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HOLOCELLULOLYTIC HYDROLASES PRODUCTION BY FILAMENTOUS FUNGI USING OIL CAKES AS SUBSTRATE

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ABSTRACT

This work evaluated the use of cottonseed, sunflower and macauba oilcakes as sources of carbon for the production of cellulolytic and xylanonytic enzymes using submerged fermentation. *Aspergillus tubingensis* AN1257 e *Penicillium*sp T1.1, isolated from environmental samples, were tested. *Trichoderma reesei* CCT2768 was used as a referential lineage. The cottonseed oilcake distinguished itself because it provided the highest endoglucanase production with the all evaluated fungi. The enzymatic extract obtained from the *A. tubingensis* AN1257 cultivated with cottonseed oilcakes exhibited values of 0.694 U mL^{-1} for endoglucanase, 0.620 U mL^{-1} for β-glucosidase, 0.048 U mL^{-1} for FPase after 96 hours of fermentation, and 37 U mL^{-1} for xylanase after 72 hours of fermentation. The production of endoglucanase by the *A. tubingensis* AN1257, using cottonseed oil cakes, was 13.6% higher than the amount produced by *T. reesei* CCT2768. These preliminary results point to the promising use of isolated *A. tubingensis* lineages and cottonseed oil cakes for the production of cellulases and xylanese.

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INTRODUCTION

Economic value and diverse use of agroindustrial residues are part of the market logic and of the effort in sustainability biomass-based production chains. The use of agroindustrial residues in bioprocesses, besides being economically interesting, contributes for minimizing the environmental problems which derive from discarding them in nature (Nigan and Singh, 2011; Singhania *et al*., 2010). Many bioprocesses have been developed using alternative raw materials for the production of molecules with high added value, highlighting basic and applied research in microbial cellulases and xylanases (Ncube *et al*., 2012; Shimokawa *et al*., 2012; Visser *et al*., 2011).

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Cellulolytic and xylanonytic enzymes have been commonly used by the textile, paper, food and beverage, and pet food industries. The demand for these biocatalystshas been rapidly growing, powered by the new second-generation ethanol market (Balat, 2011; Gottschalk *et al*., 2010; Maeda *et al*., 2013). The hydrolysis of lignocellulosic materials is a fundamental step in the process of production of second-generation ethanol, whose principle is to allow the conversion of cellulose and hemicellulose into fermentable sugars which, in turn, can be converted into ethanol by specific microorganisms. However, the enzymatic hydrolysis still forms a major cost factor in the conversion of lignocellulosic raw materials to ethanol or other sugar-based energy carriers and platform chemicals (Viikari *et al*., 2012). In order to overcome this problem is the technological development for reduce the costs per unit of enzymes and produce enzymes more efficient. The selection of new microorganisms is part of

this effort. Filamentous fungi are especially importante because, how decomposers of biomasses in nature, they produce enzymes with several specificities and physicochemical characteristics, besides having a synthetic capacity in big scale and an excellent enzymatic secretion system (Iwashita, 2002). Another relevant parameter for the production of enzymes is the composition of the fermentation medium which contributes considerably for the final price of the product, and makes it necessary to search for low cost substrates. The development of biotechnological processes which permit the use of residual biomasses with lignocellulosic compositions - such as corn husk, wheat and rice, sawdust, sugarcane bagasse, residues from the paper and cellulose industry, and coproducts of the biodiesel chain - have been researched around the world (Balat, 2011; Herculano*et al*., 2011; Bansal *et al*., 2012; Pandya and Gupte, 2012). In this context, the goal of this paper was to evaluate the inducing potential of cottonseed (*Gossypiumhirsutum* L.), sunflower (*Helianthus annuus* L.), and macauba (*Acrocomiaaculeata* Jacq.) oil cakes, coproducts of the production chain of vegetable oils, in the production of cellulases and xylanases when used as carbon sources in submerged fermentation processes with isolated filamentous fungi.

