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Enhancement of fructosyltransferase and fructooligosaccharides production by *A. oryzae* DIA-MF in Solid-State Fermentation using aguamiel as culture medium

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HIGHLIGHTS

- Aguamiel is a potential source for production of fructosyltransferases enzymes.
- FOS production in aguamiel as substrate is a viable alternative.
- Fructosyltransferase and FOS production by Solid State Fermentation is a viable and economic alternative.

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ABSTRACT

The aim of this work was to improve the production of fructosyltransferase (FTase) by Solid-State Fermentation (SSF) using aguamiel (agave sap) as culture medium and *Aspergillus oryzae* DIA-MF as producer strain. SSF was carried out evaluating the following parameters: inoculum rate, incubation temperature, initial pH and packing density to determine the most significant factors through Box-Hunter and Hunter design. The significant factors were then further optimized using a Box-Behnken design and response surface methodology. The maximum FTase activity (1347 U/L) was obtained at 32 °C, using packing density of 0.7 g/cm^3. Inoculum rate and initial pH had no significant influence on the response. FOS synthesis applying the enzyme produced by *A. oryzae* DIA-MF was also studied using aguamiel as substrate.

1. Introduction

Fructooligosaccharides (FOS) are bioactive compounds with important beneficial effects, which promote a good absorption of mineral in the body, regulate blood glucose and cholesterol levels and the most important activity is the induction of the growth of probiotic microorganisms in the intestinal tract contributing to the prevention of colonic cancer (Lim et al., 2005; Rodrigues et al., 2011; Lateef and Gueguimkana, 2012; Ganaie et al., 2013; Dominguez et al., 2014). FOS are composed by one sucrose molecule linked to 1–3 units of fructose through links β-2-1 between molecules (Ganaie et al., 2014) and are commonly called 1-kestose, 1-nistose and 1-β-fructofuranosylnistose (Monsan and Ouarné, 2009; Yildiz, 2011; Barthi et al., 2013; Chen et al., 2014; Panesar et al., 2014).

Nowadays, FOS are produced commercially through microbial enzymes called β-fructofuranosidases (FFases, EC.3.2.1.26) and fructosyltransferases (FTases EC.2.4.1.9) (Hidaka et al., 1988; Pons et al., 2000; Mussatto and Teixeira, 2010; Batista et al., 2013) that have been found in fungi such as *Penicillum*, *Aerobasidium*, *Fusarium* and principally *Aspergillus* species (Antošová and Polakovič, 2001; Hernalsteens and Maugeri, 2008; Maiorano et al., 2008; Driouch et al., 2010). FFases enzymes catalyze hydrolytic and transfructosylating reactions, but their ability for transfer is only with higher sucrose amounts. On the other hand, FTases possess only transfructosylating activity, acting on the β-1-2 link of sucrose and transfer a fructose molecule to an acceptor such as other sucrose molecule leading to generation of FOS and release glucose in the reaction (Antošová and Polakovič, 2001; Ganaie et al., 2013; Šedová et al., 2014). Recent studies have been focused

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on the use of natural materials or by-products that representing an economic alternative for the FOS production, due to the increased demand by the consumption of these bioactive ingredients in the food and pharmaceutical products.

Studies on the influence of various parameters on FTase have been evaluated by different authors (Yun, 1996). However, until today, few studies are focused on Solid-State Fermentation (SSF) for the production of FTase enzyme and FOS (Mussatto et al., 2013; Sangeetha et al., 2004).

Previous studies have demonstrated that SSF is an excellent alternative to improve FOS and FTase production; however, is necessary to establish the optimal conditions to maximize the FOS and FTase production. On the other hand, the use of aguamiel as culture medium allows reducing the production costs compared with a culture medium enriched with commercial sucrose (Muñiz et al., 2015).

The aim of this experimental section was to determine the best conditions of FTase production by SSF and to improve the FOS production by the FTase produced using aguamiel (a by-product obtained the maguey and is used in Mexico for the pulque production) as substrate.

2. Materials and methods

2.1. Reagents and standards

FOS standards 1-kestose (GF_2), 1-nystose (GF_3) and 1^F-fructofuranosylnystose (GF_4) were purchased in Wako Pure Chemical Industries, Ltd. (Japan Company). Sugars fructose (F), glucose (G) and sucrose (S) were obtained from Sigma Aldrich (St. Louis, MO, USA).

