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# Solid state fermentation process for polygalacturonase production using cashew apple

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**Abstract:** The aim of this work was the optimization of solid state fermentation for polygalacturonases production using cashew apple dry bagasse as substrate and *Aspergillus niger* CCT0916. For this, it was observed the influence of moisture content, spore concentration, ammonium sulfate concentration and fermentation temperature on polygalacturonase activity. It was observed that moisture is the limiting factor in the process. Maximum polygalacturonase activity (33 U/g) was obtained with 50 %w.b initial moisture, $10^6$ spores/g, 1.5 %(w/w) ammonium sulfate and temperature of 35°C. The models for 21, 29 and 54-hours of fermentation were statistically significant at 95% confidence level.

**Keywords:** Pectinase, *Anacardium Occidentale* L., Response surface methodology, *Aspergillus niger*

## 1. Introduction

In solid state fermentation process, several factors are decisive for the production of desired bioproduct. One of the most important is the medium composition. The appropriate balance between nitrogen sources is important for nutritional requirements of microorganism as well as the effects of environmental conditions on mycelial growth [14].

However, the limiting factor is water present in the system. The amount of water, expressed by moisture content, corresponds to the water percentage in total mass of medium. Its determination in process is closely related to the substrate nature, the needs of organism and the type of product desired [13].

Temperature is also considered a critical factor due to the accumulation of metabolic heat generated during fermentation, directly affecting microorganism germination and product formation [13].

Reference [5] observed the influence of spore concentration on the fermentation process. They found that a smaller spore concentration resulted in a prolonged lag phase. This variable is also important because it is linked with enzyme biosynthesis.

Pectinase is a general term for enzymes that hydrolyze pectic substances. According to their ability for substrate utilization (pectin and pectic acid) and operation mechanism (hydrolyze or trans-elimination), this enzymes could be divided into different groups: polygalacturonase, pectin esterase, pectin lyase and pectate lyase [3]. Pectic enzymes alone account for about one quarter of food enzyme production worldwide [9].

Polygalacturonase is the main hydrolytic enzyme. For most industrial uses, fungal polygalacturonase is useful for high activity and optimal activity at low pH range, serving for most applications in the food industry [16].

Cashew farming is one of the most important economic activities in Northeast Brazil, generating employment and income. The primary product derived from the fruit is its nut. Thus, several studies have emerged regarding full exploitation of this fruit, starting with the use of apple, making it a significant source of income.

This study is a continuation of earlier work. It was studied pectinase production using cashew apple dry bagasse as substrate and microorganism *Aspergillus niger* CCT0916 as microorganism in a solid state fermentation process, showing the influence of initial moisture and ammonium sulfate concentration. In the factorial design, maximum polygalacturonase activity (11 U/g) was obtained with 50 %(w.b) initial moisture and 0.5 %(w/w) ammonium sulfate at 71 hours of fermentation [2].

This study aim to characterize physicochemical cashew apple dry bagasse (*Anarcardium occidentale* L.) for its subsequent use as substrate in a solid state fermentation process, using *Aspergillus niger* CCT0619 as microorganism, by studying the influence of initial moisture content of the medium, inoculated spore concentration, ammonium sulfate concentration as nitrogen source and fermentation temperature on response polygalacturonase activity, using a $2^4$ factorial experimental design. Furthermore, it was observed the parameters of moisture, pH, reducing sugar and polygalacturonase activity along the evaluation of parameters throughout the fermentation.

# 2. Materials and Methods

### *2.1. Substrate*

Cashew apple bagasse was obtained from FrutNat, a fruit pulp company in Paraiba State, Brazil. Humid bagasse was dried in an over with air renewal and circulation at 55ºC. After the drying process, the bagasse was ground at TECNAL knife mill.

