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Production, characterization and application of inulinase from fungal endophyte CCMB 328

Production, characterization and application of inulinase from *fungal endophyte* CCMB 328

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ABSTRACT

Inulinase (β-2,1-D- fructan fructanohydrolase), EC 3.2.1.7, targets the β-2,1 linkage of inulin, a polyfructan consisting of linear β-2,1 linked fructose, and hydrolyzes it into fructose. This use provides an alternative to produce fructose syrup through the hydrolysis of inulin. The objective of this work was to study the production, characterization and applications of inulinases from the *fungal endophyte* CCMB 328 isolated from the Brazilian semi-arid region. Response Surface Methodology (RSM) was employed to evaluate the effect of variables (concentration of glucose and yeast extract), on secreted inulinase activities detected in the culture medium and also in the inulin hydrolysis. The results showed that the best conditions for inulinase production by CCMB 328 are 9.89 g / L for glucose and 1.09 g / L for yeast extract. The concentration of 0.20 mol/L of NaCl and KCl increased the activity of inulinase from CCMB 328 by approximately 63% and 37%, respectively. The results also showed that the inulinase has potential for inulin hydrolysis, whose conversion yields roughly 72.48 % for an initial concentration of inulin at 1% (w/v).

Key words: Inulin, inulinase, yeasts, semi-arid, fungal endophyte.

INTRODUCTION

Inulinase (β-2, 1-D- fructan fructanohydrolase), EC 3.2.1.7, targets the β-2,1 linkage of inulin, a polyfructan consisting of linear β-2,1 linked fructose, and hydrolyzes it into fructose (Gong et al. 2008).

Inulinase can be derived from many micro-organisms. In the last three decades, significant efforts have been made to find the best microbial source for the extraction of inulinase (Ricca et al. 2007, Vijayaraghavan et al. 2009).

Conventional fructose production from starch needs at least three enzymatic steps, including α-amylase, amyloglucosidades, and glucose isomerase action, yielding only 45% of fructose solutions. An alternative to this process is the hydrolysis of inulin by inulinases. The enzymatic

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formation of fructose from inulin has a single enzymatic step and yields up to 95% of fructose (Figueiredo-Ribeiro et al. 2007). The use of microbial inulinases (EC 3.2.1.7), which yield up to 95% of pure fructose in a single-step enzymatic reaction, is therefore a viable alternative for the production of high-fructose syrup (Gill et al. 2006).

Inulinases have also been used for the production of inulooligosaccharides–low caloric saccharides by acting as a growth factor for beneficial microorganisms in the intestinal flora. They are considered a prebiotic agent (Skowronek and Fiedurek 2006).

The Brazilian semi-arid region represents a large area for bioprospection since it fosters naturally occurring microorganisms adapted to a tropical semi-arid environment, with high temperature and low humidity throughout the year that may possess some features of great industrial interest (Uetanabaro and Góes-Neto 2006).

In this study, we analyzed the production, characterization and application of extracellular inulinases obtained from *fungal endophyte* (CCMB 328) isolated in the Brazilian semi-arid region.

MATERIALS AND METHODS

CHEMICALS

Inulin, bovine serum albumin, and 3,5-dinitro-salicylic acid were purchased from Sigma Chemical Co. (St Louis, MO, USA). All the other chemicals used were also of high-quality analytical grade.

MICROORGANISMS

The yeast strains *fungal endophyte* (CCMB 328) is from Culture Collection of Microorganisms of Bahia (CCMB) of Universidade Estadual de Feira de Santana, Brazil (Uetanabaro and Góes-Neto 2006), and was identified in our previous work (Lima et al. 2009).

The yeast strains were maintained in YM agar (3% yeast extract (w/v), 3% malt extract (w/v), 5% peptone (w/v), 10% glucose (w/v), and 20% agar (w/v), pH 6.2).

