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Rapid microbiological tests for prospecting new fungal strains with high potentiality for the pectinolytic enzymes production

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Prospecting of new isolates producers of pectinolitic enzymes were performed with cultivation of substrates of rice, soybean and corn bran. Pectinolitic enzymes were observed in cultures of the isolates NFC 1, NFC 2, NFC 4, NFC 5 and NFC 6 identified as belonging to the genus *Aspergillus* sp. Isolates NFR 1, NFR 2 and NFS1 were identified as belonging to the genus *Rhizopus* and NFC 3 belonging to the genus *Cladosporium*; however, pectinolitic enzymes production was not observed. The polygalacturonase (PG) production in submerged fermentation of *Aspergillus* NFC 2 in culture medium proposed (SM) at 28 and 37°C was quantified by agar diffusion and spectrophotometric methods. The highest PG production was obtained at 28°C by greater formation of halo degradation pectin and the activity measured by spectrophotometric methods. The activity of PG obtained was 7 U.mL^{-1} with volumetric productivity greater in the first 24 h of fermentation both at 28 and 37°C. A decrease in PG synthesis occurred after 48 to 120 h. The study allowed, by means of rapid microbiological tests, the selection of new isolates fungi for PG production and the definition of crucial stages for cultivation in liquid medium.

Key words: Filamentous fungi, *Aspergillus* sp, pectinolytic enzymes, polygalacturonase, submerged fermentation.

INTRODUCTION

The search for new biomolecules requires the isolation of microorganisms and the understanding of its genetic heterogeneity and metabolic (García and Bianchi, 2015; Pedrosa et al., 2013). The fungi are considered as promising sources of new biomolecules and have been employed for therapeutic use (Strobel and Daisy, 2003). These microorganisms represent an important genetic source for biotechnology, having stimulated the interest

of the scientific community due to the production of biomolecules with applications, mainly in the food and pharmaceutical industry (Strobel and Daisy, 2003). There are more than 50,000 secondary metabolites obtained by culture of microorganisms, being that, 12,000 are known antibiotics. Of these, 55% were produced by actinomycetes and 22% by filamentous fungi (Demain, 1999). Brazil offers a wide biodiversity, having a wide field to be explored in search of promising microorganisms in the production of biomolecules. The Tocantins State is located in the geographical zone of transition between the cerrado and the Amazon forest, and in both biomes, are characterized for being potential sources of microorganisms (Silva et al., 2015).

Microbial enzymes are widely used in industrial processes possessing a wide application in the conversion of food, in pharmaceutical production of medicines, leather industry, detergents, textiles and in the management of industrial waste (Lima, 2014), may be obtained by bacteria, yeasts and mainly by filamentous fungi from genus *Aspergillus* that are capable of producing enzymes in culture medium of low cost, secreting extracellularly. These fungi present formation of septate and branched mycelium, white or yellowish coloration with formation of stalks (Guimarães et al., 2006; Lelis et al., 2012; Haas et al., 2013). Pectinase production on an industrial scale by *Aspergillus* sp. presents advantage by high capacity for conversion with around 90% of products formed during fermentation (Fontana and Silveira, 2012; Sandri et al., 2015).

The pectinases are typical examples of enzymatic groups with wide applicability in the market, because they have demand and properties that meet the technical and economical requirements in industrial scale (Rehman et al., 2016). The classification of this group of enzymes is based on enzymatic attack to galacturonic skeleton; in preference for a particular substrate; in the mode of action by hydrolysis and in the cleavage of the substrate, random or terminal (Carvalho et al., 2013; Kant et al., 2013).

