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Optimisation of solid state fermentation of potato peel for the production of cellulolytic enzymes

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ABSTRACT

This paper analyses the effects of water content, temperature and time on the kinetic activity of cellulolytic enzymes produced during the solid state fermentation of potato peel, using *Aspergillus niger*. Three main analytical steps – analysis of variance, regression analysis and plotting of response surface – were performed to obtain an optimum condition for enzymatic activity. The statistical results indicated that the best activity time for enzyme CMCase (carboxymethylcellulase) is 82.88 h, with water content of 51.48% and temperature of 29.46 °C; for FPase (filter paperase), the best activity time is 80.62 h, water content of 50.19% and temperature at 30.00 °C; for xylanase, time is 81.92 h, water content is 50.72% and temperature is 28.85 °C. Pareto charts have shown that all variables were significant in enzymatic activity for CMCase and xylanase. On the other hand, FPase shows that time and temperature have significant effect for this response variable.

1. Introduction

Waste output and byproducts are inherent to all productive sectors. With the improvement of ecological awareness by the end of the 20th century, it became clear that humankind's major challenge for the coming decades is to balance the production of goods and services with economic growth, social equality and environmental sustainability (Galembeck, Barbosa, & Sousa, 2009; Pelizer, Pontieri, & Moraes, 2007). Environmental concern leads to the feasibility of projects that promote the sustainability of production systems. Contrary to what happened in the past when waste was improperly disposed of, today's concepts of minimisation, recovery and reuse of byproducts are being increasingly disseminated (Laufenberg, Kunz, & Nystrom, 2003).

In Brazil, the quantity of agro-industrial byproducts such as bagasse, bran, peel and seeds in general is expressive, and nowadays, concepts involving minimisation, recovery and reuse of such coproducts are being increasingly disseminated. In the last decade there was a significant increase in residue production in the potato processing industry, due primarily to the supply to the fast food industry (Pereira et al., 2005). These residues have high organic matter content. Approximately 40% of potatoes are wasted, representing approximately 10 tons/day of residue (Barampouti & Vlyssides, 2005; Misha & Arora, 2004). Much of these residues consist of polysaccharides such as cellulose, hemicellulose and lignin. Its use as feedstock for bioprocesses has therefore become feasible due to its low economic cost (Couto & Sanroman, 2006; Holker, Hofer, & Lenz, 2004; Soccol et al., 2010).

The cellulase hydrolysis process takes place via an enzymatic complex of cellulases (Cao & Tan, 2002). Such enzymes are biocatalysers working in synergy to release sugars. Of these, glucose attracts most of the interest from industry, due to the possibility of converting it into ethanol (Lee, Paul, Willem, Van, & Isak, 2002; Soccol et al., 2010). Cellulolytic microorganisms are known as true cellulolytic microorganisms, which are able to degrade natural cellulose. Free cellulases can be produced by fungi or bacteria, and fungi enzymes dominate commercial applications due to their high levels of expression and secretion (Chang, 2007; Dienes, Egyházi, & Réczey, 2004). The two main strategies for the production of cellulases are solid state fermentation (SSF) and submerged fermentation (SF), which differ with respect to environmental conditions and forms of conduction. One of the most exalted parameters in differentiating these types of processes is unquestionably the analysis of the volume of water present in the reaction (Mazutti et al., 2010; Pandey, 2003). The activity level of water for the purpose of ensuring growth and metabolism of cells, on the other hand, does not exceed the maximum binding capacity of the water with solid matrix. The filamentous fungus *Aspergillus* is considered of great economic importance due to its production of metabolites such as enzymes (Graminha, Gonçalves, Pirota, Balsalobre, & Gomes, 2008; Pelizer et al., 2007; Sharma, Chisti, & Banerjee, 2001).

