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Screening and evaluation of filamentous fungi potential for protease production in swine plasma and red blood cells-based media: qualitative and quantitative methods

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ABSTRACT

Many fungi excrete proteases to the medium when stimulated. We aimed to submit filamentous fungi cultures to a screening so as to evaluate their proteolytic capacity. Spore solution of these fungi was inoculated in the center of plates, each one containing medium composed of dehydrated UHT milk, plasma or red blood cells. We qualitatively evaluated the enzyme index (EI) measuring the ratio of hydrolysis halo to mycelium diameters within 22 cultures of *Penicillium*, *Aspergillus*, *Cenococcum*, *Cochliobolus*, and *Rhizopu* genera. Proteolysis halo has occurred in 14 of the 22 evaluated strains (64%) using a traditional medium with milk. We observed 5 strains (23%) and 8 strains (36%) expressing proteolytic activity by the formation of proteolysis halo in the medium containing plasma and red blood cells, respectively. Regarding the EI, the pure strains of *A. brasiliensis, P. citrinum, A. niger, A. rhizopodus* as well as 3 strains of *Penicillium* sp. stood out. The cultures which presented the most promising results were used in solid-state fermentation in a medium composed of malt residue, plasma, and red blood cells. The enzyme activity was between 150 and 2,383 U.mL^{-1}. Overall, the qualitative method is a viable alternative to select stimulated cultures to produce protease by the presence of the protein sources. Additionally, plasma or red blood cells are promising for the high activity proteases production by different genera and species of fungi.

1. Introduction

Protease is a enzymes group applied in several areas and responsible for 60% of the enzymes marketed in the world, particularly the microbial origin ones (Sharma et al., 2017). They are used in the production of food, drinks, tanneries, detergents, cosmetics, pharmaceuticals, textile, and bioactive compounds, in the residue treatment, recovery of silver from photographic and X-ray films, diagnostics, medical treatment for inflammation and injuries, and also in the treatment of protein intolerance (Abidi et al., 2014; Bahet al., 2016; Banerjee and Ray, 2017; Castro and Sato, 2014a,b; Souza et al., 2017; Zhu et al., 2013). Specific enzymes with high purity index, low production cost, and great stability, are obtained by microbial production. These aspects are aligned with sustainability and minimal environmental impact aspects, especially when using agro-industrial residues as a substrate and/or growth support (Schuster and Kempka, 2017).

The proteolytic activity can be determined qualitatively by computing the enzymatic index (EI) (involving the formation of

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proteolysis halo in the Petri dish) and quantitatively by the extract activity produced by fermentation. Screening by EI is a cost-effective experimental method, allowing the selection of microorganisms that have the ability to excrete protease in the medium with protein sources. This is, therefore, indicated to the cases whether the microorganism's ability to excrete the enzyme is unknown or the protein sources somehow stimulate the production of extracellular protease. The quantitative determination of protease activity can be a relatively expensive method because it demands complex cultivation media, equipment and high purity analytical reagents. These aspects stop researchers from using this method for screening so as to assess the proteolytic potential of a large number of fungi (Fernandes, 2009; Freitas, 2009; Souza, 2015). To the best of our knowledge, some of the microorganisms studied in the present paper have not yet been studied regarding their proteolytic potential. Additionally, there are no published studies considering media containing plasma or red blood cells for protease production by filamentous fungi.

For the quantitative determination of the production of fungal proteases, the use of solid-state fermentation (SSF) is traditional. It allows the production of more concentrated metabolites, due to the lesser amount of water in the medium, reducing the production time and the cost of the enzyme recovery process (Pandey et al., 2000; Rodarte, 2005). In this method, the use of residues and by-products as a medium for supporting and supplying nutrients to the microorganism is also well-known. The production medium of protease by fungi in SSF may support their fixing, as well as provide nutrients and water. Some studies report that protease production depends on the availability of nitrogen and carbon in the medium, both exercising regulatory effects on the synthesis of the enzyme (Belmessikh et al., 2013; Castro and Sato, 2014a,b, 2015; D'Alessandro et al., 2007; Hajji et al., 2008).

Many microorganisms (including filamentous fungi) assimilate, absorb, or utilize organic nitrogen from amines to obtain energy and for their development. The amines have their amino acids linked by peptide bonds or small peptides, polypeptides, and proteins. On the other hand, protein and peptides can be hydrolyzed in amino acids and used as nitrogen sources for microorganisms in biotechnological processes. Some complex and conventionally used sources in fermentation are yeast extract, hydrolyzed from protein-rich plants, vegetables, food residues, meat by-products, animal tissues and bodily fluids, fish waste, feathers, serum permeate, and grass press extract. The hydrolysis of these protein-rich materials can be catalyzed by extracellular proteases excreted by microorganisms in the medium or by their previous hydrolysis (Pleissner and Venus, 2016).