INULINASE PRODUCTION

The yeast was previously grown on YM agar at 28°C for 48 h, as described in Oliveira (2007), and diluted in sterile distilled water to a concentration of about 108 colony-forming units/ml. 10% (v/v) of the diluted growth medium was inoculated in flasks containing (per 1 L) mineral medium ($CaCl_2$, 0.25 g; $(NH_4)_2SO_4$, 3 g; KH_2PO_4, 4.5; $MgSO_4$, 0.25g) and supplemented with yeast extract, 1 g; glucose, 10 g; pH 5.0, for fermentation (Patching and Rose 1969). After the incubation at 28°C for 48 h in an orbital shaker at 150 rpm, the cells were separated by centrifugation at 10,000 g for 10 min at 48°C, and the supernatant liquid media was used as the extracellular fraction.

BIOMASS QUANTIFICATION

The cells, which were previously separated by centrifugation, were used to determine the dry weight at 80°C.

ENZYME ASSAYS

The inulinase activity was measured spectro-photometrically using the dinitrosalicylic reagent, as reported by Miller (1959). The reaction mixture consisted of 900 µL of 2 % (w/v) inulin in 0.05 M acetate buffer, pH 5.5, and 100 µL of culture supernatant. The mixture was incubated for 15 min at 50°C. After incubation, 1 mL dinitrosalicylic reagent was added, and the mixture was boiled at 100°C for 10 min and cooled with 10 mL of distilled water. One unit of enzyme activity of inulinase was defined as the amount of the enzyme that catalyzed the formation of 1 µmol fructose/min.

PROTEIN DETERMINATION

Total protein determination was performed according to Bradford (1976), using bovine serum albumin as the standard.

DOEHLERT EXPERIMENTAL DESIGN FOR
ENZYME PRODUCTION

The Doehlert experimental design, with two variables (concentration of glucose and yeast extract) and three replicates at the center of the domain leading to a total of 9 experiments (Table I), was used to assess the effect of glucose and yeast extract concentration on the production of the enzyme.

Thus, the concentration of glucose was studied in 5 levels (7.5 to 12.5 g/L), and the concentration of yeast extract was studied in three levels (0.5 to 1.5 g/L). The experimental errors were evaluated from the replication of the central point. The experimental data were processed by using the STATISTICA software. All the experiments in this step were carried out in a random order.

To estimate the lack of fit of the model to the experimental data, an analysis of variance (ANOVA) was performed, using Design Statistics, version 7.0.

RESPONSE SURFACE OPTIMIZATION OF
TEMPERATURE AND PH

Response surface modeling was applied to cultures of yeast to determine the optimum temperature and pH for the inulinases obtained. This statistical technique for experimental design has advantages over methods that investigate only one variable at a time (Hanrahan and Lu 2006).

The experimental design, with two variables (temperature and pH) and three replicates at the center of the domain leading to a total of 9 experiments (Table II) was used to get the effect of temperature and pH of the inulinase activity.

The pH was studied in 5 levels (5 to 9), and temperature was studied in three levels (30 to 70°C). The experimental errors were evaluated from the replication of the center point. The experimental data were processed by using the STATISTICA

TABLE I
Results of the Doehlert design for inulin production from CCMB 328

Experiment	Concentration of glucose (g/L)	Concentration of yeast extract (g/L)	Experimental values (UA)	Activities predicted (UA)
1	7.5 (**-0.5**)	1.5 (**+0.866**)	2.473600	2.485039
2	12.5 (**+0.5**)	1.5 (**+0.866**)	2.426600	2.415161
3	5 (**-1**)	1 (**0**)	1.153200	1.141417
4C	10 (**0**)	1 (**0**)	4.006000	4.004029
4C	10 (**0**)	1 (**0**)	4.014800	4.004029
4C	10 (**0**)	1 (**0**)	3.990600	4.004029
5	15 (**+1**)	1 (**0**)	0.847500	0.858596
6	7.5 (**-0.5**)	0.5 (**-0.866**)	1.601300	1.613470
7	12.5 (**-1**)	0.5 (**-0.866**)	1.594900	1.582730

