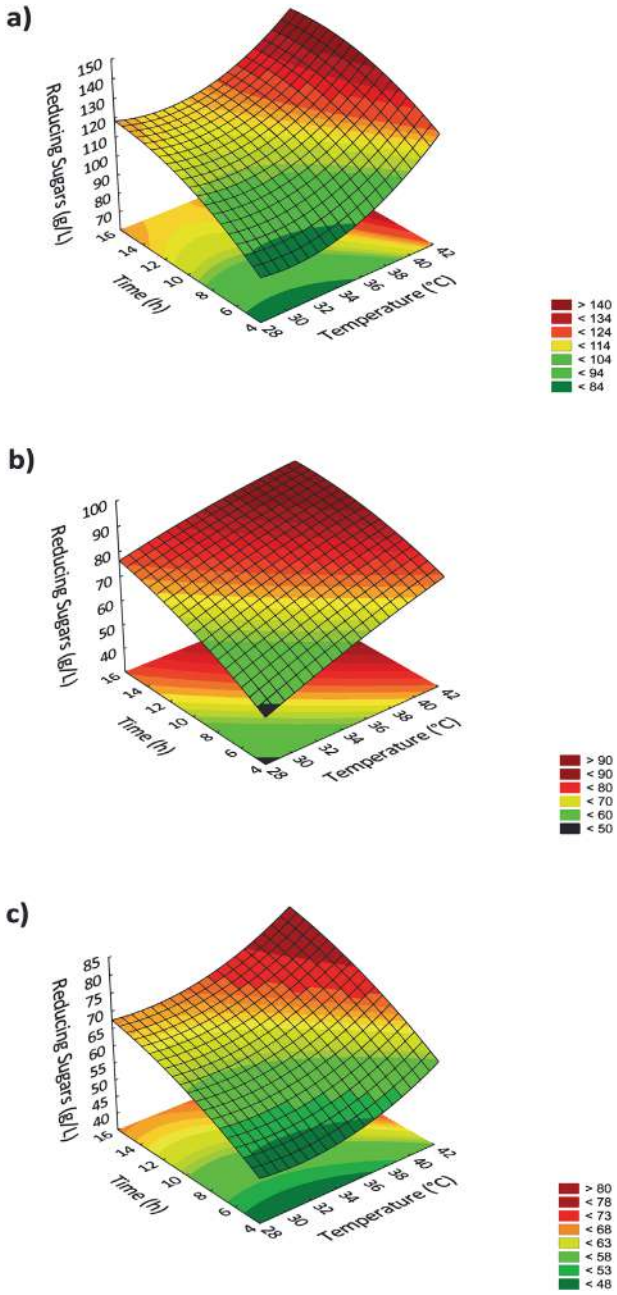

Figure 5. Effect of temperature and time on reducing sugars production, at enzyme concentration of 0.0002 L/L (0.02 v/v %) and pH of 4.5, for three agave varieties: (a) salmiana; (b) crassispina; and (c) americana.