### *2.2. Analytical Determinations*

Measurements of pH, moisture content and mineral waste (MW) followed the standards Brazil [7]. The pectin amount (PC) was determined by the gravimetric precipitation method using calcium pectate [15]. Reducing sugars (RS) and saccharine were determined by HPLC (High performance liquid chromatography). Soluble solids concentration (SS) was obtained by direct reading refract meter after adding 9 mL of distilled water to 1g of dry bagasse. It was used 100 g of residue to determine the density. This mass was placed in a graduate to determine volume occupied, without compression. Size distribution was performed using 100 g of residue in a Cotengo-Pavitest sieve shaker for 10 minutes in 14, 20, 24, 35, 48 and 60 mesh trays. The result was expressed as weight percentage. Protein was determined by semi-micro Kjeldhal method for nitrogen adjusted by spectrophotometer [10]. All characterizations were performed in triplicate. The standard deviation (SD) was based on means values.

### *2.3. Microorganism*

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

### *2.4. Fermentation Process*

Substrate was hydrated with distilled water to obtain moisture content and ammonium sulfate was added to this volume. In 250 mL Erlenmeyer flask, it was weighed 10 g of sterilized humidified medium. After spore inoculation, the medium was incubated at fermentation temperature by experimental design for 78 hours.

### *2.5. Enzyme extraction and Polygalacturonase Activity (PG)*

Enzyme extraction for 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 through Wattman 1 filter paper.

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

### *2.6. Evaluation of parameters throughout the fermentation*

Tests for the evaluation were performed following the description in Table 1, using initial moisture content of the medium (U), spore concentration (E), ammonium sulfate concentration (N) and fermentation temperature (Tf) as factors. Moisture content, pH, reducing sugar and polygalacturonase activity parameters were observed for 78 hours of fermentation. Moisture content, pH and reducing sugars were determined following the method described previously.

### *2.7. Factorial Experimental Design*

A $2^4$ factorial experimental design was conducted with 7 experiments at the central point to determine the influence of spore concentration (E), initial moisture (U), ammonium sulfate concentration (N) and fermentation temperature (Tf) on polygalacturonase activity response (Table 1).

***Table 1.*** *Concentrations and tests from factorial design*

| Tests | U %(w.b) | E (mL/g) | N (%w/w) | Tf (°C) |
|---|---|---|---|---|
| 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) |
| 21 | 40 (0) | $10^7$ (0) | 1.0 (0) | 30 (0) |
| 22 | 40 (0) | $10^7$ (0) | 1.0 (0) | 30 (0) |
| 23 | 40 (0) | $10^7$ (0) | 1.0 (0) | 30 (0) |

# 3. Results and Discussion

### *3.1. Characterization of Cashew Apple Dry Bagasse*

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

***Table 2.*** *Physicochemical characterization of cashew apple dry bagasse*

| Parameters | Unit | Value ± SD |
|---|---|---|
| Moisture | %w.b | 15.23 ± 0.13 |
| MW | %w.b | 2.56± 0.04 |
| pH | --- | 3.58 ± 0.19 |
| RS | g/100g | 32.97 ± 0.00 |
| Saccharine | g/100g | 1.26 ± 0.00 |
| SS | °Brix | 35.00 ± 0.00 |
| PC | %calcium pectate | 14.26 ± 1.03 |
| Protein | % | 7.67 ±0.70 |
| Density | g/mL | 0.646 ± 0.006 |

The pH and density found for cashew bagasse are close to literature values [2,18]. Bagasse was dried until low moisture content (15.23 %w.b). This was subsequently increased to values corresponding with experimental design. No deterioration was observed during storage, demonstrating that the material is completely fermentable. Reducing sugar values were consistent with those observed in literature for cashew in region, differently to the observed for pectin [2]. Soluble solids were higher than those reported in literature [6,11]. The amount of protein observed in cashew bagasse was lower than reported in literature [1,16].

Fig. 1 shows size distribution of cashew apple dry bagasse residue. It is observed that 63 %(w/w) of bagasse was retained in the 20 and 24-mesh sieves, corresponding to 0.85 and 0.7 mm respectively. This particle size can be used in solid state fermentation process with *Aspergillus niger*, as described in literature [18,19].