TABLE II
Results of the Doehlert design for temperature and pH on inulinase from CCMB 328

Experiment	pH	Temperature (°C)	Experimental values (UA)	Activities predicted (UA)
1	6 (-0.5)	70 (+0.866)	2.833800	2.843550
2	8 (+0.5)	70 (+0.866)	2.934800	2.925050
3	5 (-1)	50 (0)	2.608000	2.598250
4C	7 (0)	50 (0)	3.210200	3.209333
4C	7 (0)	50 (0)	3.244500	3.209333
4C	7 (0)	50 (0)	3.173300	3.209333
5	9 (+1)	50 (0)	2.717000	2.726750
6	6 (-0.5)	30 (-0.866)	2.906400	2.916150
7	8 (+0.5)	30 (-0.866)	2.972900	2.916150

software. All the experiments in this step were carried out in a random order.

To estimate the lack of fit of the model to the experimental data, an analysis of variance (ANOVA) was performed using Design Statistica, version 7.0.

EFFECT OF CATIONS

The effect of Na^+ and K^+ on the inulinase activity was studied. The concentrations used were: NaCl (0.05, 0.1, 0.15, 0.2, 0.25 and 0.3 mol/L) and KCl (0.05, 0.1, 0.15, 0.2, 0.25 and 0.3 mol/L). The inulinase activity was determined by the standard assay as described previously.

DETERMINATION OF KINETICS PARAMETERS

To obtain the parameters of the Michaelis-Menten kinetics of inulinase for the hydrolysis of inulin, K_m and V_{max}, 0.9 µL inulin (1, 1.5, 2.0, 2.5 and 3.0% w/v) was added to 100 µL of inulinase in 0.05 M acetate buffer (pH 5.5) and incubated at 50 °C for 15 mim. The K_m and V_{max} for inulin were determined by the method of Lineweaver-Burk plots.

HYDROLYSIS OF INULIN

The extent of inulin hydrolysis (%) was calculated as $\frac{amount\ of\ fructose\ released}{amount\ of\ initial\ total\ sugars} \times 100$ (Nakamura et al. 1995). Total sugars were determined by the anthrone method (Yemm and Willis 1954), and reducing sugars were determined by the dinitrosalicylic acid method (Miller 1959). The Doehlert experimental design, with two variables (concentration of enzyme and fermentation time) and three replicates at the center of the domain leading to a total of 9 experiments (Table IV), was used to get the effect of enzyme concentration and fermentation time on the extent of inulin hydrolysis from a solution of 1% (w / v) in citrate buffer 0.05 M.

THERMOSTABILITY OF INULINASE

Samples of inulinases in test tubes (selected to be equal in weight, volume and size) were incubated in buffer at different temperatures (50°C, 60°C, 70°C, 80°C and 90°C) and for various times (0, 10, 20, 30, 40, 50 and 60 min). After the heating

TABLE III

Analysis of variance (ANOVA) for the quadratic model fitted to data from the Table I.

Variation source	SS	df	MS	F	Tabulated F (IC 95%)	R^2
Regression	12.956	5	2.5911402	712.55023	9.01	
Residual	0.011	3	0.0036364			
Lack of Fit	0.01061	1	0.01061	70.71	18.51	0.98
Pure Error	0.00030	2	0.00015			
Total SQ	12.94509	8				

process, the tubes were cooled in melting ice and the residual activity measurement was carried out at pH 6.0 and at a temperature of 50°C.

RESULTS AND DISCUSSION

Production of Inulinases by Yeasts

Table I shows the Doehlert design applied to optimize the production of inulinase by *fungal endophyte* CCMB 328. The first column describes the number of planning, with C representing the central compound. In the second and third columns we have the values of glucose concentration and the concentration of yeast extract, respectively. The fourth column represents the enzymatic activity. The last column represents the predicted values.

