Production, Characterization And Application Of Inulinase From *Pseudozyma* Sp. CCMB 300

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ABSTRACT

The use of inulinases provides an alternative way to obtain fructose syrup through the hydrolysis of inulin. The objective of this work was to study the production, characterization and application of an inulinase from *Pseudozyma* sp CCMB 300 isolated from Brazilian semi-arid region. The higher production of inulinase in a medium containing 0.86 g L^{-1} yeast extract and 14.54 g L^{-1} glucose using response surface methodology (RSM). This statistical method was also used to access the optimum pH (8.28) and temperature (54ºC) for fermentative inulinase production. The results showed that the inulinase has potential for inulin hydrolysis, where a conversion yields of roughly 87.39 % for an initial concentration of inulin of 1% (w/v).

Keywords:

Inulin, inulinase, yeasts, semi-arid, fructose.

Academic Discipline and Sub-Disciplines

Biotechnology

Subject Classification

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INTRODUCTION

The enzyme inulinase (EC 3.2.1.7) target the β-2 linkage of inulin, a polyfructan consisting of linear β-2,1 linked fructose, and hydrolyzes it into fructose [1].

Inulin is a naturally occurring polyfructan in plants consisting of linear chains of β (2,1)-linked fructose residues attached to a terminal sucrose molecule. It is produced naturally in over 36,000 plants world wide, including 1200 native grasses and cereals belonging to several genera. In addition, inulin has extensive documented historical human use through the consumption of edible plants and fruits [2].

Inulinase can be derived from plants and many microorganisms. Dandelion, chicory and Jerusalem artichoke have been proven to contain inulinase that can be purified, but according to Kochhar and Kanur [3], these sources are not as productive as the microbial one, which seem to be the only source capable of producing enough enzymes for industrial applications. For this reason, in the last three decades, significant efforts have been made to find the best microbial source for the extraction of inulinase [4].

Conventional fructose production from starch needs at least three enzymatic steps, including α-amylase, amyloglucosidades, and glucose isomerase action, yielding only 45 % fructose solutions. More concentrated fructose solution requires a dedicated chromatographic step. A viable alternative to this process is the hydrolysis of inulin by inulinases. Enzymatic formation of fructose from inulin has a single enzymatic step and yields up to 95% fructose[5].

The Brazilian semi-arid region represents a large area for bioprospection since naturally occurring microorganisms adapted to a tropical semi-arid environment, with high temperature and low humidity throughout the year, may possess some features of great industrial interest [6]

In this study, we carried out a survey of the production, characterization and application of extracellular inulinases produced by *Pseudozima* sp. from the Brazilian semi-arid region.

MATERIALS AND METHODS

Chemicals

Inulin, bovine serum albumin, and 3,5-dinitrosalicylic 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 *Pseudozyma* sp. CCMB is from Culture Collection of Microorganisms of Bahia (CCMB) of the Universidade Estadual de Feira de Santana, Brazil (Uetanabaro and Góes-Neto 2006) and was identified in our previous work [7].

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 28ºC for 48 h, as described in Oliveira (2007), diluted in sterile distilled water to a concentration of about 10^8 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), supplemented with yeast extract, 1 g; glucose, 10 g; pH 5.0, for fermentation.[8] After incubation at 28 ºC 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 ºC, 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 50ºC.

Enzyme assays

Inulinase activity was measured spectrophotometrically using the dinitrosalicylic reagent, as reported by Miller [8]. The reaction mixture consisted of 900 µL of 2 % (w/v) inulin in 0.05 M acetate buffer, pH 5.5, and 100 ⎯l culture supernatant. The mixture was incubated for 15 min at 50 ºC. After incubation, 1 mL dinitrosalicylic reagent was added, and the mixture was boiled at 100 ºC for 10 min and cooled with 10 mL 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 [10], 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 centre of the domain leading to a total of 9 experiments (Table 1) was used to obtain the knowledge of the effect of glucose and yeast extract concentration on the production of enzyme.