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According to Arantes and Saddler (2010), the enzymatic hydrolysis of cellulose is catalysed by highly specific enzymes called cellulases, which are actually an enzyme complex composed of at least three major groups of cellulases: endoglucanases (EC 3.2.1.4), which randomly cleave the internal connections of the amorphous region, releasing oligosaccharides with reducing and non-reducing ends free; exoglucanases (EC 3.2.1.91), subdivided into cellobiohydrolases, which are responsible for the hydrolysis of terminal non-reducing and reducing. Xylanases (EC 3.2.1.8) are enzymes responsible for hydrolysis of xylan, which is the main polysaccharide constituent of hemicelluloses (Yang et al., 2006).

According to Granato, Ribeiro, Castro and Masson (2010), the optimal proportions among different variables can be achieved by changing one variable at a time; however, this approach is very laborious, often fails to guarantee the determination of optimum conditions, and does not depict the combined effect of all the factors involved. One option to overcome this problem is the use of response surface methodology (RSM). Response surface methodology is an efficient statistical method for the optimisation of multiple variables employed to predict the best performance condition. The main advantages of RSM are the reduced number and cost of experiments (Bidin et al., 2009). RSM has been extensively utilised to optimise culture conditions and medium composition of fermentation processes, conditions of enzyme reaction, and processing parameters in the production of food and drugs (Qiao et al., 2009; Rodriguez-Nogales, Ortega, Perez-Mateos, & Busto, 2007). There are several experimental designs that can be applied in food companies to test ingredients and/or to prepare or reformulate a new food product, including: full factorial design, fractional factorial design, saturated design, central composite design, and mixture design. Depending on the purpose, it is necessary to use a sequence of two or more designs (Granato et al. 2010).

In that context, this study aimed to apply response surface methodology in solid state fermentation on potato peel to obtain cellulolytic enzymes by the filamentous fungus *Aspergillus niger*.

2. Materials and methods

2.1. Materials

The residue was provided by an agro-industry located in the southeast region of Bahia state, then dried to 2% humidity in an oven with air circulation and renewal of forced (SOLAB SL 102, Piracicaba-SP, Brazil) at 70 °C for 24 h and ground in a mill Wiley type in the particle size of approximately 2 mm.

2.2. Solid state fermentation

The residue was sterilised in an autoclave vertical (PRISMATEC – CS30 – Itu – SP, Brazil) at 121 °C for 15 min. The microorganism used was *A. niger* from the Laboratory of Agro-industry Waste Reuse. The sporulated culture (inclined, acidified PDA HIMEDIA pH 5.02) was suspended in 1% Tween 80 (VETEC) solution. The number of spores in suspension was counted using a double mirror Neubauer chamber and a binocular microscope (BIOVAL L1000, São Paulo – SP – Brazil). The quantity of 10^7 spores per gram of dry basis substratum was added to the suspension. The solid-state fermentation occurred within a temperature range (25, 30, and 35 °C) and time (24, 72, and 120 h). The incubations were conducted in bacteriological incubator refrigerated (SOLAB SL 222/CFR Piracicaba, SP – Brazil).

2.3. Enzymatic extraction of the compounds

Following the fermentation process, the enzyme extract was mechanically extracted using a sodium citrate buffer solution (VETEC) with a pH of 4.8 at 50 mM. The enzyme extract that resulted from the fermentation was centrifuged at 80g for 10 min at 4 °C (CIENTEC CT – 6000R Piracicaba, SP – Brazil).