METHODOLOGY

Acquisition and preparation of the oil cakes: The oilcakes were acquired at oleaginous benefaction mills. All the oilcakes were initially degreased using ethyl ether for extracting residual oil, which was still abundant. After that, the degreased oil cakes were dried at 60°C in anoven with forced air circulation for 48 hours, comminuted, sieved using a 0.2 mm steel mesh, and reserved for chemical analyses and use as carbon source in the fermentation processes.

Characterization of the oil cakes: The chemical characterization of the oil cakes was done through the determination of the moisture, ashes, proteins, and total lipids (AOAC, 1992), soluble fiber in neutral detergent (FDN), soluble fiber in acid detergent (FDA), cellulose, hemicellulose and lignin (Van Soest, 1967), total soluble sugars (Dubois *et al*., 1956), reducing sugars (Miller, 1959), and starch (McCready *et al*., 1950).

Microorganisms: The lineages of filamentous fungi *Aspergillus tubingensis* AN1257 e *Penicilliumsp* T1.1 were obtained by screening of ambiental samples in a carboxymethyl celluloseagar medium in previous work (not published) and storage in microbial cultures collection from local research laboratory of Universidade Federal dos Vales do Jequitinhonha e Mucuri, Brazil. A lineage of *Trichoderma reesei* CCT2768, known as a celulase producer, was acquired from Tropical Culture Collection of the Fundação André Tosello (Brazil) and used howreference. The lineages of *T.reesei*CCT2768 were kept in stock on oat extract agar slantsunder refrigeration (4±1ºC) after growth at 30°C for 14 days. The others two lineageswere keptin stock onpotato dextrose agar slantsunder refrigeration (4±1ºC) after growth at 30°C for 7 days.The cultures were maintained by subculturingeach trhee months on the same medium and conditions.

Innoculumpreparation: The microorganisms stock cultures were picked in Vogel's solid medium (Vogel, 1964) in Petri dishes, in triplicate, and incubated at 30°C for 14 (*T.reesei* CCT2768) and 7 days (*Penicilliumsp* T1.1 and *A. tubingensis* AN1257) for conidia production. The conidia were washed and collected assepticallywith 10mL of sterile distilled water, filtered through of gauze, and counted in a Neubauer chamber for determining its concentration.

Evaluation of oil cakes and fungi leneages for enzymesproduction: The selected filamentous fungi were evaluated according to the production of endoglucanase and xylanase enzymes under submerged fermentation process with media elaboratedusing oil cakes derived from cottonseed, sunflowerand *macauba* oil extraction processes, as carbon sources, andAvicel/CMC (proportion 1:1) as reference of substract. The fermentation process was carried out in 125 mL conical flasks containing 50 mL of base medium consisting of yeast extract (0.4 gL^{-1}), NaCl(0.1 gL^{-1}), $MgSO_4$-$7H_2O$ (0.2 gL^{-1}), KH_2PO_4 (0.4 gL^{-1}), K_2HPO_4 (0.1 gL^{-1}), and 0.5 g of one of the carbon sources mentioned above. Conidia suspensions of each one of the microbial strains were inoculated so as to obtain a final concentration of 1.0×10^5 conidiam L^{-1}. The fermentation was conducted at 30°C for 120 hours in a shaker with orbital agitation of 150 rpm. The experiments were conducted in triplicate. The enzymatic activities were determined every 24 hours. Were prepared fermentation flasks to each samplingtime.

Enzymatic extracts: The total content of each culture flask was filtered in a Büchner funnel with filter paper, previously weighed, with particle retention of 7 to 12µm, to obtain the enzyme extract. The filtrate was collected in test tubes immersed in ice contained within Kitasato flask. The filtrate was kept on ice until the activities of enzymes of interest were determined.