2.2. Microorganism and culture medium

Aspergillus oryzae DIA-MF was selected in the previous step and was obtained from culture collection of molds of Food Research Department of University Autonomous of Coahuila (Mexico). Firstly, the strain was activated on potato dextrose agar (PDA) at 30 ± 1 °C for 5 days. For the production of spores, the fungus was cultivated in flasks containing PDA medium at 28 ± 1 °C for 5 days. A sterilized solution of 0.01% (w/v) Tween 80 was used for harvesting the spores. The amount spores in the suspension was determined by employing a Neubauer chamber. The volume needed to give a number of spores of 2×10^7 spores/g support was calculated and the suspension was prepared under sterile conditions.

2.3. Aguamiel characterization

Aguamiel is a fluid rich in sugars obtained from maguey. In this study, the aguamiel obtaining from *Agave salmiana* (green maguey) was collected in February 2013 in the Mangas locality, which is close to Saltillo Coahuila, Mexico. Aguamiel was first collected and filtered with strainer, then was stored in cold temperature and transported. Afterwards it was filtered through filter paper fine pore and stored at −18 °C. Before the physicochemical characterization, the stored aguamiel was sterilized at 15 lb/121 °C during 15 min. Thermal treatment was employed as method of preservation due to that aguamiel was used as culture medium (Muñiz et al., 2015).

2.3.1. Physicochemical analysis

Analysis of aguamiel consisted in the determination of total sugars (TS) by method Anthrone (Dreywood, 1946; Pearson et al., 1984 and Campana et al., 2006), reducing sugars (RS) by the method previously reported by Miller (1959) using 3-amino-5-dinitrosalicylic acid reagent with some modifications (Gonçalves et al., 2010), protein by Bradford's method (Bradford, 1976), lipids, ashes (i.e. mineral content), total solids, moisture and pH using the AOAC methods (AOAC, 1990) and degrees brix and density with a densitometer (Anton Paar, DMA 35N, Austria). All analysis were carried out in triplicate.

2.3.2. Determination of sugars by HPLC analysis

High performance liquid chromatography (HPLC) analysis of aguamiel consisted in identification and quantification of fructose, glucose, sucrose using a Perkin Elmer Series 200 HPLC according with the methodology described by Mussatto et al., 2009.

2.4. Fermentation conditions

Firstly a study of fermentation was performed in order to determine the maximum fructosyltransferase and hydrolase activities. For the assays, aguamiel of maguey (*A. salmiana*) previously sterilized was used as culture medium in the fermentation (Muñiz et al., 2015). Fermentation was performed in column reactors and polyurethane foam was used as inert support. Aguamiel (initial pH 4.5) was inoculated with 2×10^7 spores/g of support. The inoculated reactors were incubated at 30 °C with 70% initial moisture. Fermentation process was carried out for 48 h and samples were withdrawn at regular time intervals (0, 18, 24, 30, 42 and 48 h of fermentation). At the end of fermentation, crude enzymatic extract was obtained by mechanical compression of the fermented material. All experiments were carried out in duplicate. Crude extracts were filtered and analyzed by HPLC as well as for the enzymatic activities determination.

2.5. Fructosyltransferase and hydrolase activities

For the fructosyltransferase and hydrolase activities determination, 100 μL of crude extract were mixed with 900 μL of sucrose solution (4% in acetate buffer, pH 5.0, 50 mM). The reaction mixture was incubated at 30 °C for 20 min. The reaction mixture was stopped in boiling for 3 min. Control was prepared with sucrose and buffer without enzyme. One enzymatic unit of fructosyltransferase (U) was defined as the amount of the enzyme necessary to form 1 μmol of kestose per min under certain conditions. On the other hand, one enzymatic unit of hydrolase (U) was defined as the amount of the enzyme necessary to liberate 1 μmol of glucose per min under certain conditions.