The polygalacturonase (EC 3.2.1.15) is pointed out as the main hydrolase enzyme and is classified as a result of its action mechanism on the substrate in two groups: Endo-polygalacturonase and exo-polygalacturonase (Pan et al., 2015; Rehman et al., 2012). This enzyme is commonly produced by *Aspergillus*, *Agrobacterium*, *Aureobasidium*, *Bacillus, Colletotrichum*, *Clostridium*, *Fusarium, Geotrichum, Penicillium, Rhizopus*, *Saccharomyces* and *Trichoderma* (Favela-Torres et al., 2008; Fontana and Silveira, 2012; Lelis et al., 2012; Sandri et al., 2015).

Thus, the present work has the objective of prospecting new fungal isolates from cereals originated in the geographical zone of transition between the cerrado and the Brazilian Amazon forest and the characterization of species by means of rapid microbiological tests that comprise potential in the PG production before complete identification. Besides, this work contributed to definition of crucial stages for cultivation in liquid medium.

MATERIALS AND METHODS

Microorganisms, production of inoculum and culture conditions

The microorganisms tested for production of pectinolitic enzymes were filamentous fungi isolated from rice (NFR), soybean (NFS) and corn bran (NFC) obtained from cereals originating from the geographical zone of transition between the cerrado and the Brazilian Amazon forest, Gurupi city, Tocantins, Brasil. The prospecting of new fungi from cereals was based on the possibility of application in animal feeding. Coordinates study was 11° 43' 45" S, 49° 04' 07" W.

All the cereals were packaged in closed containers with 100 g of each and soaked with 100 mL of nutrient solution containing 120 $g.L^{-1}$ sucrose and 30 $g.l^{-1}$ of NaCl. The flasks soaked with nutrient solution were incubated for 5 days at 25°C. Sample of cultivation of each substrate was transferred in potato dextrose agar medium (PDA) and incubated at 25°C for five (5) days. In this step, ten (10) fungi isolates were obtained. After isolation, the bank of the culture of each fungi isolate was prepared and stored in ultra-freezer at 80°C (Sanyo - VIP vertical 519L). In all the experiments for PG production, the activation of fungi occurred in PDA medium at 25°C for three (3) days. This work used *Aspergillus parasiticus* as positive control for PG production. This reference fungi was provided by Integrated Pest Management Laboratory of the Federal University of Tocantins.

Reproductive structures and morphological aspects

The identification of fungi isolates was performed after cultivation on PDA culture medium. Sample of spores was fixed on slides and analyzed in binocular optical microscope (Nikon, Eclipse E200). Analyzes of the structures of spores and septa mycelium were observed with increase at 1000X. After analysis, the classification of each fungi isolate was performed to identify up to genus (Griffin 1994).

The submerged fermentation

A new culture medium was proposed for cultivation in submerged fermentation (SM) composed of 2 $g.l^{-1}$ of calcium chloride; 5 $g.l^{-1}$ of ammonium nitrate; 0.5 $g.l^{-1}$ of potassium chloride; 0.02 $g.l^{-1}$ of magnesium sulphate; 0.02 $g.l^{-1}$ of iron sulphate; 20 $g.l^{-1}$ of glucose; 10 $g.l^{-1}$ of pectin; 7.5 $g.l^{-1}$ of yeast extract and 2 $g.l^{-1}$ of peptone of meat. The effects of temperature on the production of PG were

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evaluated with cultivation at 28 and 37°C for five (5) days. Supernatant of the samples were analyzed by measure of the halo degradation pectin and the activity of PG production by spectrophotometric methods.

Qualitative test of pectinolitic enzymes

The qualitative test of PG production was performed by means of culture in agar diffusion with evaluation of halo formation of pectin degradation. The culture medium in agar diffusion was composed of 1 $g.l^{-1}$ of corn bran; 2 $g.l^{-1}$ of citrus pectin; 2.2 $g.l^{-1}$ glucose; 0.005 $g.l^{-1}$ yeast extract; 0.5 $g.l^{-1}$ iron sulphate and ammonium; 0.05 $g.l^{-1}$ of magnesium sulphate; 0.25 $g.l^{-1}$ of monopotassium phosphate ; 62 $\mu g.l^{-1}$ of zinc sulphate; 1 $\mu g.l^{-1}$ of sulphate manganese and 2 $g.l^{-1}$ of agar. The pH was adjusted to pH 4 (Fontana and Silveira, 2012). After activation of fungal isolates on PDA culture medium, samples of crops were evaluated in agar diffusion to 28°C observing the formation of halo of pectin degradation. In the first step of fermentation, submerged samples were evaluated in agar diffusion at 28 and 37°C observing the formation of halo degradation pectin. The center point of the plate was determined as a referential for the measurement of the halos of degradation of pectin. In the second step of the submerged fermentation, the production of pectinolitic enzymes were determined by quantitative test.

