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Strategy for Biotechnological Process Design Applied to the Enzymatic Hydrolysis of Agave Fructo-oligosaccharides To Obtain Fructose-Rich Syrups

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A strategy to optimize biotechnological process design is illustrated for the production of fructose-rich syrups via enzymatic hydrolysis of agave fructo-oligosaccharides. The optimization process includes ecological studies from natural fermentations leading to the selection of a strain with capacity for inulinase synthesis, and variable optimization for the synthesis, and enzymatic hydrolysis using the response surface methodology. The results lead to the selection of *Kluyveromyces marxianus*, endogenous strains isolated from *aguamiel* (natural fermented sugary sap from agave plants), as the main strain with high capacity for enzyme synthesis with inulinase activity. Production optimization at bioreactor level revealed that operation at 30.6 °C, 152 rpm, 1.3 VVM of aeration, and pH 6.3 leads to maximum inulinase synthesis, whereas 31 °C, 50 rpm, and pH 6.2 leads to maximum hydrolysis of agave fructo-oligosaccharides. HPLC analysis of the fructose-rich syrups obtained at these optimal conditions showed an average composition of 95% of fructose and 5% of glucose and the absence of sucrose. The analysis also revealed that the syrups are free of residues and toxic compounds, an undesirable occurrence often present when traditional methods based on thermal or acid hydrolysis are applied for their obtainment. Therefore, the product may be suitable for use as additive in many applications in the food and beverage industries.

KEYWORDS: Agave; oligosaccharides; hydrolysis; fructose; syrups

INTRODUCTION

The design of a biotechnological process implies the definition of the biological system to be used, the substrate to be transformed, and the operation conditions that maximize the metabolic activities and increase the main product yield. In addition, if the main product is an enzyme, the conditions that maximize the enzymatic activity should also be considered (*1*−*3*). Thus, the integral design of such a process should consist of guidelines for ecological studies leading to the selection of the strains more suitable for a given application as well as tools for process variable optimization for bioreactor operation. The strategies to achieve these goals rely mainly in statistical techniques for optimization, such as experimental designs and surface-response analysis.

In this contribution, an illustration of the application of these techniques for the integral process design of the enzymatic hydrolysis of agave fructo-oligosaccharides to obtain fructose-rich syrups is presented.

Fructose-rich syrups have received increasing attention as food additives due to their beneficial health effects, such as those associated with calcium absorption stimulation in postmenopausal women, iron absorption increase in children, and colon cancer prevention, and their low glycemic index (*4*). Nowadays, fructose-rich syrups are widely preferred as food and beverage additives instead of sucrose-rich alternatives. Inulin hydrolysis has been identified as an alternative for fructose-rich syrup production (*5*−*7*). Several plant materials, such as dahlia, chicory, and Jerusalem artichokes, have already been reported as effective raw materials for high fructose production due to their high fructo-oligosaccharide or inulin content (*7*, *8*). Other potential alternatives are the agave plants, due to their concentration of fructo-oligosaccharides comparable to those in chicory, dahlia tubers, dandelion, and Jerusalem artichoke (*5*). Agave plants are known as raw materials for the production of alcoholic beverages such as tequila, mezcal, sotol, and bacanora (*9*) that rely on the fermentation of sugars generated by thermal hydrolysis of agave fructo-oligosaccharides during the cooking stage (*10*). Another fermented beverage obtained from agave plants is the *aguamiel*. Aguamiel is the sugary sap obtained from some species of agave (for example, *Agave atrovirens*, *Agave americana*, or *Agave salmiana*) transformed by the metabolic activity of strains naturally present in the agave juice, and it is commonly used to obtain