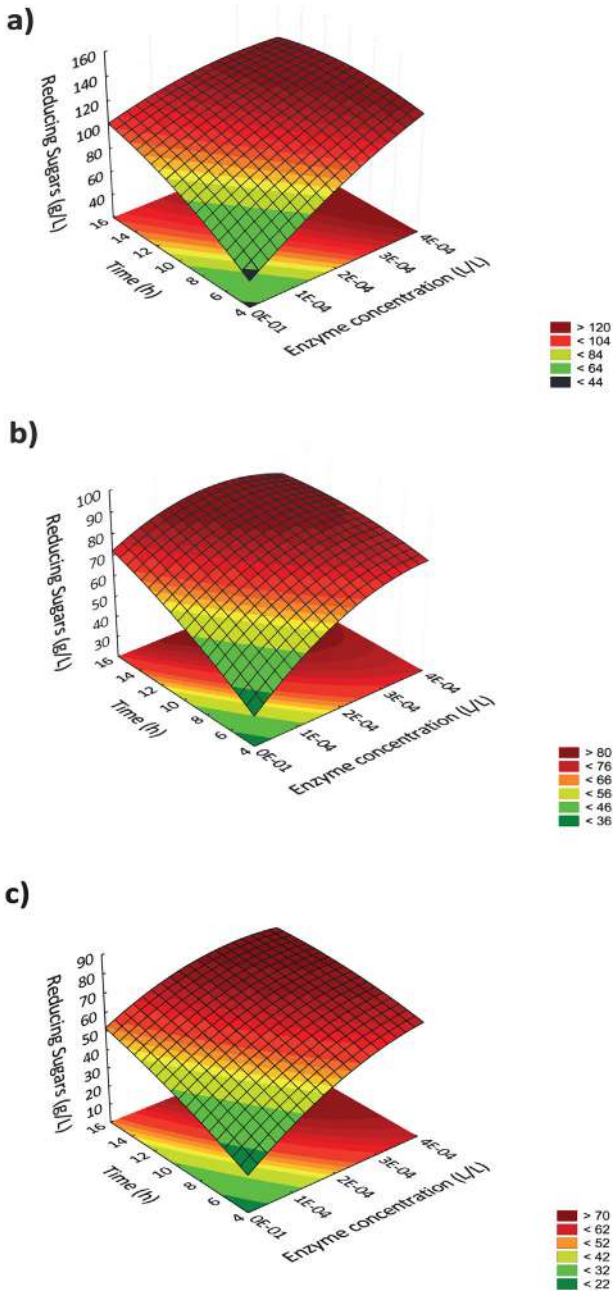

Figure 6. Effect of enzyme concentration and time on reducing sugars production, at temperature of 35 °C and pH of 4.5, for three agave varieties: (a) salmiana; (b) crassispina; and (c) americana.

causes limitations to the mass transfer rate of the immobilized enzyme systems as well as an increased cost due to the immobilization process; for what is suggested that the fructan hydrolysis in agave leaf juice for producing reducing sugars must be studied using immobilized inulinase, in order to establish a more economical process. In this sense, the selection of the better design must be optimized by balancing costs and benefits.

Optimization of Reducing Sugars Production

In order to optimize the process conditions for reducing sugars production by using a numerical optimization technique, the main criteria for constraints optimization was the maximum possible concentration of reducing sugars. Table 5 shows the optimum operating conditions for the production process in order to achieve the maximum reducing sugars production under the conditions of this study. At these conditions for the process variables, the predicted values of reducing sugars concentration were found to be 145.0, 95.8, and 83.35 g/L for the agave varieties salmiana, crassispina, and americana, respectively.

The results of optimization were confirmed by conducting the experiments in triplicate at the above-optimized values using an Erlenmeyer flask (validation experiment) and a batch stirred tank bioreactor (scaling up experiment); thus, the experimental values for reducing sugars concentration were found to be 139.31, 93.61, and 83.15 g/L for the agave varieties salmiana, crassispina, and americana, respectively, using an Erlenmeyer flask; and 136.12, 90.34, and 80.07 g/L using a batch stirred tank bioreactor.

Michel-Cuello et al.[16] reported a qualitative and quantitative characterization of nonstructural carbohydrates in raw and hydrolyzed juices extracted from *Agave salmiana* stems and leaves, indicating that the raw leaf juice is a potential source of reducing sugars. They extracted juice from different parts of the agave leaf, which were subsequently processed via acid hydrolysis to produce reducing sugars and the following results were reported: 106.4, 103.3, 67.2, and 62.9 g/L of reducing sugars from juice extracted from the leaf base, leaf neck, leaf wing, and leaf tip, respectively. Certainly, acids yielding syrups in which 75–98 % of the fermentable sugars are fructose readily hydrolyze fructans. However, there are disadvantages to the use of acid hydrolysis. Although the acid is cheap, its use increases the already high ash content, whose removal is expensive.[32]

There are various efforts in the use of agave waste for the production of the value added chemical. Recently, Saucedo-Luna et al.[13] studied the chemical and enzymatic saccharification of the lignocellulosic residue from *Agave tequilana* bagasse to produce ethanol by fermentation. They developed a sequential saccharification process that consists of two steps. Firstly, the saccharification of bagasse of *Agave tequilana* was carried out at 147 °C with 0.02 L/L (2 v/v %) sulphuric acid for 15 min, yielding 25.8 g/L of fermentable sugars. In the second step, the remaining lignocellulosic material was hydrolyzed using commercial enzymes, which were incubated for 72 h at 40 °C rendering 41 g/L of fermentable sugars. Certainty, this process required the use of milling and sieving operations in the adequacy of the raw material. Therefore, it is possible to observe that the processing of both the agave bagasse and the agave leaf juice require the same unit operations. In this sense, the combination of the use of agave bagasse with the use of agave leaf juice could lead to a process economically more attractive, allowing the total use of agave waste.