Enzyme assays: The FPase, endoglucanase, and β-glucosidase activities were determined according to the method described by Ghose(1987), using as substrate, respectively, n◦1 Whatmanpaper filter (50 mg), cellobiosein acetate buffer 50 mmol L^{-1}, (1%, pH 5) and carboxymethylcellulosein acetate buffer 50 mmol L^{-1}, (1%, pH 5). The enzyme extract was dilutedin acetate buffer 50 mmol L^{-1}, pH 5, when necessary. The reducing sugars, expressed as glucose, which are released in the reactions of FPase and endoglucanase, were quantified based on their reducing power according to the method described by Miller (1959), using a 3,5-dinitrosalycilic acid (DNS) as a reactive, glucose as standard, and spectrophotometric reading at 540 nm.Oneunit of endoglucanase or of FPase activitywas defined as the volume of enzymaticextract that releases 1 µmol of reducing sugar,per minute of reaction, under the assay conditions. The glucose liberated during the reaction of β-glucosidase was measured using a commercial enzymatic determination kit based in glucose-oxidase/peroxidase reactions (Laborlab®), with spectrophotometric reading at 510 nm. One unit of β-glucosidase activity was defined as the volume of enzymatic extractthat releases 1 µmol of glucose, per minute of reaction, under the assay conditions. The xylanolytic activity was determined according to Bailey *et al*. (1992), using birchwoodxylan (Sigma) in acetate buffer 50 mmol L^{-1} (1%, pH 5) as substrate. The reducing sugars released during the xylanase reaction were quantified according to the method described by Miller (1959), using xyloseas standard. One unit of xylanase activity was defined as the volume of enzymatic extractthat releases 1 µmol of reducing sugars, per minute of reaction, under the assay conditions. The protease activity was

determined using the method described by Leigton *et al.* (1973) which consists in the hydrolysis of a 1% azocasein solution in a Tris-HCl buffer 100 mmolL^{-1}, pH 8.0, in the presence of enzymatic extract at 25 ^{0}C. One unit of protease activity was defined as the volume of enzymatic extract required to produce an absorbance change of 1 unit at 440 nm in 60 minutesunder the assay conditions.All enzyme assays were performed in triplicate and results are presented as mean values.

Enzymes production pattern from the more productive combination between researched oil cake and microbial lineage: A new fermentation was carried out in triplicate, using only the microbial lineage and the carbon source that stood out in production of endoglucanasic and xylanasic activities. The progress of secretion of endoglucanase, β-glucosidase, FPase, xylanase and protease activities over a period of 144 hours of submerged fermentation, in the same condictions previously described, was evaluated. Besides of determination of the enzymatic activities, the quantification of soluble proteins was performed using the method of Lowry *et al.* (1951), the variation in reducing sugars concentration throughout the fermentation process was quantified according to the method described by Miller (1959), the pH of the medium was determined every 24 hours and the solid residues retained in the filter paper to obtainment of enzymatic extract was also quantified by gravimetry, after dried in an oven at 60°C for 48 hours.

RESULTS AND DISCUSSION

Proximate composition of the oil cakes: The proximate composition of the cottoseed, sunflower and *macauba*oil cakes is presented in Table 1.

Table 1. Chemical composition of Cottonseed, Sunflower seed and Macauba oil cakes

Sources	Cottonseed (M% ± SD)	Macauba (M% ± SD)	Sunflowerseed (M% ± SD)
Moisture	3.27 ± 0.08	7.80 ± 0.23	0.46 ± 0.01
Lipids	13.38 ± 0.06	4.70 ± 0.15	0.03 ± 0.01
Ash	3.70 ± 0.13	4.19 ± 0.16	5.62 ± 0.03
TSS	4.44 ± 0.55	11.48 ± 0.62	7.76 ± 0.93
Starch	2.39 ± 0.35	23.16 ± 0.95	4.04 ± 0.38
FDA	45.32 ± 1.80	14.12 ± 0.91	32.90 ± 0.66
FDN	52.19 ± 0.14	23.72 ± 0.36	53.02 ± 0.76
Cellulose	33.29 ± 1.13	11.49 ± 1.08	23.22 ± 0.30
Hemicellulose	7.56 ± 0.92	9.60 ± 0.79	20.55 ± 1.22
Lignin	15.98 ± 0.52	4.33 ± 0.12	9.77 ± 0.67

