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# Scale Up of Polygalacturonase Production by Solid State Fermentation Process

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Additional information is available at the end of the chapter

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## 1. Introduction

Polygalacturonases are pectinolytic enzymes that catalyze the cleavage of polygalacturonic acid chain with the introduction of water through hydrogen bonds. These enzymes have technological, functional and organic applications on food processing and plant-fungus interactions.

They are produced by plants, fungi, bacteria and yeasts. However, the fungi are preferred in industrial scale, since about 90% of enzymes produced may be secreted into the medium and produce large amounts of enzymes [1,2].

Polygalacturonases involved in hydrolysis of pectic substances are endo-polygalacturonase and exo-polygalacturonase. Exo-polygalacturonase act on polygalacturonic acid monomers terminals, producing monogalacturonic acids. Endo-polygalacturonase randomly on polygalacturonic acid, producing oligogalacturonic acid [6].

Pectic enzymes alone are responsible for one quarter of food production enzymes in the world [3]. These are widely used in fruit juices industry to reduce viscosity, improve and increase the efficiency of filtration and clarification [4], preliminary treatment of grape wine industry, extraction of tomato pulp and among others applications [5].

For fruit juices production with pome (e.g. citrus), and red fruit (e.g. grape) extracts require addition of enzymes to convert viscous macerated or triturated fruit semi gelled (caused by the partial solubility of pectins and the ability high water retention of solids) to maximize the extraction of juice during pressing, subsequent step of process. Pectinases capable to degrade pectins depolymerize with high methylation are more suitable and include endopolygalacturonase and pectin methylesterase [7].

Principal application of enzymes that pectins hydrolyze is clarification or extraction of juices. The turbidity may be desirable in some juices (e.g. orange), but not for apple and grape juice, in which are translucent over sold. Turbidity is conferred by colloidal particles consisting of proteins coated with pectin. Pectinases depolymerize pectins, promoting flocculation and facilitating clarification [7].

Polygalacturonases produced by fungi are more active in pH range of 3.5 to 6.0 and temperature of 40-55°C. The practical result of activity of these enzymes is that middle lamella is disrupted and the viscosity of pectin solutions is decreased as the action of enzyme is maintained [7]. In fact, the characteristics of enzyme related with temperature and pH effects will depend on factors of production process, e.g. microorganism used, available nutrients, fermentation temperature, among others.

Industrially, pectinases are produced either by submerged fermentation and solid state fermentation with *Aspergillus niger* strains, however, the solid state fermentation technique is generally considered more susceptible to higher yields of pectin esterase and polygalacturonase. Some authors state that this preference occurs because the solid state fermentation allows the production of crude enzymes more concentrated and therefore a lower cost of extraction and purification [5,8].

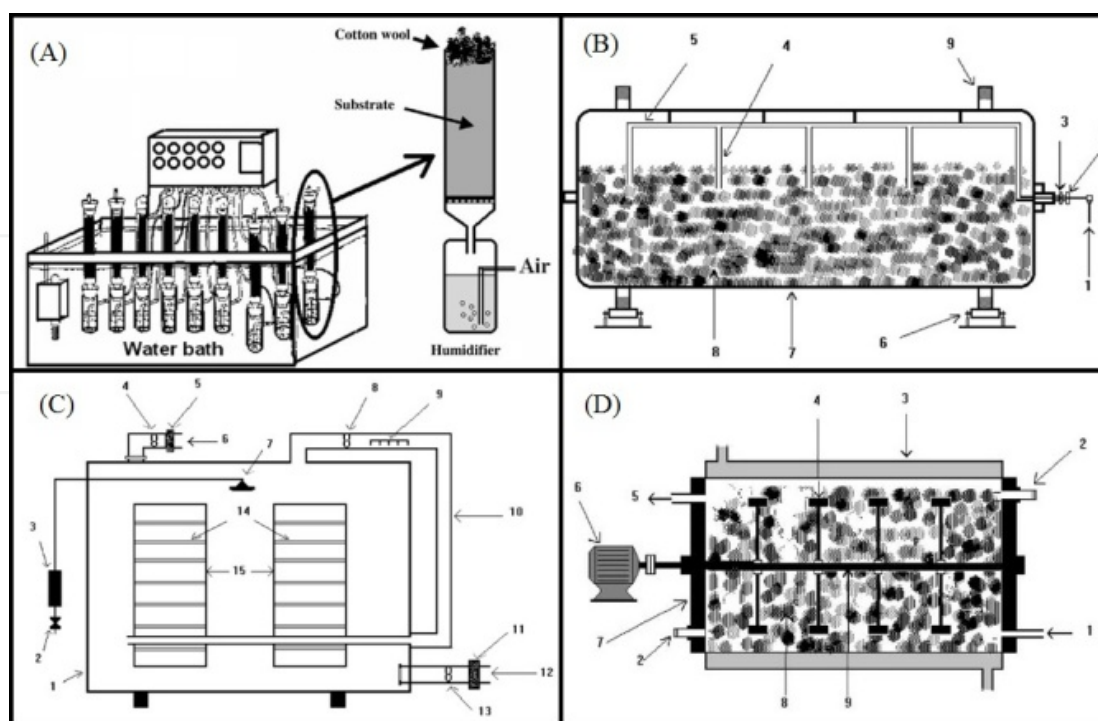
Solid state fermentation process is defined as a process that occurs over a non-soluble material, acting as support and nutrients source, with small quantity of water, under the action of fermenting agent [9].