2.6. FOS and sugars determination

Crude extracts were filtered through a 0.45 μm nylon membrane (Millipore) and were used for determination of FOS (1-kestose, 1-nystose and 1^F-fructofuranosylnystose) and residual sugars (fructose, glucose and sucrose). Samples were analyzed by high performance liquid chromatography (HPLC, Perkin Elmer Series 200) using a Prevail Carbohydrate ES Column (5 μm, 250 × 4.6 mm, Grace) at 30 °C. A mixture of acetonitrile/water 70:30 (v/v) was used as mobile phase at a flow rate of 1 mL/min with a pressure of 1700 psi. A refractive index detector (RID) was operated at 35 °C. The response of the RID was recorded and integrated using the TOTALCHROM WS V6.3 software. The quantification of sugars and FOS in samples was determined by using standards curves made with known concentrations of each compound (Mussatto et al., 2009).

2.7. Studies on the influence of various parameters

The influence of 4 variables (inoculum, temperature, initial pH and packing density) affecting the fructosyltransferase production

was evaluated using a Box-Hunter and Hunter experimental design. Then, the variables that had a significant effect on the enzymatic activity (FTase) were evaluated in a Box-Behnken design. The high and low values of the variables in the two experiments are shown in Table 1.

The Box-Behnken design was used to evaluate the significant factors on the response (FTase production). The variables studied included the inoculum (2×10^7, 3×10^7 and 4×10^7 spores/g support), temperature (28, 30 and 32 °C) and packing density (0.3, 0.5 and 0.7 g/cm^3). Table 2 shows the levels used to each factor (coded values). The highest level (+1) and lowest level (−1) besides a central level (0) was used in this design.

2.8. Statistical analysis

Statistica 7.0 software was used to identify the variables with statistically significant effect on the response. The regression coefficients for linear, quadratic and interactions for each variable were determined and adjusted to a polynomial second order equation (Eq. (1))

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_1 X_1^2 + \beta_2 X_2^2 + \beta_1 X_1 X_2 + \beta_2 X_1 X_2 \quad (1)$$

where Y = fructosyltransferase activity (FTase in U/L of crude extract), X_1 and X_2 are the significant variables (temperature and packing density, respectively) and β_i is the regression coefficient for each factor.

A design randomized complete block was also used in the experiments corresponding to application of FTase on aguamiel for the FOS synthesis. Here, SAS software version 9.0 was employed for such analysis.

2.9. FOS synthesis using FTase enzyme

2.9.1. Fermentation study under the best process conditions

In this section, a fermentation study under the best conditions of FTase production after optimization was performed. SSF assays were performed using aguamiel as culture medium (Initial pH 4.5) and polyurethane foam (PUF) as inert support (Mexican polyurethanes, Ramos Arizpe, Coahuila, Mexico). Column bioreactors were used and the following process conditions were applied: 70% moisture, inoculum of 2×10^7 spores/g of support, packing density 0.7 g/cm^3, 32 °C. Samples were withdrawn each 4 h for 32 h. All experiments were done in triplicate.

For FTase activity assay, 100 μL crude enzymatic extract were mixed with 900 μL sucrose (4% in acetate buffer, pH 5.0, 50 mM) and incubated at 30 °C for 20 min in a water bath. The reaction was stopped by boiling during 3 min. As control was used a sucrose solution in buffer without source enzyme. Quantitative analysis of the product formed (kestose) was done by HPLC under the above described conditions. Calibration curve was established using commercial HPLC grade standard. Total FOS concentration was also determined.

Enzyme production was carried out under the best fermentation conditions (described above) during 28 h. Aluminum trays were used for fermentation in order to obtain the greatest possible amount of crude extract for application on aguamiel for FOS synthesis enzymatic.

Table 1
Coded and real levels of factors evaluated in the Box-Hunter and Hunter design.

Factor	Lowest level (−)	Highest level (+)	Units
Inoculum	2×10^5	2×10^7	spores/g support
Temperature	25	30	°C
Initial pH	4.5	5.5	pH units
Packing density	0.2	0.5	g/cm^3

Table 2
Experimental matrix obtained in Box-Behnken design.

Trial	Inoculum	Temperature	Packing density	FTase activity (U/L)
1	−1	−1	−1	0.00
2	−1	0	1	834.08
3	−1	1	0	1088.90
4	0	−1	1	359.00
5	0	0	0	634.50
6	0	1	−1	646.44
7	1	−1	0	0.00
8	1	0	−1	157.58
9	1	1	1	1347.58

After obtained the extract was concentrated by lyophilization (batch 10 mL), subsequently dissolved in 1.5 mL acetate buffer pH 5.0, 50 mM, filtered through Microcon Centrifugal filters 30 kDa and centrifuged at 14,000*g* for 12 min at 4 °C. Three fractions were tested to determine FTase activity: (1) lyophilized extract; (2) upper extract, and (3) bottom extract.