The application of blood and its derivatives is restricted in the food industry due to health and legal issues as well as limitations concerning, especially, coloring. However, raw blood can be separated after the centrifugation or filtration into a fraction of plasma and a fraction of red blood cells. The first is composed of water and soluble substances, showing good emulsifying properties and thermal coagulation (Pérez-Gálvez et al., 2011), as well as translucency. However, it also contains significant content of salts, affecting organoleptic properties when used in foods (Del Hoyo et al., 2008). In spite of containing quality proteins in large quantities (represents 70–75% of the total protein in the blood), red blood cells have limited application in foods for human consumption, due to their intense brown color. Thus, research is necessary to take more advantage of this product and all the benefits which this rich nutritional composition could provide.

Dehydrated swine plasma has a protein content varying from 70% to 85%, moisture between 10% and 13%, and 1.5% of lipids (Niu et al., 2018). Red blood cells are composed of globular proteins, including hemoglobin, whose structure contains Fe^{2+} ions. Hemoglobin represents, approximately, 31% of the blood cellular fraction and 90–95% on a dry basis of the protein fraction of red blood cells (Pérez-Gálvez et al., 2011).

The use of agroindustrial residues rich in protein for protease production medium is a practice which results from the increase in the production of this enzyme when microorganisms are stimulated to excrete it in the medium to obtain carbon or nitrogen from protein hydrolysis. Despite the high concentration of protein in dehydrated plasma and red blood cells, their use by filamentous fungi is not yet known. It is necessary to evaluate if the microorganism will adapt to this medium, the protein is in the form in which it can be assimilated by the fungus and it is bioavailable. Bah et al. (2016) studied the production of bioactive peptides from the hydrolysis of blood from cattle, deer, sheep, and swine. The authors observed that the production was increased with fungal protease than with plant protease (papain and bromelain), especially from swine blood. This information indicates that fungi can produce proteases able to hydrolyze swine blood.

Fungal enzymes have been industrially produced as they present greater viability to obtain enzymes in high concentration in the fermentation medium and presenting easier downstream processing when compared to bacterial production (Siala et al., 2012). However, it is still primary to reduce costs and optimize production by the selection of more efficient media and microorganisms. Many fungal species are studied for the production of proteases, among which we can mention: *Penicillium italicum* (Abidi et al., 2014), *Aspergillus niger* (Bensmail et al., 2015; Castro and Sato, 2014a; Castro et al., 2015a,b; Gnanadoss et al., 2011), *Aspergillus oryzae* (Belmessikh et al., 2013; Chutmanop et al., 2008; Kumura et al., 2011; Murthy and Kusumoto, 2015), *Aspergillus foetidos* (Souza et al., 2015), *Penicillium* sp. (Agrawal et al., 2004; Benluvankar et al., 2015; Cunha et al., 2016; Germano et al., 2003), *Penicillium digitatum* (Aissaoui et al., 2014), *Penicillium roqueforti* (Fernández-Bodega, 2009), *Aspergillus clavatus* (Hajji et al., 2008), *Eupenicillium javanicum* (Hamin-Neto, 2012). However, we did not find reports of fungal protease production in media with plasma or red blood cells, only the use of fungi for production of protein hydrolysates and bioactive compounds by the action of fungi (Bah et al., 2016; Lee and Song, 2009) and the use of blood meal as supplement for protease production by microorganisms undergoing genetic mutations (Zheng et al., 2014).

The malt bagasse is the residue from the brewery industry formed by the grains with barley shells malted and grounded, subjected to mashing for sugars extracting from the enzymatic conversion of the grain's starch. This by-product represents approximately 85% of the total residues generated, contributing, on average, from 30% to 60% of the biochemical oxygen demand and suspended solids generated by the breweries (Fillaudeau et al., 2006). The malt bagasse generated represents, on average, 31% of the original weight of malt. Therefore, it is necessary to highlight all the possibilities available for recycling this waste for economic processing and bioconversion to more valuable products (Nigam, 2017).

According to Nigam (2017), barley residues can be used as a source of carbon in the fermentation for the cultivation of microbial biomass and production of microbial enzymes. It is a lignocellulosic material containing, approximately, 17% of cellulose, 28% of non-cellulosic polysaccharides, and 28% of lignin. Barley malt bagasse is available in large quantities throughout the year, but its main application was limited to animal nutrition (Mussatto et al., 2006). The concentration of protein and other nutrients in the malt bagasse is dependent on factors such as type of cereal used, adding of adjuncts to the production process, and grinding and mashing conditions. Also, it ranges from 10 to 28% on a dry basis, according to Robertson et al. (2010). It contains up to 70% of dry fiber (Mussatto et al., 2006) and absorbs large amounts of water, apart from having the ideal heterogeneous granulometry to avoid medium compacting and large surface area for the utilization of nutrients by microorganisms. In addition, the present shell is a good medium to fix filamentous fungi (Mathias and Mello, 2014), which led to the option of using it as a basis for fixing of the fungus and main source of carbon of the medium to SSF.