The response surface graphics (Figures 1 and 2) shows the influence of glucose and yeast extract concentrations in the production of inulinase of CCMB 328. From the analysis of the area chart in Figures 1 and 2 it can be concluded that the production of inulinase by CCMB 328 strains clearly peaks for glucose concentrations in excess of 10 g/L glucose for the whole range of concentrations of yeast extract tested.

Equation 1 illustrates the relation of these two variables and the enzyme activity (UA), where G is the glucose concentration g/L and YE is the yeast extract concentration g/L:
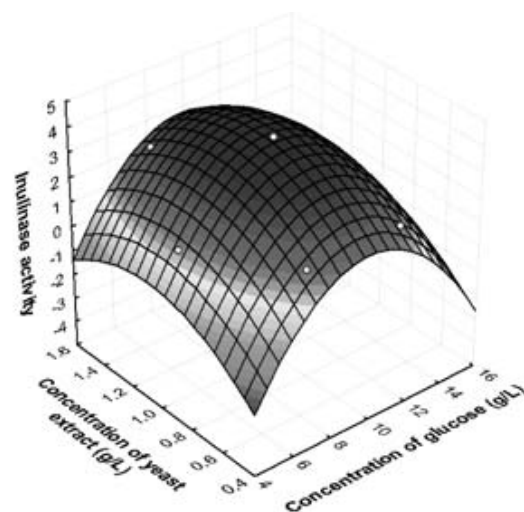

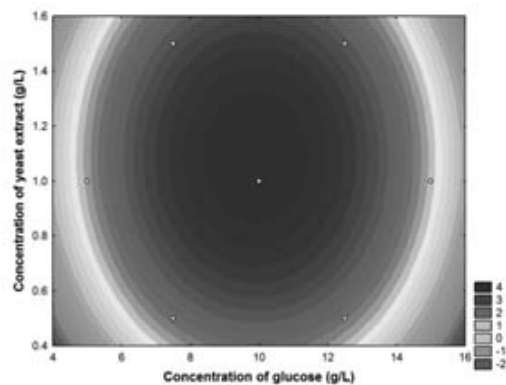**Figure 1.** Response surface for yeast extract

Figure 2. Level curves for yeast extract concentration versus glucose concentration

$$UA = -13.6433 + 2.3911(G) - 0.1202 \ (G)^2 + 10.7575 \ (YE) - 4.9122 \ (YE)^2 - 0.00812 \ (G).(YE).$$

Through the derivation of this equation, the media composition, regarding glucose and yeast extract concentrations, which are expected to allow for the production of maximum enzymatic activity, can be obtained. For CCMB 328 the conditions are 9.89 g / L for glucose and 1.09 g /L for yeast extract.

Kalil (2004) used the technique of factorial design and response surface analysis to optimize the culture medium for inulinase production by *Kluyveromices marxianus* According to his results, the best concentration of yeast extract was 10 g / L.

Schneider (1996) studied the production of inulinase by *Kluyveromices marxianus* and found that the presence of yeast extract is essential for the growth of microorganisms and enzyme production.

The chart of Paretto (Figure 3) shows that the variables glucose concentration and yeast extract concentration are linearly and quadratically significant for the production of inulinase *fungal endophyte* CCMB 328, because both variables have a P value greater than 0.05.

The statistical significance of the regression can be assessed by the ratio of the mean square regression and mean square of the residue, as well as by comparing these sources of variation using the Fisher distribution (F test) (Table III). Thus, a statistically
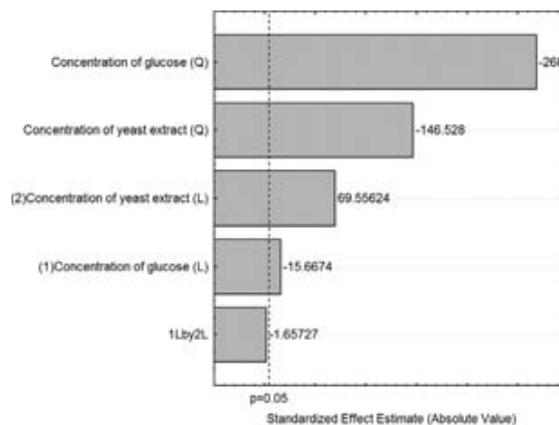

Figure 3. Pareto Chart for inulinase production CCMB 328

significant value of this ratio must be greater than the tabulated value for F. The calculated F value from the ANOVA table (712.55) was higher than the F tabulated (9.01), showing that the function is well suited to the answers.