Quantitative tests of pectinolitic enzymes production

The quantitative test of PG production was performed by the method of the increase of the groups reducers formed after the action of the enzyme with dinitrosalicylic acid (DNS). The mixture contained 0.8 mL of acetate buffer 0.2 M, pH 5.0, containing 1% of pectin (Sigma-Aldrich 212.2 MM) and 0.2 mL of gross enzyme solution. The pre-incubation was performed at 45°C for 15 min in a bain marie. The reaction was blocked by adding 1 mL of DNS, and tubes were agitated immediately. The test tubes were placed in bain marie for 5 min at 45°C. The reducing sugar released acid (D-galacturonic) was quantified by the method of 3.5-acid dinitrosalicylic (DNS) proposed by Miller (1959). The standard curve was performed with solution of galacturonic acid Sigma-Aldrich G5269 with concentration of 0.9 to 4.2 µmol. It is defined thus: a unit of enzyme activity is the amount of enzyme capable of generating 1 µmol galacturonic acid per minute in the reaction conditions. Supernatant of the samples were analyzed by Spectrophotometer (UV visivel - Biospectro SP-220) at 540 nm, all the tests were performed in triplicate and with three replications.

Calculation of the activity and PG volumetric productivity

The standard curve of galacturonic acid Sigma-Aldrich G5269 with corresponding concentration of 0.9 to 4.2 µmol was used to define the enzyme concentration. Considering Equation 1 of the standard curve and the absorbance values (*y*) of each time of cultivation, the enzyme concentration (*x*) was obtained. In Equation 2, (x) is enzymatic concentration, (t) time of the reaction (in minutes) and ($\frac{1}{0,05}$) conversion factor (µmol to mL).

$$y = 0.2227x - 0.2181 \quad (1)$$

$$V = \frac{x}{t} \cdot \frac{1}{0.05} \quad (2)$$

Statistical analysis

All experiments were conducted using a totally randomized design. Analysis of variance (ANOVA) and Tukey test with 5% of significance was performed to evaluate statistical differences of the formation of halo of degradation pectin and the statistical differences of activity and volumetric productivity of PG in submerged fermentation. All experiments were performed in triplicate and with three replications.

RESULTS

The isolation of new filamentous fungi was obtained in three (3) cultivation system: rice (NFR), soybean (NFS) and corn (NFC) (Figure 1). The production of pectinolitic enzymes quantified in agar diffusion were observed in cultures of the isolates NFC 1, NFC 2, NFC 4, NFC 5 and NFC 6 identified as belonging to the genus *Aspergillus* sp. The isolates NFR 1, NFR 2 and NFS1 were identified as belonging to the genera *Rhizopus* and NFC 3 belonging to the genus *Cladosporium*, without enzymatic production (Figure 1). The initial experiments showed that the isolated NFC 2 obtained for substrate of corn bran obtained greater formation of halo degradation of pectin (2.3±0.27 cm) and the lowest production was obtained on the cultivation of isolated NFC 4 (1.6±0.2 cm) (Figure 1). In this step, the reference strain was used *Aspergillus parasiticus* as positive control of pectinolitic enzymes in the proposed system of study showing the formation of halo of pectin degradation. Halo degradation pectin of *A. parasiticus* was 1.0±0.2 cm (Figure 2). After quantification by agar diffusion methods, the submerged fermentation of *Aspergillus* NFC 2 in culture (SM) at 28 and 37°C was performed to evaluate the increase in PG production.