Thus, the objective of this study was to evaluate the feasibility of adapting the conventional qualitative methodology replacing milk for the protein source of interest (plasma and swine red blood cells) to select

Table 1
Identification code, genus/species, and origin of the strains used in the screening of fungi with proteolytic potential.

Identification code	Genus/species	Source institution
D36	*Cenococcum geophilum*	UFSC
D210	*Penicillium bilaiae*	UFSC
D211	*Aspergillus niger*	UFSC
D214	*Penicillium camemberti*	UFSC
D215	*Penicillium roqueforti*	UFSC
LMA 071	*Cochliobolus kusanoi*	CBMAI-UNICAMP
LMA 103	*Aspergillus flavus*	CBMAI-UNICAMP
LMA 105	*Penicillium* sp.	CBMAI-UNICAMP
LMA 141	*Penicillium* sp.	CBMAI-UNICAMP
CBMAI 2135	*Penicillium citrinum*	CBMAI-UNICAMP
LMA 239	*Penicillium* sp.	CBMAI-UNICAMP
LMA 240	*Aspergillus parasiticus*	CBMAI-UNICAMP
LMA 264	*Penicillium* sp.	CBMAI-UNICAMP
LMA 878	*Penicillium* sp.	CBMAI-UNICAMP
LMA 1300	*Penicillium* sp.	CBMAI-UNICAMP
CBMAI 2138	*Talaromyces cf. francoae*	CBMAI-UNICAMP
CBMAI 2140	*Aspergillus unguis*	CBMAI-UNICAMP
CBMAI 2133	*Aspergillus giganteus*	CBMAI-UNICAMP
CBMAI 2132	*Aspergillus rhizopodus*	CBMAI-UNICAMP
Rh CAV	*Rhizopus stolonifer*	CAV – UDESC
A.B - LB	*Aspergillus brasiliensis*	SENAI – SC
A.N - LB	*Aspergillus niger*	BIOLAB - UDESC

cultures that presented proteolytic activity in their presence.

2. Materials and methods

2.1. Reagents and agro-industrial inputs

The reagents used were all analytical degree (purity $\geq$ 95%). Ultrapure water (Milli-Q, Millipore, Bedford, MA, USA) was used for the preparation of solutions. The azocasein and trichloroacetic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). The media PDA and Sabouraud were purchased from Merck KGaA (Darmstadt, Germany).

The malt bagasse was donated by Dalla Microcervejaria (Chapecó, SC, Brazil), all residue from Pilsen beer production. The bagasse was dried at 45 °C in a stove with air circulation of up to moisture <6%, then classified with a Mesh 8 sieve (mesh opening from 1.68 to 2.38 mm) for granulometry standardization. The plasma and red blood cells were donated by APC do Brazil Ltda (Chapecó, SC, Brazil). They were fractions from the blood of swine slaughtered for food purposes, subjected to the ultrafiltration separation process, followed by spray drying.

2.2. Substrates characterization

The methodologies of the AOAC (AOAC, 2016) were used for the physicochemical characterization of substrates as follow: moisture (by mass difference), protein content (by Kjeldahl total nitrogen), lipid content (by Soxhlet extraction) and ashes (by incineration in muffle at 550 °C). The carbohydrate content was considered as the difference between the total weight and the other components analyzed previously. All analyses were performed in triplicate and the results expressed as mean and standard deviation.

2.3. Microorganisms

The strains of microorganisms used to determine the proteolytic capacity were made available by the Laboratory of Bioprocesses (BIOLAB - UDESC), Center of Biological Sciences - Department of Microbiology, Immunology and Parasitology of the Federal University of Santa Catarina (UFSC), the Laboratory of Microbiological Analysis of the National Industrial Apprenticeship Service (SENAI Chapecó-SC), the Brazilian Collection of Environment and Industry Microorganisms (CBMAI/DRM UNICAMP), and the Micoteca of the Center of Agro-veterinary Sciences (CAV-UDESC). Table 1 presents the microorganisms used in the screening, their identification code, genus/species, and institution of origin.