RESPONSE SURFACE OPTIMIZATION OF TEMPERATURE AND PH

Table III shows the Doehlert design applied to the optimization of temperature and pH inulinase obtained by *fungal endophyte* CCMB 328.

The influence of temperature and pH on the activity of the enzyme was investigated for CCMB 328 by the surface response methodology. The results are shown in Figures 4 and 5.

TABLE IV
Analysis of variance (ANOVA) for the quadratic model fitted to data from the Table II.

Variation source	SS	df	MS	F	Tabulated F (IC 95%)	R^2
Regression	0.392	5	0.0783983	75.717366	9.01	
Residual	0.003	3	0.0010354			
Lack of Fit	0.000570	1	0.000570	0.4498	18.51	0.99
Pure Error	0.002536	2	0.001268			
Total SQ	0.391421	8				

stables from pH 3.5 to 6.5 ours results indicate a maximum inulinase activity at pH 7.11. In relation to the reaction temperature, it was observed that the maximum enzymatic activity at 48.35°C. Similar values were found in Cazetta et al. (2005).
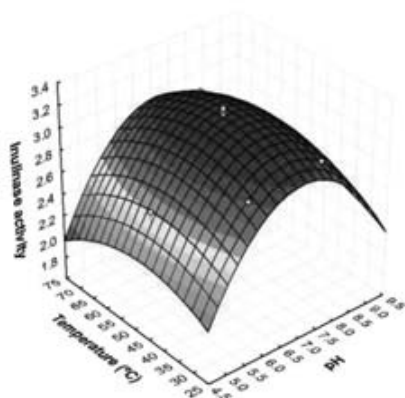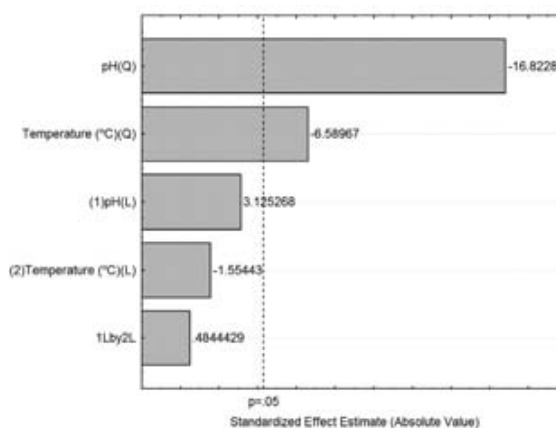

Figure 4. Response surface for pH versus temperature
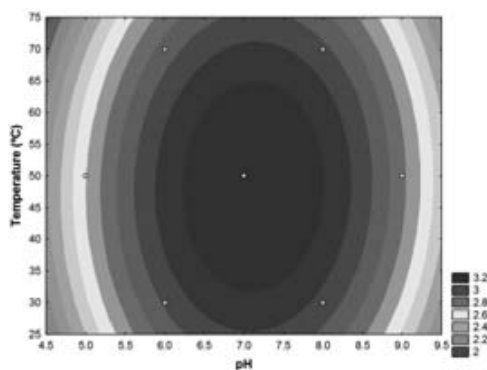
Figure 5. Level curves for pH versus temperature

Figure 6. Pareto Chart for pH x temperature surface response CCMB 328

The regression model provided for the enzyme activity in relation to pH and temperature in the experimental design is expressed by Equation 2:

$$UA = -4.4934+1.9239(pH) -0.1367(pH)^2 + 0.0356(T) -0.0004(T)^2+0.0004(pH).(T)$$

This equation illustrates the relationship of these two variables with the enzyme activity (UA) where P is the pH and T is the temperature (°C). Through the derivation of this equation, the points of maximum enzymatic activity could be obtained. According to this methodology, the optimal pH and temperature values were 7.11 and 48.35°C, respectively.