One of the important factors in pectinases production by solid state fermentation is the medium composition. Appropriate balance between sources of nitrogen and carbon is so important to the nutritional requirement of microorganism that the effects of environmental conditions which affect mycelial growth [10].

Water in the system is a limiting factor. This amount of water is related to the medium through of moisture, as regards the percentage of water in total mass. Determination of its value in process is closely related to the substrate nature, requirements of microorganism used and type of end product desired. If moisture level is high, it will result in a decrease of substrate porosity and it will result in lower oxygen diffusion within the medium, consequently, decrease in gas exchange and increases the risk of contamination, especially bacterial. For lower levels of moisture needed by the microorganism, there will be greater difficulty in diffusion of nutrients, resulting in a lower growth and, consequently, lower production of desired product [11].

Temperature is also considered a critical factor, as well as moisture, due to the accumulation of metabolic heat generated during fermentation, which directly affects the microorganism germination and product formation. In composting process, this effect is desirable, however, for biotechnology processes, such as enzyme production, the heat must be dissipated immediately, so that temperature increase does not adversely affect the desired fermentation [11].

There are several types of reactors used in solid state fermentation process. Although there are many projects to industrial bioreactors, these have a limited extent for this type of process [12].



**Figure 1.** Type of bioreactors: (A) Column; (B) Rotating drum; (C) Koji-type; (D) Stirred horizontal [12].

Tray reactor consists of a chamber in which temperature and humidity air controlled circulates around a series of trays. Each tray contains a thin layer of depth. It is noteworthy that intermittent mixing of medium can be performed, but this generally occurs only once daily [13].

Scale-up using tray reactor cannot be simply by increasing the medium thickness, as this leads to problems of overheating. For this reactor type, scaling-up must be done by increasing the area of trays, which can be achieved using larger trays [14].

As described above, tray reactors are limited by heat transfer, but also by mass transfers, and may develop high temperatures and internal gas concentration gradients in height above 40 mm of substrate [15]. Still about the medium temperature, it is almost impossible to maintain this variable in the optimum value for production. However, fermentative process will vary in temperature that can be of 10°C above the optimum value [16].

Although there are numerous projects for industrial bioreactors, it is observed that these have limited development to processes using solid state fermentation. This occurs because there are some limitations of this process, such as the difficulty to remove heat generated by microbial metabolism, heterogeneity of mixture during fermentation, which makes the control of cell growth and various parameters such as temperature, pH, agitation, aeration, concentration of nutrients and products, making difficult the automation [17].

Despite these difficulties, the use of semi-solid medium may be advantageous, it allows the use of industrial residues (flours, bark and cake) as substrate, which is abundant raw material and low cost at Brazil.



**Figure 2.** Cashew fruit [22].

Industrial wastes have been used in bioproducts production through fermentation processes. Among these residues may be mentioned the cashew apple (*Anacardium occidentale* L.), which is rich in sugars, organic acids and fiber, that is why it has been used in the production of phenols [18], bioethanol, cashew wine [19], protein enrichment [20], pectinases [21], cellulases, among others.

Therefore, this study aims to characterize the scaling-up of solid state fermentation process for polygalacturonases production, using cashew apple dry bagasse as substrate, *Aspergillus niger* CCT 0916 as microorganism and tray reactor as operating system. For this, it will be performed to characterize of substrate used concerning the physical-chemical properties. It will be constructed and adjusted adsorption isotherms of substrate, showing the relationship with fermentation process. Initially, there will be characterization of factors that most influence the fermentation process in a laboratory scale. By setting these factors, it will be made to scaling-up of solid state fermentation process using tray reactor. Finally, it will be characterized the crude enzyme extract and its stability over temperature and pH.

## 2. Material and methods

### 2.1. Substrate

Cashew apple bagasse was obtained from fresh cashew fruit acquired at Empresa de Abastecimento e Serviços Agrícolas (EMPASA) at Campina Grande City, Brazil. First, cashew nut was removed. Next, apple was triturated and pressed to separate the juice. Humid bagasse was dried with air renewal and circulation at 55°C. After drying process, bagasse was ground in TECNAL knife mill.

## 2.2. Substrate characterization

Measurements of pH, moisture content and mineral waste (MW) followed the standards Brazil [23]. The pectin amount (PC) was determined by gravimetric precipitation method using calcium pectate [24]. Reducing sugars (RS) and saccharose were determined by HPLC (High performance liquid chromatography). Concentration of soluble solids (SS) was obtained by direct reading in refractometer after adding 9 mL of distilled water to 1 g of dry bagasse. It was used 100 g of material 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. Result was expressed as weight percentage. Protein was determined by semi-micro Kjeldhal method for nitrogen adjusted by spectrophotometry [25]. All characterizations were performed in triplicate. Standard deviation (SD) was based on means values.

## 2.3. Substrate adsorption isotherms

Triplicate samples were weighed approximately 1 g of product in aluminium crucibles and stored in airtight containers containing saturated salt solutions until reached the equilibrium moisture content for a certain relative humidity range (Table 1). Temperatures of 25, 30, 35 e 40°C were supplied by environmental chamber. Samples were weighed every 24 hours, reached constant weight. After that, samples were transferred to stove at 100°C for determination of dry weight. Equilibrium moisture content ( $x_{eq}$ ) was calculated by Equation 1.

$$x_{eq} = \left( \frac{m_i - m_s}{m_s} \right) \times 100 \quad (1)$$

In which:  $x_{eq}$  = equilibrium moisture, % dry basis;  $m_i$  = initial weight of sample, g;  $m_s$  = dry weight of sample, g.