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Table 1. Centroid Simplex Mixture Design for Strain Selection

assay	X_1	X_2	X_3	X_{12}	X_{19}	$[Y^a]_{24}$
1	1	0	0	0	0	0.84
2	0	1	0	0	0	10.62
3	0	0	1	0	0	11.4
4	0	0	0	1	0	0.53
5	0	0	0	0	1	0.98
6	0.5	0.5	0	0	0	6.58
7	0.5	0	0.5	0	0	5.15
8	0.5	0	0	0.5	0	0.46
9	0.5	0	0	0	0.5	0.72
10	0	0.5	0.5	0	0	9.90
11	0	0.5	0	0.5	0	0.49
12	0	0.5	0	0	0.5	3.31
13	0	0	0.5	0.5	0	1.89
14	0	0	0.5	0	0.5	0.69
15	0	0	0	0.5	0.5	1.6
16	0.33	0.33	0.33	0	0	5.08
17	0.33	0.33	0	0.33	0	0.59
18	0.33	0.33	0	0	0.33	1.22
19	0.33	0	0.33	0.33	0	0.69
20	0.33	0	0.33	0	0.33	0.84
21	0.33	0	0	0.33	0.33	0.76
22	0	0.33	0.33	0.33	0	4.69
23	0	0.33	0.33	0	0.33	0.61
24	0	0.33	0	0.33	0.33	0.31
25	0	0	0.33	0.33	0.33	2.12
26	0.25	0.25	0.25	0.25	0	2.01
27	0.25	0.25	0.25	0	0.25	2.28
28	0.25	0.25	0	0.25	0.25	2.15
29	0.25	0	0.25	0.25	0.25	0.54
30	0	0.25	0.25	0.25	0.25	0.88
31	0.2	0.2	0.2	0.2	0.2	0.51

[a] Fructose concentration (g/L).

pulque, a Mexican indigenous alcoholic beverage (*11*). Previous contributions have demonstrated that aguamiel contains some strains with capacity for inulinase synthesis (*12*). On the basis of these observations, in this contribution, microbial ecology studies of aguamiel strains are performed to select those with capacity for hydrolysis of agave fructo-oligosaccharides via inulinase synthesis. These studies are followed by optimization of the bioreactor critical variables leading to the production of enzymatic extracts with high enzymatic hydrolysis activity on the fructo-oligosaccharides from agave. In this manner, an integral approach to the design of a biotechnological process to obtain fructose-rich syrups by enzymatic hydrolysis of agave fructo-oligosaccharides is achieved.

MATERIALS AND METHODS

Agave Juice. Fresh "heads" or "pines" (plants without leaves) from *Agave tequilana* weber var. *azul* of a single batch were chopped, mixed with hot water (near boiling point), and pressed to obtain agave juice containing 120 g/L (average) of fructo-oligosaccharides. The agave juices were diluted to obtain a solution with 80 g/L of fructo-oligosaccharides, which was used in all experiments.

Aguamiel. Two samples of aguamiel obtained from traditional rural producers of pulque of Guanajuato state in Mexico were used for microbial ecology studies.

Strain Isolation. Samples of aguamiel were taken and inoculated on Petri dishes containing nutritive agar (Difco, Detroit, MI) for bacteria, potato dextrose agar (Difco, Detroit, MI) for yeast, and selective media formulated with aguamiel or agave extract, plus bacteriological agar at 1.5% (v/w). One milliliter of aguamiel samples was spread onto the medium surface in Petri dishes and incubated at 28, 32, and 37 °C for 24, 48, and 72 h. Developed colonies with different morphologies were transferred to fresh agar mediums (nutritive, potato dextrose, and selective) and incubated again. The procedure was repeated until pure cultures were obtained (*13*).

Strain Purity and Preliminary Identification. To ensure the purity of the isolated colonies, during the whole isolation strategy and at the end of every incubation time, the colonies developed were analyzed by microscopic (model DMRAX2, Leica Microsystems GmbH, Wetzlar, Germany) methods using stain techniques and biochemical tests using an API Biochemical card (API CAUX, API 20E, and 20NE) (*13*, *14*).