2.4. Determination of CMCase activity

The method chosen to determine the activity of CMCase and that represents the dosage of endoglucanases is based on the dose of reducing sugars produced (Ghose, 1987) by the degradation of carboxymethylcellulose (CROMOLINE) at 2% (p/v), previously diluted in a sodium citrate solution with pH of 4.8 at 50 mM. The dinitrosalicylic acid method has been used for quantification (DNS) (Miller, 1959). Reaction assays were conducted by adding 0.5 mL of sodium citrate buffer solution with a pH of 4.8 at 50 mM, 0.5 mL of enzyme extract, and 0.5 mL of CMC (2% per volume) to an assay tube. The reaction control was carried out in another tube, to which 0.5 mL of the same buffer solution and 0.5 mL of enzyme extract have been added. The blank assay contained 0.5 mL of DNS and 0.5 mL of buffer solution. The samples were incubated in a bacteriological incubator (SOLAB SL 222/CFR Piracicaba – SP – Brazil) at 50 °C and 10g, for 10 min. The reaction was interrupted by the addition of 0.5 mL of DNS. After that, the tubes were submerged into boiling water, for 5 min, and shortly after, 6.5 mL of distilled water were added for a subsequent measurement of absorbance – in the 540 nm range – carried out using a spectrophotometer (BEL PHOTONICS SF200DM – UV Vis – 1000 nm, Osasco – SP – Brazil).

2.5. Determination of FPase activity

The FPase activity, i.e., the filter paper activity, comprises a mixture of endoglucanases and exoglucanases resulting from the degradation of a strip of Whatman filter paper No.1, which measures are 1.0 cm × 6.0 cm (Ghose, 1987). One millilitre of a sodium citrate buffer solution with pH of 4.8 at 50 mM, 0.5 mL of enzyme extract and a filter paper strip have been added to the tube containing the reaction assay. Another tube received the addition of 1 mL of the same buffer solution and 0.5 mL of enzyme extract. The third tube, which was the substratum control, received the addition of a 1.5 mL buffer solution and a filter paper strip. The blank assay contained 0.5 mL of buffer solution and 0.5 mL of DNS; thus, the samples were left in an incubator at 50 °C for 1 h (SOLAB SL 222/CFR Piracicaba – SP – Brazil). The reaction was interrupted by the addition of 3 mL of DNS. The tubes were then heated in boiling water for 5 min and 20 mL of distilled water were shortly after added for the subsequent measurement of absorbance in the 540 nm range, and finally carried out using a spectrophotometer (BEL PHOTONICS SF200DM – UV Vis – 1000 nm, Osasco – SP – Brazil).

2.6. Determination of xylanase activity

The activity of the enzyme xylanase (Ghose, 1987) was determined according to Miller (1959). The reaction consists of mixing 1 mL of culture supernatant (enzyme extract), 1 mL of 1% xylan (SIGMA) in 0.05 M acetate buffer pH 5.0, and 2 mL of acid 3,5-Dinitrosalicylic (DNS) was incubated at 50 °C for 30 min (SOLAB SL 222/CFR Piracicaba – SP – Brazil), and enzyme–substrate system was shaken. The tubes containing the reactions were measurement of absorbance in the 540 nm range, and finally carried out using a spectrophotometer (BEL PHOTONICS SF200DM – UV Vis – 1000 nm, Osasco – SP – Brazil).

2.7. Calibration curve

The standard curve for CMCase and FPase was built from the determination of glucose concentrations from 0.1 to 2.0 g/L by the method of DNS (Miller, 1959). Xylanase for the curve was

Table 1
Coded levels and "real" values for each factor under study.

	Factorial planning of the one in codified values			Factorial planning of the one in real values		
Ensaio	Time (X_1, h)	Temperature (X_2, °C)	Water content (X_3, %m/m)	Time (X_1, h)	Temperature (X_2, °C)	Water content (X_3, %m/m)
1	−1	−1	−1	24	25	40
2	−1	+1	−1	24	35	40
3	+1	−1	−1	120	25	40
4	+1	+1	−1	120	35	40
5	−1	−1	+1	24	25	60
6	−1	+1	+1	24	35	60
7	+1	−1	+1	120	25	60
8	+1	+1	+1	120	35	60
9	0	0	−1	72	30	40
10	0	0	+1	72	30	60
11	−1	0	0	24	30	50
12	+1	0	0	120	30	50
13	0	−1	0	72	25	50
14	0	+1	0	72	35	50
15	0	0	0	72	30	50
16	0	0	0	72	30	50
17	0	0	0	72	30	50
18	0	0	0	72	30	50

constructed from the determination from 0.1 to 2 g/L xylose produced per minute. The unit of enzyme activity (U) was defined as the amount of enzyme capable of releasing 1 μmol reducing sugar per minute at 50 °C, where the enzyme activity expressed as U/mL. The absorbance was measured in a spectrophotometer (BEL SF200DM PHOTONICS – UV Vis – 1000 nm, Osasco – SP – Brazil) at 540 nm for CMCase and FPase, for xylanase was measured at 550 nm.