2.9.2. Application of enzyme (FTase) on aguamiel for the synthesis FOS

FOS synthesis was carried out using aguamiel as substrate (sucrose content 37 g/L) and different conditions of incubation. Lyophilized extract resuspended in acetate buffer pH 5.0, 50 mM was used in this stage.

The effect of incubation temperature on FOS synthesis by FTase was studied by adjusting the initial pH to 5.0. Three incubation temperatures were tested: 30, 40 and 50 °C. First, 100 μL of concentrated extract were mixed with 900 μL aguamiel and incubated in a water bath for 1 h. Finally, the reaction was stopped by heating in a water bath for 3 min. As control it was used the enzyme in aguamiel at time 0 (reaction stopped immediately). The concentration of kestose formed was determined by HPLC as described above.

The major incubation temperature was selected to monitor the effect of initial pH. FOS production in aguamiel was studied using three different pHs: 5.0, 5.5 and 6.0. The reaction was done as described above. A temperature of 30 °C was established in the first stage of application.

2.9.3. Kinetic study of the FOS enzymatic synthesis

Finally, a kinetic study was studied for the FOS enzymatic synthesis with the parameters of temperature and pH previously established (30 °C with initial pH 5.0). The FOS synthesis was measured at a regular interval of 20 min up to 180 min of incubation. All experiments were made in triplicate.

3. Results and discussion

3.1. First kinetic study

Table 3 shows the enzymatic activities by *A. oryzae* DIA-MF in SSF. The maximum fructosyltransferase activity was at 42 h; however, hydrolytic activity was also present during fermentation,

Table 3
Hydrolase and fructosyltransferase activities by *Aspergillus oryzae* DIA-MF.

Enzymatic activities (U/L)		
Fermentation time (h)	Hydrolase (Uh)	Fructosyltransferase (Ut)
0	0	0
18	3424 ± 266	2496 ± 309
24	3049 ± 134	3085 ± 1314
30	4914 ± 602	2402 ± 267
42	5564 ± 1524	3625 ± 777
48	6764 ± 1830	3585 ± 173

increasing significantly between 24 and 48 h. Transfructosylating activity at 24 h was 3085 U/L, similar to the hydrolytic activity (3049 U/L). Therefore, 24 h of fermentation was selected because hydrolytic activity after this time was major than fructosyltransferase activity.

Table 4 shows the FOS production during fermentation. Maximum yield, 22.6 g/L, was obtained at 24 h of fermentation with a productivity of 0.94 g/L h. FOS production using aguamiel of maguey as culture medium is a good alternative because aguamiel is a economic by-product, besides, Solid State Fermentation is a bioprocess inexpensive and easy to operate. Some works have been reported for the FOS production using sucrose as carbon source (Mussatto and Teixeira, 2010).

3.2. Screening of significant variables by Box-Hunter and Hunter design

The first kinetic study showed that 24 h of fermentation was the optimal time for the expression of fructosyltransferase and hydrolase enzymes. After, a Box-Hunter and Hunter design was applied to study factors that influence the response (FTase activity). Inoculum, packing density and temperature had a significant effect on the fructosyltransferase activity. On the other hand, initial pH had no effect on the enzymatic activity (FTase) indicating that pH of 4.5 is suitable for transfructosylation activity. Yun (1996) mentioned that values of pH between 4.5 and 5.5 are optimal for this enzyme. Since the three factors, named inoculum, packing density and temperature, were found to have a significant effect on the response, their optimal levels were established through a response surface methodology (RSM).

3.3. Optimization studies of significant variables using a Box-Behnken design

To evaluate the quadratic effects and interaction between the factors, a Box-Behnken design was used. An analysis of variance (ANOVA) (Table 5) revealed that incubation temperature (X_2) and packing density (X_3) had a linear effect statistically significant at a 95% confidence level on the FTase production. A mathematical model was then obtained (Eq. (2)):

$$Y = (634.504) + (453.986)(X_2) + (310.266)(X_3) \quad (2)$$

The maximal theoretical value was reached with the highest temperature (32 °C) and the highest packing density (0.7 g/cm^3). Coefficient of determination (R^2 = 0.91) was obtained. Lower values of these two factors significantly affected the response.