In order to assess a system of cultivation in submerged fermentation, tests were carried out by proposing cultivation in culture medium (SM) with isolated *Aspergillus* NFC 2 of greater potential for the enzyme production. Figure 2A to C presents the first step of the submerged fermentation of isolated *Aspergillus* NFC 2 in medium SM 28°C with evidence of enzymatic production observed by pectin degradation of the medium. It is possible to observe a halo of degradation of isolated *Aspergillus* NFC 2 in this condition of cultivation (Figure 2A). Cultivation of the reference fungi showed the enzymatic production by observation of pectin degradation of the medium; however, with minor intensity (Figure 2B). The negative control did not show degradation of pectin medium (Figure 2C). These results confirmed a higher potential of *Aspergillus* NFC 2 in the production of pectinolitic enzymes.

In another step of process definition, cultivations were performed with the *Aspergillus* NFC 2 at 28 and 37°C. After activation in PDA medium, an inoculum was obtained and transferred in Erlenmeyer's flasks 500 mL with 25ml of culture medium SM incubated in static system for 5 days at 28 and 37°C. One aliquot was collected at the end of the cultivation and evaluated in the agar diffusion method. Halo degradation pectin of isolates

a

New fungal strains	*Brans*	*Degradation halo formation*	*Agar diffusion essay with pectina 1% (cm)*	*Fungal identification*
NFR 1	RICE	0(3)	Wp	*Rhizopus sp* *(g-h)*
NFR 2	RICE	0(3)	Wp	*Rhizopus sp* *(g-h)*
NFS 1	SOY	0(3)	Wp	*Rhizopus sp* *(g-h)*
NFC 1	CORN	3(3)	1.5(0.04)	*Aspergillus sp* *(b-e)*
NFC 2	CORN	3(3)	2.3(0.27)	*Aspergillus sp* *(b-e)*
NFC 3	CORN	0(3)	Wp	*Cladosporium sp* *(f)*
NFC 4	CORN	3(3)	1.6(0.2)	*Aspergillus sp* *(b-e)*
NFC 5	CORN	3(3)	1.8(0.04)	*Aspergillus sp* *(b-e)*
NFC 6	CORN	3(3)	2(0.55)	*Aspergillus sp* *(b-e)*
NFC 7	CORN	0(3)	Wp	*Aspergillus sp* *(b-e)*

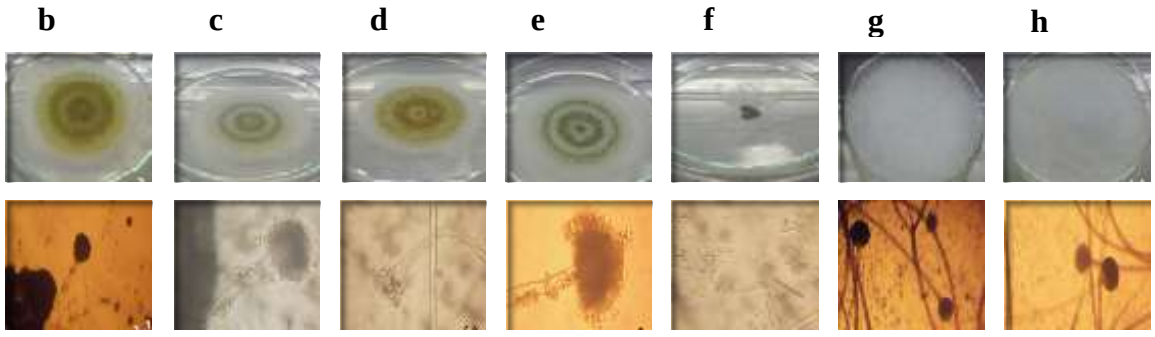

Figure 1. New fungal strains producers of pectinolytic enzymes. a- identification and characterization of fungi producers of pectinolytic enzyme. b-h- corresponding to the species by morphology and reproductive structure. Mean (standard error). NFR– new fungal strain of rice; NFS– new fungal strain of soy; NFC- new fungal strain of corn. Wp– without production. 0(3) absent in three replications and 3(3) present in three replications.

