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Agave syrup as a substrate for inulinase production by *Kluyveromyces marxianus* NRRL Y-7571

Luana Paula de Azevedo de Oliveira[1], Tiago Felipe de Oliveira[1], Jonas Contiero[2] and Márcia Luciana Cazetta[3*]

*[1]Centro de Ciências Agrárias, Ambientais e Biológicas, Universidade Federal do Recôncavo da Bahia, Cruz das Almas, Bahia, Brazil. [2]Universidade Estadual Paulista "Júlio de Mesquita Filho", Rio Claro, São Paulo, Brazil. [3]Centro de Ciências Exatas e Tecnológicas, Universidade Federal do Recôncavo da Bahia, Rua Rui Barbosa, 710, 44380-000, Cruz das Almas, Bahia, Brazil. *Author for correspondence. E-mail: malucz@ufrb.edu.br*

ABSTRACT. The factorial planning was used to plan and optimize inulinase production by the yeast *Kluyveromyces marxianus* NRRL Y-7571. The experiments were conducted using a Central Composite Design (CCD) 2^2, at different concentrations of agave syrup (3.6 to 6.4%) and yeast extract (2.2 to 3.0%). After 96 hours of fermentation, the best condition for the inulinase production was 5% agave syrup and 2.5% yeast extract, which yielded an average of 129.21 U mL^{-1} of inulinase. Partial characterization of the crude enzyme showed that the optimal pH and temperature were 4.0 and 60°C, respectively. The enzyme showed thermal stability at 55°C for 4 hours.

Keywords: yeast, microbial enzymes, agricultural substrate.

Xarope de agave como substrato para produção de inulinase por *Kluyveromyces marxianus* NRRL Y-7571

RESUMO. O planejamento fatorial foi utilizado para planejar e otimizar a produção de inulinase pela levedura *Kluyveromyces marxianus* NRRL Y-7571. Os experimentos foram conduzidos por meio de Delineamento Composto Central Rotacional (DCCR) 2^2 em diferentes concentrações de xarope de agave (3,6 a 6,4%) e extrato de levedura (2,2 a 3,0%). Depois de 96 horas de fermentação, a melhor condição para produção de inulinase foi xarope de agave 5% e extrato de levedura 2,5%, com uma produção média de 129,21 U mL^{-1}. A caracterização parcial do extrato enzimático bruto mostrou que a enzima apresenta pH e temperatura ótimos de 4,0 e 60ºC, respectivamente. A enzima mostrou estabilidade térmica a 55ºC durante 4 horas.

Palavras-chave: levedura, enzimas microbianas, substrato agrícola.

Introduction

Inulinases are 2,1-β-D-fructanfructanohydrolases (EC 3.2.1.7), which act on the 2,1-β-D-fructosidic linkages of inulin. The application of inulinases in high-fructose syrup production has been investigated for decades (Vandamme & Derycke, 1983; Parekh & Margaritis, 1985; Etallibi & Baratti, 2001). In addition, these enzymes have many other applications, such as in the production of fructooligosaccharides, which are fermentable sugars that enhance alcoholic beverages production, such as tequila, and biofuels production from inulin-rich substrates (Chi et al., 2011; Lim et al., 2011; Waleckx et al., 2011; Flores-Gallegos, Morlett-Chávez, Aguilar, & Rodríguez-Herrera, 2012).

Microorganisms are the main sources of inulinases. Although high levels of inulinase production have been reported for *Aspergillus niger* (Poorna & Kulkarne, 1995; Singh & Gill, 2006; Mutanda, Wilheimi, & Whiteley, 2008; Dinarvand et al., 2012), yeasts of the genus *Kluyveromyces* have also attracted great interest. Besides being considered as GRAS (Generally Recognized as Safe), those yeasts have been described as some of the best producers of inulinase in several fermentative processes (submerged and solid fermentation) with a wide range of substrates (Selvakumar & Pandey, 1999; Silva-Santisteban & Maugeri, 2005; Xiong, Jinhua, & Dongsheng, 2007; Treichel, Mazutti, Maugeri, & Rodrigues, 2009; Neagu & Bahrim, 2011).