M: Mean; SD: Standard Deviation; TSS:Total soluble sugars; FDA:soluble fiber in acid detergent; FDN: Soluble fiber in neutral detergent

The values found for the contents of cellulose and hemicellulose allow the conjectures that the cottonseed and sunflower oil cakes, subproducts of the extraction of oil from the respective seeds, have potential to be used in the production of second-generation ethanol and also as a carbon source for the production of lignocellulosic enzymes. The *macauba*oil cakes, in particular, presented high contents of starch, 23.2%, which qualifies it for the production of first-generation ethanol. The high percentage of total proteins found in cottonseed and sunflower oil cakes suggests its possible contribution as sources of nitrogen in the composition of culture media to enzymes production.

Evaluation of perspective to produce lignocellulolitic enzymes from examined oil cakes and fungi lineages: The Table 2 presents values of the maximum activities of endoglucanase found in enzymatic extracts produces by the *Aspergillus tubingensis* AN1257, *Penicillium sp*.T1.1 and *Trichoderma reesei* CCT2768 lineages, using submerged fermentation processes with medium elaborated using cottonseed, sunflower and *macauba*oil cakes, and Avicel/CMC commercial substrate as carbon sources. Among the three evaluated lineages, the one that most produced the endoglucanase activity was the *A. tubingensis* AN1257, with activity of 0.71 ± 0.07 U mL^{-1}, after 96 hours of fermentation. This value is close to the one observed by Shimokawa*et al.* (2012) in his study with fungal lineages of the same species, and using oat and rice bran (proportion 9:1) as carbon sources. It isalso possible to observe that the production of endoglucanase for the AN1257 lineage was 13.6% higher than the used reference lineage, *T.reesei* CCT2768,which presented maximum activity of 0.62±0.04 UmL^{-1} of endoglucanase after 96 hours of fermentation. Among the carbon sources which were used, the cottonseed oil cake was the one that best induced the production of endoglucanase, both with*A. tubingensis* AN1257 and with*T.reesei* CCT2768 lineages (Table 1). Cottonseed oil cakes were also the best option of carbon sources for the production of xylanolytic enzymes (Table 2). This biomass was more efficient in inducing the production of xylanolytic enzymes, even though it presents lower quantities of hemicellulose (15.19 ± 1.49%) when compared to the sunflower oil cakes (20.55 ± 1.22%). Among the evaluated fungi lineages, *A. tubingensis* AN1257 was an excellent producer of xylanases, producing 36.51±5.13 UmL^{-1} of xylanolytic activity in 72 hours (Table 2). The reference lineage (*T. reesei* CCT2768) produced 38.93±6.55UmL^{-1} of xylanolytic activity in the same fermentation conditions applied to the *A. tubingensis* AN1257, but with 96 hours of process (Table 2). Therefore, the volumetric productivity of xylanases from *A. tubingensis* AN1257 was 25% greater than that observed for the *T. reesei* CCT2768 (Table 2). Adhyaru *et al.* (2015), in work of xylanase production optimization by*A. tubingensis* FDHN1, reported maximum production of 4,105 U g^{-1} using SSF with sorghum straw as substrate.The specific production value (enzymatic activity obtained per gram of oil cake) obtained for the xylanasic activity produced with*A. tubingensis* AN1257 and cottonseed oil cake in non optimized condition was 3,651 U g^{-1} (Table 2).