Pure Culture Propagation. The pure strain isolated, bacteria and yeast, were cultured in potato dextrose (for yeast) and nutritive (for bacteria) agar slants at 28 °C for 48 h (better conditions for growth). Biomass samples were taken from each slant and transferred to 250 mL Erlenmeyer flasks containing 100 mL of agave juice supplemented with 1% of yeast extract, mixed, and incubated at 28 °C and 100 rpm for 48 h on a rotary shaker (model 4520, Forma Scientific, Marietta, OH) for culture propagation.

Enzymatic Extracts. The biomass obtained from the Erlenmeyer flask propagation was centrifuged at 10000*g* (Hermle, model Z383). The supernatant obtained was used as a source of extracellular enzyme or crude enzymatic extract.

Quantification of Synthesized Enzyme. All crude enzymatic extracts obtained were analyzed according to the Bradford method (*15*) to quantify the synthesized enzyme.

Experimental Strategy for Microbial Ecology Studies. A centroid simplex experimental mixture design was developed to discriminate unnecessary strains and select only those with potential for inulinase synthesis (*3*, *16*). The design included the analysis of crude enzymatic extracts from pure cultures, as well as mixed extracts from more than one culture to evaluate synergic effects (**Table 1**). The results were analyzed using a statistical framework and were expressed via a mathematical model used to predict which enzymatic extracts have significant inulinase activity for the agave fructo-oligosaccharide hydrolysis.

Considerations for the Centroid Simplex Experimental Mixture Design. Every assay included in the experimental strategy was performed on the basis of the restrictions [$0 < X_i < 100\%$ and $X_1 + X_2 + X_3 + ... + X_p = 100\%$], where 100% corresponds to 50 mL of enzymatic extract. That is, in all assays the volume of enzymatic extract is 50 mL, and it will be integrated by the extract from a pure culture (for example, the first experimental assay must contain 50 mL of enzymatic extract from supernatant of the pure culture X_1) or by mixtures of extracts from different cultures (for example, experimental assay 6 must contain a mixture of 25 mL of enzymatic extract from X_1 and 25 mL from X_2). Each enzymatic extract was placed, to evaluate its activity, in an Erlenmeyer flask containing 100 mL of agave extract with 80 g/L of fructo-oligosaccharides and incubated on a rotary shaker at 28 °C and 100 rpm for 24 h for fructo-oligosaccharide hydrolysis.

Strain Identification. Once the cultures yielding high fructose concentrations were selected, strain identification was performed. Several techniques can be used for strain identification; among them, molecular biology tools are the most commonly used. In this study, the ribosomal sequence analysis of the 5.8S rRNA gene and two ribosomal internal transcribed spacers ITS1 and ITS2 were amplified by Polymerase Chain Reaction (PCR), followed by restriction analysis using endonucleases. The method is based on genomic DNA extraction from selected strains according to the protocol proposed by Ausubel et al. (*17*). For rRNA gene amplification the DNA extracted was mixed with PCR super mix (Invitrogen, Carlsbad, CA) containing high-fidelity DNA polymerase (Invitrogen). PCR was performed at the following conditions: initial denaturalization at 95 °C 5 min, 35 cycles with 1 min of denaturation at 95 °C, 2 min of annealing at 53 °C, 2 min of extension at 72 °C, and a final extension for 10 min at 72 °C. The PCR products were purified with the QIAEX II kit (Qiagen, Hilden, Germany) and digested with restriction enzymes (Invitrogen). Once purified, the amplified fragment was cloned on Topo TA 2.1 (Invitrogen) and sequenced. The sequence was compared with those reported on the National Center for Biotechnology Information (NCBI) database using the "Blastx" algorithm for strain identification.