Despite their various applications, high costs of production have limited the use of inulinases (Flore-Gallegos et al., 2012). Thus, several raw materials, such as agro-industrial residues (Sguarezi et al., 2009; Treichel et al., 2009; Chen et al., 2011) and extracts from plants that store inulin (Ertan, Aktac, Kaboglu, Ekinci, & Bakar, 2003; Cazetta, Martins,

Monti, & Contiero, 2005; Sharma, Kainth, & Gill, 2006; Singh & Bhermi, 2008), have been explored as alternative substrates for the production of these enzymes. Extracts obtained from chicory, Jerusalem artichoke and dahlia are among the most studied industrially important plant extracts (Ávila-Fernandez et al., 2007). Another plants alternative to obtain inulin rich extracts is species from the genus *Agave*, which are originated from Mexico and whose saps have high inulin content (Garcia-Aguirre, Saenz-Alvaro, Rodriguez-Solo, & Vicente-Magueyal, 2009). Fructans present in the blue agave (*Agave tequilana*) have a degree of polymerization ranging from 3 to 29 units of fructose and contain mostly 2,1-β linkages in their linear chain and 2,6-β linkages in the ramifications (Lopez, Mancilla-Margalli, & Mendoza-Diaz, 2003). Agave syrup is traditionally used in tequila production, and it was hypothesized, based on its composition, that it could be a good substrate for inulinase production. Thus, in this study, we used agave syrup as a carbon source for inulinase production by *Kluyveromyces marxianus* NRRL Y-7571 in submerged fermentation, using Response Surface Methodology (RSM) for process optimization.

Material and methods

Microorganism and culture conditions

The yeast *K. marxianus* NRRL Y-7571 was acquired from the André Toselo Institute in Campinas, São Paulo, Brazil (reference 7073) and stored in a freezer at -80°C in a liquid medium composed of (%): yeast extract, 0.3; malt extract, 0.3; bacteriological peptone, 0.5; glucose, 1.0; and glycerol, 20.

Pre-inoculum, inoculum, and fermentation

Initially, the yeast was incubated in Petri dishes containing Yeast Malt Agar (YMA) and incubated for 24 h at 28 ± 2°C to obtain a young cell culture for starting the fermentation process. Then, the yeast culture was transferred to a test tube containing 5 mL of pre-inoculum medium composed of the basal medium (%): sucrose, 1.0; $MgSO_4$, 0.07; KH_2PO_4, 0.5; KCl, 0.12; NH_4Cl, 0.15; yeast extract, 0.5; and peptone, 1.0. The medium was sterilized at 121°C for 15 min. Sucrose was sterilized separately by vacuum filtration and combined with the medium. The pre-inoculum culture was incubated overnight at 30°C, with agitation at 150 rpm. Afterwards, the content of the pre-inoculum tube was transferred to a 250 mL Erlenmeyer flask containing 50 mL of inoculum medium, which was the same as above but sucrose was replaced with agave syrup at 1% for adaptation of the microorganism. This culture was grown for 24 hours in the same conditions of temperature and agitation. Later, this culture was used as inoculum (10%, v/v) for the fermentation flasks.

Fermentations were conducted in 125 mL Erlenmeyer flasks containing 30 mL of the basal medium, without sucrose, and added of agave syrup and yeast extract at different concentrations, according to the Central Composite Design (CCD) 2^2 (Rodrigues & Iemma, 2009). The pH was adjusted to 5.0 with orthophosphoric acid, and the medium was sterilized for 15 min at 121°C. Fermentation was carried out at 28 ± 2°C, 150 rpm, for 96 hours. Every 24 hours, samples were removed, and the fermented broth was centrifuged at 5,000 rpm for 20 min. The obtained supernatant was used for the determination of enzymatic activity, and the biomass was used for the quantification of cell growth.

Factorial designs

RSM was applied to optimize the production of inulinase (dependent variable) using yeast extract (X_1) as the source of nitrogen and agave syrup (X_2) as the carbon source (independent variables). For this, we used CCD 2^2, resulting in 11 runs total. Two CCDs were carried out as follows: in CCD 1, independent variable concentrations ranged from 0.3 to 1.7% for yeast extract and from 3.0 to 17.0% for agave syrup (Table 1); CCD 2 was carried out based on the results obtained in CCD 1, with concentrations varied in the range of 2.0-2.8% for yeast extract and 3.6-6.4% for agave syrup (Table 4). This model is represented by a second-order polynomial regression Equation (1):

$$Y = b_0 + b_1X_1 + b_2X_2 + b_{12}X_1X_2 + b_{11}X_1^2 + b_{22}X_2^2 \quad (1),$$

where Y is the predicted response [inulinase activity (U mL^{-1})]; X_1 and X_2 are the encoded values of the independent variables (yeast extract and agave syrup, respectively); b_0 is a constant; b_1 and b_2 are the linear coefficients; b_{12} is the interaction coefficient; b_{11} and b_{22} are the quadratic coefficients. The test factors were coded according to the following Equation (2):

$$Xi = (Xi - X0)/\Delta xi \quad (2),$$

where xi is the encoded value; Xi is the current value of the independent variable; X0 is the current value of the centerpiece, and Δxi is the step change value.