Saturated salt solutions Temperature (°C)				
	Relative humidity (%)			
25	30	35	40	
K(C <sub>2</sub> H <sub>3</sub> O <sub>2</sub> )	23	23	23	23
MgCl <sub>2</sub>	33	32	32	31
K <sub>2</sub> CO <sub>3</sub>	43	42	41	40
NaBr	57	57	57	57
NaCl	75	75	75	75
KCl	86	84	84	83

**Table 1.** Equilibrium relative humidity of saturated salt solutions [26]



It was used the BET model [27] to fit experimental data (Equation 2):

$$\frac{x_{eq}}{x_m} = \frac{C \cdot a_w}{1 - a_w} \left[ \frac{1 - (n + 1)(a_w)^n + n(a_w)^{n+1}}{1 - (1 - C)a_w - C(a_w)^{n+1}} \right] \quad (2)$$

In which:  $x_{eq}$  = equilibrium moisture, % dry basis;  $a_w$  = water activity, adimensional;  $C$  = BET constant;  $x_m$  = moisture in the molecular monolayer;  $n$  = number of molecular layers.

Criteria used to observe the fit were the coefficient of determination ( $R^2$ ) between observed responses and predicted by the fitted model and average percentage deviation ( $P$ ) (Equation 3). The best fits were those with the highest  $R^2$  and lowest value of  $P$  [28].

$$P = \frac{100}{n} \sum_{i=1}^n \frac{|X_{exp} - X_{teo}|}{X_{exp}} \quad (3)$$

In which:  $n$  = observation numbers;  $X_{exp}$  = humidity of experimental material;  $X_{teo}$  = humidity calculated by adjusted models.

#### 2.4. Fermentation process in laboratory scale

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.

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

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

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

One unit of polygalacturonase activity was defined as the amount of enzyme that releases 1  $\mu$ mol of galacturonic acid per minute of reaction at 35°C for 30 minutes.

#### 2.5. Fermentation process using tray reactor

Microorganism used in this stage was also used for optimization in the laboratory scale. Spores concentration, moisture content and ammonium sulphate concentration were determined based on experiments performed on laboratory scale.

Substrate was hydrated with distilled water to obtain the initial moisture content and it was diluted ammonium sulphate in this water. On polypropylene trays (Figure 3), it was weighed 500 g of sterilized medium. Substrate thickness was equal to 40 mm. After spore inoculation, the medium was incubated at 23°C for 77 hours. Substrate thickness and fermentation temperature had been selected taking into account studies reported in literature [15,16].

Test	Variables			
	U %(w.b.)	E (mL/g)	N %(w/w)	T <sub>f</sub> (°C)
1	30 (-1)	10 <sup>6</sup> (-1)	0.5 (-1)	25 (-1)
2	50 (+1)	10 <sup>6</sup> (-1)	0.5 (-1)	25 (-1)
3	30 (-1)	10 <sup>8</sup> (+1)	0.5 (-1)	25 (-1)
4	50 (+1)	10 <sup>8</sup> (+1)	0.5 (-1)	25 (-1)
5	30 (-1)	10 <sup>6</sup> (-1)	1.5 (+1)	25 (-1)
6	50 (+1)	10 <sup>6</sup> (-1)	1.5 (+1)	25 (-1)
7	30 (-1)	10 <sup>8</sup> (+1)	1.5 (+1)	25 (-1)
8	50 (+1)	10 <sup>8</sup> (+1)	1.5 (+1)	25 (-1)
9	30 (-1)	10 <sup>6</sup> (-1)	0.5 (-1)	35 (+1)
10	50 (+1)	10 <sup>6</sup> (-1)	0.5 (-1)	35 (+1)
11	30 (-1)	10 <sup>8</sup> (+1)	0.5 (-1)	35 (+1)
12	50 (+1)	10 <sup>8</sup> (+1)	0.5 (-1)	35 (+1)
13	30 (-1)	10 <sup>6</sup> (-1)	1.5 (+1)	35 (+1)
14	50 (+1)	10 <sup>6</sup> (-1)	1.5 (+1)	35 (+1)
15	30 (-1)	10 <sup>8</sup> (+1)	1.5 (+1)	35 (+1)
16	50 (+1)	10 <sup>8</sup> (+1)	1.5 (+1)	35 (+1)
17	40 (0)	10 <sup>7</sup> (0)	1.0 (0)	30 (0)
18	40 (0)	10 <sup>7</sup> (0)	1.0 (0)	30 (0)
19	40 (0)	10 <sup>7</sup> (0)	1.0 (0)	30 (0)
20	40 (0)	10 <sup>7</sup> (0)	1.0 (0)	30 (0)
21	40 (0)	10 <sup>7</sup> (0)	1.0 (0)	30 (0)
22	40 (0)	10 <sup>7</sup> (0)	1.0 (0)	30 (0)
23	40 (0)	10 <sup>7</sup> (0)	1.0 (0)	30 (0)

**Table 2.** Concentrations and tests from factorial design





**Figure 3.** Tray reactor

Enzyme extraction for fermented complex was performed by adding 5.0 mL/g of fermented medium, using 200 mM acetate buffer pH 4.5. Samples were left in water bath for 1 hour at 30°C and filtered on Wattman 1 paper filter.