2.8. Statistical analysis

A 2^{3-1} fractional factorial planning added of 4 repetitions in the central point was implemented in order to evaluate the influence of temperature, water content and time in the enzymatic active of CMCase, FPase, and xylanase. The variable level values are shown in Table 1. Three main analytical steps – analysis of variance (ANOVA), regression analysis and plotting of response surface – were performed to obtain an optimum condition for the enzymatic active.

First, the results obtained from experiments were submitted to ANOVA Variance analysis, and effects were considered significant at $p < 0.02$. With a second order polynomial model (Eq. (1)), experimental data and regression coefficients were adjusted and regression coefficients were obtained by multiple linear regression

$$EA = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j + \sum \beta_{ijk} X_i X_j X_k \quad (1)$$

where β_0, β_i, β_{ii}, β_{ij}, and β_{ijk} represent the overall constant process effect, the linear and the quadratic effects of X_i, and the interaction effect between X_i and X_j, X_i, X_j, and X_k on enzymatic active, respectively. By the response surface methodology, best conditions of enzymatic active were determined for intervals of utilised experimental conditions. All statistical analysis was conducted using *Statistical Analysis System*® 9.0 version, RSREG procedure (SAS Institute Inc., Cary, NC, USA).

According to Granato et al. (2010), to validate the adjusted model, the optimised values of the independent variables (X_1 and X_2) should be used in the same initial experimental procedure, in order to verify the prediction power of the developed models by comparing theoretical predicted data to the experimental ones. In this work, triplicate of biotransformation using the optimised variables were prepared and analysed.

3. Results and discussion

In order to evaluate which factors had significant effect on the enzymatic active of CMCase, FPase, and xylanase, an ANOVA (Table 2) and parameters estimative analysis were conducted for the 2^{3-1} fractional factorial.

The analysis of variance (ANOVA) for the models was performed and the model significance was examined using Fisher's statistical test (F-test) applied to significant differences between sources of variation in experimental results, i.e., the significance of the regression (SOR), the lack of fit (LOF), and the coefficient of multiple determination (R^2). Since the full second-order models (models containing both parameter interactions) were not accepted by the mentioned tests, they were improved by the elimination of the model terms until the determined conditions were fulfilled. All factors that were not significant at 10% were then pooled into the error term and a new reduced model was obtained for response variables by regression analysis using only the significant factor previously listed.

The outcome of the ANOVA can be visualised in a Pareto chart (Fig. 1), in which the absolute value of the magnitude of the standardised estimated effect (the estimate effect divided by the standard error) of each factor is plotted in decreasing order and compared to the minimum magnitude of a statistically significant factor with 90% of confidence ($p = 0.10$), represented by the vertical dashed line. From this figure it can be observed that all variables were significant in the enzymatic active for CMCase and xylanase. On the other hand, the Pareto chart regarding the FPase active shows that time and temperature have a significant effect for this response variable.

For all cases, the interactions with the variables time, temperature, and water content were not significant to the enzymatic activity.

The reduced models can be described by Eqs. (2)–(4), in terms of uncoded values.

$$AC_1 = 25.61154 + 3.41369X_1 + 1.50245X_2 - 1.11489X_3 - 7.45472X_1^2 - 5.06567X_2^2 - 5.19840X_3^2 \quad (2)$$

$$AC_2 = 16.50989 + 2.04927X_1 + 0.22829X_2 - 5.20710X_1^2 - 6.18927X_2^2 \quad (3)$$

$$AC_3 = 16.32000 + 2.10063X_1 + 0.46313X_2 - 0.67402X_3 - 5.11916X_1^2 - 3.21701X_2^2 - 1.45959X_3^2 \quad (4)$$

where AC_1, AC_2, and AC_3 stand for the activity of CMCase, FPase, and xylanase, respectively.