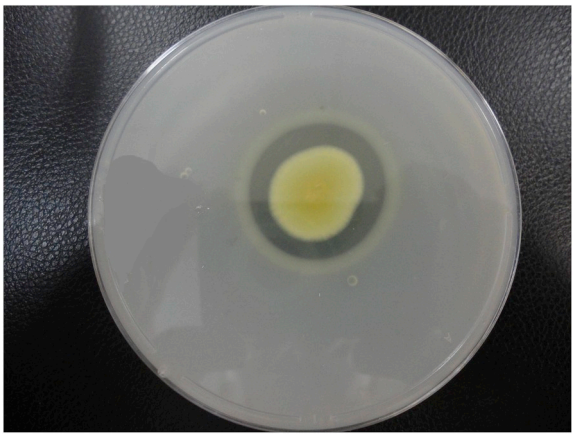


Fig. 1. Mycelium and the translucent halo of fungi presenting proteolytic potential.

2.4. Development of cultivation media for identification of fungi with potential for production of proteolytic enzymes

The cultivation media used to identify protease-producing strains consisted of a standard medium (M) of agar-milk according to conventional methodology (Fernandes, 2009; Freitas, 2009; Souza, 2015), and two developed media based on the standard medium. In these cases, milk was replaced by plasma (P) or red blood cells (R) of swine-origin (spray dried).

Aqueous solutions of agar-agar (2% m.v^{-1}) and gelatin (1% m.v^{-1}) were mixed to obtain the M medium. The mixture was heated for 15 s in a microwave at average power for dissolution. The medium was autoclaved at 121 °C, 1 atm for 15 min and then cooled in a water bath until 58 °C. Then, under aseptic conditions, 10% of lactose-free skimmed UHT milk was added and the pH was adjusted to 5.0 with a 1% tartaric acid solution. Approximately 20 mL of medium were shed in sterilized 80 mm diameter Petri dishes, leaving them to cool completely for later use (Fernandes, 2009; Freitas, 2009; Souza, 2015). According to Morris et al. (2012), the use of milk with lactose can generate false-positive results due to the formation of a hydrolysis halo from the conversion of lactose into lactic acid (acid hydrolysis) and not due to the production of protease.

To obtain the media P and R, we followed the same procedure of medium M. Separately, we dissolved 2.5 g of dried plasma (for the medium) and 2.5 g of dehydrated red blood cells (for the medium) in 25.0 mL of sterile Tween 80 0.3% solution, aiming to obtain better dispersion of proteins in the medium. The solution of plasma and red blood cells was added to the medium, under aseptic conditions and separately. The media achieved a final concentration of plasma or red blood cells of 1%. The addition of the protein source to the sterile medium and the correction of pH after addition became necessary due to the denaturation of the protein caused by the heat and isoelectric point. Then, the procedure was the same as carried out for standard medium M.

2.5. Screening of fungi with proteolytic potential for the formation of a proteolysis halo - qualitative method

The determination of the enzymatic index, obtained by the formation of proteolysis halo, followed the methodology described by Souza et al. (2015), Fernandes (2009), and El-shora and Metwally (2008), with adaptations. The microorganisms were cultivated in a PDA (Potato Dextrose Agar) medium in a tilted tube for 10 days at 28 °C. Then, we

removed the spores with sterile Tween 80 0.3% solution and glass beads and diluted to obtain a solution containing 1×10^6 spores.mL^{-1}. The spore count was carried out in an optical microscope and a Neubauer Chamber. Growing media M, P, and R were prepared and we inoculated, in the central region of the dish, 10 μL of suspension with 10^6 spores. mL^{-1} of the studied strains.

The dishes were incubated at 28 °C for 120 h. We measured the diameter of the mycelium and the translucent halo (halo of proteolytic activity) every 12 h of incubation using a pachymeter, as shown in Fig. 1. The presence of the halo characterized proteolytic activity in the fungus. The EI (enzymatic index) was obtained by the ratio between the diameters of the halo (mm) divided and the mycelium (mm) (Fernandes, 2009). All experiments were performed in triplicate and the results presented as average ± standard deviation.

2.6. Evaluation of the potential for protease production by SSF - quantitative method

The cultures that presented proteolytic halo in the P and R medium were submitted to SSF and the protease activity was determined quantitatively. To do so, each strain was cultivated in tubes (sloped medium) containing 10 mL of the PDA medium at 2% and pH 5 (corrected using 0,1 M tartaric acid). Incubation was carried out at 28 °C for 10 days for complete development of spores. After this time, the tubes were kept refrigerated (4 °C–7 °C) for at most 14 days and young cultures were subsequently used in all experiments (Castro et al., 2015; Fernandes, 2009; Silva et al., 2011).

