CHEMICAL ENGINEERING TRANSACTIONS Volume 24, 2011
Editor Sauro Pierucci
 ISBN 978-88-95608-15-0 ISSN 1974-9791
DOI: 10.3303/CET1124159

Influence of Spores Concentration, Moisture, Ammonium Sulphate Concentration and Temperature on Polygalacturonase Production Using Cashew Apple in the Solid State Fermentation Process

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The pectinases are one of the most used enzymes in food industry. The aim of this work was the polygalacturonases production using the cashew apple dry bagasse such as substrate and the microorganism *Aspergillus niger* CCT0916 in a solid state fermentation process, checking the influence of spores concentration, moisture, ammonium sulphate concentration and temperature, using a response surface methodology, observing the polygalacturonase activity response. The maximum polygalacturonase activity (33 U/g) was obtained with 50 %(w.b.), 10^6 spores per gram of humid medium, 1.5 %(w/w) of ammonium sulphate and 35ºC at 29 hours of fermentation. The model for 29 hours of fermentation is statistically significant with 95% confidence. The moisture is the variable that most influences the process, having a positive effect on response, as the temperature of fermentation. The concentration of inoculums resulted in negative effect and the nitrogen concentration do not show any effect on polygalacturonase activity.

1. Introduction

Pectinase is a general term for enzymes which hydrolyse pectic substances and according to their ability for substrate utilization (pectin and pectic acid) and operation mechanism (hydrolyze or trans-elimination) divided into different groups: polygalacturonase, pectin esterase, pectin lyse and pectate lyase (Bari et al., 2010). Pectic enzymes alone account for about one quarter of the word's food enzyme production (Gomes et al., 2010).

The polygalacturonase is the main hydrolytic enzyme. For most industrial uses, the polygalacturonase produced by fungi to be useful for the high activity and optimal activity at low pH range, serving for most applications in the food industry (Santos et al., 2008).

This work is a continuation of work done previously. Alcântara et al. (2010) studied the pectinases production using the cashew apple dry bagasse as substrate and the microorganism *Aspergillus niger* CCT0916 in a solid state fermentation process,

Please cite this article as: Alcantara S.R. and Silva F.L.H., 2011, Influence of spores concentration, moisture, ammonium sulphate concentration and temperature on polygalacturonase production using cashew apple in the solid state fermentation process, Chemical Engineering Transactions, 24, 949-954
DOI: 10.3303/CET1124159

verifying the influence of initial moisture and ammonium sulphate concentration. In the factorial design, maximum polygalacturonase activity (11 U/g) was obtained with 50 %(w.b.) of initial moisture and 0.5 %(w.w) of ammonium sulphate at 71 hours of fermentation.

The aim of this work was the optimization of solid state fermentation for polygalacturonases production using cashew apple dry bagasse as substrate and as fermenting agent the *Aspergillus niger* CCT0916, verifying the influence of amount spores inoculated, initial moisture of the medium, concentration of a nitrogen source and fermentation temperature.

2. Method and Materials

2.1 Substrate

Cashew peduncle bagasse was given by FrutNat, a fruit pulp industry at Brazil. Humid bagasse was dried with air renewal and circulation at 60ºC. After the drying process, the bagasse was ground at the TECNAL knife mill.

2.2 Physical-chemical characterization

Measurements of pH and moisture followed the standards Brasil (2005). Pectin amount (PC) was determined by gravimetric precipitation method using calcium pectate (Rangana, 1979). Reducing sugars (RS) were determined with 0.5 g of sample by DNS methodology (Miller, 1959) in spectrophotometer with glucose solution as standard. The concentration of soluble solids (SS) was obtained by direct reading refractometer in after adding 9 mL of distilled water to 1 g of dry bagasse. To determine the density, we used 100 g of material. This mass was placed in a graduate to determine the volume occupied, with no compression. Size distribution was performed using 100 g of residue in a Cotengo-Pavitest sieve shaker for 10 minutes in with 14, 20, 24, 35, 48 and 60 mesh trays. The result was expressed as weight percentage. All characterization was performed in triplicate. Based on the average (A), it was calculated the standard deviation (SD) and coefficient of variation [CV(%) = (SD/A)x100].

2.3 Fermentative process

The microorganism used was *Aspergillus niger* CCT0916, donated by Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA, Fortaleza – Brazil). Spore concentration was ajusted according to experimental design.

