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Pectinases production by solid state fermentation with cashew apple bagasse: water activity and influence of nitrogen source

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Pectinases are one of the most used enzymes in food industry, mainly in the extraction, clarification and the removal of pectin from fruit juices. The aim of this study was to produce pectinases using the cashew apple dry bagasse as substrate and the microorganism *Aspergillus niger* CCT0916 in a solid state fermentation process, verifying the influence of water quantity and nitrogen source. In a previous substrate characterization, it was observed that sugars (20.26 g/100g) and pectin (8.39%) concentrations needed to be adjusted. Adsorption isotherms were constructed and adjusted and the GAB model was the best fit. In the factorial design, maximum polygalacturonase and pectinolytic activities (11 U/g) were obtained with water activity above 0.99. There was inhibition by the presence of ammonium sulphate at concentrations from 1.5% (w/w), had made negative effect on the enzymatic activities. Initial moisture, however, had positive effect. The highest enzymatic activity values were obtained with 50 %(w.b) of initial moisture and 0.5 %(w/w) of ammonium sulphate.

1. Introduction

Solid-state fermentation (SSF) is defined as a process that occurs on a non-soluble material that acts both as support and a source of nutrients, with a reduced among of water, under the action of fermenting agent (Couto and Sanromán, 2006).
The most important factor is water, since this is limited in this process. The amount of water is related with two variables: moisture and water activity. Moisture is defined as the percentage of water in the total mass of medium. Water activity (aw) is defined as the amount of water available for microbial growth (Del Bianchi et al., 2001). Water activity could be related to substrate moisture through sorption isotherms for a given temperature.
Many residues have been used in bioproducts production through fermentation process. One of these residues is the cashew apple (*Anacardium occidentale* L.), which is rich in sugars, organic acids and fibre. Although Brazil is the second largest producer of cashew, however the apple consumption is still limited despite the fact that the juice is usually accepted by the population.

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As one of the most widely used industrial enzymes, pectinolytics were pioneers in the preparation of wines and fruit juice. According to Antier et al. (1993), the SSF technique was considered more susceptible to higher yields pectin estereases and polygalacturonases.
The aim of this work was to produce pectinases using the cashew apple dry bagasse as substrate and the microorganism *Aspergillus niger* CCT0916 in solid-state fermentation process, verifying the influence of water quantity and nitrogen source.

2. Methods and Materials

Substrate
Cashew peduncle bagasse was obtained natural cashew fruit acquired at Empresa de Abastecimento de Serviço Agrícolas (Empasa) in the city of Campina Grande City, Brazil. First, the cashew nut was removed. Next, the peduncle was triturated and pressed to separate the juice. Humid bagasse was dried with air renewal and circulation at 60ºC. After the drying process, the bagasse was ground at the TECNAL knife mill.

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. 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.

Adsorption isotherms
Adsorption isotherms were constructed using the gravimetric state method with saturated solutions at 25, 30, 35 and 40°C (Rockland and Beuchat, 1987). Temperatures were supplied by B.O.D model 347. The BET (Brunauer et al., 1938) and GAB (Simatos and Milton, 1985) were adjusted to experimental data. Better adjustment was determined by average relative deviation (P) and the coefficient of determination (R^2).

Fermentative process
The microorganism used was *Aspergillus niger* CCT0916, donated by Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA, Fortaleza - Brazil). Spore concentration was 10^7 spores per gram of wet medium.
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 30ºC for about 79 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 30°C and filtered on Wattman 1 filter paper.
One unit of polygalacturonase activity was defined as the amount of enzyme that releases 1 µmol of galacturonic acid per minute of reaction ate 35°C for 30 minutes.

The pectinolytic unit was defined as the amount of enzyme able to reduce the initial viscosity of pectin 1% (w/v) solution by 50% in 10 minutes of reaction ate 35°C.
A 2x2 factorial experimental design was conducted with 3 experiments at the centre point to determine the influence of ammonium sulphate concentration (N) and initial moisture (U) in the medium on enzyme activities (Table 1).

Table 1 Concentrations and tests from the factorial design

Tests	Variables	
	N (% w/w)	U (% w.b.)
1	0.5 (-1)	30 (-1)
2	1.5 (+1)	30 (-1)
3	0.5 (-1)	50 (+1)
4	1.5 (+1)	50 (+1)
5	1.0 (0)	40 (0)
6	1.0 (0)	40 (0)
7	1.0 (0)	40 (0)

3. Results and discussion

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
Moisture	%d.b	11.69±0.19
pH	---	3,66±0.032
RS	g/100g	20.26±0.22
PC	%calcium pectate	8.39±0.21

The pH value found is close to those cited in literature (Santos et al., 2008). For moisture, water must be added for water activity above 0.93 (Antier et al., 1993). The RS value is greater than that reported in literature (Matias et al., 2005), as observed by the amount of pectin (Santos et al., 2008). The results suggest that the addition of inducer pectin to this residue fraction to increase the enzyme activity. It was observed that 60% of residue was retained in 24 and 35 mesh trays, corresponding to 0.7 and 0.42 mm, values compatible with those found in the literature (Santos et al., 2008).

Adsorption isotherms
Table 3 shows parameters average percentage deviation (P) and coefficient of determination (R^2) values for each temperature.