## 2.6. Enzymatic stability

To check the stability concerning temperature, crude extract samples were taken to a water bath for 20 min at temperatures between 10 and 90 ° C. For pH, crude extract was diluted in the buffers listed below, thus verifying the pH influence on polygalacturonase activity. After reaching the corresponding pH, samples were incubated for 24 hours at 2°C [46]: 0.1 M glycine-HCl pH 2.5; acetate buffer 200 mM pH 3.5-6.5; 0.1 M tris-HCl pH 7.5-8.5; 0.1 M glycine-NaOH pH 9.5. Results of thermostability and stability as to pH were expressed as relative activity (%).

## 3. Results and discussion

### 3.1. Physicochemical characterization of cashew apple dry bagasse

In a solid state fermentation process for enzymes production, it is important to know about the substrate composition, because the microorganism uses such as nutrient, for growth and reproduction so as to produce.

In case of pectinases, the inducing substance is pectin. Microorganism will adapt to the environment and for its maintenance, it will produce the enzyme needed to break these substan-

ces. Other substances also welcome in process are reducing sugars. These sugars (glucose, fructose, etc.) are sources of quick energy, and are also consumed by microorganism during fermentation process [21].

However, there must be a balance between nutrient sources. The literature indicates that high concentrations of sugars in fermentation medium, supplying the microorganism needs for its growth, and pectin little used, hence there is little enzymes production [29].

Cashew has on average 30% of reducing sugars (glucose and fructose) and about 10% of pectin in your composition. This implies the need for addition of inducer in a bagasse for use in a pectinases production.

Some authors observed that for pectinases production, using *Aspergillus niger* T005007-2 and wheat bran as substrate through a solid state fermentation process, the citrus pectin addition, up to 16% (w/w) led to increased enzyme production [30]. Based on these data, and in the proximity of same amount with sugar in cashew bagasse, it was decided not to add inductors in fermentation process.

In general, pH is an important variable in any biological process, with optimum values for microorganism growth. Generally, fungi prefer low pH between 4.5 and 5.0 [31,32]. Average value observed for cashew dry bagasse is 4.0, confirming proximity to other values found in the literature for cashew cake dry [17,33]. Thus, bagasse pH characterized can promote the pectinases production without requiring adjustment using buffer solution. Moreover, acid pH favors the storage at room temperature without contamination problems.

Bagasse was dried to below 15% (w.b), it became necessary to store a reasonable amount, noting that below 15%, the organic materials retain their properties over time and makes it difficult to contamination by bacterias.

Water amount in fermentation medium is a limiting factor and it directly affects the microorganism needs, and the final product type. To use this residue, it will be necessary to adjust the moisture content, since some microorganisms that produce pectinases require higher levels. This quantity of water is related with the environment via two variables: moisture and water activity.

Moisture regards percentage of water in the total mass of medium. And determination of this value in process is closely related to substrate nature, the requirements of microorganism used and the type of end product desired [11]. Water activity indicates that the organism may grow by fermentation, ensuring product quality.

Microorganism growth depends on the water activity, due to influence of osmotic pressure by on exchange membranes. And it can be related to moisture in the substrate used in fermentation using sorption isotherms for a given temperature [32].

Some authors [8,31] cite the use of various substrates for pectinase production by *Aspergillus niger* with water activity above 0.93. Importantly, low levels of water activity means low availability of water molecules near the cell, making exchange of solutes in solid phase, reducing metabolism and generating lower rates of growth or synthesis of metabolites. In con-

trast, high levels of water activity hinder the diffusion of air through solid particles, leading to a reduction in microbial growth [35]

Crude protein value is approximately 8% and it is close to the value observed by some authors [36,37]. This value is important for characterization as serve as a nitrogen source for microorganism. And from that data, it can be observed the necessity of supplementation with alternative sources of nitrogen such as urea or ammonium sulphate.

Physical characteristics with respect to substrate morphology are essential, particularly, in size and porosity, as these properties governing the accessible surface area of microorganism [17].

For granulometric distribution, 80% of bagasse was retained in sieves 20, 24 and 35 mesh, corresponding to particle size of 0.85, 0.70 and 0.42 mm, respectively. This particle size can be used in a solid state fermentation process by *Aspergillus niger*, which was already described in literature, particle sizes for pectinases production from 0.5 to 0.7 mm [39,40].

Average particle size of residual fermentation media must be obtained so that there have been no particles large or small. Particles of small size promote greater surface area and consequently a higher degree of processing. However, the process itself needs to have a particle size allowing the circulation of air through the mass and waste gases and heat produced, which could harm the efficiency of process [11]. Particles larger interparticles promote more space, reducing the efficiency of nutrients absorption for microorganisms. Furthermore, particle size analysis is important in enzyme complex extraction, since finely divided solid carriers facilitate access by the solvent [29].

It is important to remember that, in general, the crops characterization crops can vary dramatically depending on time of harvest, agricultural practices and phenomena related to planting. Is then very important to characterize the substrate for solid state fermentation process and therefore the adjustment of certain parameters, which is also an additional challenge is to be considered in using this process.

### 3.2. Adjust of adsorption isotherms from cashew apple dry bagasse

Data water activity ( $a_w$ ) and average equilibrium moisture ( $x_{eq}$ ) of material at temperatures studied (25, 30, 35 and 40°C) was adjusted BET model.

Table 3 are the values of equation BET parameters, average deviation percentage (P) and correlation coefficient ( $R^2$ ) for each temperature.

From Table 3, there is the BET model appropriately fit to experimental data because P value indicates a good fit when it is less than 10% and  $R^2$  must be as close to unity [38].