Using the response surface method (RSM), with the temperature value fixed in the optimal condition, the relations between factors and response can be better understood, showing that time and water content affect the behaviour of enzymatic active. With

Table 2
ANOVA for the response surface quadratic model for cellulolytic enzymes production.

Source	DF	SS	MS	*F*-value	*p*-Value
AC_1					
Model	6	1135.49687	189.24948	12.29	0.0003
Error	11	169.32320	15.39302		
Lack of fit	8	137.4376666	17.1797083	1.62	0.3778
Pure error	3	31.885530	10.628510		
Total	17	1304.82007			
$R^2 = 0.8702$					
AC_2					
Model	4	490.83584	122.70896	28.40	<0.0001
Error	13	56.17044	4.32080		
Lack of fit	10	48.2108561	4.8210856	1.82	0.3407
Pure error	3	7.9595798	2.6531933		
Total	17	547.00628			
$R^2 = 0.8973$					
AC_3					
Model	6	370.7574623	26.4826759	31.68	0.0079
Error	11	10.51922	0.95629		
Lack of fit	8	8.0112153	1.0014019	1.20	0.4885
Pure error	3	2.5080035	0.8360012		
Total	17	373.26547			
$R^2 = 0.9718$					

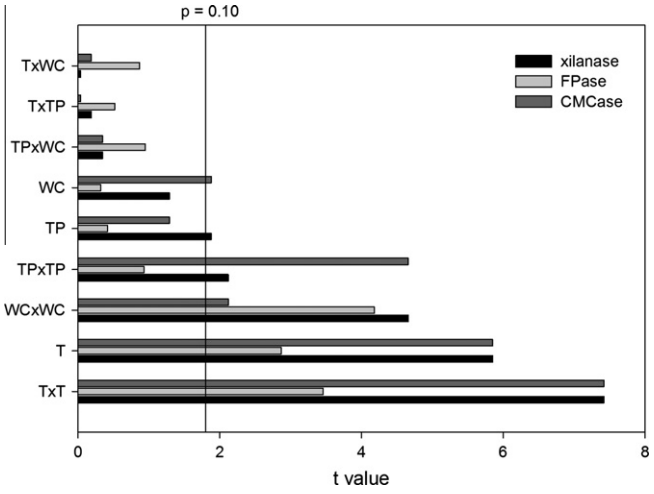

Fig. 1. Pareto chart showing the significance of variables time fermentation, temperature fermentation and water content on the CMCase, FPase and xylanase production.

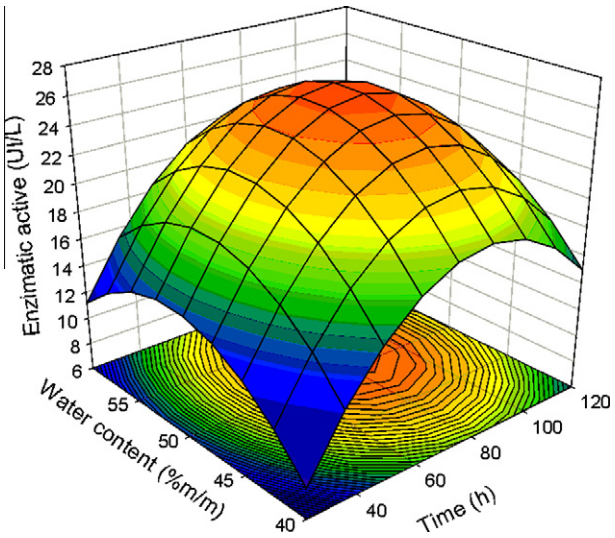

Fig. 2. Response surface plots for enzyme CMCase as a function of time, fermentation and water content.