were measured (Figure 2D). The occurrence of growth was observed in both cultivation systems. However, the enzymatic production measured by degradation of pectin was greater at 28°C ($p>0.02$) (3.37cm), and lower at

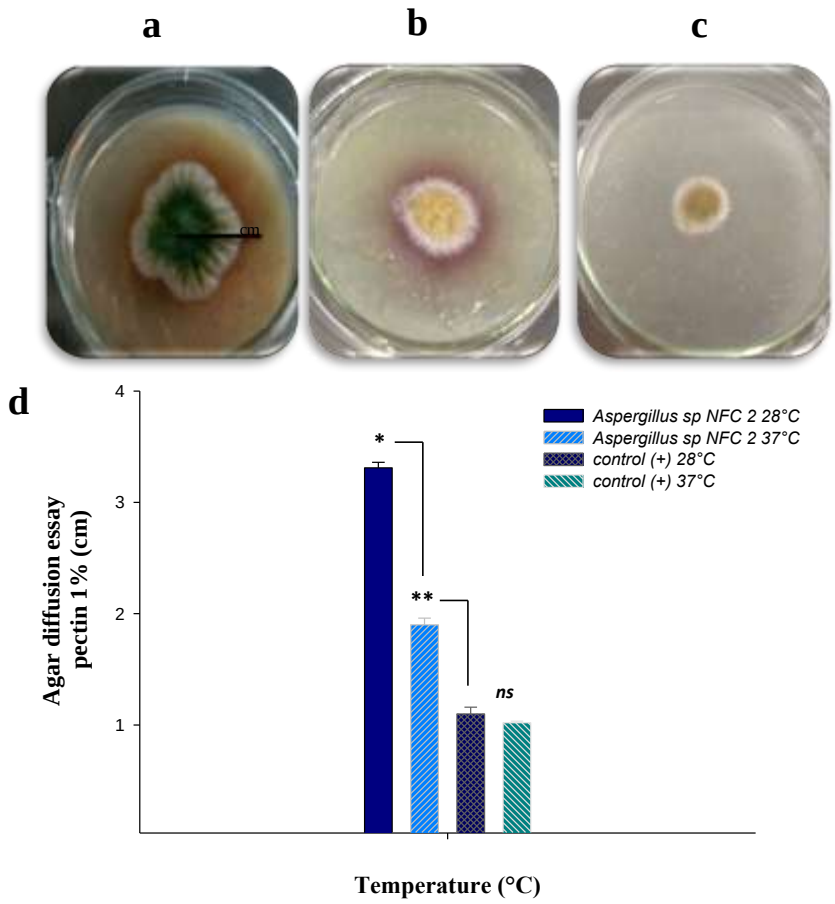

Figure 2. Analysis of the pectinolytic enzymes production in agar diffusion assay. Morphology of *Aspergillus* NFC 2 at 28°C (a). Morphology of positive control *Aspergillus parasiticus* at 28°C (b) and morphology of negative control *Aspergillus* at 28°C (c). Agar diffusion assay of *Aspergillus* NFC 2 and *Aspergillus parasiticus* (control +) in medium culture with measure of the degradation of pectin 1% at 28 and 37°C (d). *p>0.02; **p>0.007.

37°C (1.87 cm). This enzyme production in the medium SM was greater than the values of degradation of pectin obtained in previous experiments (Figure 1). The enzymatic production of *Aspergillus* NFC 2 in submerged cultivation was significantly higher (p>0.007) with reference to the positive control (1.90 cm) both at 28 and 37°C (Figure 2). In the cultivation of the reference fungi, significant differences was not observed between the temperatures evaluated. These results confirm the optimization of production of isolate NFC 2 in medium SM 28°C.