Regarding to the pH of the enzymatic reaction, although the microbial inulinases are described as

The Paretto chart (Figure 6) shows that the temperature concentration and pH variables are independent for the production of inulinase from *fungal endophyte* CCMB 328 because this had a p value less than 0.05.

The statistical significance of the regression can be assessed by the ratio of the mean square regression and the mean square of the residue, as well as by comparing these sources of variation using the Fisher distribution (F test) (Table IV). Thus, a statistically significant value of this ratio must be greater than the tabulated value for F. The F value calculated from the ANOVA table (75.71) was higher than the F tabulated (9.01), showing that the function is well suited to the answers.

EFFECT OF CATIONS

The effect of salts NaCl and KCl on the activity of inulinase is presented in Figure 7. The concentration

and 2.34 µmol/mL.min^{-1}, respectively (Figure 8). While assessing the kinetic characterization of the inulinase from *A. niger* 20 OSM, Showronek and Fiedureki (2006) reported K_m and V_{max} values of 6.7 g/L and 0.0476 mg/mL/min, respectively.
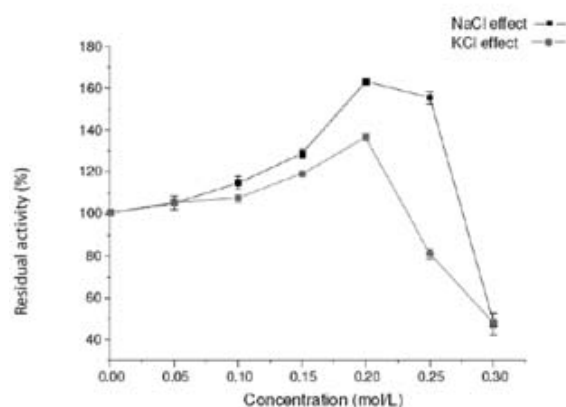

Figure 7. Effect of salts on inulinase activity

of 0.20 mol/L of NaCl and KCl increased the activity of inulinase from CCMB 328 by approximately 63% and 37%, respectively. A KCl concentration higher than 0.20 mol/L decreases the activity of the enzyme (Figure 7).

Our results were similar to those observed by Sheng (2007) who reported an increase in the enzymatic activity at 62.2% in the presence of KCl.

Ohta et al. (2002), using inulinases produced by *Rhizous*, noted that the presence of KCl did not change the enzyme activity.

KINETICS PARAMETERS

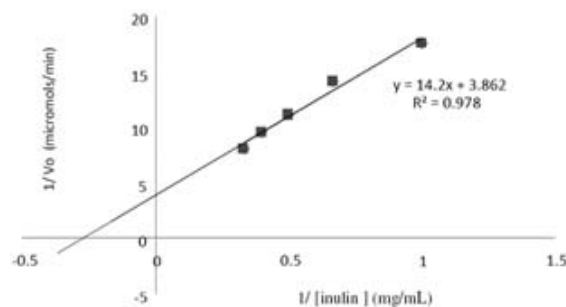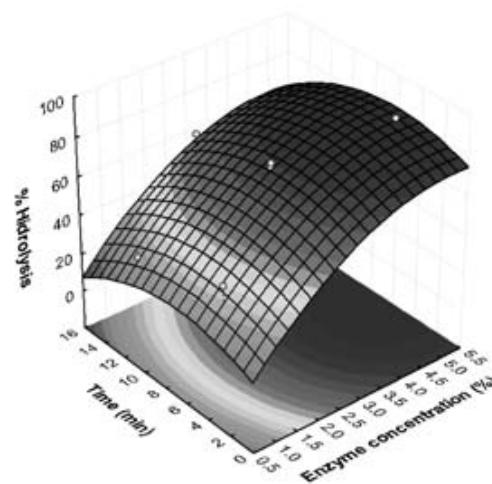The Lineweaver-Burk plot showed that the K_m and V_{max} values of the enzyme inulinase were 7.53 mg/mL

Figure 8. Influence of substrate concentration on the inulinase activity.