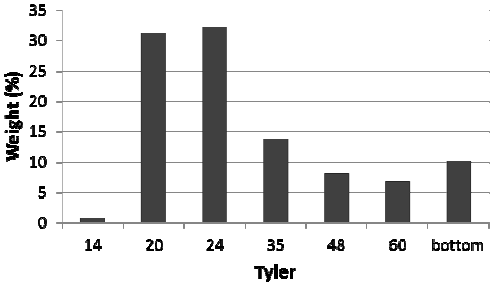


***Figure 1.*** *Particle size of cashew apple dry bagassse.*

### *3.2. Evaluation of parameters throughout the fermentation*

About kinetic fermentation, tests 1, 3, 5, 9, 11, 13 and 15, all with 30 %w.b, did not ferment. In these tests, it was observed that none of kinetic parameters varied significantly, remaining close to their initial values. Fig. 2 shows fermentation kinetics for test 1, representing the other tests mentioned.

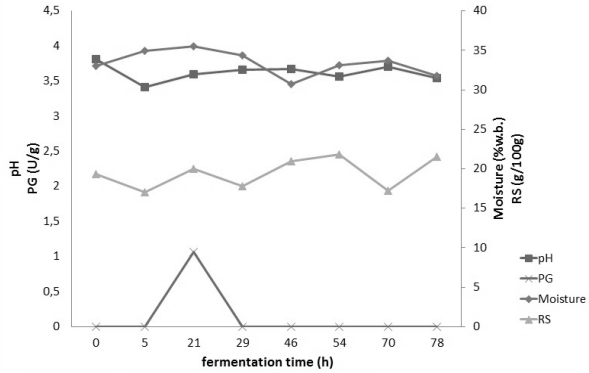


***Figure 2.*** *Fermentation kinetics of test 1 (U = 30 %w.b, E = $10^6$ spores/g, N = 0.5 %w/w, Tf = 25ºC).*

According to [8], in a medium without water available to microorganisms, fungi undergo changes in their cell membranes, leading to transport limitations, thereby affecting microbial metabolism.

The only test with 30 %w.b initial moisture which showed little enzyme production was test 7 (Fig. 3). For this test, the moisture kinetic showed a small increase, which can be attributed to microbial respiration. The pH and reducing sugar (RS) fell throughout kinetics. This confirms the consumption of RS by microorganism. Peak polygalacturonase activity (5 U/g) was observed at 46 hours of fermentation.

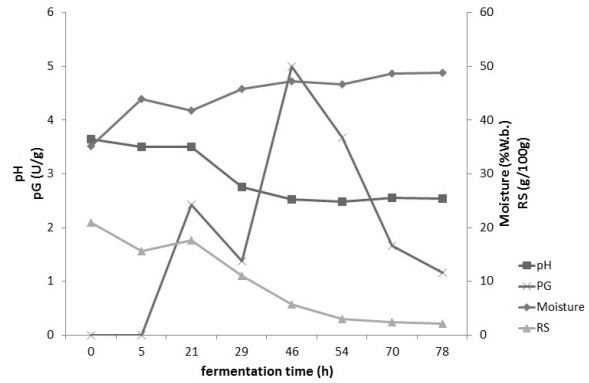


***Figure 3.*** *Fermentation kinetics of test 7 (U = 30 %w.b, E = $10^8$ spores/g, N = 1.5 %w/w e Tf = 250C).*

Moisture content remained unchanged in tests 2, 6, 14 and 16. Such tests have 50 %w.b initial moisture content. It was observed that pH decrease as of 21 hours of fermentation for all tests. This pH values could be related to the production of citric acid by the microorganism [5]. In addition, polygalacturonase produced is an acid enzyme. Reducing sugar was consumed at values close to zero in all tests, varying only the beginning of consumption.

In tests 2 and 6, peak polygalacturonase activity was observed at 54 hours of fermentation (15 U/g). These two tests have different ammonium sulfate concentration (0.5% and 1.5 %w/w, respectively), indicating that this variable has no influence the process when initial moisture content was 50 %w.b, spore concentration was $10^6$ spores/g and

fermentation temperature was 25ºC.

Test 14 showed increased polygalacturonase activity (33 U/g) (Fig. 4). Similar behavior was observed between pH and reducing sugars, since both values decreased as of 21 hours of fermentation.