In the third step of optimization, the cultivation of *Aspergillus* NFC 2 was performed in medium SM 28°C with quantification of the activity and volumetric productivity of PG by spectrophotometric method. Figure 3 shows the activities of PG of *Aspergillus* NFC 2 in medium SM 28°C during 120 h of fermentation. The activity of PG observed during 120 h was close to 7 $U.mL^{-1}$. There was no significant difference in the activity of PG between the temperatures evaluated (Figure 3). However, volumetric productivity of PG was higher (p<0.05) in the first 24 h in both cultivation systems. At 37°C, it showed greater decay of volumetric productivity of PG along the fermentation. Statistics data are shown in Tables 1 and 2.

The identification of filamentous fungi has been performed based on analysis of their microscopic structures and morphologic aspects. Microscopic observations allow identifying characteristics of hyphae, shape, arrangement, reproductive structures, conidia and the formation of spores. The macroscopic and microscopic observation of fungi allows the definition of the genus quickly and efficient definition of cultivation system based on information of the genera of the species (Vecchia and

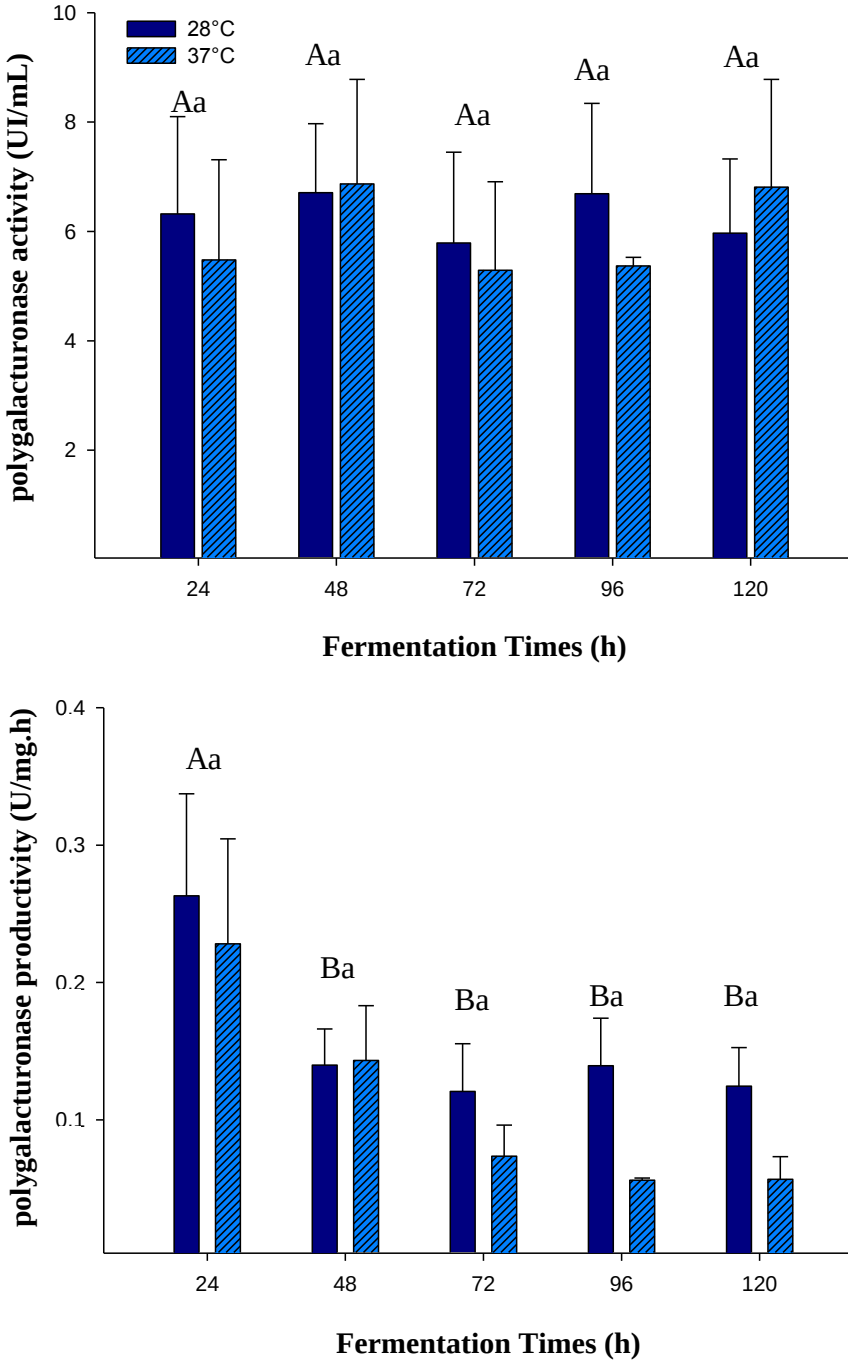

Figure 3. Enzymatic activity of the new fungal strain *Aspergillus* NFC 2. Polygalacturonase activity (a) and volumetric productivity (b) versus temperature. Means followed by the same capital letters (fermentation times) and lower case letters (temperature) do not differ significantly by the Tukey test ($p < 0.05$).

Castilhos-Fortes, 2007). Griebeler et al. (2015) made the selection of filamentous fungi producers of various compounds, including pectinase. Of their selections, 32.7% were described as microorganisms producers of pectinolytic enzymes with major halo degradation pectin to the species *Aspergillus* (2.8 cm) and *Penicillium* (3 cm) for agar medium with 1.25% of pectin. Marchi et al. (2006) analyzed activities of pectinolytic of 45 isolates