The substrate was hydrated with distilled water to obtain the moisture content and it was diluted ammonium sulphate in this volume. In a 250 mL Erlenmeyer flask, were weighed 10 g of sterilized humidified medium. After spore inoculation, the medium was incubated at temperature by experimental design for 78 hours.

Enzyme extraction for the fermented complex was performed by adding 2.5 mL/g of fermented medium using 200 mM acetate buffer pH 4.5. The samples were then left in water bath for 1 hour at 30ºC and filtered on Wattman 1 filter paper.

One unit of polygalacturonase activity was defines as the amount of enzyme that releases 1 µmol of galacturonic acid per minute of reaction at 35ºC for 30 minutes.

A 4x2 factorial experimental design was conducted with 4 experiments at the centre point to determine the influence of spores concentration (E), initial moisture (U),

ammonium sulphate concentration (N) and fermentation temperature (T_f) on enzyme activities response (Table 1).

Table 1: Concentrations and tests from factorial design

Tests	Variables			
	U %(w.b.)	E (mL/g)	N %(w/w)	T_f (ºC)
1	30 (-1)	10^6 (-1)	0.5 (-1)	25 (-1)
2	50 (+1)	10^6 (-1)	0.5 (-1)	25 (-1)
3	30 (-1)	10^8 (+1)	0.5 (-1)	25 (-1)
4	50 (+1)	10^8 (+1)	0.5 (-1)	25 (-1)
5	30 (-1)	10^6 (-1)	1.5 (+1)	25 (-1)
6	50 (+1)	10^6 (-1)	1.5 (+1)	25 (-1)
7	30 (-1)	10^8 (+1)	1.5 (+1)	25 (-1)
8	50 (+1)	10^8 (+1)	1.5 (+1)	25 (-1)
9	30 (-1)	10^6 (-1)	0.5 (-1)	35 (+1)
10	50 (+1)	10^6 (-1)	0.5 (-1)	35 (+1)
11	30 (-1)	10^8 (+1)	0.5 (-1)	35 (+1)
12	50 (+1)	10^8 (+1)	0.5 (-1)	35 (+1)
13	30 (-1)	10^6 (-1)	1.5 (+1)	35 (+1)
14	50 (+1)	10^6 (-1)	1.5 (+1)	35 (+1)
15	30 (-1)	10^8 (+1)	1.5 (+1)	35 (+1)
16	50 (+1)	10^8 (+1)	1.5 (+1)	35 (+1)
17	40 (0)	10^7 (0)	1.0 (0)	30 (0)
18	40 (0)	10^7 (0)	1.0 (0)	30 (0)
19	40 (0)	10^7 (0)	1.0 (0)	30 (0)
20	40 (0)	10^7 (0)	1.0 (0)	30 (0)

3. Results and Discussion

3.1 Physical-chemical characterization

Table 2 shows the parameters observed and standard deviations for the physical-chemical characterization of cashew apple dry bagasse.

Table 2: Physical-chemical characterization of cashew apple dry bagasse

Parameter	Unit	Value ± SD	CV (%)
Moisture	%w.b.	15.23 ± 0.13	0.85
pH	---	3.58 ± 0.19	5.31
RS	g/100g	33.02 ± 1.30	3.94
SS	ºbrix	35.00 ± 0.00	0.00
PC	%calcium pectate	14.26 ± 1.03	7.22
Density	g/mL	0.646 ± 0.006	0.92

The pH and density found for the cashew bagasse are close to the values cited in the literature. The drying of bagasse at low moisture content was obtained for subsequent updating of content to values corresponding to the experimental design, though that is not observed deterioration of nutrients in it, being a fully fermentable material. The values of reducing sugar also consistent with those observed in the literature for cashew in the region, unlike the observed values for quantity of pectin (Santos et al., 2008; Alcântara et al., 2010). The soluble solids are higher than those reported in the literature (Brandão et al., 2003; Matias et al., 2005).

3.2 Influence of moisture, spore concentration, ammonium suphalte concentration and temperature on polygalacturonase activity (fermentation kinetics)

Table 3 shows the values obtained for polygalacturonase activity (PG) for each test of the factorial experimental design, at intervals of about 16 and 8 hours alternately.

The highest polygalacturonase activity (33.27 U/g) was found with 50 %(w.b.) of initial moisture content, 10^6 spores per gram of humidity medium, 1.5 %(w/w) of ammonium sulfate and 35ºC at 29 hours of fermentation.