For protease production by SSF, different cultivation media containing plasma and red blood cells, called SP and SR media, respectively, and control medium (C) were used. The SP medium consisted of 9 g of malt bagasse added of 10% (of the total mass) of dehydrated swine plasma (1 g). The plasma was dissolved in 10 mL of dispersant solution (0.3% Tween 80 and 0.9% NaCl) and shook at 60 rpm for 10 min. Then, it was spread over the malt bagasse. The medium's moisture was standardized to 65% by the addition of ultrapure water and the inoculum. For the SR medium, the same procedure used for the SP medium was followed, replacing the plasma by dehydrated swine red blood cells (10%). For the C medium, the same procedure used for the SP and SR medium was followed using only malt bagasse (i.e. without plasma or red blood cells).

All media were prepared in 600 mL glass beakers, covered with aluminium foil and soft tissue. The media were sterilized in autoclave 121 °C at 1 atm for 20 min. After cooling, 1 mL of the suspension with 10^6 spores.mL^{-1} was inoculated in the medium and incubated in a BOD incubator at 28 °C for 72 h. An ultrasonic humidifier was used to set the relative moisture inside the BOD at 85–90%.

After the fermentation time, 100 mL of ultrapure water was added (Milli-Q® system, Millipore). The solution was homogenized and stirred, in orbital shaker, for 1 h at 60 rpm. The mixture was filtered in paper filter weighting 80 g m^{-2}, and the filtrate was collected and centrifuged at 3,500 rpm for 10 min to remove impurities and cells in suspension. The obtained supernatant was the gross enzymatic extract (Benluvankar et al., 2015; Castro et al., 2014; Germano et al., 2003).

2.7. Determination of protease activity

Protease activity was determined according to the method proposed by Charney and Tomarelli (1947), described by Souza (2015) and Castro et al. (2014). 500 μL of the crude filtered extract was added in Eppendorf tubes, as well as 500 μL of azocasein at 0.5% (m.v^{-1}) in a buffered solution of sodium acetate at 0.2 M pH 5.0. It was incubated at 37.5 °C and, after 40 min, the reaction stopped by the addition of 500 μL of trichloroacetic acid (TCA) 10% (m.v^{-1}) for precipitation of non-hydrolyzed casein. We centrifuged it at 10,000 g for 15 min at 4 °C and 1 mL of the supernatant was transferred to a new tube. 1 mL of KOH 5.0 N was added for alkalinization of the medium to induce the formation of an orange coloration from the azo group and the absorbance was measured in a spectrophotometer (Pro-Tools V 1200) at 428 nm. To avoid interference due to the different colorations of extracts in the growth medium, a blank was used for each sample, obtained by addition of 500 μL of TCA in 500 μL of the enzymatic extract before the addition of the substrate (azocasein). A unit of enzyme activity was defined as the quantity of enzyme capable to produce an increase in the difference between absorbance of the blank and of the sample in 0.001. min^{-1}. The activity was determined according to Equation (1), where: A is the protease activity (U.mL^{-1}), A_{ba} is the sample absorbance, A_{bb} is the sample's blank absorbance, t is the incubation time (minutes), multiplied by 100 due to the use of 100 mL of ultrapure water in the extraction.

$$A = \frac{Aba - Abb}{0,001.t} * 100 \quad (1)$$

Table 2
Maximum enzyme index (EI) followed by incubation time for the strains evaluated in the screening, grown in growth media with milk, plasma, and red blood cells.