Table 1. Volumetric productivity of PG by *Aspergillus* NFC 2 in submerged fermentation conditions.

Treatment	37°C		28°C	
	Volumetric productivity	Standard error	Volumetric productivity	Standard error
T1	0.2282^{Aa}	±0.04402	0.2631^{Aa}	±0.0428
T2	0.1432^{Ba}	±0.0230	0.1399^{Ba}	±0.0151
T3	0.0736^{Ba}	±0.0130	0.1208^{Ba}	±0.0200
T4	0.0560^{Ba}	±0.0009	0.1395^{Ba}	±0.0199
T5	0.0568^{Ba}	±0.0094	0.1245^{Ba}	±0.0162

Evaluation of capital letters in the columns and lowercase letters in the lines. Means followed by the same letter are not significantly different at the level of 5% probability by the Tukey test. Legend: treatment is fermentation times.

Table 2. Activity of PG by *Aspergillus* NFC 2 in submerged fermentation conditions.

Treatment	37°C		28°C	
	PG activity	Standard error	PG activity	Standard error
T1	6.32Aa	±1.0290	5.48^{Aa}	±1.0577
T2	6.71Aa	±0.7286	6.87^{Aa}	±1.1077
T3	5.80Aa	±0.9604	5.30^{Aa}	±0.9386
T4	5.80Aa	±0.9604	5.38^{Aa}	±0.09145
T5	5.98Aa	±0.7813	6.81^{Aa}	±1.1396

Evaluation of capital letters in the columns and lowercase letters in the lines. Means followed by the same letter are not significantly different at the level of 5% probability by the Tukey test. Legend: treatment is fermentation times.

of *Alternaria solani* by agar medium diffusion evaluated by halo degradation of 1.48 cm with 3% pectin. Farias et al. (2015) also analyzed the pectinolytic enzymes production. The results showed values greater than 1.40 cm halo degradation pectin at 1.25%. In this work, higher halo degradation of (3.37 cm) with agar medium was obtained at 1% of pectin.