Analysis of variance (ANOVA) was used to estimate statistical parameters. The significance of the regression coefficients was determined by the Student's test. The equation of the second-order model was determined by the Fisher test. The

variance explained by the model was given by the determination coefficient (R^2). The Statistica software, version 7.0 (StatSoft, Inc.), was used for the graphical analysis and regression testing.

Biomass production

The biomass (g L^{-1}) was determined by optical density using a spectrophotometer at λ = 600 nm and it was calculated by correlating the dry matter and optical density according to a standard curve. All analyses were carried out in triplicates.

Enzymatic activity

The enzymatic activity was determined in the supernatant according to Suzuki, Ozawa, and Maeda (1988). Total reducing sugars released after the incubation of 1 mL of the enzyme with 2% sucrose in 0.05 M citrate-phosphate buffer pH 4.0, were determined using the 3,5-dinitrosalicylic acid reagent according to Miller (1959). Glucose (1 g L^{-1}) was used as a standard.

Partial characterization of the crude enzymatic extract

The optimal temperature for the enzyme was established by determining the enzymatic activity at temperatures from 45 to 70°C. The optimal pH was evaluated by performing the enzymatic assay at pH values ranging from 2.0 to 10.0, using the following buffers (50 mM): glycine-HCl for pH 2.0 and 3.0, sodium citrate for pH 3.0 to 6.0, sodium phosphate for pH 6.0 to 8.0, Tris-HCl for pH 8.0 and 9.0, and glycine-NaOH for pH 9.0 and 10.0. The thermal stability was determined by incubation of the crude extract at temperatures of 50, 55, and 60°C, for 5 hours, and testing the residual activity.

Results and discussion

The CCD 1 obtained results indicated that the enzyme was produced in a range of 0.01-118.62 U mL^{-1} (Table 1).

The results showed that the maximum enzyme production was achieved in run 2, with 5.0% agave syrup and 1.5% yeast extract, after 96 h of fermentation, resulting in a productivity of 118.62 U mL^{-1}. The second high production, 71.05 U mL^{-1}, was obtained after 72 hours of fermentation (run 7), with 3.0% agave syrup and 1.0% yeast extract.

It can be observed that the best production of inulinase was obtained at lower concentrations of agave syrup (runs 2 and 7), while the increase of the concentration resulted in negative effects, with a significant reduction of enzyme production, independent of the nitrogen source concentrations. These observations were confirmed by regression analysis where the linear terms of agave syrup ($p = 0.0268$) and the interaction between the variables ($p = 0.0459$) were negative and statistically significant at 5% ($p < 0.05$). Yeast extract at the studied concentrations did not influence the response (Table 2).

According to the ANOVA (Table 3), the *F*-value (11.9) shows that the used model was significant. The correlation coefficient (R^2) of 0.82 indicates that the proposed model can explain 82% of the obtained data variability, showing a good correlation between observed and predicted values.

According to the obtained results, the largest enzyme production within the studied range of substrates was found at low levels of the carbon source (-1 and -1.41) and high levels of the nitrogen source (+1 and +1.41).

A new round of CCD 22 was conducted with new concentrations of agave syrup (3.6 to 6.4%) and yeast extract (2.0 to 3.0%). According to the results (Table 4), the enzyme activity varied from 59.03 U mL^{-1} (run 8) to 144.166 U mL^{-1} (run 10). The maximum values were achieved in the runs of the center point (9, 10, and 11), at concentrations of 5.0% agave syrup and 2.5% yeast extract, with an average of 129.21 U mL^{-1}. Concentration changes of both variables to levels below or above the central point values resulted in a decrease on the enzyme production, showing that the established concentrations in this range were the most favorable.

Table 1. Central Composite Design 2^2 of inulinase production by *Kluyveromyces marxianus* NRRL-Y 7571, during 96 hours.