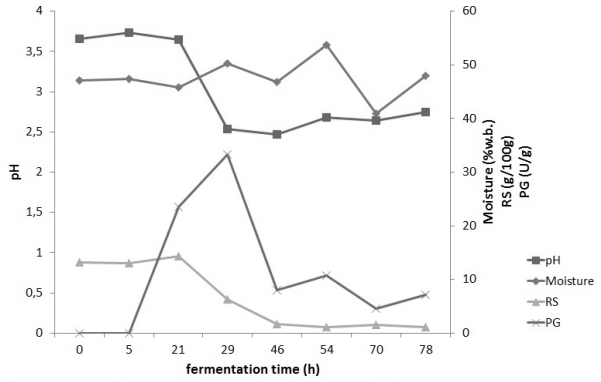


***Figure 4.*** *Fermentation kinetics of test 14 (U = 50 %w.b, E = $10^6$ spores/g, N = 1.5 %w/w, Tf = 35ºC).*

A comparison between tests 14 and 16, different inoculated spore concentration ($10^6$ and $10^8$ spores/g, respectively), shows that organism started production later (21 hours) with a lower amount of inoculum. However, with a peak at 29 hours, showing that consumption of RS was practically for enzyme production. In contrast, consumption of RS in first hours of fermentation for test 16 indicates that microorganism used it for maintenance and reproduction, since the peak of highest activity is observed only at 70 hours (Fig. 5).

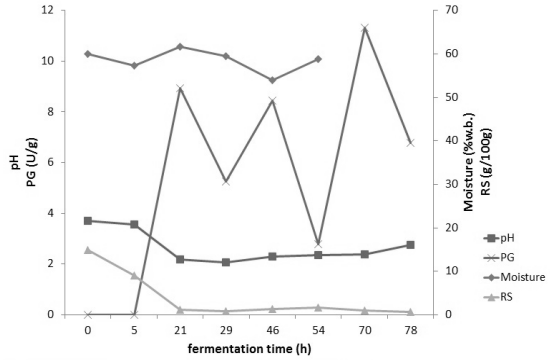


***Figure 5.*** *Fermentation kinetics of test 16 (U = 50 %w.b, E = $10^8$ spores/g, N = 1.5 %w/w, Tf = 35ºC).*

In tests 4, 8, 10 and 12, also with 50 %w.b initial moisture, a small increase throughout moisture kinetics as well as decrease in pH and RS was observed at the onset of fermentation. Peak polygalacturonase activity (10 U/g) occurred between 29 and 46 hours, except for test 10, with peak of 24 U/g over the same time period.

With respect to tests 17 to 23, there is little variation in moisture throughout kinetics. The pH and reducing sugars decreased during the fermentation, noting that enzyme production occurred in all tests. Polygalacturonase activities (PG) for these tests could be found in Fig. 6. These behaviors demonstrate the difficulty in reproducing experiments in solid state fermentation process. Reference [17] attributed this difficulty to substrate heterogeneity, usually organic residues.

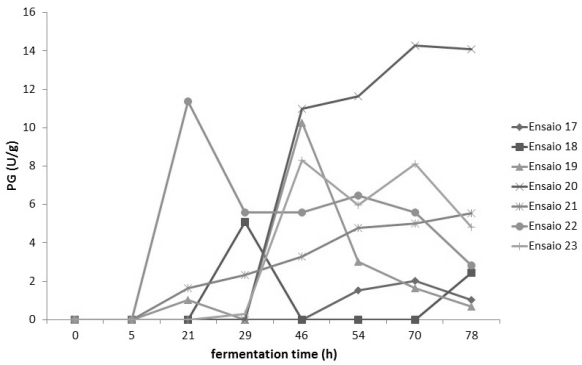


***Figure 6.*** *Kinetics of polygalacturonases activity (PG) for tests 17 to 23.*

Reference [18] observed moisture content, RS, pH and polygalacturonase activity until 72 hours of fermentation, using passion fruit peel as substrate and *Aspergillus niger* CCT0916 as microorganism, with 40 %w.b initial moisture and 1 %(w/w) ammonium sulfate concentration. Authors found that pH remains constant throughout kinetics. Maximum polygalacturonase activity (21 U/g) occurred at 66 hours of fermentation. Reducing sugars were consumed as of 22 hours of fermentation and moisture content did not significantly change.