Table 01: Results of the factorial analysis for inulinase production CCMB 300

N°	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)	1.0242	1.0409
2	12.5 (+0.5)	1.5 (+0.866)	1.7904	1.7736
3	5 (-1)	1 (0)	1.3198	1.3030
4C	10 (0)	1 (0)	2.3734	2.3935
4C	10 (0)	1 (0)	2.4376	2.3935
4C	10 (0)	1 (0)	2.3697	2.3935
5	15 (+1)	1 (0)	2.6988	2.7155
6	7.5 (-0,5)	0.5 (-0.866)	1.7022	1.7189
7	12.5 (-1)	0.5 (-0.866)	2.4155	2.3987

Thus, concentration of glucose was studied in 5 levels (7.5 to 12.5 g L^{-1}) and yeast extract was studied in three levels (0.5 to 1.5 g L^{-1}). The experimental errors were evaluated from replication of central point. The experimental data were processed by using the STATISTICA software. All the experiments in this step were carried out in random order.

To estimate the lack of fit of the model to the experimental data an analysis of variance (ANOVA) was performed (Table 2), using Design Statistics, version 7.0.

Table 02 Analysis of variance

Variation source	SQ	gl	MQ	F	Tabulated F (IC de 95%)	R^2
Regression	2.669513	5	0.533903	348.3017	9.01	
Residual	0.004599	3	0.001533			
Lack of Fit	0.001683	1	0.001683	1.155	0.394970	0.99
Pure Error	0.002915	2	0.001458			
Total SQ	2.674111	8				

Response surface optimization of temperature and pH

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

The experimental design, with two variables (temperature and pH) and three replicates at the centre of the domain leading to a total of 9 experiments (Table 3) was used to obtain the knowledge of the effect of temperature and pH of inulinase activity.

Table 03. Results of the factorial for optimization of temperature and pH CCMB 300

N°	pH	Temperature °C	Experimental values (UA)		Activities predicted (UA)
1	6 (-0.5)	70 (+0.866)	1.5104	1.5743	
2	8 (+0.5)	70 (+0.866)	1.9197	1.8557	
3	5 (-1)	50 (0)	1.8427	1.7787	
4C	7 (0)	50 (0)	2.1973	2.2147	
4C	7 (0)	50 (0)	2.2279	2.2147	
4C	7 (0)	50 (0)	2.2189	2.2147	
5	9 (+1)	50 (0)	2.1971	2.2610	
6	6 (-0.5)	30 (-0.866)	1.0458	1.1097	
7	8 (+0.5)	30 (-0.866)	1.3744	1.3104	

The pH was studied in 5 levels (5 to 9) and temperature was studied in three levels (30 to 70ºC). The experimental errors were evaluated from replication of central point. The experimental data were processed by using the STATISTICA software. All the experiments in this step were carried out in 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 inulinase activity was studied. The concentrations used were: NaCl (0.05, 0.1, 0.15, 0.2, 0.25 and 0.3 mol/L), 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 ºC for 15 min. The K_m and V_{max} for inulin was determined by the method of Lineweaver-Burk plots.

Hydrolysis of inulin

The extent of inulin hydrolysis (%) was calculated as $\dfrac{amount\ of\ fructose\ released}{amount\ of\ initial\ total\ sugars} \times 100$ [11]. Total sugars were determined by antrone method 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 centre of the domain leading to a total of 9 experiments (Table 4) was used to obtain the knowledge of the effect of enzyme concentration and fermentation time on the extent of inulin hydrolysis from a solution 1% (w / v) in citrate buffer 0.05 M.

Statistical analysis

All experiments were carried out in triplicate. One-way analysis of variance was used to compare the specific activity of inulinase among the strains. Where significant differences were detected between strains, the means were compared using Tukey's test. For all statistical analyses, the level of significance was set at 5%, and the analyses were performed using the standard statistical software SPSS for Windows (release 6.1.3, 1995; SPSS, Brazil, 1995).

Results and Discussion

Production of inulinases by yeasts

The table 01 shows the Doehlert design applied to optimize the production of inulinase by *Pseudozyma* sp. CCMB 300. The first column describes the number of planning, with C representing the central compound. In the second and third column are the values of glucose concentration and the concentration of yeast extract, respectively. The fourth column represents the enzymatic activity. The last column represents predicted