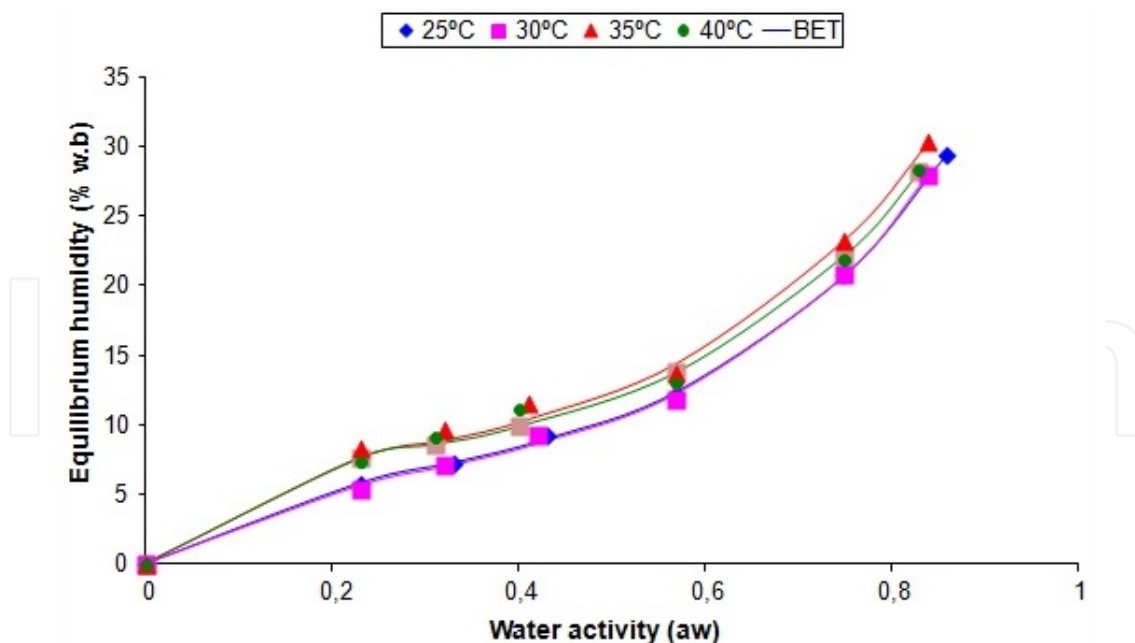
When comparing the monolayer humidity values of humidity ( $X_m$ ) by BET equation, it is noted that the range of 25-30°C to 35-40°C,  $X_m$  increased by approximately 0.4%. This is not a common behaviour, but can be explained by two mechanisms: (1) increase of temperature may cause changes in product physical structure, providing a larger number of active sites with affinity for water molecules, (2) or may cause an increase in intrinsic solubility of solute to the product, causing a greater number of water molecules is retained on monolayer [39].

Observed that the curves 25 and 30°C overlap, indicating a similar behaviour as regards the adsorption of water to these their temperatures, in which case this difference (5°C) showed no influence of temperature on these isotherms, unlike curves 35 and 40°C, in which there is small, but clearly influence of this variable. There is also a greater effect of temperature interval 25-30°C to 35-40°C. An analysis of the equilibrium moisture behaviour in relation to water activity shows similarity to temperature behaviour.

Can be found in the literature several studies that relate initial content of water activity with development of various microorganisms responsible for synthesis products obtained in solid state fermentation process.

In particular, *Aspergillus niger* is described as the organism that best fits to the fermentation process, being around 0.7 the value of minimum water activity for development of their metabolic activities [11].

Thus, minimum value being 0.7 from water activity to grow *Aspergillus niger*, when using cashew apple dry bagasse as substrate in solid state fermentation process and adsorption isotherms obtained (Figure 4), has an indicative that moisture in substrate at intervals of 25-30°C and 35-40°C should not be less than 17.5 and 19.0%(d.b), respectively.



**Figure 4.** BET model to adsorption isotherms of dry cashew apple bagasse

Parameters	Temperature (°C)				
	25	30	35	40	
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 <sup>2</sup>		0.9998	0.9986	0.9948	0.9941
P (%)		0.86	2.70	4.79	4.11

**Table 3.** Adsorption isotherms fitting parameters of cashew peduncle dry bagasse to the BET model

For pectinases production by *Aspergillus niger* in solid state fermentation process, several authors describe fermentation processes in which it can be seen that water activity which best favourable to synthesis of product is above 0.90. This implies that substrate moisture must be greater than 35%(d.b).

It is therefore extremely important to understand the hygroscopic behaviour of semisolid product used as substrate in a fermentation process, since the quantity of available water in through the microorganism to grow and synthesizing reactions during the production process is a limiting factor.

**3.3. Most influential factor in solid state fermentation process on laboratory scale**

As previously noted, there are many factors that affect a solid state fermentation process: amount of water available to microorganism can be quantified by moisture of medium (U), amount of inoculum necessary to overcome the adaptation phase and microorganism to produce the desired products (E), addition of nitrogen source such as ammonium sulphate (N) and fermentation temperature ( $T_f$ ). These are variables that will be studied as previously described. It was conducted an experimental design 2<sup>4</sup>, taking an answer the polygalacturonase activity. Objective was to determine which variable most affects the process and maximize the amount of enzyme produced.

The highest activities found for each assay and the fermentation time it was noted, are available in the Table 4. The greatest polygalacturonase activity (33.27 U/g) found, during the execution of experimental design, was obtained under the initial conditions: 50% (w.b) initial moisture, 10<sup>6</sup> spores/g, 1.5% (w/w) ammonium sulfate and 35°C at 29 hours of fermentation.