Kinetic Analysis of Fructan Hydrolysis Process

During the experimentation, it was observed that the temperature, enzyme concentration, and the hydrolysis time are critical factors for the fructan hydrolysis process. In fact, the highest levels of temperature, enzyme concentration, and the hydrolysis time were conducted to optimize the production of reducing sugars in all cases studied. Concerning hydrolysis, time is a factor that strongly affects the process economy, so it was considered important to examine in more detail the effect of the hydrolysis time on the reducing sugars production. For this purpose, a batch stirred tank bioreactor was used at the optimal conditions of temperature, pH, and enzyme concentration previously obtained, at 200 rpm and a working volume of 2 L. Samples were taken every 1.5 h over a 15 h period, and analyzed immediately.

Figure 7a shows the kinetics of reducing sugars production and fructan consumption for all agave varieties studied. The trends shown by experimental data exhibit a monotonic behaviour. Due to the high complexity of the fructan hydrolysis via endo-inulinase, exo-inulinase, and sucrose, and to the fact that this study is oriented to provide a technology implementation, the fructan bioconversion model was, therefore, fitted simply by the Michaelis-Menten equation:

$$-.\frac{dS}{dt} = \frac{V_m S}{K_m + S} \quad S = S_0 \text{ at } t = 0\text{h} \tag{11}$$

$$\frac{dP}{dt} = -Y_{P/S}\frac{dS}{dt} \quad P = P_0 \text{ at } t = 0\text{h} \tag{12}$$

where S is the fructan concentration, P is the reducing sugars concentration, Vm is the maximum hydrolysis rate, K_m the substrate affinity constant, and $Y_{P/S}$ is the yield coefficient of substrate. Notice $Y_{P/S}$ is constant, and V_m and K_m are effective parameters, because of the complexity of enzyme used.

Table 5. Optimal conditions of process variables for the enzymatic hydrolysis of fructans

	Level		
Factor	Salmiana	Crassispina	Americana
Temperature (°C)	40	40	40
pH	4.5	4	4
Enzyme concentration (L/L)	0.0003	0.0003	0.0003
Hydrolysis time (h)	15	15	15
Production values predicted (g of reducing sugars/L of juice)	145.05	95.85	83.35
Production values obtained* (g of reducing sugars/L of juice)	139.31	93.61	83.15

*Experimental results obtained at the optimal operating conditions using an Erlenmeyer flask.