Fig. 1 shows the response surface plots obtained by the Statistical program. In Fig. 1A is observed the effect of the temperature and packing density on the FTase production.

Enzymatic activity was not affected with inoculum rates used in this study. However, Fig. 1B and C, reveal that there is a trend of increased the enzymatic activity when increased packing density with inoculum rate (B) and incubation temperature with inoculum (C) but, in general, the interactions between factors tested did not show a significant effect in the response ($p < 0.05$). In a recent work, Mussatto et al. (2012) studied the FTase and FOS production with the optimization of some factors, obtaining maximum values of FTase production at 30 °C, 2 × 10^6 spores/g dry material and 70% moisture content. However, they used *Aspergillus japonicus* as producer fungus. Sangeetha et al. (2004) evaluated the FTase production in SSF by *A. oryzae* CFR 202 with various agro-industrial by-products enriched with 60% of sucrose. Based on their results, the rice bran was major substrate for the FTase production reaching about 22 U/mL. In the present study were obtained 1347 U/L at 24 h of fermentation with initial concentration of sucrose of 35 g/L while Sangeetha et al. (2004) used 600 g/L of sucrose as substrate. Finally, 32 °C incubation temperature, 0.7 g/cm^3 packing density and 2 × 10^7 spores/g of support were selected for futures studies.

Table 4
FOS production by *Aspergillus oryzae* DIA-MF by Solid-State Fermentation.

Fermentation time (h)	Total FOS (g/L)	Productivity (g/L h)
0	1.50 ± 0.09	0
18	15.92 ± 0.83	0.88
24	22.63 ± 0.12	0.94
30	4.11 ± 0.19	0.13
42	2.16 ± 0.15	0.05
48	0	0

3.4. Enzymatic synthesis of FOS

3.4.1. Kinetic study under the best conditions in optimization studies

After selected the best process conditions, other kinetic study was done but sampling each 4 h under same conditions of fermentation above mentioned. Fig. 2a, shows the production of FTase during 48 h of fermentation by *A. oryzae* DIA-MF. Here, maximum enzymatic activity was reached at 28 h with 1431 U/L. Similar results were found in the optimization studies at 24 h of fermentation (1347 U/L). Therefore, the present study is reproducible under conditions tested. For obtaining enzymatic extract in tray, we selected 28 h of fermentation. In this section, also, protein in the extract was determined (Fig. 2b). Maximum protein content was also achieved at 28 h followed by an appreciable decrease at 32 h. Probably exist a correlation between the two responses. However, more detailed experiments are needed to confirm these assumptions.

An analysis of FOS produced during the fermentation was also carried out. Fig. 2c shows measurement profiles of FOS. An increase of FOS was observed between 12 and 16 h of incubation (15.5 g/L) with a productivity of 1.29 g/L h. Several studies have focused in the FOS production obtaining yields of 50–60% from initial sucrose, but they particularly using high levels of sucrose between 15 and 60%, while in this work only is used the sucrose present in the aguamiel (35 g/L) that corresponds to 3.5% (Sangeetha et al., 2004, 2005; Mussatto et al., 2009).

Crude extract in tray was obtained and was prosecuted in three steps. Results of the enzymatic activity (FTase) obtained at different steps, clearly were major activity in the lyophilized extract. Therefore, the lyophilized extracts were selected for the application of enzyme on aguamiel for the FOS production due to a major concentration of protein with FTase activity.

3.4.2. Application of enzyme (FTase) on aguamiel for the synthesis of FOS

In this part of the study, the FOS enzymatic synthesis was carried out for 1 h using aguamiel as substrate and lyophilized enzymatic extract. In Table 6 the effect of incubation temperature in the FOS synthesis is shown. As observed the FOS concentration was similar between temperatures tested with a FOS yield of 9 g/L in 1 h of reaction. Then, 30 °C was using for the following experiments because no significant difference between treatments. Yun (1996) mentioned that FTase activity is major when incubation temperatures between 50 and 60 °C are used.

The effect of initial pH in the FOS enzymatic synthesis was monitored at 5.0, 5.5 and 6.0 (Table 6), the results showed no significant effect in the FOS synthesis under conditions tested. This agrees with the reported by Dhake and Patil (2007) who found an optimum activity fructosyltransferase of *Penicillum purpurogenum* at pH 5.5. However they mentioned that intracellular and extracellular FTase show stability in the pH range of 4.5–7.0.