Test	PPG (U/g)	t (h)
1	1.06	21
2	14.93	54
3	0.42	70
4	15.91	46
5	0.59	5
6	15.38	54
7	5.00	46
8	10.78	21
9	0	---
10	24.23	46
11	0	---
12	7.69	29
13	1.66	54
14	33.27	29
15	0	---
16	11.31	70
17	2.01	70
18	5.07	29
19	10.28	46
20	14.29	70
21	5.53	78
22	11.37	21
23	8.30	46

**Table 4.** Highest polygacturonase activities (PG) observed for which solid state fermentation assay.



From regression of polygalacturonase activity data and values of factors studied, it was constructed a first order model with 95% confidence for peaks of enzymatic activities.

$$PPG = 8.66 + 7.80U - 2.50E + 0.86N + 0.88T_f - 2.76UE + 0.14UN + 1.56UT_f - 0.48EN - 2.52ET_f + 0.93NT_f \tag{4}$$

Model validation was done using Test F. This test shows the ratio between calculated value of F and F tabulated, knowing that the latter was equal to 2.75. When this ratio is greater than 1, regression is significant. Not only to a statistically significant regression, but also predictive value of ratio between this two F's must be greater than four [40].

Regarding the determination coefficient ( $R^2$ ), it is known that the maximum value is 1, meaning that between the experimental data and curve, there is no waste and any variation about the mean is explained by regression.

In Equation 4, calculated F was equal to 7.99. Thus, the ratio between calculated F and tabulated is equal to 2.91, meaning that this equation is statistically significant. The  $R^2$  was equal to 0.8695, showing a good fit of experimental data to this equation.

Figure 5 indicates the profile of curve representing synergistic effect of studied factors on peaks of polygalacturonase activity response. These figures show the influence of initial moisture content (U), concentration of spores in inoculation medium (E), ammonium sulfate concentration (N) and fermentation temperature ( $T_f$ ) on polygalacturonase activity response (PPG).

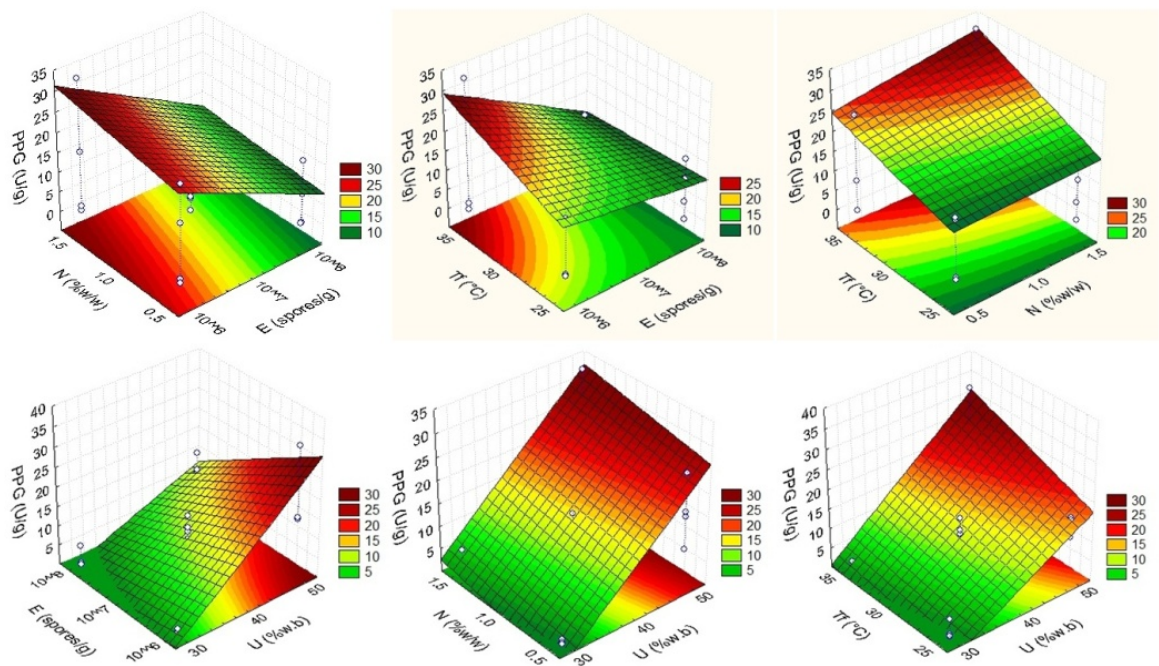
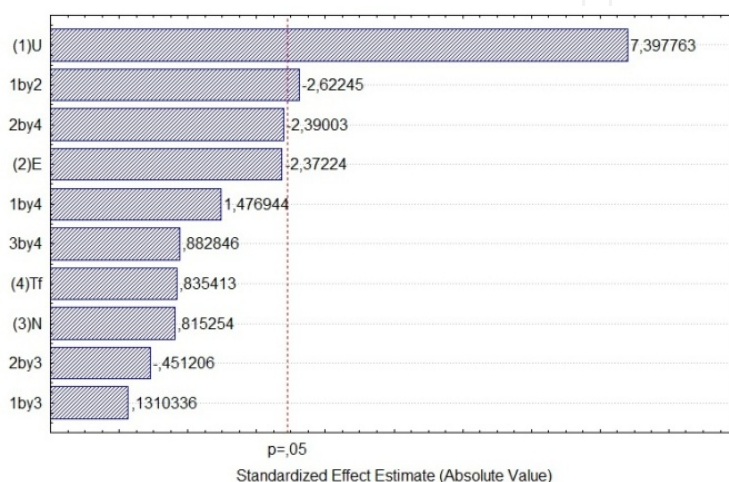


Figure 5. Response surface for polygalacturonase activity.