The response surface graphics (Figures 01 and 02) shows the influence of glucose and yeast extract concentrations in the production of inulinase of CCMB 300. From the analysis of the area chart in Figures 01 and 02 it can be concluded that the production of inulinase by CCMB 300 strains clearly peaks for glucose concentrations in excess of 10 L^{-1} L glucose, for the whole range of concentration of yeast extract tested.
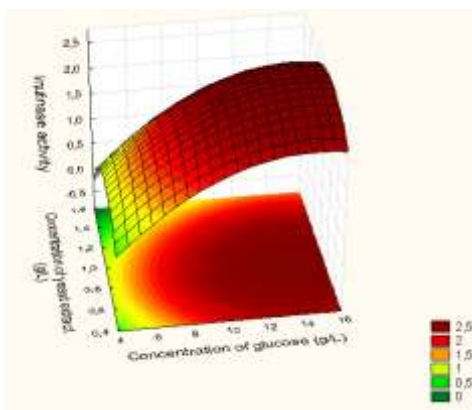
Fig. 01. Response surface for yeast extract concentration versus glucose concentration CCMB 300
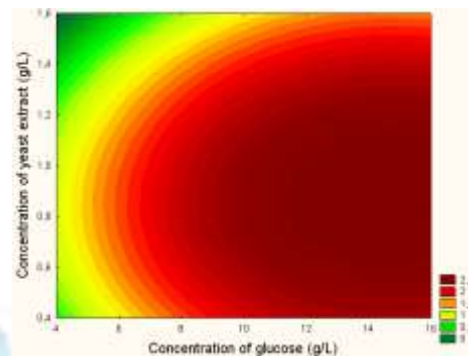
Fig. 02. Level curves for yeast extract concentration versus glucose concentration CCMB 300

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.

$$(UA) = -2.056 + 0{,}438(G) - 0.0153(G)2 + 3.758(YE) - 2.258(YE)2 + .0106(G) \times (YE). \qquad \text{(Equation 1)}$$

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 300 the conditions are 14.54 g L^{-1} for glucose and 0.86 g L^{-1} for yeast extract.

Different nitrogen sources were studied for *Cryptococccus aureus* inulinases. Yeast extract was the better and increased inulinase production [12]. According to these authors, vitamins and trace elements present in the yeast extract may enhance inulinase production.

The chart of Paretto (Figure 03) shows that both linearly (L) and quadratically (Q), the variables glucose concentration and yeast extract concentration are significant for the production of inulinase by *Pseudozyma* CCMB 300, because both variables had a p value greater than 0.05.
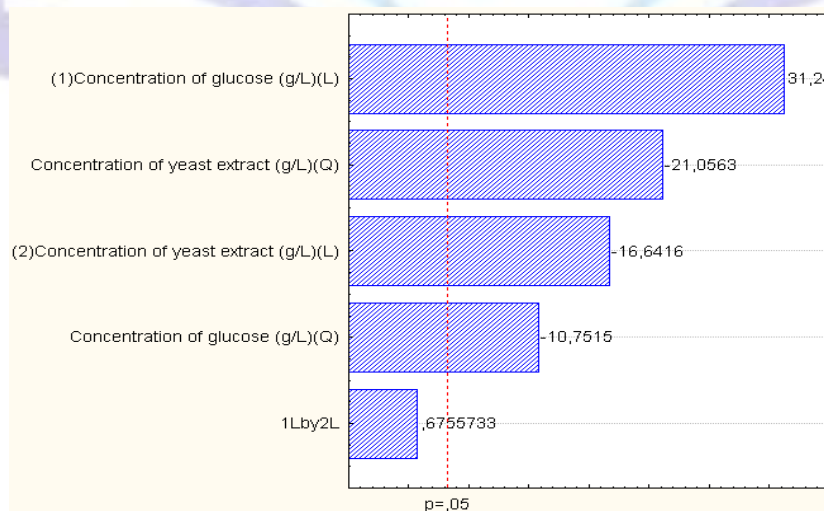
Figure 03. Pareto Chart for inulinase production CCMB 300

The statistical significance of the regression (Table 02) can be assessed by the ratio of the mean square regression and mean square of the residue and by comparing these sources 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. It is obtained by the ANOVA table that calculated F (348.30) is higher than the F tabulated (9.01) showing that the function is well suited to the answers.

Response surface optimization of temperature and pH

The table 03 shows the Doehlert design applied to the optimization of temperature and pH inulinase obtained by *Pseudozyma* sp. CCMB 300.