Fungus	EI ± SD - time (h)		
	M medium	P medium	R medium
A. brasiliensis	$1.71^{bc*B} \pm 0.04$ – 84 h	$3.76^{aA**} \pm 0.5$ – 48 h	$1.55^{bcdB} \pm 0.18$ – 108 h
P. citrinum (CBMAI 2135)	$1.94^{abA} \pm 0.01$ – 36 h	$1.48_{bA} \pm 0.08$ – 48 h	$1.93^{bA} \pm 0.45$ – 48 h
Penicillium sp. (LMA1300)	$1.70^{bcA} \pm 0.15$ – 60 h	$1.55^{bA} \pm 0.03$ – 60 h	$1.42^{cdeA} \pm 0.13$ – 72 h
A. rhizopodus (CBMAI 2132)	$1.44^{cA} \pm 0.01$ – 72 h	$1.51^{bA} \pm 0.03$ – 120 h	$1.21^{deB} \pm 0.06$ – 108 h
Penicillium sp. (LMA 878)	$0.70^{eB} \pm 0.11$ – 48 h	$1.00^{cA} \pm 0.09$ – 48 h	$1.10^{dA} \pm 0.12$ – 48 h
Penicillium sp. (LMA 239)	$1.69^{bcA} \pm 0.05$ – 72 h	$1.78^{bA} \pm 0.32$ – 84 h	***
A. niger (A.N LB)	$1.58^{cB} \pm 0.01$ – 96 h	***	$2.93^{aA} \pm 0.27$ – 48 h
A. niger (D211)	$1.55^{cA} \pm 0.24$ – 72 h	***	$1.78^{bcA} \pm 0.07$ – 48 h
Aspergillus sp. (LMA1798)	$1.57^{cA} \pm 0.09$ – 120 h	***	$1.50^{defA} \pm 0.23$ – 60 h
P. bilaiae (D210)	$1.64^{bcA} \pm 0.26$ – 48 h	***	$1.49^{defA} \pm 0.15$ – 60 h
A. giganteus (CBMAI 2133)	$1.43^{cA} \pm 0.03$ – 72 h	***	***
A. parasiticus (LMA 240)	$1.1^{dA} \pm 0.04$ – 72 h	***	***
Talaromyces cf. francoae (CBMAI 2138)	$1.48^{cA} \pm 0.02$ – 72 h	***	***
Penicillium sp. (LMA 264)	$1.70^{bcA} \pm 0.11$ – 48 h	***	***
R. stolonifera (Rh CAV)	$2.02^{aA} \pm 0.06$ – 120 h	***	***
A. flavus (LMA 103)	***	***	***
P. camemberti (D 214)	***	***	***
P. roqueforti (D215)	***	***	***
Penicillium sp. (LMA 105)	***	***	***
Penicillium sp. (LMA 141)	***	***	***
Cenococcum geophilum (D36)	***	***	***
Cochliobolus kusanoi (LMA 071)	***	***	***

SD = standard deviation. * Means followed by lower case letters in the same column do not differ statistically by the Tukey test at 5% probability. ** Means followed by equal capital letters on the same line do not differ statistically by the Tukey test at 5% probability. *** No halo formation during 120 h incubation.

2.8. Molecular characterization of the protease fungi producers (dendrogram)

The DNA sequences generated by automated sequencing in ABI 3500XL equipment were used for the construction of distance genetic dendrograms, aiming the taxonomic identification. The assembly of the consensus sequences (contig) was performed using the Bioedit program. Afterwards, the "consensus" sequence was compared with others available in the databases, using NCBS Blast and Pairwise sequence alignment tools from CBS (Westerdijk Fungal Biodiversity Institute). After the selection of the sequences, preferably those of Type lines, the set was aligned using the program CLUSTAL X (Thompson et al., 1997) within BioEdit 7.2.6 (Hall, 1999). The generated file was then used for the construction of the dendrogram considering the evolutionary distances (Kimura, 1980), made by the Neighbor-Joining method (Saitou and Nei, 1987). The bootstrap values were calculated from 1,000 re-samples in the MEGA version 6.0 program (Tamura et al., 2013).

2.9. Statistical analysis

The values are expressed as the arithmetic average ± standard deviation. Tukey test was used to determine the significant differences between the groups analyzed, with 95% confidence. When $p < 0.05$, the differences were considered significant. The Statistica® software version 13.3 (Statsoft Inc.) was employed to compare the results statistically.

3. Results and discussion

3.1. Screening by the qualitative method of protease-producing filamentous fungi

Table 2 shows the results of EI for microorganisms grown in the M, P, and R media. We verified that there was a statistically significant difference ($p < 0.05$) between the strains and the media. The fungi showed different growth rates in the media, some manifested proteolytic halo in the first hours of incubation, while others took more time for the halo formation.

By determining EI over 120 h of incubation, we found that among the 22 strains evaluated, 14 showed the formation of proteolytic halo in at least one of the formulated media. According to results shown in Table 2, it is possible to observe that the isolates tested exhibited variation about the potential for protease production and the time required to achieve the maximum EI. The fungi that stood out were *A. brasiliensis*, *P. citrinum* (CBMAI 2135), *A. rhizopodus* (CBMAI 2132), *Penicillium* sp. (LMA 1300 and LMA 239), *Rhizopus stolonifera* (in the milk medium), and *A. niger* (in the red blood cells medium).

In the traditional medium containing milk (M medium), there was the formation of proteolysis halo in 14 of the 22 evaluated strains (64%). In the P medium, added of plasma, 5 strains (23%) expressed proteolytic activity by the formation of proteolysis halo, while in the R medium, added of red blood cells, 8 strains (36%) presented proteolysis halo in the studied conditions. Some fungi that presented proteolytic activity did not manifest this ability in the presence of plasma and red blood cells. This characteristic can be related to the fact that protease production may be conditioned to the availability of nitrogen and carbon in the medium, both exercising regulatory effects on the synthesis of the enzyme (Belmessikh et al., 2013; Castro and Sato, 2014, 2015; D'Alessandro et al., 2007; Hajji et al., 2008) or because the ability of the fungus to use certain carbon or nitrogen sources differs from one microorganism to the other (Castro and Sato, 2014; Freitas, 2009; Schuster et al., 2017).