Runs	Coded values		Real values		Enzymatic activity (U mL^{-1})			
	X_1	X_2	Yeast extract (%)	Agave syrup (%)	24 h	48 h	72 h	96 h
1	-1	-1	0.5	5.0	2.12	1.189	0.516	0.164
2	1	-1	1.5	5.0	16.21	57.990	67.239	118.62
3	-1	1	0.5	10.0	1.35	0.719	0.411	0.278
4	1	1	1.5	10.0	1.47	14.95	1.57	1.11
5	-1.41	0	0.3	7.0	0.18	0.10	0.59	0.08
6	+1.41	0	1.7	7.0	5.05	9.07	12.48	0.10
7	0	-1.41	1.0	3.0	12.87	30.39	71.06	55.20
8	0	+1.41	1.0	17.0	0.01	0.11	0.12	0.19
9	0	0	1.0	7.0	0.11	0.16	0.89	0.22
10	0	0	1.0	7.0	0.84	1.03	0.95	0.37
11	0	0	1.0	7.0	0.70	1.09	0.91	0.68

Table 2. Regression coefficients of inulinase production by *Kluyveromyces marxianus* NRRL-Y 7571

Factors	Regression coefficient	Standard error	t(5)	p
Mean	0.359	12.856	0.028	0.9788
Yeast extract (L)	14.959	7.884	1.897	0.1163
Yeast extract (Q)	3.906	9.408	0.415	0.6952
Agave syrup (L)	-24.443	7.884	-3.100	0.0268
Agave syrup (Q)	17.791	9.408	1.891	0.1172
Yeast extract × Agave syrup	-29.406	11.134	-2.641	0.0459

L= linear; Q=quadratic; R^2=0.82

Table 3. Analysis of variance (ANOVA) of inulinase production by *Kluyveromyces marxianus* NRRL-Y 7571.

Source	Sum of square	DF	Mean square	F-value	Pr > F
Regression	11792.39	3	3930.7967	11.09	0.004
Residue	2479.2	7	354.17143		
Total	14271.59	10	4284.9681		

DF = Degree of freedom; R^2 = 0.82; $F_{3;7;0.05}$ = 4.35

Table 4. CCD 2^2 of inulinase production and biomass by *Kluyveromyces marxianus* NRRL Y- 7571, after 96 hours of culture.

	Variables				Measured Responses	
	Coded values		Real values		Enzymatic Activity ($U \cdot mL^{-1}$)	Biomass ($g \cdot L^{-1}$)
Run	X_1	X_2	Yeast extract (%)	Agave syrup (%)	96 h	
1	-1	-1	2.2	4.0	65.91	13.70
2	1	-1	2.8	4.0	71.07	17.24
3	-1	1	2.2	6.0	74.08	20.65
4	1	1	2.8	6.0	70.49	17.24
5	-1.41	0	2.0	5.0	103.42	14.67
6	1.41	0	3.0	5.0	73.64	12.32
7	0	-1.41	2.5	3.6	97.57	14.62
8	0	1.41	2.2	6.4	59.03	17.75
9	0	0	2.5	5.0	103.16	15.86
10	0	0	2.5	5.0	144.17	13.38
11	0	0	2.5	5.0	140.30	20.60

The results showed that there was 18% increase in the enzyme production at the second CCD central point and an enzyme production increment at all second CCD assays when compared to the first CCD. In both factorial designs, high concentrations of agave syrup resulted in a decrease of enzyme production, which could be related to catabolic repression. The production of inulinase is described to suffer from catabolic repression at high substrate concentrations, and therefore, the highest production of this enzyme is usually observed at the end of the growth phase (Parekh & Margaritis, 1985; Jing, Zhengyu, & Augustine, 2003; Cazetta, Monti, & Contiero, 2010; Singh & Lotey, 2010).

Table 5 shows, through regression coefficients, that both agave syrup (p = 0.02) and yeast extract (p = 0.04), in their quadratic terms, negatively affected the enzyme production. Therefore, variation of substrates concentrations from a minimum to a maximum decreased enzyme production, as could be observed in runs 5 and 6 for the nitrogen source and runs 7 and 8 for the carbon source (Table 3). The variables linear terms and their interaction did not show a significant influence on the response ($p > 0.05$).