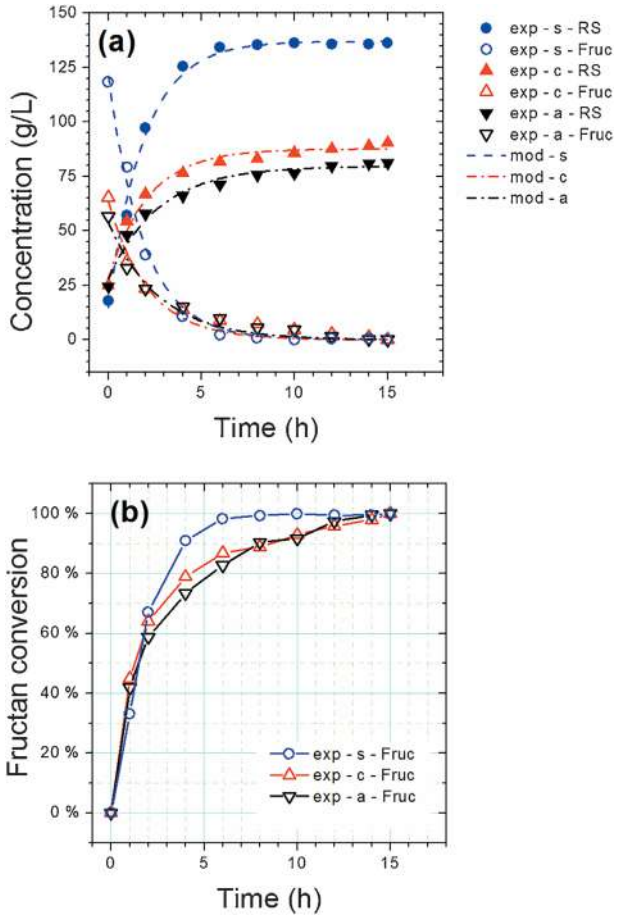

Figure 7. (a) Reducing sugars production and fructan consumption in agave leaf juice using a commercial brand of inulinase. (b) Percentage conversion of fructan to reducing sugars. Nomenclature: exp = experimental; mod = model; RS = reducing sugars; Fruc = fructans; s = salmiana; c = crassispina; a = americana.

To estimate the optimal model parameters, a nonlinear regression technique assisted by a code developed in Matlab (The MathWorks, Natick, MA) was used to minimize the deviation between the model and the experimental data. For calculation of model predictions, a system of differential equations describing the enzymatic kinetics was solved by an integration program based on Runge-Kutta-Fehlberg.[33] The optimization program for the direct search of the minimum of a multivariable function was based on the Broyden-Fletcher-Goldfarb-Shanno method.[34] The minimization criteria used in the program is as follows:

$$\mathrm{SSWR} = \sum_{i=1}^{n}\sum_{j=1}^{m}\left(\frac{dij}{W_j}\right)^2 \qquad (13)$$

where SSWR represents the sum of squares of the weighed residues, i and j represent the number of experimental data points and the number of variables respectively, W_j represents the weight of each variable (maximum value of each variable), and d_{ij} denotes the difference between the model and the experimental value. The results of parameter estimation as well as the corresponding values of r^2 are summarized in Table 6. In general, the model presents a good correlation between the experimental data and the model predictions.

In all cases, it is possible to observe $K_m >> S$, therefore Equation (11) can be simplified to the following first-order kinetic model:

$$-.\frac{dS}{dt} = kS \quad S = S_0 \,\mathrm{at}\, t = 0\mathrm{h} \qquad (14)$$

where $k = V_{\max}/K_m$ is the specific rate constant. This indicates that fructan hydrolysis rate is only a function of the fructan concentration; i.e. there is a high affinity between the hydrolases used and the fructans of agave leaf juice. Therefore, the enzymatic hydrolysis of fructans can be properly described by a first-order kinetic model with a specific rate constant in the range of 0.360–0.510 h^{-1}. In all agave varieties, the yield coefficient is close to one, especially for the variety salmiana, which indicates that fructans were totally hydrolyzed to reducing sugars. With respect to the specific rate constant, this can be ranked from highest to lowest in the following order: salmiana, crassispina, and americana, which reveals that the commercial enzyme used is more specific to the salmiana agave fructans. Figure 7b exhibits that a period of 7.5 h practically achieved the bioconversion of fructans to reducing sugars (at least 90 % of fructan conversion); i.e. there is a higher productivity ($\Delta P/\Delta t$) in a such time period. So, a time longer than 7.5 h is not desirable because the productivity will be decreased. In fact, this is a very significant reduction of hydrolysis time for all varieties studied, which must be considered for the sake of the process' economy, because it minimizes the energy requirement in the mixing operation. Additionally, it is found that the more promising agave leaf juice is obtained from the variety salmiana, because the reducing sugars content obtained via hydrolysis and the specific rate constant are the highest ones among the three agave varieties studied.