The influence of temperature and pH on the activity of the enzyme was investigated for CCMB 300 by surface response methodology. The results are shown in Figures 04 and 05.
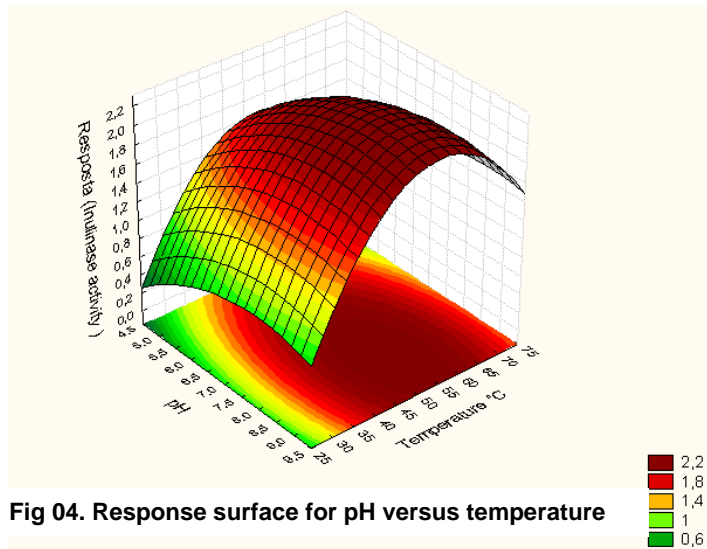

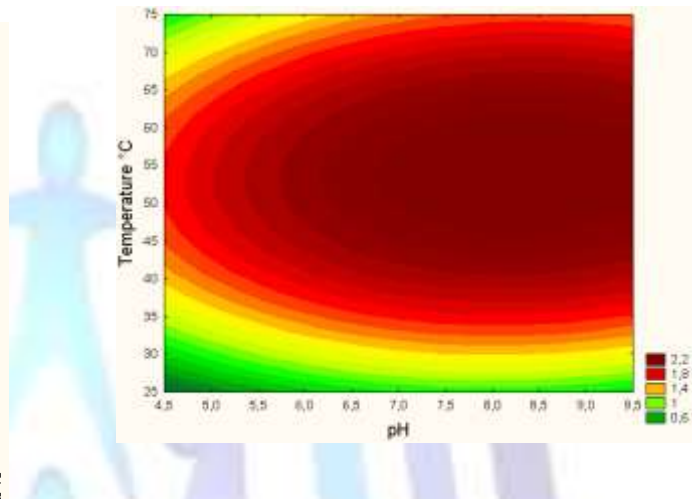**Fig 04. Response surface for pH versus temperature**

Fig 05. Level curves for pH versus temperature

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

(UA) = -5.6887+ 0.7514(P)-0.0486(P)2+0.1814(T) -0.00175(T)2+0.00101(P).(T).   (Equation 2)

This equation illustrates the relationship of these two variables with enzyme activity (UA), where: P is the pH and T is the temperature ºC. Through the derivation of this equation, the points of maximum enzymatic activity can be obtained. According to this methodology, the optimal pH and temperature values were of 8.28 and 54°C, respectively.

Many works in the literature deal with the effects of temperature and pH on inulinase activity. It is certain that the response of the enzyme activity to these variables depends mainly on the strain used as a source for enzyme production. The studies of Pandey et al[13] showed that fungal inulinases exhibited an optimum pH between 4.5 and 7.0, yeasts inulinases between 4.4 and 6.5 and bacterial inulinases between 4.8 and 7.0. Inulinases preparations from *A. niger* strains have also been shown to have pH and temperature optima in the ranges of 4.35 to 5.35 and 45 to 60ºC [14] Information about the effect of temperature and pH on inulinase activity is very important for assessing the feasibility industrial application, and to the development of bioprocesses and to the selection of bioreactors.

The Paretto chart (Figure 06) shows that both linearly and quadratically, temperature and pH are exerting influence on the enzyme activity of inulinase *Pseudozyma* CCMB 300, because both variables had a p value greater than 0.05.
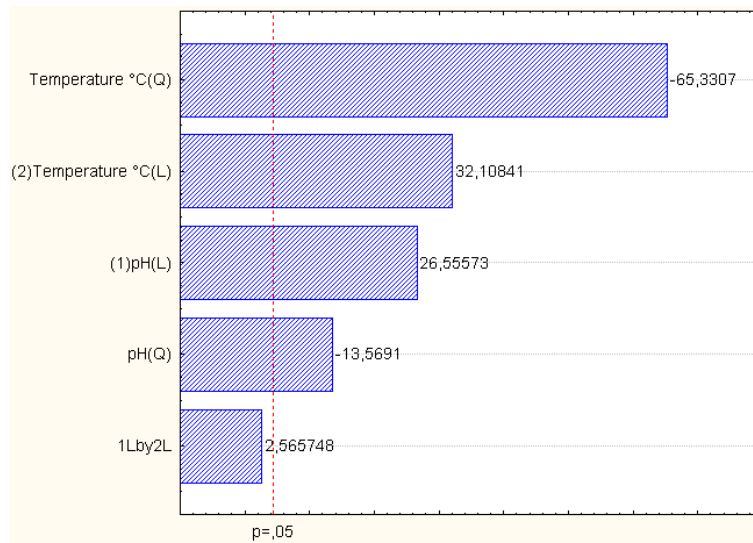
Fig. 06. Paretto Chart