Figure 9. Response surface for hydrolysis of inulin

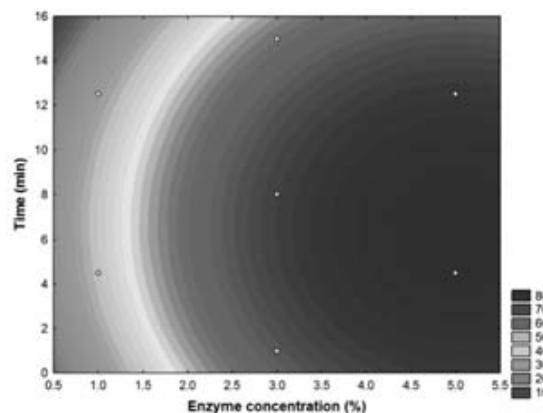**Figure 10.** Level curves for hydrolysis of inulin

HYDROLYSIS OF INULIN

Table V shows the Doehlert design applied to optimize the hydrolysis in function of time of reaction and enzyme concentration of inulin by *fungal endophyte* CCMB 328 inulinase.

The hydrolysis time was studied in five levels (1 to 15 minutes), and enzyme concentration was studied in three levels (1 to 5%) (Figures 9 and 10).

TABLE V
Results of the Doehlert design for inulin hydrolysis

Experiment	Enzyme Concentration (%)	Time (min.)	Experimental values (% Hydrolysis)	Response predicted (% Hydrolysis)
1	5 (+**0.866**)	4.5 (**-0.5**)	87.84000	81.9350
2	5 (+**0.866**)	11.5 (+**0.5**)	70.63000	76.5350
3	3 (**0**)	1 (**-1**)	60.40000	66.3050
4C	3 (**0**)	8 (**0**)	69.36000	71.1200
4C	3 (**0**)	8 (**0**)	72.48000	71.1200
4C	3 (**0**)	8 (**0**)	71.52000	71.1200
5	3 (**0**)	15 (+**1**)	62.37000	56.4650
6	1 (**-0.866**)	4.5 (**-0.5**)	42.23000	36.3250
7	1 (**-0.866**)	11.5 (+**0.5**)	25.98000	31.8850

The experimental errors were evaluated from the replication of the central point. The experimental data were processed by using the STATISTICA software. All the experiments in this step were carried out in a random order.

Equation 3 illustrates the relationship of these two variables with the extent of hydrolysis (% H), where C is the Enzyme Concentration (% v/v) and T is time (min). Through the derivation of this equation, the points of maximum enzymatic activity were obtained.

$$\% \ H = 2.3208 + 29.5811 \ (C) - 3.0040 \ (C)^2 + 2.5787 \ (T) - 0.1986 \ (T)^2 - 0.0342 \ (C)(T)$$

Through the derivation of this equation, the points of maximum enzymatic activity were obtained. In this work the highest productivity, based on the extent of hydrolysis, was observed for 4.90 % of enzyme and at 6.0 minutes of incubation. There are few studies of inulin hydrolyses using the same conditions and RSM analysis, as performed in this work.
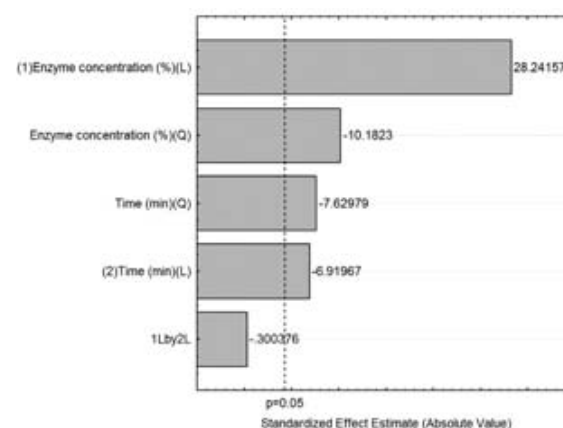