Waleckx et al.[35] reported the use of inulinases to improve fermentable carbohydrate recovery during tequila production. They made a comparison among three different commercial enzymes: Fructozyme L (Novozyme), Oligofructse 3000 (Beldem Food Ingredients), and Invertasa S (ENMEX). Among the most important findings were: (1) Fructozyme L was the only enzyme prepared able to significantly hydrolyze agave water soluble carbohydrates; (2) the optimum temperature found was between 55–65 °C, and optimum pH values were between 4.0 and 4.5; (3) Fructozyme concentrations ranging from 0.000125 to 0.0012 L/L (0.0125 to 0.12 v/v %) of cooking honey (i.e. agave juice generated during the cooking involved in tequila production), which allows efficient hydrolysis of the fructans contained in the cooking honey (≥90 %); and (4) for comparative purposes for this study, they obtained at least 90 % of fructan hydrolysis in a 9 h treatment using a Fructozyme L concentration of 0.0003 L/L (0.03 v/v %). In this study, at least 90 % of fructan hydrolysis was obtained using an enzyme concentration of 0.0003 L/L (0.03 v/v %) at 4, 9, and 9 h treatment for mezcal agave varieties: salmiana, crassispina, and americana, respectively (see Figure 7b). In this sense, this comparison of results shows that the commercial enzyme used in this study is as competitive as the Fructozyme L enzyme.

Future Prospects

There are two reasons for the choice of a low temperature (30–40 °C) during the hydrolysis of the agave fructan: (1) to make a fructan hydrolysis process economically feasible using low heating energy requirements; and (2) to lay the groundwork for ethanol production from agave leaf juice using simultaneously fructan hydrolysis with free enzymes and alcoholic fermentation with immobilized yeast cells. Certainly, this bioprocessing

Table 6. Model parameters for enzymatic hydrolysis of fructans in agave leaf juice

Variety	K_m (g/L)	v_{max} (g/(h · L)	$Y_{P/S}$	S_0 (g/L)	P_0 (g/L)	$k = V_{max}$ (1/h)	r^2
Salmiana	1.508×10^4	7.696×10^3	0.999	121.1	14.9	0.510	0.994
Crassispina	1.578×10^6	7.155×10^5	0.938	63.4	28.2	0.453	0.983
Americana	1.753×10^6	6.318×10^5	0.960	53.8	28.0	0.360	0.980

strategy could decrease dramatically the cost of input power required by the mixing operation during the bioreaction process, which allows achieving a biological process that is economically feasible and environmental friendly for the ethanol production from mezcal and tequila agave wastes.

CONCLUSIONS

The waste from mezcal-agave leaves are a potential source of green energy and/or an ideal feedstock for the development of sustainable bioindustries as we have shown. This is due to the fact that the agave leaf juice can be processed enzymatically in order to produce fermentable sugars using a low-cost commercial hydrolase at low temperatures. Hence, by this methodology, a volume of 1 m^3 of agave leaf juice can be processed to produce 80.07–136.12 kg of reducing sugars in an aqueous solution at an enzyme cost of $40.80 US dollars in a period of time similar to that reported for Fructozyme L enzyme. Finally, studies on the use of waste materials are very important from the economic and the environmental points of view, because they reduce the amount of waste that is habitually burned, and present the potential for such waste to be used as a source of energy.

ACKNOWLEDGEMENTS

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NOMENCLATURE

A	pre-exponential factor (1/h or L/(g · h))
Adj MS	adjusted mean square
ANOVA	analysis of variance
β_0	value of fitted response at the centre point
β_i	linear regression coefficients
β_{ij}	quadratic and cross-product regression coefficients
DF	degree of freedom
d_{ij}	difference between the model and the experimental value
E	enzyme concentration (g/L)
e_i	statistical error
E_i	activation energy (J/mol)
ES	enzyme-substrate complex
E_t	total enzyme concentration (g/L)
F	Fisher's F-test statistic
k_i	rate constants (1/h or L/(g · h))
K_m	substrate affinity constant (g/L)
P	reducing-sugars concentration (g/L)
p	probability
P_0	reducing-sugars initial concentration (g/L)
R	gas constant (J/(mol · K))
RSC	reducing sugars concentration (g/L)
RSM	response surface modelling
S	fructan concentration (g/L)
S_0	fructan initial concentration (g/L)
Seq SS	sequential sum of squares
T	sum of squares of the weighed residues
t	temperature (°C)
t	time (h)
T_{opt}	optimal temperature (°C)
$V_{\max}$	maximum hydrolysis rate (g/(L · h))
W_j	weight of each variable
x_i	independent variables
y	response variable
$Y_{P/S}$	yield coefficient of substrate

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