The statistical significance (Table 04) of the regression can be assessed by the ratio of the mean square regression and mean square of the residue and by comparing these sources 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. It is obtained by the ANOVA table that calculated F (35.67) is higher than the F tabulated (9.01) showing that the function is well suited to the answers.

Table 04 Analysis of variance

Variation source	SQ	gl	MQ	F	Tabulated F (IC de 95%)	R^2
Regression	1.486667	5	0.297333	35.67048	9.01	
Residual	0.025007	3	0.008336			
Lack of Fit	0.024512	1	0.024512	99.111	0.009940	0.98
Pure Error	0.000495	2	0.000247			
Total SQ	1.511674	8				

Effect of cations

The effect of salts NaCl and KCl on the activity of inulinase are shown in Figure 07. The activity was maximal at a concentration of 0.15 mol/L of NaCl and KCl. Thconcentrations of 0.15 mol L^{-1} of NaCl and KCl increased the activity of inulinase from CCMB 300 by approximately 40%. When concentration higher than 0.15 mol L^{-1} were used, a decrease in the activity of the enzyme was observed.
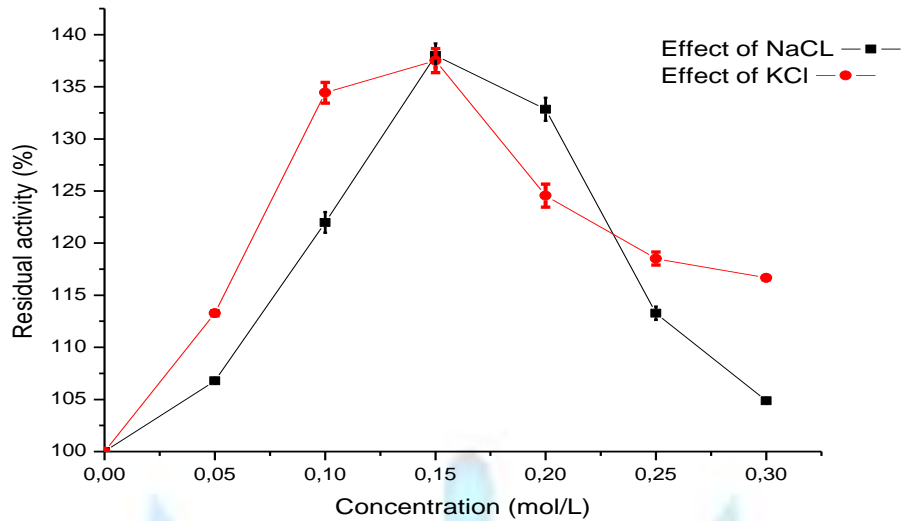
Fig. 7. Effect of salts on CCMB 300 inulinase activity