### *3.3. Factorial Experimental Design*

Polygalacturonase activity was observed throughout the fermetation until 78-hours for all tests described (Table 1). The highest polygalacturonase activity (33.27 U/g) was found with 50 %(w.b) initial moisture content, $10^6$ spore/g, 1.5 %(w/w) ammonium sulfate concentration, fermentation temperature at 35ºC and 29 hours of fermentation.

***Table 3.*** *Empirical models for polygalacturonase activity in 21, 29 and 54-hours of fermentation.*

| Empirical models | $R^2$ | F calculated | F test |
|---|---|---|---|
| PG21 = 3.74 + 3.95U – 0.90E + 1.93N + 2.09Tf – 0.95UE + 1.64UN + 2.41UTf – 0.0006EN – 1.79ETf – 0.19NTF | 0.7464 | 3.533 | 1.285 |
| PG29 = 5.52 + 6.93U – 2.80E + 0.004N + 1.64TF – 2.97UE – 0.17UN + 1.81UTf – 0.77EN – 2.71ETf + 0.89NTf | 0.8000 | 4.800 | 1.746 |
| PG54 = 4.78 + 4.12U – 2.81E – 0.44N – 0.06Tf – 3.06UE – 1.11UN + 0.19UTf + 0.21EN – 0.08ETf – 0.49NTf | 0.8398 | 6.289 | 2.290 |

F tabulated for 95% confidence level = 2.75.

A first-order model was constructed based on regression of polygalacturonase activity data and observed factors with 95% confidence level. Model validation was performed using the F test. This test shows the ratio between calculated and tabulated F. When this ratio is greater than 1, regression is statistically significant with respect to the relationship between the independent variables. For a regression not only statistically significant but also useful for predictive purposes, the ratio between both Fs should be at least greater than 4 [4]. The tabulated F for a 95% confidence level, for this experimental design, was 2.75. Coefficient of determination ($R^2$) maximum value is 1, there was therefore no residue between the curve and experimental points and all the variation from the mean can be explained by the regression [16].

The 21, 29 and 54-hours fermentation models (Table 3) were considered statistically significant, as were the coefficients in bold face.

Fig. 7, 8 and 9 indicate the curve profile representing the synergistic effect of factors studied on the response. These figures show the influence of moisture (U), inoculated spore concentration (E), ammonium sulfate concentration (N) and fermentation temperature (Tf) on the response polygalacturonase activity (PG) for 21, 29 and 54-hours of fermentation, respectively.