Agar diffusion methods have been used for analysis of detection of biomolecules production. Palumbo and O'Keeffe (2014) analyzed different species of genus *Aspergillus* employing this technique. Each isolate filamentous fungi were analyzed for the production of enzyme pectinase with employment of the agar diffusion method and the observation of halo of characteristic degradation by consumption of pectin in the medium. Zhang et al. (2015) used methods of agar diffusion for analysis up to species, identifying *Aspergillus fumigatus*. The initial analysis of the isolated fungus was performed by the agar diffusion method with the image collection and analysis of morphogenesis by optical microscopy. The identification until species was confirmed by molecular techniques with DNA sequencing where PCR primers were used. In other works, techniques for obtaining identification until species were combined. Niazi et al. (2014) employed techniques for the identification of fungi which cause aspergillosis with analyzes of macro and microscopic structures of the fungi, followed by PCR techniques, LightCycler and Elisa.

The employment of practical methods in the selection of new isolates for the production of microbial enzymes relates to the consumption of specific substrate. Robl et al. (2013) analyzed the enzymatic production of hemicelullases and related enzymes, with use of agar aesculin and agar with specific substrate evaluating the halo degradation for the initial selection. Glinka and Liao (2011) analyzed the production of pectin metilesterase (PME), polygalacturonase (PG) and pectin lyase (PL) through activity tests in the agar diffusion assays. Downie et al. (1998) also examined the quantification of pectin metilesterase activity by agar diffusion method.

The enzymatic activity and volumetric productivity of PG of *Aspergillus* NFC 2 in submerged fermentation were higher than 28°C with 24 h of fermentation. Menezes et al. (2006) studied the enzymatic activity and volumetric productivity of PG in solid fermentation using wheat bran (66.75%), humidity of 62.5% and with 64 h of fermentation of isolate of *A. niger*. The enzymatic activity and volumetric productivity obtained were of 12.01 and 0.09 $U.mL.h^{-1}$, respectively. Barman et al. (2015) evaluated the production of pectinase by *A. niger*, the

activity of PG obtained was 6.6 $U.mL^{-1}$. In this study, the volumetric productivity of PG of *Aspergillus* NCF 2 in submerged fermentation with medium SM 28°C was three (3) times more with values close to 0.3 $U.mg.h^{-1}$. The optimization of production processes is required to achieve high product yields (Brandi et al., 2014).

Zeni et al. (2011) evaluated the selection of filamentous fungi producers of PG. Out of total of 107 isolates, 15 isolates were previously identified as *A. niger*, *Penicillium* sp. and selected as potential producers of this enzyme. The production of PG obtained was greater than 3 $U.mL^{-1}$. After study of optimizing the enzymatic activity was 13 times higher than the initial values.

The enzymatic activity and volumetric productivity of PG obtained in this work characterizes the *Aspergillus* NCF 2 as a benchmark for the production of pectinolitic enzymes, therefore, their production was superior to the other remaining fungal isolates and higher than those researched in the literature. New studies of process optimization will certainly lead to higher incomes in the production of pectinolitic enzymes.

Conclusion

The tests employed in this study made it possible to obtain new fungal isolates characterizing the isolate NFC 2 with high potential for the production of pectinolitic enzymes. It allowed in a practical and efficient way, advance in the definition of cultivation systems of filamentous fungus with high productivity in submerged fermentation.

Conflict of Interests

The authors did not declare any conflict of interests.

ACKNOWLEDGEMENT

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Abbreviation

ANOVA, Analysis of variance; **DNA,** deoxyribonucleic acid; **DNS,** dinitrosalicylic acid; **NFC,** new fungal strain of corn; **NFR,** new fungal strain of rice; **NFS,** new fungal strain of soy; **PCR,** polymerase chain reaction; **PDA,** potato dextrose agar; **PG,** polygalacturonase; pH, hydrogen potential; **PL,** pectin lyase; **PME,** pectin metilesterase; **SM,** culture medium proposed.

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