Kinetics Parameters

The Lineweaver-Burk plot showed that the K_m and V_{max} values of the enzyme for inulin were 23 mg/mL and 28.33 µmol/mL.min, respectively (Fig. 08). These results demonstrate that the inulinase from CCMB 300 displayed affinity for inulin and are in agreement with other studies. The K_m value for inulinase from *Aspergillus ficuum* JNPSP was 25.6 mg mL^{-1} [15].
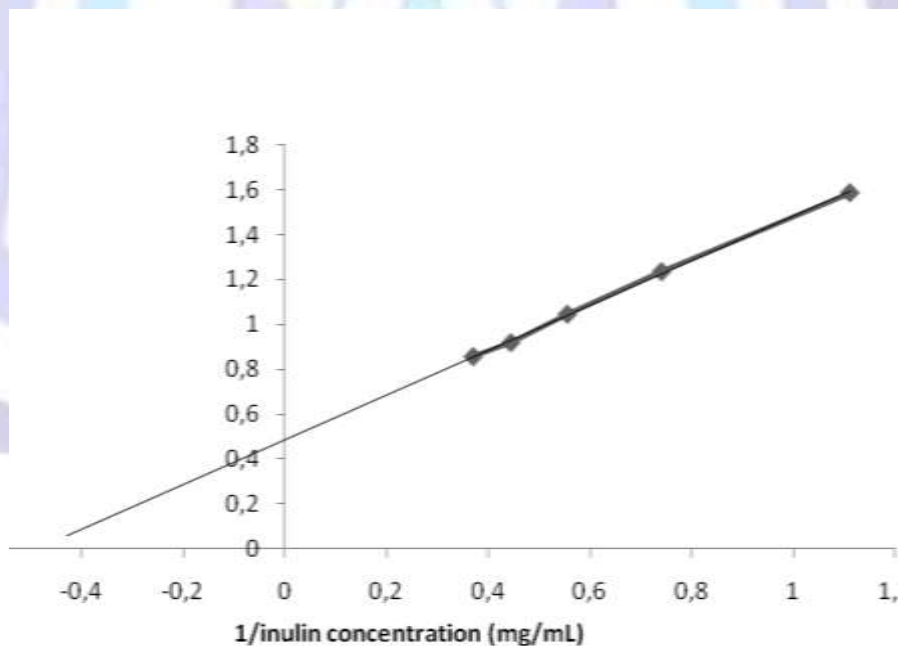

Fig. 08. Influence of substrate concentration on the inulinase activity

Hydrolysis of inulin

The Table 05 shows the Doehlert design applied to optimize the hydrolysis in function of time of reaction and enzyme concentration of inulin by *Pseudozyma sp.* CCMB 300 inulinase.

Table 05: Results of the factorial for inulin hydrolysis

N°	Enzyme Concentration (% v/v)	Time (min)	Experimental values (% Hidrolysis)	Response predicted (% Hidrolysis)
1	5 (+0.866)	4.5 (-0.5)	64.543	63.12755
2	5 (+0.866)	12.5 (+0.5)	49.7088	51.12425
3	3 (0)	1 (-1)	51.6736	53.06016
4C	3 (0)	8 (0)	87.0309	87.38700
4C	3 (0)	8 (0)	88.5784	87.38700
4C	3 (0)	8 (0)	86.0895	87.38700
5	3 (0)	15 (+15)	51.0665	49.21775
6	1 (-0.866)	4.5 (-0.5)	48.6767	47.26125
7	1 (-0.866)	12.5 (+0.5)	41.6217	43.03715

The hydrolysis was studied in five levels (1 to 12.5 minutes) and enzyme concentration was studied in three levels (1 to 5 %) (Figure 09 and 10).The experimental data were processed by using the STATISTICA software. All the experiments in this step were carried out in random order.
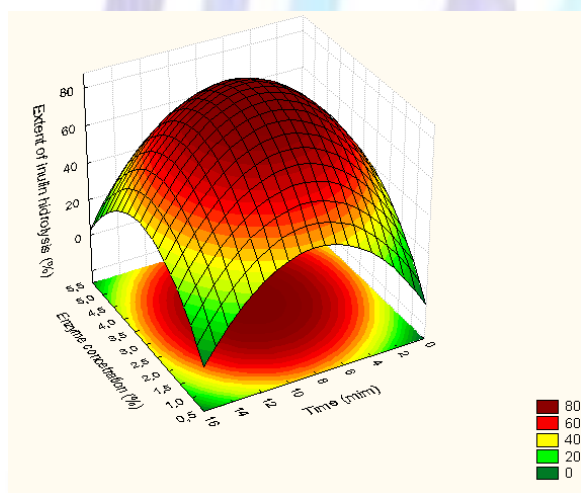

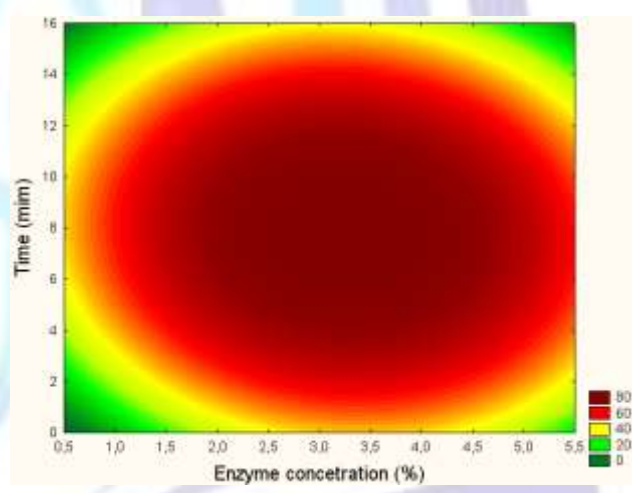Fig. 09. Response surface for hydrolysis of inulin