Experimental Design for Inulinase Synthesis Maximization at Bioreactor Level. Using the starter inoculum previously defined from the ecological studies, an experimental strategy for maximization of inulinase

Table 2. Central Composite Design for Inulinase Synthesis Maximization at Bioreactor Level

assay	V_1:temp (°C)	V_2:pH	V_3:agit (rpm)	V_4:air (Lpm)	$[Y^a]_{24}$
1	0	0	0	0	26.85
2	−1	−1	−1	−1	14.67
3	1	−1	−1	−1	21.83
4	−1	1	−1	−1	15.43
5	1	1	−1	−1	24.56
6	−1	−1	1	−1	26.93
7	1	−1	1	−1	13.87
8	−1	1	1	−1	7.32
9	1	1	1	−1	26.53
10	−1	−1	−1	1	25.72
11	1	−1	−1	1	18.39
12	−1	1	−1	1	18.81
13	1	1	−1	1	21.63
14	−1	−1	1	1	27.19
15	1	−1	1	1	23.92
16	−1	1	1	1	24.01
17	1	1	1	1	26.68
18	−2	0	0	0	8.56
19	2	0	0	0	24.99
20	0	−2	0	0	9.45
21	0	2	0	0	20.66
22	0	0	−2	0	5.12
23	0	0	2	0	28.87
24	0	0	0	−2	4.48
25	0	0	0	2	22.79
26	0	0	0	0	26.49

[a] Fructose concentration (g/L).

synthesis on a stirred vessel bioreactor was designed. The following variables were considered: V_1, temperature; V_2, acidity (expressed as pH); V_3, agitation; V_4, aeration. These variables were singled out on the basis of their relationship with the biomass growth and metabolic activities (temperature and pH) and their relationship with the heat and mass transfer limitations in the batch reactor (agitation and aeration). Agitation and aeration are essentially collinear variables affecting the oxygen transfer rate, which implicitly affects microbial growth, substrate transformation, and synthesis of product. The feasible ranges for these variables were defined as 25−31 °C for V_1, pH 3−7 for V_2, 50−150 rpm for V_3, and 0.5−1.5 VVM for V_4, on the basis of a preliminary screening design (not shown). A central composite design for four variables with axial points ($\pm\alpha_i$) was constructed (**Table 2**) as described by Montgomery (*16*). In this scheme, the variable levels are coded (−1 for the lower level, +1 for the upper level, and 0 for the mean value) on the basis of the following equation: (factor level − mean factor value)/[(factor upper level − factor lower level)/2]. In all experimental assays, the starter inoculum concentration (2.2 g/L), volume medium (1.5 L), and composition medium (0.3 g/L of potato flour, 0.46 g/L of sucrose, and 0.13 g/L of inulin) were kept constant.

Experimental Design for Maximization of Fructo-oligosaccharide Hydrolysis Using Inulinase Obtained in Situ. For maximization of fructo-oligosaccharide hydrolysis, as a result of the enzymatic treatment, on a stirred vessel bioreactor the following variables were considered: V_5, temperature; V_6, agitation; V_7, acidity (expressed as pH). These variables were singled out on the basis of their relationship with the enzymatic activity (temperature and pH), whereas agitation is important due to the enzyme−substrate interaction induced by mixing and inactivation induced by the shear force, as well as the fact that the agave juice (substrate) is constituted by a mixture of both insoluble and soluble fructo-oligosaccharides that must be held in suspension. The feasible ranges for these variables were defined as 30−40 °C for V_5, 100−200 rpm for V_6, and pH 3−7 for V_7, on the basis of a preliminary screening design. A central composite design for three variables with axial points ($\pm\alpha_i$) was constructed (**Table 3**) as described by Montgomery (*16*). The variable levels were coded as before. In all of these assays, the enzymatic extract and the substrate (agave extract) were mixed in a 1:2 ratio, and the process yield was evaluated as a function of the fructose concentration present in the solution measured via HPLC after 24 h of enzymatic treatment.