Pattern analysis ofprocess carried out with *A. tubingensis* AN1257 and cottonseed oil cake: The process conduced with *A. tubingensis*AN1257 and cottonseed oil cake presented the betters resusts of volumetric productivity to endoclucanase and xylanase and, therefore, it was chosen for the study of enzyme productionprofile, including other activities not anlysed in initial approach. The graphs presented in Figure 1 show the fermentative profile for the production of lignocellulosic enzymes, in which the endoglucanase, β-glucosidase, FPase, and xylanase activies are registered, and also the protease, total soluble proteins, reducing sugars intake, pH variation, and dry weight of insoluble residues, was resgistaredin the period of 144 hours of submerged fermentation using cottonseed oil cake as carbon source and the *A. tubingensis* AN1257 lineage as innoculum. It was possible to observe that the studied lineage presented capacity for the production of several cellulolytic enzymes, exhibiting values of 0.694 U mL^{-1} for the endoglucanasic activity, 0.620 UmL^{-1} for the ***β***-glucosidasic activity and 0.048 U mL^{-1} for the FPasic activity after 96 hours of fermentation (Figure 1). The peak of xylanasic activity (37 U mL^{-1}, 72 hours) happened before the maximum production of cellulolytic activities (Figure 1).

Table 2. Values of production (U mL^{-1}), specific production (U g^{-1} cake) and volumetric productivity (U L^{-1} h^{-1}) in the times of maximum production of endoglucanase and xylanase enzymes by *Aspergillus tubingensis* AN1257, *Penicillium* sp. T1 and *Trichoderma reesei* CCT2768 strains in submerged fermentation process

Strain	CarbonSource	Endoglucanase				Xylanase			
		Time (h)	U mL^{-1}	U g^{-1}cake	U L^{-1} h^{-1}	Time (h)	U mL^{-1}	U g^{-1}cake	U L^{-1} h^{-1}
Aspergillus tubingensis AN1257	Cottonseedcake	96	0.71±0.07	71.3±6.9	7.43±0.72	72	36.51±5.13	3,651±513	507±71
	Sunflowerseedcake	96	0.34±0.04	33.5±3.5	3.49±0.37	72	18.16±2.98	1,816±298	252±41
	Macaubacake	96	0.30±0.03	30.2±2.7	3.15±0.28	72	6.13±0.89	613±89	85±12
	Avicel/CMC	96	0.27±0.04	27.4±3.8	2.85±0.40				
*Penicillium*sp. T1.1	Cottonseedcake	120	0.30±0.04	29.6±4.0	2.47±0.33	96	18.25±3.75	1,824±375	190±39
	Sunflowerseedcake	120	0.25±0.02	25.0±1.5	2.08±0.13	96	2.01±0.29	201±29	21±3
	Macaubacake	120	0.21±0.03	21.0±3.2	1.75±0.27	96	1.10±0.25	110±25	11±2
	Avicel/CMC	120	0.18±0.04	18.0±3.6	1.50±0.30				
Trichodermareesei CCT2768	Cottonseedcake	96	0.62±0.04	61.6±4.0	6.42±0.42	96	38.93±6.55	3,893±655	405±68
	Sunflowerseedcake	96	0.34±0.03	34.1±2.9	3.54±0.30	96	10.15±1.51	1,014±151	106±16
	Macaubacake	96	0.29±0.02	29.2±1.5	3.02±0.16	96	7.02±0.99	702±99	73±10
	Avicel/CMC	96	0.41±0.03	41.0±3.1	4.27±0.32				

Avicel: microcrystalline cellulose. CMC: carboxymethylcellulose

Table 3. Parameters of enzymatic production in the process carried out with *Aspergillus tubingensis* AN1257 and cottonseed oil cake

Activities	Production (U mL^{-1})	$Y_{P/S}$ (U g^{-1})	Specific production (U g^{-1})	Specific activity (U mg^{-1} protein)
Endoglucanasic	0.694	198.3	69.4	0.875
β-glucosidasic	0.620	177.1	62.0	0.775
FPasic	0.048	13.7	4.8	0.060
Xylanasic	37	10,571	3,700	70

$Y_{P/S}$(yield product-substrate) - ratio between the produced enzyme and the quantity of cottonseed oil cake consumed.