Fig. 10. Level curves for hydrolysis of inulin

Equation 3 illustrates the relation of these two variables with the extent of hydrolysis (% H), where: C is the enzyme concentration (% v/v) and T is the time (min).

$$\% H = -27.148 + 41.197(C) -6.0227(C)^2 +12.291(T) -0.739(T)^2 -0.2431(C)(T) \quad \text{(Equation 3)}$$

Through the derivation of this equation, the points of maximum enzymatic activity are obtained. In this work the highest productivity, based on the extent of hydrolysis, was observed for 3.26 % of enzyme and 7.7 minutes of incubation. The higher conversion yields was 87.39 %. There are few studies of inulin hydrolysis using the same conditions and SRM analysis, as performed in this work.

The Paretto chart (Figure 11) shows that both linearly and quadratic, variables: enzyme concentration and time of hydrolysis are significant for the hydrolysis of inulin *Pseudozyma sp* CCMB 300, because both variables has a p value greater than 0.05.
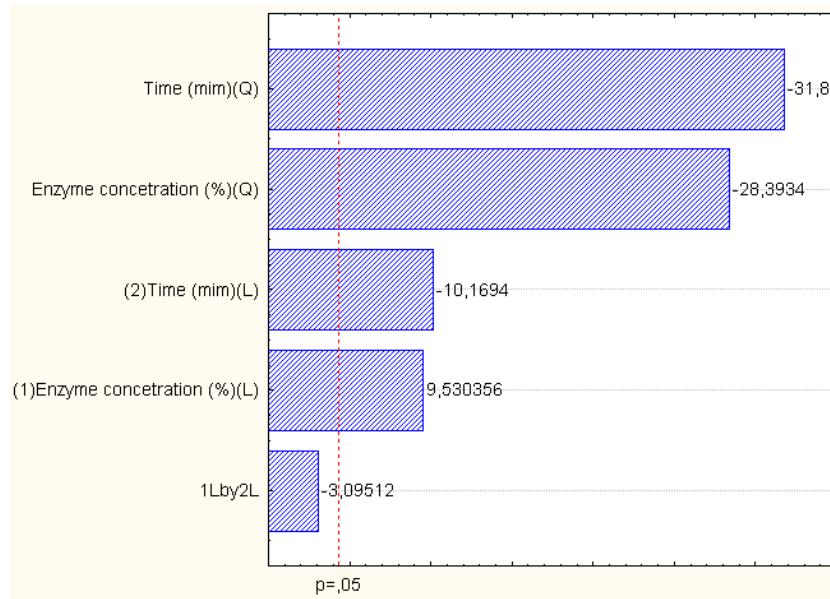
Fig. 11. Pareto Chart for inulin hydrolysis CCMB 300

The statistical significance (Table 06) e of the regression can be assessed by the ratio of the mean square regression and mean square of the residue and by comparing these sources 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. It is obtained by the ANOVA table that calculated F (103.46) is higher than the F tabulated (9.01) showing that the function is well suited to the answers.

Table 06. Analysis of variance

Variation source	SQ	gl	MQ	F	Tabulated F (IC de 95%)	R^2
Regression	2859.761233	5	571.9522	103.4639	9.01	
Residual	16.584105	3	5.528035			
Lack of Fit	13.426	1	13.426	8.501	0.100253	0.99
Pure Error	3.159	2	1.579			
Total SQ	2876.345	8				

CONCLUSION

In this work the production and characterization of the inulinase by *Pseudozyme* sp. CCMB 300 was effectively performed. The enzyme was afterwards used in the hydrolysis of inulin, where a conversion yields of roughly 90% 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 *Pseudozyme* sp. This corresponds to a medium containing 0.86 g L^{-1} yeast extract and 14.54 g L^{-1} glucose. This statistical method was also used to access the optimum pH (8.28) and temperature (54⁰C 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 300 for fructose production at pilot scales.

This optimization through RMS can be used in future industrial scale fermentation in promoting the rational use of enzyme and substrate in the process.

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Author' biography with Photo

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