Table 3. Central Composite Design for Fructo-oligosaccharide Hydrolysis Maximization

assay	V_5:temp (°C)	V_6:agit (rpm)	V_7:pH	$[Y^a]_{24}$
1	0	0	0	26.11
2	−1	−1	−1	13.20
3	1	−1	−1	29.02
4	−1	1	−1	11.73
5	1	1	−1	19.26
6	−1	−1	1	19.98
7	1	−1	1	5.98
8	−1	1	1	4.09
9	1	1	1	0.79
10	−1.68	0	0	16.19
11	1.68	0	0	8.46
12	0	−1.68	0	29.07
13	0	1.68	0	18.17
14	0	0	−1.68	0.79
15	0	0	1.68	10.15
16	0	0	0	23.38

[a] Fructose concentration (g/L).

Fermentation System. A stirred tank bioreactor (ADI autoclavable bioreactor, Applikon Dependable Instruments, Schiedam, The Netherlands) of 3 L capacity with a dished bottom with Rushton turbine impellers was used for both inulinase synthesis and fructo-oligosaccharide hydrolysis. The system includes a BioConsole (ADI 1035, Applikon Dependable Instruments), a BioController (ADI 1030, Applikon Dependable Instruments) for accurate parameter setting, and a bidirectional serial communication port for supervisory control and data acquisition (BioXpert software, Applikon Dependable Instruments).

High-Performance Liquid Chromatography (HPLC) Analysis. For the bioreactor optimization, the response function was evaluated as the fructose concentration present in the hydrolyzed solution measured via HPLC. The hydrolyzed extract was filtered on Millipore membranes of 45 μm. Samples consisting of 20 μL of hydrolyzed extract were injected to the HPLC. The equipment used for the HPLC assays was a system standalone module that includes an LC1120 advanced spindle-driven pump, an LC1205 programmable UV−vis detector, a refractive index detector, and a WinChrom Chromatography Management System for computer control (GBC Scientific Equipment, Dandenong, Victoria, Australia). The sugars concentration was measured using an SS Exsil amino column (SGE Inc., Austin, TX). The solvent elution was operated at 1.0 mL/min of a mixture containing 70% acetonitrile and 30% water. The separation was performed at 40 °C, and the sugars were monitored using a refractive index detector. Retention time comparison to external standards of fructose, glucose, and sucrose, followed by concentration estimation using the relative percentage area covered in the chromatogram, was used to calculate sugar concentrations. The experimental results described below were performed in duplicate, and the reported values represent the mean values of these duplicated experiments.

Statistical Analysis. The results from the experimental designs were analyzed using a statistical framework on the basis of analysis of variance procedures (Statgraphics Graphics Plus Corp., V 2.1, 1996), followed by mathematical model construction via least-squares using lineal regression.

RESULTS AND DISCUSSION

Microbial Ecology Studies. Nineteen ($X_1 + X_2 + X_3 + ... + X_{19}$) pure cultures were isolated from aguamiel. All of them were cultured, and their capacity for inulase synthesis, both extra- and intracellular, was evaluated. The results showed that five (X_1; X_2; X_3; X_{12}; X_{19}) strains exhibited the capacity to synthesize extracellular inulinase and to decompose the agave polysaccharides into sugars, mainly fructose. The enzymatic extracts from these five strains were evaluated in a centroid simplex design (**Table 1**) to test symbiotic relationships among the different enzymatic extracts to maximize fructo-oligosaccharide hydrolysis, expressed as a function of fructose concentration.