Table 5
Analysis of variance (ANOVA) for Box-Behnken design.

	Sum of squares	Degrees of freedom	Mean squares	*F*-value	*p*-value	
Inoculum (1L)	58,190	1	58,190	1.56385	0.242636	NS
Inoculum (1Q)	2442	1	2442	0.06563	0.803562	NS
Temperature (2L)	2,473,243	1	2,473,243	66.46791	0.000019	*
Temperature (2Q)	3993	1	3993	0.10732	0.750708	NS
Packing density (3L)	577,590	1	577,590	15.52262	0.003407	*
Packing density (3Q)	53,387	1	53,387	1.43476	0.261581	NS
1L × 2L	85,687	1	85,687	2.30282	0.163451	NS
1L × 2Q	5205	1	5205	0.13987	0.717066	NS
Error	334,886	9	37,210			
Total SS	3,970,113	17				

NS = statistically not significant at 95% confidence interval.
* Statistically significant at 95% confidence interval.

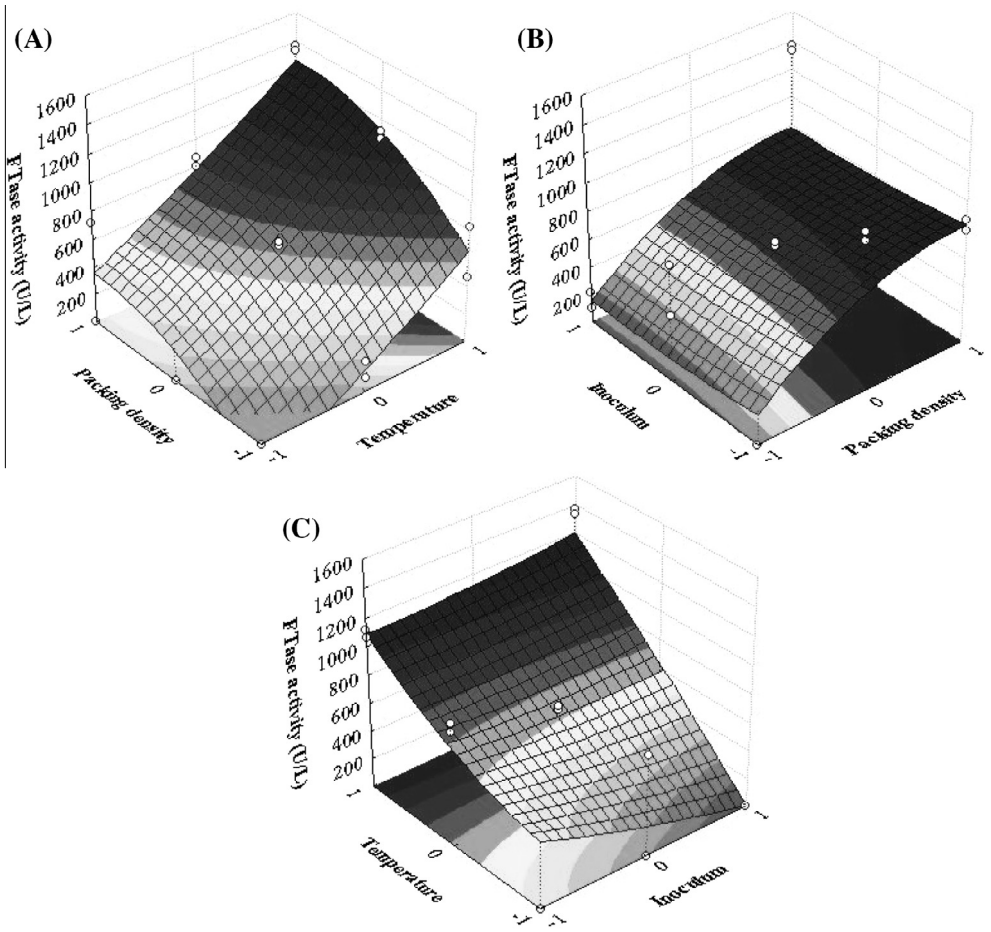

Fig. 1. Response surface plot of FTase production as function of packing density and temperature (A), inoculum and packing density (B) and temperature and inoculum (C).