Table 5. Regression coefficients of the inulinase production using agave syrup and yeast extract by *Kluyveromyces marxianus* NRRL Y-7571

Fators	Regression coefficients	Standard error	t(5)	p
Average	129.258	11022	11	0.0001
Yeast extract (L)	-5.065	7.206	-0.702	0.513
Yeast extract (Q)	-23.694	8.599	-2.755	0.04
Agave syrup (L)	-5.861	7.206	-0.813	0.452
Agave syrup (Q)	-28.839	8.599	-3.353	0.02
Yeast extract × Agave syrup	-2.185	10.176	-0.214	0.838

L=linear; Q=quadratic

According to the ANOVA (Table 6), the *F*-value (21.34) showed that the regression was statistically significant at the 95% confidence level. The determination coefficient (R^2=0.70) implies a satisfactory process representation by the model, in spite of the great variability inherent to biological processes involving enzymes and microorganisms, especially when using complex substrates. Equation 3 describes the enzyme production as a function of the variables studied in the re-standardized model, which contains only statistically significant terms:

$$\text{Enzyme activity} = 129.258 - 23.694 \cdot x_2 - 28.839 \cdot x_2^2 \quad (3)$$

Table 6. The ANOVA of the inulinase production by *Kluyveromyces marxianus* NRRL Y-7571, using agave syrup and yeast extract.

Source	Sum of square	DF	Mean Square	*F*-value	Pr > F
Regression	6090.356	1	285.426	21.337	0.001
Residual	2568.84	9	6090.356		
Total	8659.196	10			

DF = Degree of freedom; R^2 = 0.70; $F_{9;1;0.05}$= 5.12

The enzyme production was optimized at the center point (Figure 1), using 5.0% agave syrup and 2.5% yeast extract. The concentration ranges of the agave syrup and yeast extract, that were needed to reach the maximum inulinase production were wide, allowing variation around the optimal point and keeping the process in an optimized condition. This is very important, since it allows a greater variability of the substrate amount without reducing the production, especially with regard to complex substrates, in which a considerable variation in composition can occur.

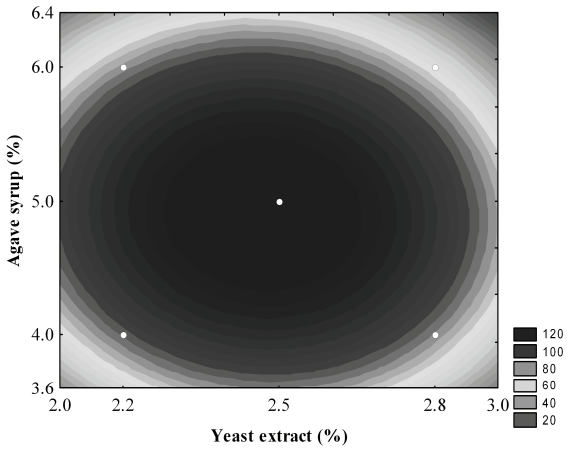

Figure 1. Contour curves of Central Composite Design 2^2, showing the inulinase production optimization by *Kluyveromyces marxianus* NRRL Y-7571.

The results obtained in the present study are similar or superior to those obtained in studies where defined substrates, such as sucrose (Kalil, Suzan, Maugeri, & Rodrigues, 2001; Silva-Santisteban & Maugeri, 2005) or commercial inulin (Kumar, Kunamneni, Prabhakar, & Ellaiah, 2005; Singh, Sooch, & Puri, 2007; Singh & Lotey, 2010) were used, which are more expensive than agave syrup. According to Silva-Santisteban, Converti, & Maugeri. (2009) and Chen et al. (2011), inulinase production can vary widely, because its biosynthesis depends on the used sources of carbon and nitrogen, on their medium concentration, as well as on the used microorganisms and strains. Considering this, the agave syrup proved to be a promising substrate for inulinase production by *K. marxianus*.

Regarding the biomass, it was observed that its maximum production reached 20.0 g L^{-1} and showed no significant influence on the variations of carbon and nitrogen source concentrations in the studied ranges. The enzyme production was not followed by biomass production, suggesting that the former is not associated with cell growth (Table 4).

The profiles of optimum pH and temperature for inulinase activity in the crude enzymatic extract are shown in Figures 2a and b. The enzyme showed the best enzymatic activities at temperatures from 45 to 60°C, with the optimum temperature being 60°C. The maximal activity was reached at pH 4.0, but the enzyme was still more than 80% active in the pH range from 2.0 to 5.0. At pH values above 6.0, the activity declined, and at alkaline pH (8.0 to 10.0), the enzyme activity was close to zero. Other authors have also reported these pH and temperature optimums for inulinase produced by *K. marxianus* (Treichel et al., 2009; Cazetta et al., 2010; Risso et al., 2010). Inulinases of other fungal genera feature similar biochemical characteristics, such as the enzymes from *Pichia guilliermondii* (Chi et al., 2009), *Geotrichum candidum* (Erdal et al., 2011), *Aspergillus ochraceus* (Guimarães et al., 2007), and *A. niger* (Dinarvand et al., 2012; Yewale et al., 2013). These features are very interesting for the industrial sector, since more acidic pH values and high temperatures reduce the contamination risks (Yewale et al., 2013).