The results obtained (last column of **Table 1**) were analyzed using a combination of both descriptive and inductive statistics. In the first instance, a quadratic model was constructed, using least-squares, to locate the optimum. The following expression was obtained:

$$Y_1 = 1.19X_1 + 10.99X_2 + 11.62X_3 + 0.45X_{12} + 1.16X_{19} - 2.06X_1X_2 - 8.59X_1X_3 - 4.01X_1X_{12} - 2.62X_1X_{19} - 8.51X_2X_3 - 20.03X_2X_{12} - 16.36X_2X_{19} - 13.86X_3X_{12} - 24.33X_3X_{19} + 5.18X_{12}X_{19}$$

The model was analyzed by statistical tools based on the analysis of variance, which suggests that the model exhibits a statistically significant relationship between fructose concentration and the enzymatic extract (variables) used. On the basis of this model, the strain selection that maximized the fructo-oligosaccharide hydrolysis was accurately computed via standard constrained optimization techniques based on the Levenberg−Marquardt method and the restrictions [$0 \leq X_i \leq 1.0$; $X_1 + X_2 + X_3 + X_{12} + X_{19} = 1.0$] of the system (*18*). The solution gave the following results: $X_1 = 0.0\%$, $X_2 = 0.0\%$, $X_3 = 100.0\%$, $X_{12} = 0.0\%$, and $X_{19} = 0.0\%$. That is, only the enzymatic extract from a pure culture (X_3) appears as the best candidate for agave fructan hydrolysis, yielding solutions with maximum fructose concentration. However, the model also showed that the coefficients of the linear terms for X_2 and X_3 are very similar, indicating that these enzymatic extracts by themselves will achieve similar yields for fructose concentration. Additional verification assays demonstrated the superior performance of the extracts from the culture X_3. The verification assays yielded 11.8 g/L for X_3, which compares favorably against the predicted model value of 11.67 g/L.

Strain Identification. Once the strain X_3 was identified as the culture that maximizes fructose yield, we proceeded with the strain identification. Strain X_3 was identified, with a 99.8% identity score (0% expectancy), as *Kluyveromyces marxianus*. This strain has been previously reported as an inulinase producer and studied to obtain fructose syrups from inulin of Jerusalem artichoke (*12*, *19*).

Optimization Variables for Inulinase Synthesis Maximization. Once the selection of *Kluyveromyces marxianus* as the best strain for inulinase production is completed, the experimental strategy for bioreactor synthesis optimization can be applied. The results, expressed as fructose concentration based on fructo-oligosaccharide hydrolysis (response function), are shown in the last column of **Table 2**. These results, under an analysis of variance, revealed that, for a confidence interval of 0.05, none of the variables exhibited a significant effect within their defined limits and seemingly suggest the presence of curvature and the optimum proximity. Furthermore, the experimental results were used to construct a second-order polynomial model using the least-squares method (*16*), describing the relationship that exists among the variables. For this case, the regression model obtained is the following:

$$Y_2 = 26.67 + 2.09V_1 + 1.14V_2 + 2.62V_3 + 2.99V_4 - 1.43{V_1}^2 + 3.15V_1V_2 - 0.39V_1V_3 - 1.72V_1V_4 - 2.64{V_2}^2 - 0.45V_2V_3 - 0.04V_2V_4 - 1.4{V_3}^2 + 1.19V_3V_4 - 2.22{V_4}^2$$

Here, the variables are specified in their coded units. In this equation, it is implicitly assumed that the dependence of the response variable with respect to the factors is nonlinear and that the effects of these factors are additive. Statistical analysis of the model indicates that these assumptions are adequate. The location of the optimum provides a simple example of locating an extreme for a multidimensional system via the derivation of the second-order model and solving the resulting set of linear equations:

$$\frac{\partial[Y_2]}{\partial V_1} = 2.09 - 2.86V_1 + 3.15V_2 - 0.39V_3 - 1.72V_4 = 0$$

$$\frac{\partial[Y_2]}{\partial V_2} = 1.14 + 3.15V_1 - 5.28V_2 - 0.45V_3 + 0.04V_4 = 0$$

$$\frac{\partial[Y_2]}{\partial V_3} = 2.62 + 0.39V_1 + 0.45V_2 - 2.8V_3 + 1.19V_4 = 0$$

$$\frac{\partial[Y_2]}{\partial V_4} = 2.99 + 1.72V_1 + 0.04V_2 - 1.19V_3 + 4.44V_4 = 0$$