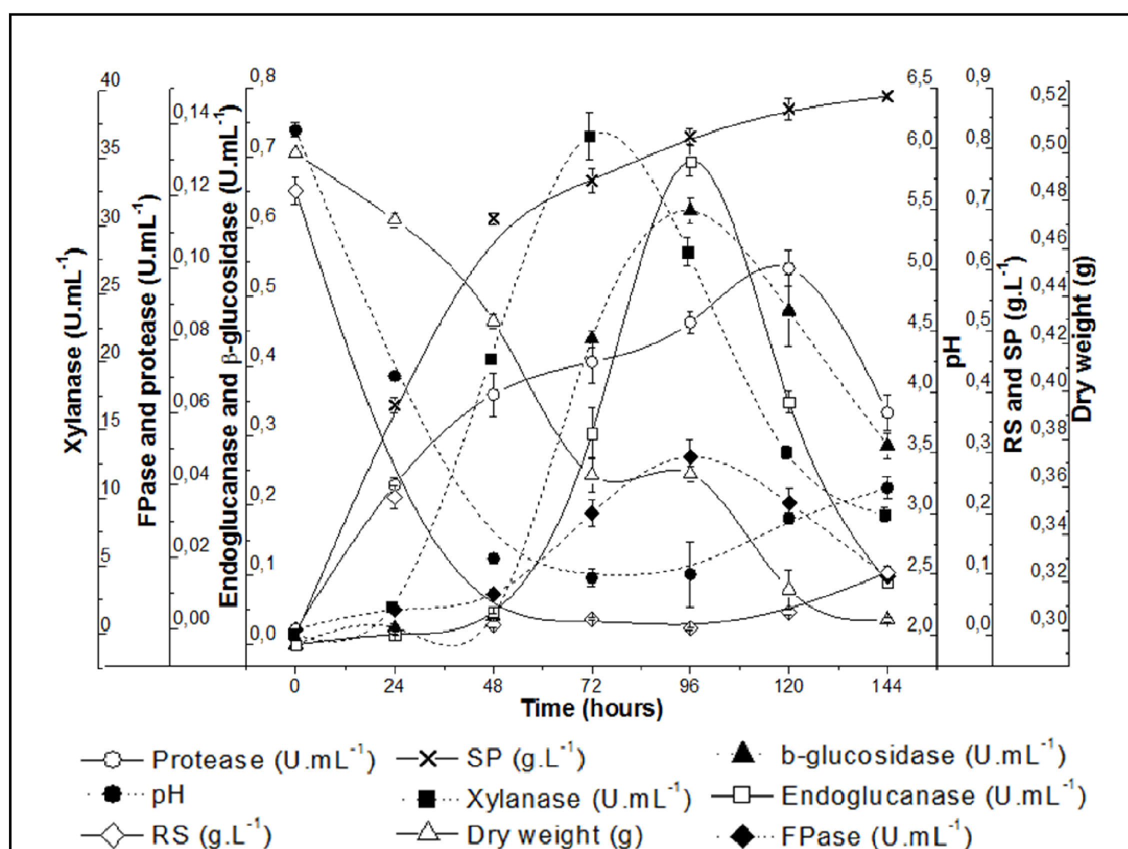

Figure 1. Activity profiles of endoglucanase, β-glucosidase, FPase, xylanase, protease, soluble proteins, reducing sugars, pH and dry weight variation in the period of 144 hours of submerged fermentation using cottonseed oil cake as carbon source and the strain *Aspergillus tubingensis* AN1257 as fermentative agent. SP: soluble protein; RS: reducing sugars