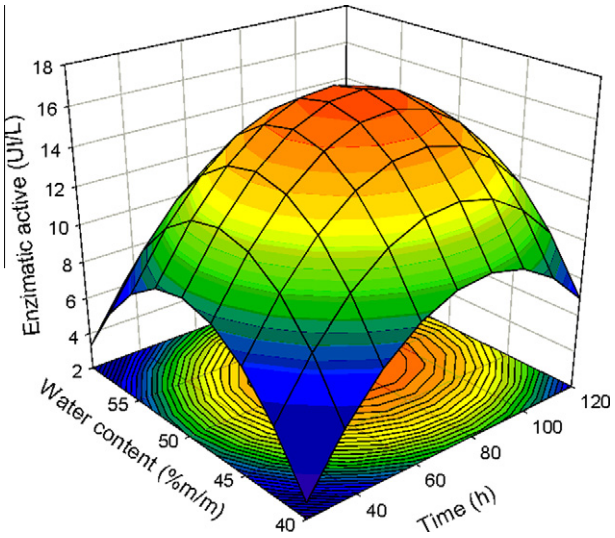

Fig. 3. Response surface plots for enzyme FPase as a function of time, fermentation and water content.

data obtained from the Surface Response Graph, using the optimal value for temperature, a tendency can be observed of the enzymatic active as a function of time and water content.

Figs. 2–4 illustrate combinations of the effects of independent variables on enzyme activity; through the derivatives of Eqs. (2)–(4), it can be observed that the optimal activity point for enzyme CMCase is at time 82.88 h, water content 51.48% and temperature 29.46 °C, whereas FPase at time 80.62 h, water content was 50.19% and temperature of 30.00 °C, for enzyme xylanase the optimal activity point was at time 81.92 h, water content 50.72% and temperature was 28.85 °C. It is necessary to take into consideration that *A. niger* synthesised the enzyme with the potato waste and water at various concentrations, thus demonstrating that it is a constitutive enzyme.

It was found that in this experiment, fermentation time significantly influenced enzyme production, which lasted approximately 80 h for all enzymatic activities. One hypothesis for this result would be that the presence of nutrients dispersed throughout the fermentation may have contributed to the growth of the microorganism, and the decay of these nutrients over time may have affected enzyme activity, and it was the decay of the microbial production and therefore the enzyme production.

Water content is a very significant factor in the fermentation process. High water activity causes the decrease in porosity of the substrate, thereby reducing the exchange of gases. On the other hand, low water activity may result in the reduction of microbial growth and consequent lower production of the enzyme (Mahanta, Gupta, & Khare, 2008). It was noted that approximately 50% moisture was ideal for obtaining the enzyme studied here. In the other water activities studied, the values ranged between 40% and 60%, with a decrease in fungal activity possibly related to inhibition of the fungus, marked by extrapolation of the ideal water level for the development of the line selected in the case of 60%, or low activity of water needed for the fungus to develop as might have occurred in 40%. These two conditions may have influenced the metabolism responsible for enzyme production. Enzymes usually have an expression control mechanism that can be stimulated or inhibited by products of the medium. The end products of a particular metabolic pathway are often inhibitors of enzymes that

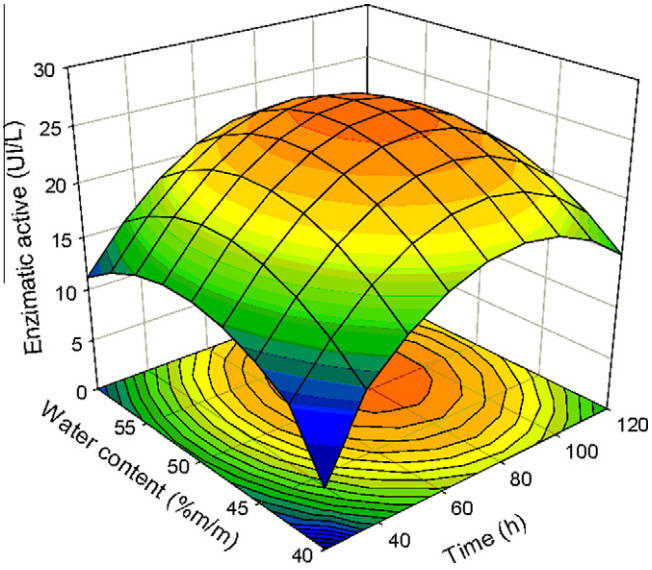

Fig. 4. Response surface plots for enzyme xylanase as a function of time, fermentation and water content.