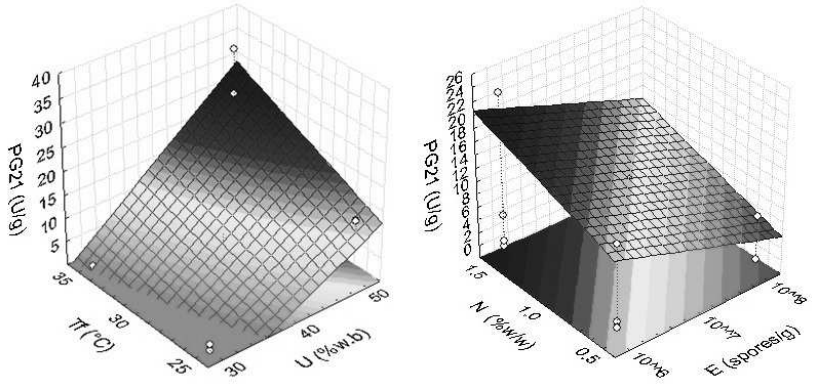


***Figure 7.*** *Surface responses for polygalacturonase activity at 21-hours of fermentation.*

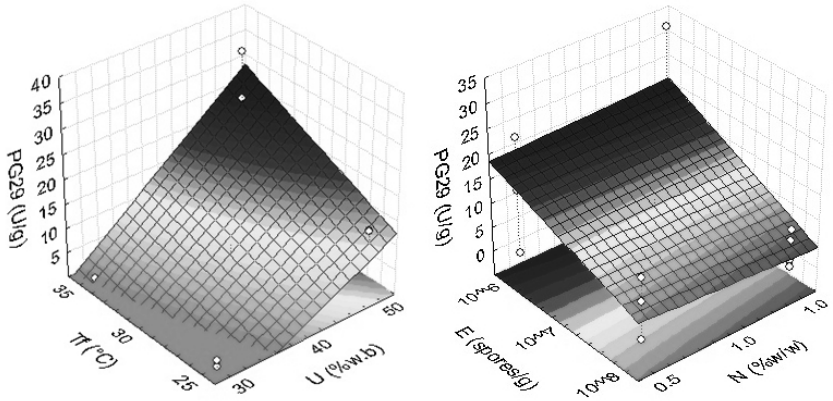


***Figure 8.*** *Surface responses for polygalacturonase activity at 29-hours of fermentation.*

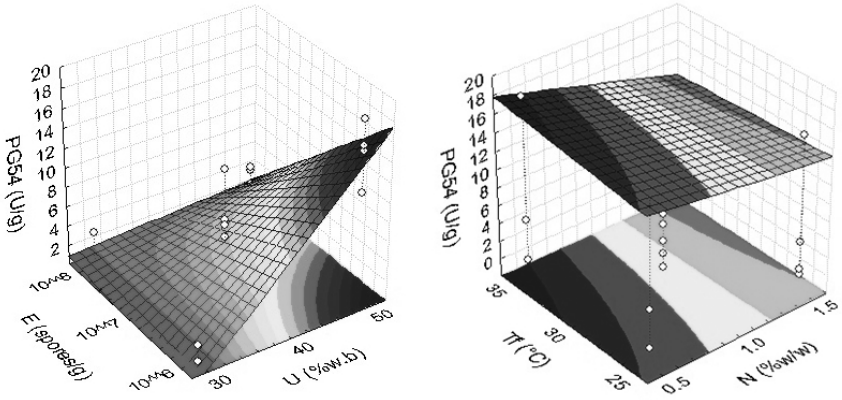


***Figure 9.*** *Surface responses for polygalacturonase activity at 54-hours of fermentation.*

In general, along the fermentation, moisture had a positive effect, being the most influential factor in process, confirming declarations reported in literature about the amount of water is a limiting factor [13,17]. Inoculated spores concentration had a negative effect, in the other words, highest activity calculated by the model was found in lower level of this concentration. Temperature also had a positive effect.

For ammonium sulfate concentration, there was differentiation according with fermentation time. For 21-hours, ammonium sulfate concentration had a positive effect. For 29-hours, the same concentration had no effect on polygalacturonase activity. And for 54-hours, it was observed that maximum activity calculated by the model on the lower level of experimental design.

Reference [5] studied the effect of temperature in solid state fermentation process, using *Aspergillus niger* A 163 and apple pomace as substrate, on drum bioreactor with 15 L of solid medium. Temperature was studied between the track 22–60°C, observing its influence on polygalacturonase activity. A process temperature of 35ºC was found to be the most suitable for polygalacturonase enzyme production.

Similar to that described in this paper, reference [3] studied the influence of ammonium sulfate concentration (0.25 – 0.45%), pH (4.82 – 6.12) and fermentation time (50 – 90h) on endopectinase production in solid state fermentation process, using date pomace and *Aspergillus niger* PC5. It was observed that ammonium sulfate concentration has a positive effect on enzyme activity but that this effect was negligible compared to fermentation time.

Polygalacturonases production using cashew bagasse as substrate is competitive with other residues reported in literature, such as passion fruit peel (21 U/g) [18], washed cashew apple dry bagasse (10.4 U/g) [16] and industrial date pomace (10.88 U/mL) [3].

# 4. Conclusion

It was observed that initial moisture is the limiting factor in solid state fermentation process, since significant changes in kinetic parameters behaviors are observed with variations of this factor. A challenge to overcome is the difficulty in reproducing the solid state fermentation process. The greatest polygalacturonase activity (33.27 U/g) was found with 50 %(w.b) initial moisture content, $10^6$ spores/g, 1.5 %(w/w) ammonium sulfate concentration and 35ºC fermentation temperature at 29 hours of fermentation, demonstrating that dry cashew apple is a promising substrate when compared to others reported in literature. The 21, 29 and 54-hours fermentation models were considered statistically significant at a confidence level of 95%. It was found that moisture content had a significant influence whereas ammonium sulfate concentration had no significant influence on polygalacturonase activity.


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