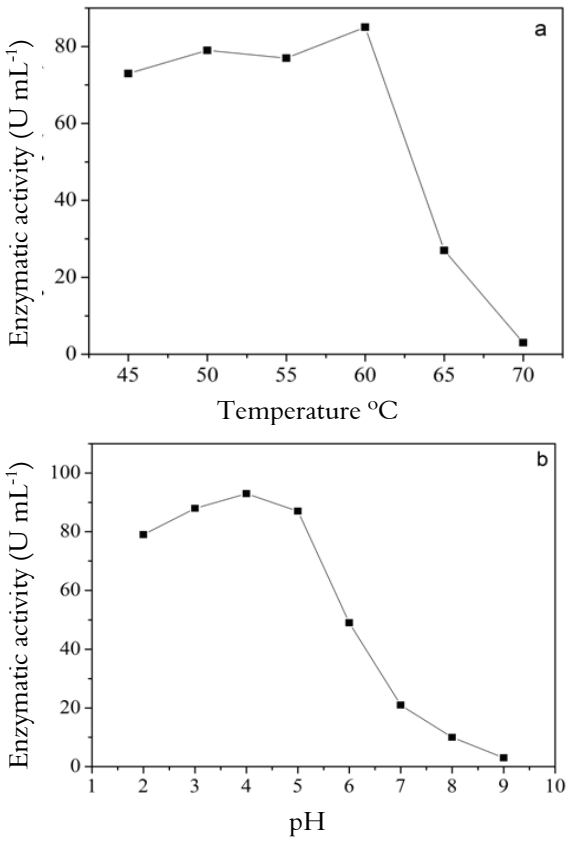

Figure 2. The effect of temperature (a) and pH (b) on the inulinase enzymatic activity from *Kluyveromyces marxianus* NRRL Y-7571, produced using agave syrup and yeast extract as substrates.

The enzyme showed good thermal stability at 50°C, maintaining its high activity for 5 hours at 55 and 60°C, the activity decreased after 15 min. at 60°C, a drastic drop in activity occurred after 1 h of incubation, and the activity was almost completely lost after 2 hours of incubation (Figure 3). In contrast to our results, there are reports in the literature showing that inulinases from *K. marxianus* are stable in a temperature range of 40 and 60°C, irrespective of the growth medium (Cazetta et al., 2005; Mazutti et al., 2010). The knowledge that the produced enzyme has a high thermal stability is very useful, not only for the product storage but also to minimize the losses during industrial processes.

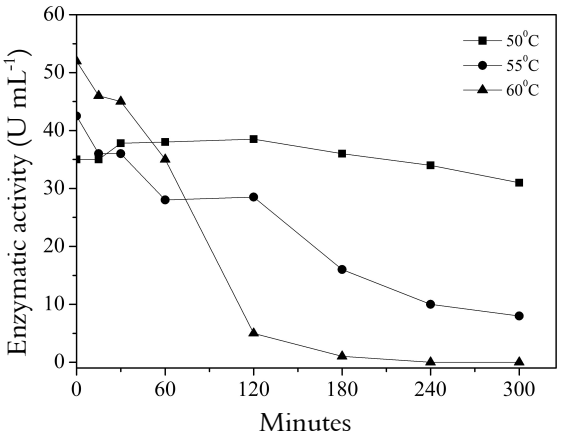

Figure 3. Thermal stability of inulinase produced by *Kluyveromyces marxianus* NRRL Y-7571, using agave syrup and yeast extract as substrates.

Conclusion

The combination of agave syrup with yeast extract proved to be a good substrate for inulinase production by *K. marxianus* NRRL Y-7571. The produced inulinase has presented interesting features for industrial applications, such as a low optimum pH and a high stability at elevated temperatures. The use of the factorial design was important for the enzyme production process optimization, with the best enzyme production achieved at the concentrations of 5% agave syrup and 2.5% yeast extract.

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