catalyse the first steps of the pathway. This mechanism is known as negative feedback (Santana, Gonçalves, Bonomo, & Franco, 2012). Biazus, Souza, Santana, and Tambourgi (2006), working with corn malt, noted that in the production of enzymes the beginning is slow, then accelerates until it reaches its maximum value; thereafter, the concentration of products generated are inhibited and its activity is reduced, which was also observed in this study. Omemu, Akpan, Bankole, and Teniola (2005) obtained higher yields of cassava starch hydrolysis by *A. niger* after 72 h of fermentation, which concurs with Alva et al. (2007), who also reported a higher enzymatic activity by *Aspergillus*. The decrease in activity with increasing incubation time may be due to the production of by-products resulting from microbial metabolism, as well as nutrient depletion, inhibiting fungal growth and enzyme formation (Shafique, Bajwa, & Shafique, 2009).

The literature shows the production of endoglucanases by actinomycetes, particularly *Streptomyces*, on different substrates. The strain of *Streptomyces* T3-1 produced 40.3 U/mL in 1.5% CMC and ammonium sulphate, urea and peptone (Jang & Chen, 2003), but these nutrients were not used with low cost substrates. *Streptomyces* sp. isolated from Canadian soil was cultivated in a solution containing Mandel peptone, 1.0% Tween 80 in crystalline cellulose and produced 11.8 U/mL of CMCase (Alani, Anderson, & Moo-young, 2008); however, *Thermomonospora sp.* (George, Ahmad, & Rao, 2001) when grown in medium containing cellulose paper powder, yeast extract and Tween 80, showed a peak of 23 U/mL, whereas when grown on wheat bran activity was 8.5 U/mL.

Jorgensen and Olsson (2006) working with *Penicillium brasilianum* IBT in a bioreactor in medium containing yeast extract and a type of pine wood subjected to steam explosion, obtained values of 0.59 U/mL FPase. *Trichoderma viride* NCIM 1051 in 1.0% of sugarcane bagasse treated with NaOH resulted in FPase activity of 0.4 U/mL (Adsul et al., 2004). *A. niger* IZ9 in medium containing sugarcane bagasse treated with sodium hydroxide (NaOH) showed peak activity of 0.2 U/mL (Aguiar & Menezes, 2000).

Lu, Lii and Wu (2003) concluded that the xylanase production by *Aspergillus sulphureus* by SSF, on a pilot scale using koji noodles (made of fermented rice) and dry environment, was strongly affected by water activity of the medium. The best moisture of the medium to reach the maximum enzyme productivity was 40–50%. Qinnghe, Xiaoyu, Tiangui, Cheng, and Qiugang (2004) obtained 24.98 U/mL of xylanase activity, using corn cob and oat *Pleurotus ostreatus* as substrate in liquid fermentation under optimised conditions. In all mentioned studies, incubation times ranged from 7 to 15 days, much longer than those used in this work.

4. Conclusion

The analysis indicates that the optimal time expected for the CMCase of *A. niger* is 82.88 h, water content of 51.48% and temperature of 29.46 °C, whereas FPase was U/L at 80.62 h, water content of 50.19% and temperature of 30.00 °C; for enzyme xylanase, the optimal activity point was U/L at 81.92 h, water content of 50.72% and temperature of 28.85 °C. SSF is a technology that can propose alternative paths for the reuse of agro-industrial waste, therefore decreasing possible environmental problems, as well as adding economic value to these co-products.

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