The system solution is the following: $V_1 = 0.86$, $V_2 = 0.66$, $V_3 = 1.06$, and $V_4 = 0.63$. Using the transformation (V_i = optimum factor value [(factor upper boundary − factor lower boundary)/2] + mean factor value), the decoded factor values can be obtained. This procedure indicates that the optimum conditions for inulinase synthesis maximization are 30.6 °C, pH 6.3, 152 rpm, and 1.3 VVM of aeration level. Under these experimental conditions confirmation assays were performed; the results yielded 9.33 mg of total protein (synthesized enzyme)/L (average) and an enzyme concentration equal to 126.3 international units (average), which was defined as the amount of enzyme that will produce 1 μmol of product per minute.

Optimization for Fructo-oligosaccharide Hydrolysis Optimization. The enzymatic extracts obtained at the previously identified optimal conditions were used as the basis to proceed with the fructo-oligosaccharide hydrolysis optimization at bioreactor level. The experimental results, expressed as fructose concentration based on fructo-oligosaccharide hydrolysis (response function), are shown in the last column of **Table 3**. These results, under an analysis of variance (**Table 3**), revealed that, for a confidence interval of 0.05, none of the variables exhibits a significant effect within their defined limits, suggesting once again the presence of curvature and the optimum proximity. As before, these experimental results were used to construct a second-order polynomial model using the least-squares method (*16*), describing the relationship that exists among the variables. For this case, the regression model obtained is the following:

$$Y_3 = 24.76 - 0.51V_5 - 3.71V_6 - 1.95V_7 + 0.30V_5V_6 - 5.08V_5V_7 - 1.23V_6V_7 - 4.43{V_5}^2 - 0.43{V_6}^2 - 6.84{V_7}^2$$

Again, the variables were specified in their coded units, and the equation implicitly assumed that the dependence of the response variable with respect to the factors is nonlinear and that the effects of these factors are additive. Statistical analysis of the model indicates that these assumptions are adequate. The set of linear equations that should be solved this time is

$$\frac{\partial[Y_3]}{\partial V_5} = -0.51 - 8.84V_5 + 0.3V_6 - 5.08V_7 = 0$$

$$\frac{\partial[Y_3]}{\partial V_6} = -3.71 + 0.3V_5 - 0.86V_6 - 1.23V_7 = 0$$

$$\frac{\partial[Y_3]}{\partial V_7} = -1.95 - 5.08V_5 - 1.23V_6 - 13.68V_7 = 0$$

The system solution is the following: $V_5 = -0.15$, $V_6 = -1.68$, and $V_7 = 0.06$. Using the transformation (V_i = optimum factor

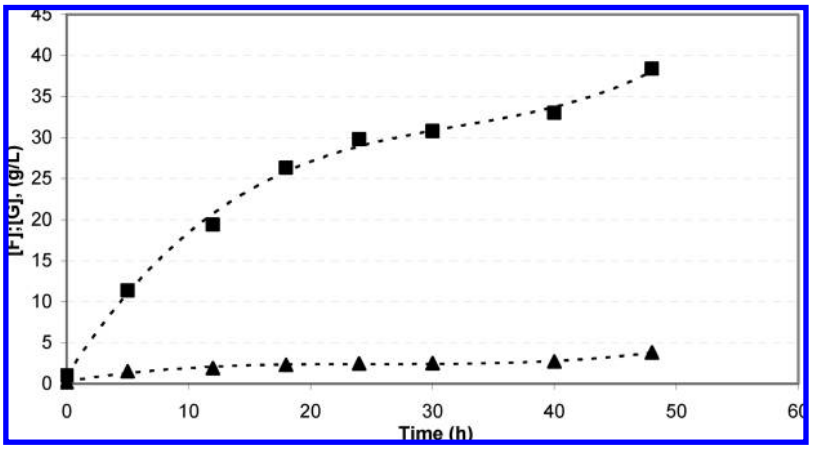

Figure 1. Profile concentration of fructose (■) and glucose (♦) as a function of the processing time during the enzymatic hydrolysis of agave fructo-oligosaccharides.