The highest peak of polygalacturonase activity calculated by the model (30 U/g) was obtained at higher levels for initial moisture content, for the lower concentration of inoculum and higher for fermentation temperature, observing that ammonium sulfate concentration did not influence significant on the response.

According to Pareto graphic (Figure 6), variable that most influenced fermentation process was initial moisture content of medium (U), and its interaction with variable concentration of inoculums (E), confirming claims reported in literature about the amount of water is thus a limiting factor [11,12].



**Figure 6.** Pareto graphic

Authors [41] examined the effect of temperature on a solid state fermentation process using *Aspergillus niger* 163 and apple pomace as a substrate in a bioreactor rotary drum with 15L of solid medium. Temperature was studied within the range 22-60°C, observing their influence on polygalacturonase activity. Inoculated spore concentration was equal to  $5 \times 10^8$  spores/ml. Temperature of 35°C was found to be more susceptible to production of polygalacturonases enzymes.

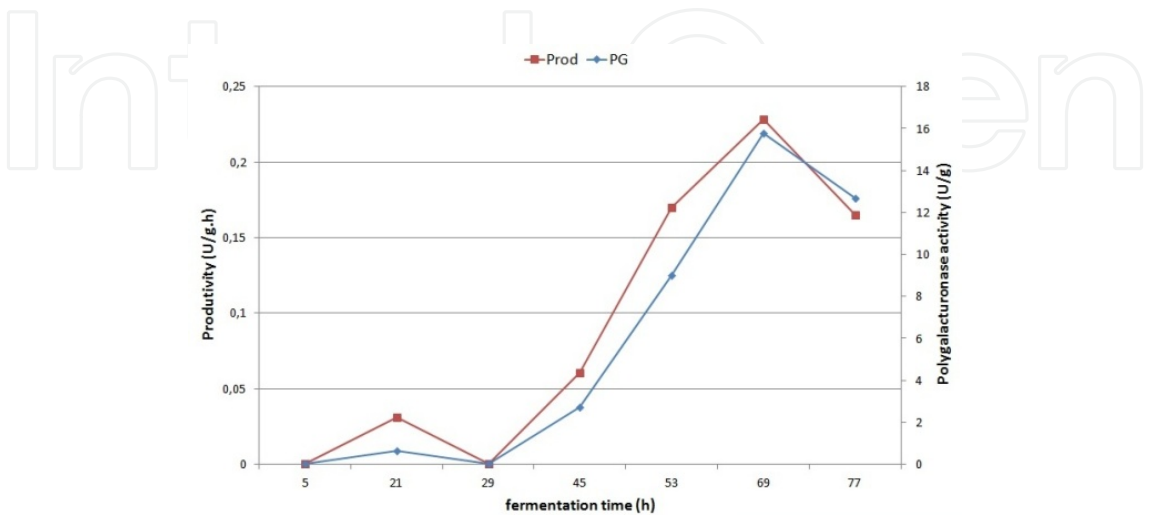
Similarly to what was described in this paper, authors [42] studied the influence of ammonium sulfate concentration (from 0.25 to 0.45%), pH (4.82 to 6.12) and fermentation time (50-90h) on endopectinase enzyme production in a solid state fermentation process, using as substrate apple pomace and *Aspergillus niger* PC5. It was observed that ammonium sulfate concentration have positive effect on enzymatic activity. However, the effect was insignificant compared to fermentation time.

### 3.4. Scaling-up of solid state fermentation process using tray reactor

It was evaluated the scale-up process using a tray reactor, setting the mass of humid medium in 500 g. Spores concentration, moisture content and ammonium sulphate concentration were determined based on experiments performed on laboratory scale. Thus the conditions of fermentation process was 50%(w.b) of initial moisture content,  $10^6$  spores/g of inoculum

concentration, 1.5%(w/w) of ammonium sulphate concentration, 40 mm of substrate thickness [15] and 23°C of fermentation temperature [16].

In Figure 7, there are the behavior of polygalacturonase activity (PG) and process productivity (Prod) as function of fermentation time.



**Figure 7.** Polygalacturonase activity and productivity versus fermentation time.

Under conditions described previously, it was observed a peak of polygalacturonase activity of 15.76 U/g at 69 hours of fermentation, corresponding to the highest productivity.

Comparing the maximum activity obtained with reactor tray, and maximum activity obtained at laboratory scale, it is clear that production was 45 times lower. In this regard, the first fact to note is the internal temperature of medium. When using tray, there is an increase of at least 10°C above of initial value. Thus, there may be denatured enzyme.

Other factors can change during fermentation. One is the medium moisture, because with increase of temperature, there is an increased in evaporation rate of water, reducing the amount available to microorganism, which complicates the production process.

It can be observed that the time for adaptation of microorganism is greatly increased. Remembering, the greatest polygalacturonase activity in laboratorial scale was obtained at 29 h of fermentation. In the fermentation with tray, the highest activity obtained was reached at 69h of fermentation.

When the subject is solid state fermentation process, a large number of studies were conducted in a laboratory scale. It is relatively easy to control certain parameters for enzyme production. On this scale, many productions show great promise. However, beyond this process to pilot-scale bioreactors, which contain greater amounts of substrate, difficulties in carrying out the fermentation are revealed. Thus, it is characterized one of difficulties in scaling-up solid state fermentation processes [28].