Figure 11. Pareto Chart for inulin hydrolysis CCMB 328

The Paretto chart (Figure 11) shows that the variables temperature concentration and pH are independent for the production of inulinase CCMB 328, because s had a p value less than 0.05.

The statistical significance of the regression (Table VI) can be assessed by the ratio of the mean square regression and mean square of the residue, as well as by comparing these sources

TABLE VI
Analysis of variance (ANOVA) for the quadratic model fitted to data from the Table V.

Variation source	SS	df	MS	F	Tabulated F	R^2
Regression	2677.247	5	535.4494	201.2	9.01	
Residual	7.985	3	2.661667			
Lack of Fit	2.878	1	2.878	1.127	18.51	0.92
Pure Error	5.107	2	2.554			
Total SQ	2685.2	8				

SS: Sum of squares; df: degree of freedom; MS: mean of square.

of variation using the Fisher distribution (F test). Thus, a statistically significant value of this ratio must be greater than the tabulated value for F. The F value calculated from (201.2) was higher than the F tabulated (9.01), showing that the function is well suited to the answers.

CONCLUSION

In this work the production and characterization of inulinase by *fungal endophyte* CCMB 328 was effectively performed. The enzyme was afterwards used in the hydrolysis of inulin whose a conversion yields of roughly 72.48 % for an initial concentration of inulin of 1% (w/v). Therefore, the inulinase from this yeast is a potential candidate for inulin hydrolysis in the food industry.

Using the response surface methodology it was possible to determine the best media composition for obtaining inulinase from *fungal endophyte* CCMB 328. This corresponds to a medium containing 9.89 g / L for glucose and 1.09 yeast extract. This statistical method was also used to access the optimum pH (7.11) and temperature (48.38°C) for fermentative inulinase production.

The combination of results obtained in this work (enzymatic characterization and hydrolysis of inulin) can provide useful guidelines for the utilization of inulinase from CCMB 328 for fructose production at pilot scales.

This optimization through response surface methodology can be used in future industrial scale fermentation and promote the rational use of enzymes and substrates in the process.

RESUMO

A enzima inulinase (EC 3.2.1.7, β-D-frutano frutanohidrolase) atua sobre as ligações β-2,1 da inulina, um polifrutano consistindo de frutose unida por ligações β-2,1. A hidrólise de inulina através do uso de inulinase é uma alternativa viável para a obtenção de xarope de frutose. O objetivo deste trabalho foi estudar a produção, caracterização e aplicação de inulinase obtidas a partir do fungo endofítico CCMB 328, isolado do semi-árido

brasileiro. A metodologia de Superfície de Resposta (MSR) foi empregado para avaliar os efeitos das variáveis (concentração de glicose e extrato de levedura) na atividade da enzima inulinase produzida em meio de cultura líquido e também para avaliar a atividade da enzima na hidrólise de inulina. Os resultados mostraram que as melhores condições para a produção de inulinase pela levedura CCMB 328 são 9,89 g/L de glicose e 1,09 g/L de extrato de levedura. A concentração de 0,20 mol/L de NaCl e KCl aumentou a atividade da enzima da CCMB 328 em aproximadamente 63% e 37%, respectivamente. Os resultados também mostraram que a enzima inulinase tem potencial para a hidrólise de inulina, apresentando rendimentos de 72,48 % para uma concentração inicial de inulina de 1% (m/v).

Palavras-chave: Inulina, inulinase, leveduras, semi-árido, fungo endofítico.

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