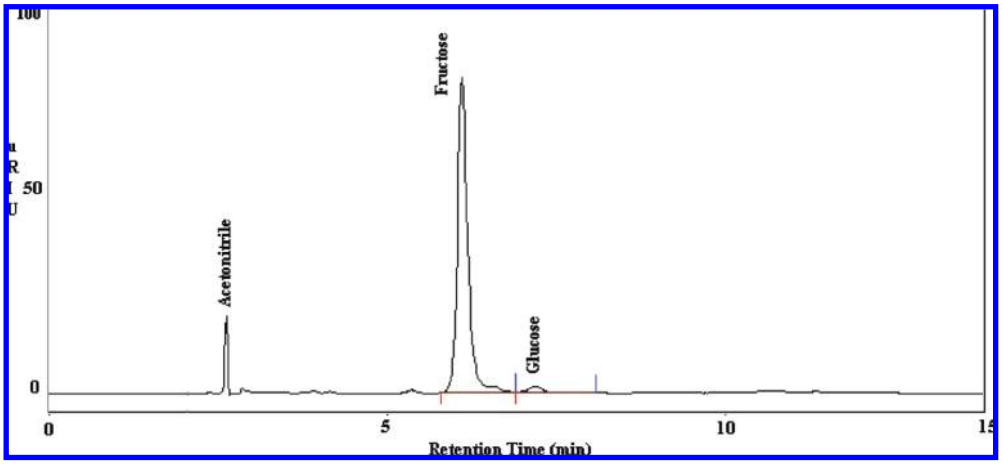

Figure 2. HPLC profile of the enzymatic catalysis product from *K. marxianus* inulinase on agave fructo-oligosaccharides (the first signal is the dissolvent response, acetonitrile).

value [(factor upper boundary − factor lower boundary)/2] + mean factor value), the decoded factor values can be obtained. Thus, the enzymatic treatment for fructo-oligosaccharide hydrolysis should take place at 34 °C, 66 rpm, and pH 5.0. These optimal conditions of pH and temperature are similar to those previously reported (*7*).

Global Process Assay at Optimal Conditions To Obtain Fructose-Rich Syrups. The previous individual steps are the basis for formulating an overall treatment process for processing agave extracts to obtain fructose-rich syrups. A general verification assay revealed (**Figure 1**) that 61% (average) of the total substrate was hydrolyzed, obtaining fructose and glucose as main hydrolysis products during the first 48 h of processing time, and that the higher enzymatic activity is presented during the first 24 h, when 64% (average) of the total conversion was reached. After this time, the product accumulation velocity diminished, possibly due to substrate depletion or perhaps as a result of the enzyme catabolic repression, as it has been shown for other strains (*12*).

HPLC Analysis of the Agave Syrups. The product obtained from the global process was analyzed by HPLC. The chromatogram obtained (**Figure 2**) showed signals that confirmed that the solution contained 95% fructose (average) and 5% glucose (average) and was sucrose free. In addition, the products obtained from this process were free of toxic contaminants such as hydroxymethylfurfural (*10*), evaluated according to the method of Martínez et al. (*20*), which are commonly present in products obtained by either thermal or acid hydrolysis of agave plants. Therefore, the inulinase from *K. marxianus* can be used for production of high-fructose syrups from inulin and inulin-containing materials. At present, fructose can also be produced from starch by enzymatic methods involving α-amylase, amyloglucosidase, and glucose isomerase (*21*). However, the procedure involving microbial inulinase appears to be very attractive: a one-step enzymatic hydrolysis of inulin yields 95% pure fructose. These results confirm that inulinase from *K. marxianus* may have great potential for use in the direct digestion of inulin involved in the food and fermentation industries.

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