3.4.3. Kinetic study of the FOS enzymatic synthesis

A kinetic study was done with an incubation temperature of 30 °C to initial pH of 5.0. Fig. 3 shows that the maximum synthesis of FOS occurred in a period of 60–120 min with yield between 8 and 11 g/L of kestose. Sucrose is considered the best carbon source for FTase enzymes (Dhake and Patil, 2007). In this work, the sucrose present in aguamiel was used after a sterilization process (approximately 35–40 g/L). According to the Fig. 3 the biotransformation of sucrose and FOS synthesis were observed in 20 min of reaction.

On the other hand, the maximum FOS synthesis occurred during 80 at 120 min. Only 43.8% of the initial sucrose was transformed in FOS with yield of product/total substrate equal to 0.30 g/g and productivity of 0.097 g/L min. Sangeetha et al. (2004) studied the FOS production by FTase from *A. oryzae* CFR 202 and obtained maximum FOS of 52% after 8 h of reaction, but high levels of sucrose were used as substrate (600 g/L).

4. Conclusions

The use of experimental designs allowed to identify the variables that significantly affected or favored the FTase production. The highest enzymatic activity (FTase) was obtained using an incubation temperature of 32 °C with a packing density of 0.7 g/cm^3. On the other hand, the inoculum rate and initial pH did not influence in this response. Also, the FOS production was possible using

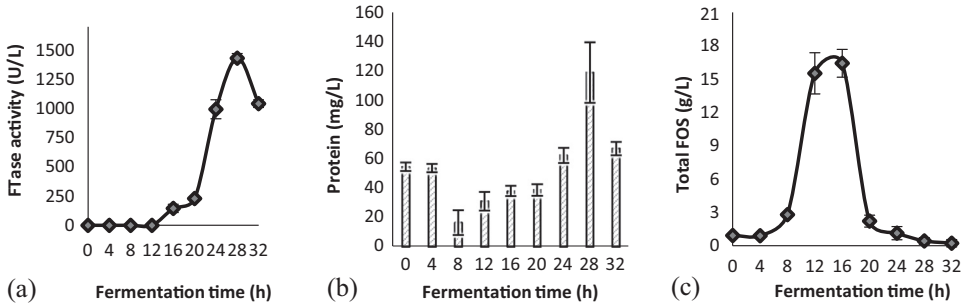

Fig. 2. (a) Kinetic study of fructosyltransferase by *Aspergillus oryzae* DIA-MF under major conditions obtained in the optimization studies. (b) Protein content during fermentation. (c) Kinetic study of FOS by *Aspergillus oryzae* DIA-MF under major conditions obtained in the optimization studies.

Table 6
Effect of incubation temperature and initial pH in the FOS production by FTase.

Factor	Temperature effect (°C)			pH effect		
	30	40	50	5	5.5	6
Yield FOS (g/L)	7.89 ± 1.10[a]	9.02 ± 0.60[a]	9.06 ± 0.49[a]	7.89 ± 1.10[a]	7.96 ± 0.62[a]	7.57 ± 0.35[a]

Significance letter. Value statistically not significant at 95% confidence interval.
Note: tested temperatures at pH 5.5. Setting the temperature (30 °C), initial pH was tested.

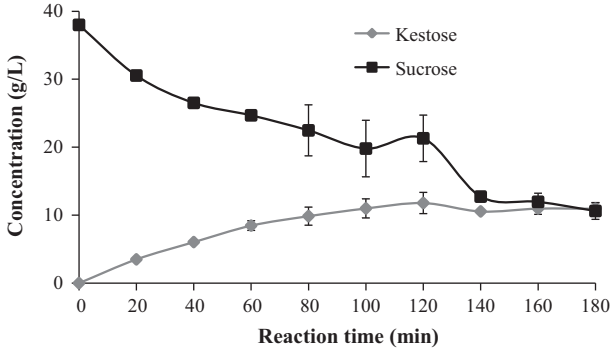

Fig. 3. FOS production during 180 min of reaction.

aguamiel as substrate and concentrated enzyme in the reaction. Here, incubation temperature and initial pH did not influence the enzymatic activity. This work, is a first study on FOS production from aguamiel as culture medium. The results obtained indicate that the aguamiel represents economical alternative for the FOS and enzyme FTase industrial production.

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