A. brasiliensis (A.B LB) reached an EI = 3.76 in 48 h of incubation in the P medium, which is greater and statistically different ($p < 0.05$) from the EI manifested by this fungus in the M and R media. This result is also statistically different ($p < 0.05$) and greater to the EI presented in the P medium by the other analyzed fungi. According to El-Shora and Metwally (2008), EI ≥ 2 is considered as a significant index of proteolytic activity. This result suggests that plasma stimulates more *A. brasiliensis* to produce and excrete the protease to the medium in the first hours of fungus development when compared to the M and R media, although there is also the formation of hydrolysis halo in their presence. There are reports of the application of this fungus for the production of naringinase enzymes (Shanmugaprakash et al., 2014), epoxide hydrolases (Beloti et al., 2013), and proteases (Novelli et al., 2016).

The *P. citrinum* (CBMAI 2135) strain also presented the formation of halo in the 3 evaluated media, being EI = 1.94, 1.48, and 1.93 in M, P, and R, respectively, in the first hours of incubation. There was no statistically significant difference ($p > 0.05$) among them, getting very close to 2, for the M and R media. This shows that protein sources stimulate the production of protease as much as the conventional methodology that uses milk. The ability to produce extracellular protease of this microorganism was previously reported by Rodarte (2005) and Rodarte et al. (2011), who isolated it from coffee beans, and by Sousa (2015), who isolated it from the Brazilian cerrado soil and determined EI in a medium with casein and protease activity of the extract produced by fermentation. The culture of *A. rhizopodus* manifested activity in both media, although the EI in the R medium is smaller and statistically different ($p < 0.05$) from the P and M media. No reports of proteolytic activity investigations were found using the enzymatic index method using this fungus.

Among the cultures of the species of *A. niger* (A.N LB and D211), both presented halo formation and did not differ statistically ($p > 0.05$) by Tukey test (95%) regarding the EI in the standard medium M. On the other hand, the presence of red blood cells or plasma resulted in significant differences among the strains of this species. We highlight the pure culture A.N LB with EI = 2.93 in 48 h of incubation in the red blood cells medium, with the EI being smaller ($p < 0.05$) in the M medium (EI = 1.58) and absent in the P medium. Culture D211 of the same species presented EI statistically equal to the M medium in the presence of red blood cells, suggesting that this protein source (red blood cells) stimulates the production of extracellular protease by these strains. This variability can be associated with affinity and adaptation that the microorganism has regarding the medium and the environment. The enzymatic activity of the metabolites of the fungi depends on the cell line, making necessary the distinction and selection of lines (Rodarte et al., 2011; Souza et al., 2015). One same microorganism may produce numerous proteases, depending on the species, or even different strains of the same species, as noted in the species *A. niger*. Proteases with different properties can be produced within the same species depending on the isolate and the factors related to the process for obtaining the enzyme (Koka and Weimer, 2000; Rodarte, 2005).

Strains LMA 1300 and LMA 878, both of *Penicillium* sp., also presented EI in the 3 medium formulations, while culture LMA 239 presented EI in the M and P media, showing the proteolytic potential of this genus.

Culture *Rhizopus stolonifer* also stood out, presenting EI greater than 2.0 in the M medium, although no activity has not been observed in the other media. When the microorganism expressed proteolytic activity in the M medium but did not in the P or R media, this result indicates that protein sources may have inhibited the production and release of the enzyme to the medium. Furthermore, the protease excreted might not be able to hydrolyze the protein, and consequently, do not present halo formation. The evaluation of a standard medium is essential to differentiate fungi that do not excrete protease from those ones which do not produce it in the medium with plasma and red blood cells.

Among the *Aspergillus* genus, only *Aspergillus flavus* did not show proteolytic activity. Fernandes (2009) also evaluated the EI of *A. flavus* isolated from peanut in a medium containing skim milk and the index for the proteolytic activity was 1.07, much lower than that of the other microorganisms in the same study. In addition, the author evaluated strains of *Penicillium* sp. isolated from meat sausages, soil, and environment, getting EIs of 1.58, 1.13, and 1.05, respectively. The peanut