Ncube*et al*. (2012), working with the microorganism *Aspergillus niger* FGSCA733 and with Jatropha curcas oil cakes as a substrate, also observed this same secretory behavior in the fermentation process.This event, from the point of view of microbial physiology, is coherent with the priority expected in the process of deconstructing of the lignocellulosic matrix.In the classical model of the lignocellulosic matrix, the hemicellulose fraction covers the microfibrils of cellulose (Beg *et al*., 2001) and, therefore, should be first subtracted for that the microbial enzymes can access to cellulose. The Figure 1 also presente the profile of the production of total soluble proteins.The increase in the concentration of soluble proteins in the enzymatic extract started before of the cellulolytic and xylanolytic activities, and probably this rate of protein

secretion was associated with the early production of proteases. In the profile of protease production it is possible to observe the presence of proteolytic enzymes with activity of 0.100 UmL^{-1} after 120 hours of fermentation process, a fact that possibly contributed for the reduction of the activities of xylanolytic and cellulolytic enzymes, as presented in the progress curves. Pandya and Gupte (2012) also related the production of proteases during production of xylanases by *Aspergillus tubingenses* JP-1. In the mentioned work was observed a maximum peak of xylanase production followed by a reduction of the activities of this enzyme. During the submerged fermentation process with the *A. tubingensis* AN1257 lineage there was a significant reduction of the pH of the medium (Figure 1). The data show that during the peak of enzymatic production, 72 hours for xylanases and 96 hours for cellulases, the pH value dropped from 6.3 to 2.5. This data agree with the fact that some species of *Aspergillus* sp., produce many organic acids and usually have their growing associated to the reduction of the pH value in the cultivation medium (Aghaie*et al*., 2009; Angumeenal and Venkappayya, 2013; Papagianni, 2007).

As to the use of reducing soluble sugars, which are present in the fermentation medium, it is possible to verify that this carbon source ended in the first 48 hours. Only then the production of xylanases and cellulases, in this order, was observed. This may be connected to the repressing effect of glucose (or other sugars) over the synthesis of enzymes of the cellulolytic complex (Beguin, 1990; Hanif *et al*., 2004). Accompanying the dry weight related to the insoluble material recovered after the paper filtering for separating the anzymatic extract, allowed a glimpse of the decomposition of the insoluble fraction of the cottonseed oil cake, directly associated to the lignocellulosic material. At the end of the fermentation process, there was a decrease of 35% of the dry weight of the insoluble fraction. The mass of insoluble material retained on the filter paper was considered here as the undecomposed mass of oil cake originally added as a carbon source. Based on the value of the carbon source mass decay was calculated the yield ($Y_{P/S}$), due to the ratio between each enzyme produced and the quantity of cottonseed oil cake comsumed (Table 3). The quantification of soluble protein concentration produced along the time also allowed the calculation of specific activities values (amount of enzymatic activity per mg of produced soluble protein) of researched enzymes (Table 3). Among the cellulases, the largest value of specific activity was found for endoglucanase (0.875 U mg^{-1}). The specific activity for xylanase was 70 U mg^{-1}. Adhyaru*et al*. (2015) reported values of specific activity from 3.68 to 18.72 U mg^{-1} when studied the effect of various agro-residues on xylanase production by *A. tubingensis* FDHN1 under SSF, highlighting the sorghum straw.

Conclusions

The studied*Aspergillus tubingensis* AN1257 proved to be a good producer of endoglucanase and xylanase in submerged fermentation process using cottonseed, sunflower and macaubaoil cakes as substrates compared to the control strain *T. reesei* CCT2768. The cottonseed oil cake was the carbon source which better induced the production of holocellulolytic enzymes by *A. tubingensis* AN1257, exhibiting values of 620 UL^{-1} for endoclucanase, 670 UL^{-1} for β-glucosidase, 48 UL^{-1} for FPase and 35,000 UL^{-1} of xylanolytic activity.

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