Table 3 Setting parameters of adsorption isotherms of cashew apple dry bagasse

Modelo		Temperature (°C)			
		25	30	35	40
BET	Xm	5.64	5.61	6.33	6.07
	C	12.59	11.28	39.72	49.85
	n	14.39	15.02	13.35	13.18
	R^2	0.9998	0.9986	0.9948	0.9941
	P (%)	0.86	2.70	4.79	4.11
GAB	Xm	7.35	6.95	7.14	6.81
	C	5.64	6.00	7.14	6.81
	k	0.8886	0.9104	0.9137	0.9180
	R^2	0.9989	0.9983	0.9971	0.9962
	P (%)	1.97	2.34	2.13	3.53

Both models are appropriately adjusted to experimental data, because the P values indicates good fit when P is less than 10%, while R^2 should be close to one (Lomauro et al., 1985). However, the best was the GAB model (Figure 1). It can be observed that monolayer moisture (Xm) of the BET model increased with temperature. The adsorption isotherms are type II curves with sigmoid shape, following the Brunauer's classification.

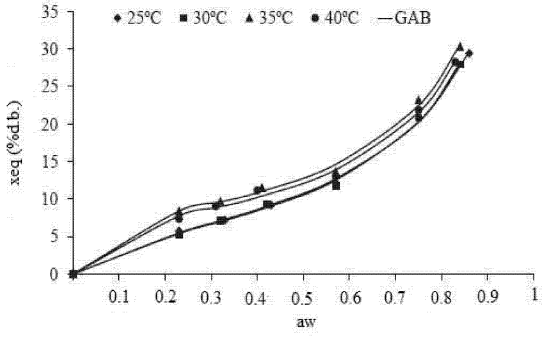

Figure 1 GAB model for adsorption isotherms of cashew apple dry bagasse

The literature contains several studies that report initial water activity and *Aspergillus niger* development in SSF in pectinases production. Thus, to produce this bioproduct with cashew apple dry bagasse as substrate, making a correlation with water activity (0.93), substrate moisture must be greater than 35 %d.b (Antier et al., 1993).

Influence of initial moisture content and ammonium sulphate concentration on enzymatic activities

Table 4 shows the values obtained for polygalacturonase (PG) and pectinolytic (Pt) activities for each test of the factorial experimental design, at intervals of about 15 and 8 hours alternately.

Table 4 Polygalacturonase (PG) and Pectinolytic (Pt) activities obtained in the experimental design

Test	Fermentation time (hours)													
	7.5		23		31.5		47		55.5		71		79	
	PG	Pt	PG	Pt	PG	Pt	PG	Pt	PG	Pt	PG	Pt	PG	Pt
1	0.2	0.0	0.0	0.0	2.1	0.2	6.3	0.0	2.2	0.0	0.0	0.0	1.1	0.0
2	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
3	0.3	0.0	7.3	0.8	8.1	1.2	10.3	4.0	8.4	3.8	10.9	5.3	7.8	3.1
4	0.2	0.0	0.4	0.1	1.2	0.4	2.0	1.6	3.5	2.7	1.5	1.9	4.8	2.6
5	0.0	0.0	1.2	0.9	9.1	5.2	7.3	11.1	9.6	4.9	9.4	4.0	7.6	5.2
6	2.2	0.0	3.4	0.0	0.5	5.4	7.2	0.4	4.5	3.4	6.5	1.1	2.0	0.8
7	0.0	0.0	1.7	0.2	2.8	0.8	0.2	0.9	7.1	9.2	7.4	11.0	1.6	0.5

The highest polygalacturonase activity was found with 50 %w.b. of moisture and 0.5 %(w/w) of ammonium sulphate at 71 hours of fermentation. For the pectinolytic activity, the highest value was obtained with moisture content of 40 %w.b. and 1 %(w/w) of ammonium sulphate at 47 and 71 hours of fermentation, corresponding to water activity above 0.99 for both enzyme activies.

For the data obtained (Table 4), first-order regressions were used with 95% confidence level for each fermentation time. However, only the Equation 1, which describes polygalacturonase activity during fermentation at 23 hours, was significant with the confidence level applied.

$$PG=\mathbf{1.99-1.72N+1.91U-1.72NU} \quad (1)$$

The coefficient signs indicate that the concentration of ammonium sulphate had a negative effect on enzyme activities, whereas moisture had a positive effect. Terms in bold are significant for 95% confidence level. The coefficient of determination (R^2) for Equation 1 was 0.9368.

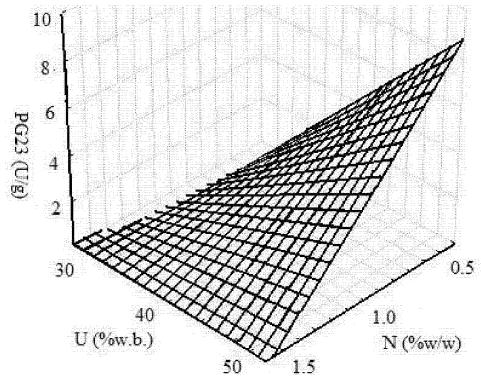

Figure 2 Surface response for polygalacturonase activity at 23 hours of fermentation

To have a high polygalacturonase activity, the medium must have high moisture content and low ammonium sulphate concentration, over the range studied (Figure 2).

4. Conclusion

Physical-chemical characterization indicates that the parameters reducing sugar and pectin must be adjusted. The best fit for adsorption isotherms was GAB model. Maximum polygalacturonase and pectinolytic activities were obtained with water activity above 0.99. This means that the moisture content was 50 %w.b. and 0.5 %(w/w) ammonium sulphate concentration.

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