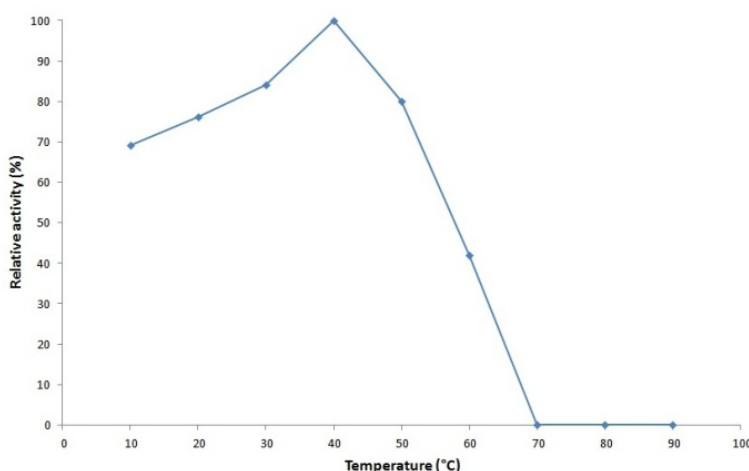
### 3.5. Enzymatic stability as temperature and pH

In summary, the enzyme is maintained by a delicate balance of noncovalent forces such as hydrogen bonds, ion pairing, hydrophobic interactions and van der Waals force [45]. Thus, variations in temperature and pH are important in the analysis of enzyme activity.

Temperature has the activation and deactivation effect on enzyme activity. Continued increases of temperature beyond the maximum or optimum for enzyme activity leads to protein denaturation, which involves the deployment of large segments of polypeptide chain [45]. At the other extreme, enzymes inactivation by cooling can occur when nonpolar forces are involved and association of polypeptides. Low temperature reduces the strength of such interactions can promote the dissociation of subunits and compromise enzyme activity [7].

With respect to pH, all of ionisable groups of proteins undergo transitions pH dependent on the basis of intrinsic pKa values of amino acid residues. Many of these transitions will cause impacts on the stability of the enzyme and, in a narrow range of pH, can act together to destabilize it completely.

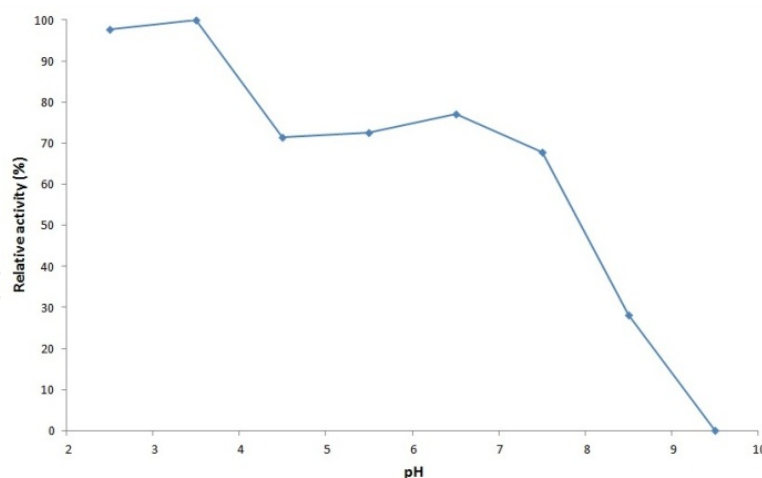
Knowing the pH and temperature stability is important for selection of enzymes compatible with the prevailing conditions for application potential so that the enzyme persist long enough to fulfil the expected function [7].



**Figure 8.** Thermostability of polygalacturonase enzyme

It can be observed (Figure 8) that temperatures of between 30 and 50°C, polygalacturonase activity remained with up to 80% of its maximum activity, and its optimum temperature at 40°C. After 50°C, relative enzymatic activity falls abruptly, reaching zero at 70°C.

Considering the greater use of pectic enzymes is in fruit juices industry, the processing of juices is normally done at 30-50°C. Thus, produced enzymes are been active during the process. For inactivation, the binomial time versus temperature must be considered, as well as chemical characteristics of juices. Usually, enzymatic inactivation is made between 70-90°C [7].



**Figure 9.** Stability of polygalacturonase enzyme in relation to pH

It is observed that the pH's of 2.5 and 3.5 (Figure 9), polygalacturonase activity was highest, almost 100%, characterizing the enzyme as acidic. Relative activity is equal to zero only at pH 9.5.

In literature some authors have reported the viability of various types of waste from processed fruits (apple, cranberry and strawberry), as substrates for polygalacturonases production, with *Lentinus edodes* as microorganism, using solid state fermentation process. These authors also observed the effects of temperature and pH on the enzymatic extract. Polygalacturonase produced has good thermal stability up to 50°C and high tolerance between pH 3.0 and 6.5 [46].

Other authors have studied the polygalacturonases production, using submerged fermentation with orange peel and passion fruit as substrate and *Aspergillus niger* as microorganism. In terms of stability, polygalacturonase produced showed the highest activity at 40°C and pH between 3.0 and 4.5 [48].

In general, polygalacturonase enzyme activity has maximum pH ranges between 3.5 - 6.0 and temperature between 40 - 55°C [7].

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]. Thus, enzyme extract studied can be applied in fruit juice processes, such as Barbados cherry (pH 3.3), orange (pH 3.0), apple (pH 3.6), passion (pH 3.4), peach (pH 3.3) and grapes (pH 3.1) [47].

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