From the regression of polygalacturonase activity data and observed factors, it was construted a first-order models to represent it with 95% confidence. However, only the model for 29 hours of fermentation (Equation 1) was considered statistically significant, and the coefficients in bold were statistically significant.

$$PG = \mathbf{6.34} + \mathbf{6.62U} - 2.48E + 0.32N + 1.33T_f - \mathbf{3.28UE} - 0.48UN + 2.12UT_f - 0.46EN - 3.02ET_f + 0.58UT_f \quad (1)$$

Model validation was performed using the Test F. This test shows the ratio between the calculated F and tabulated F. Whenever this ratios is greater than 1, the regression is statistically significant, with the relationship between the independent variables. For a regression is not only statistically significant but also useful for predictive purposes, the value of the ratio should be at least greater than 4 (Barros Neto et al., 1996). The tabulated F for 95% confidence level was equal to 2.97. Since, the calculated F was equal to 4.07. Thus, the Test F was equal to 1.37.

The coefficient of determination of Equation 1 was equal to 0.8027, knowing that the greatest possible value for R^2 is 1, which means that between the curve and the experimental points, there was any residue and that all the variation around the average is explained by the regression (Santos et al., 2008).

Table 3: Polygalacturonase activities (PG) obtained in the experimental design

Test	Fermentation time (hours)						
	5	21	29	46	54	70	78
1	0	1.06	0	0	0	0	0
2	0	0.13	12.79	9.49	14.93	5.63	5.73
3	0	0	0	0	0	0,42	0
4	0	0	12.58	15.91	4.28	7.43	4.20
5	0.59	0	0	0	0	0	0
6	0	4.92	9.41	6.42	15.38	13.13	10.40
7	5.68	9.88	6.37	5.13	0	1.11	0.53
8	7.29	10.78	7.51	0.74	0.53	1.80	0
9	0	0	0	0	0	0	0
10	1.90	12.70	23.70	24.23	18.05	15.33	13.53
11	0	0	0	0	0	0	0
12	0	6.71	7.69	5.18	4.57	3.46	3.28
13	0	0.92	0	0	1.66	0	0
14	0	23.49	**33.27**	8.09	10.73	4.57	7.19
15	0	0	0	0	0	0	0
16	0	8.93	5.26	8.43	2.77	11.31	6.79
17	0	0	0	0	1.51	2.01	1.04
18	0	0	5.07	0	0	0	2.43
19	0	1.04	0	10.28	3.02	1.65	0.69
20	0	0	0	10.99	11.64	14.29	14.08

Figure 1 indicates the profile of the curve that represents the synergistic effect of the studied factors on the response. This figure shows the influence of fermentation temperature and ammonium sulphate concentration on the response polygalacturonase activity, fixing the initial moisture in the upper level (50 %w.b) and the spores concentration in the lower level (10^6 spores/g).

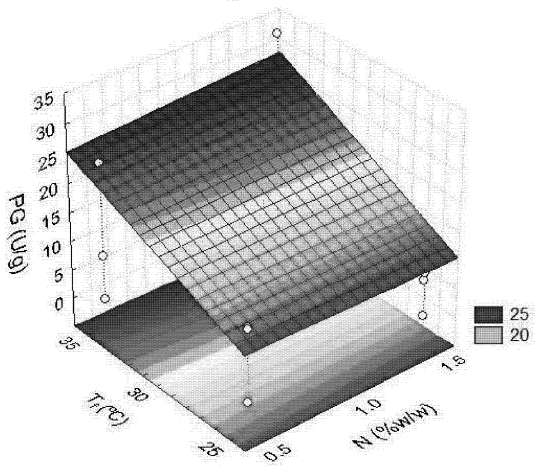

Figure 1: Surface response for polygalacturonases activity (PG) at 29 hours of fermentation: influence of ammonium sulphate concentration (N) and temperature of fermentation (T_f)

It was observed that the moisture has a greater influence on polygalacturonase activity. The highest polygalacturonase activity obtained by the model (25.77 U/g) was achieved in the higher levels for moisture (50 %w.b.) and temperature (35ºC) and inferior to the spore concentration (10^6 spores per gram of humidity medium). The nitrogen concentration had no significant influence on the response.

4. Conclusion

The highest polygalacturonase activity (33.27 U/g) was found with 50 %(w.b.) of initial moisture content, 10^6 spores per gram of humidity medium, 1.5 %(w/w) of ammonium sulfate and 35ºC at 29 hours of fermentation. The model for 29 hours of fermentation was considered statistically significant with 95% of confidence. It is observed that the moisture has a greater influence and the ammonium sulphate concentration had no significant influence on polygalacturonase activity.

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