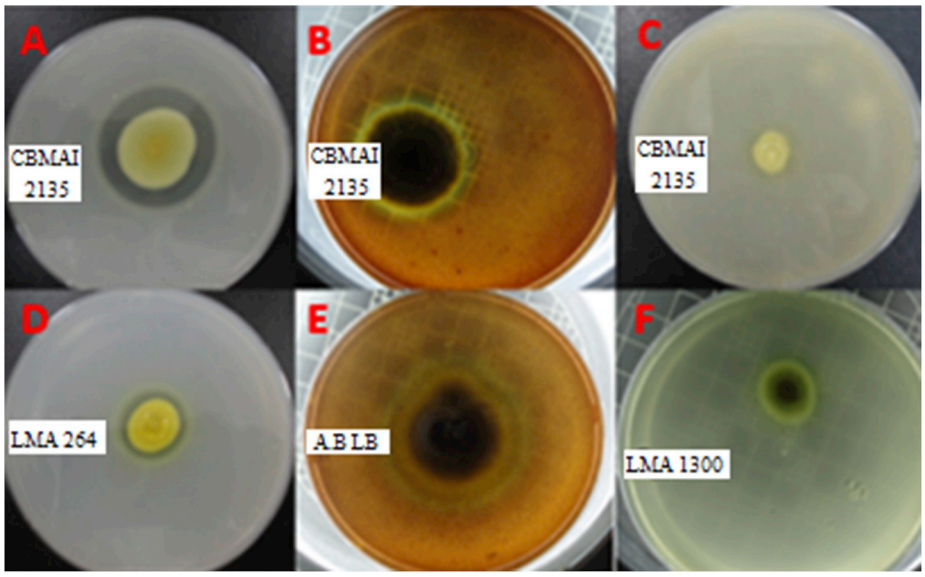


Fig. 2. Halos of protease activity in the milk, plasma, and red blood cells media for different fungi strains. A, B, and C – CBMAI 2135 in the M, R, and P media, respectively; D – LMA 264 in the M medium; E – A.B LB in the R medium; F- LMA 1300 in the P medium. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 3
Characterization of the media used in solid state fermentation (SP, SR and C) and the substrates malt bagasse, plasma and red blood cells used in the formulation of the media.

Parameter	Plasma	Red blood cells	Malt bagasse	SP	SR	C
Moisture (%)	$5.41^{a} \pm 0.07$	$4.52^{b} \pm 0.06$	$5.82^{a} \pm 0.29$	$65.00^{a} \pm 0.20$	$65.00^{a} \pm 0.02$	$65.00^{a} \pm 0.12$
Protein (%)	$78.66^{a} \pm 0.82$	$85.80^{a} \pm 0.59$	$17.23^{b} \pm 0.05$	$8.45^{a} \pm 0.61$	$8.97^{a} \pm 0.10$	$6.41^{b} \pm 0.25$
Lipids (%)	$0.80^{a} \pm 0.27$	$1.21^{a} \pm 0.36$	$4.75^{b} \pm 0.29$	$1.62^{a} \pm 0.11$	$1.64^{a} \pm 0.08$	$1.77^{a} \pm 0.05$
Ashes (%)	$10.00^{a} \pm 0.11$	$5.38^{b} \pm 0.52$	$2.84^{c} \pm 0.14$	$1.32^{a} \pm 0.07$	$1.15^{a} \pm 0.05$	$1.06^{a} \pm 0.02$
Carbohydrates (%)	$5.13^{b} \pm 0.30$	$3.09^{b} \pm 0.31$	$61.29^{a} \pm 0.17$	$23.61^{a} \pm 0.21$	$23.24^{a} \pm 0.80$	$26.46^{b} \pm 0.32$

Means followed by equal letters in the same line do not differ statistically by the Tukey test at 5% probability between the substrates (plasma, red blood cells and malt bagasse) or between formulated media (SP, SR and C).

strain *A. niger* isolated did not provide EI and the best result was 2.64 due to *Cladosporium cladosporioides*.

Evaluating the ability of enzymes production in fungi isolated in cornflour Abe et al. (2015), obtained EI of 1.53 and 1.17 for *Penicillium* sp. and *A. flavus*, respectively. The authors did not find a proteolytic activity for the fungi *Aspergillus* sp. and the greater EI was of 2.33, also for *Cladosporium cladosporioides*.

Fig. 2 presents examples of the manifestation of halo in different media by different fungi, which show this difference in growth rate and intensity of the proteolytic halo.

3.2. Substrate characterization

The characterization of the substrates is presented in Table 3, the results of which are expressed on a dry basis, as a mean of triplicate ± standard deviation. It is verified a high protein content in the plasma and red blood cells can be used as a source of nitrogen in fermentation processes. Malt bagasse is the supplier of carbon to the energy demand required by the fungi in their development, while plasma and red blood cells mainly supply nitrogen for fungus growth. For the characterization of the SP, SR and C media, after adjusting them to 65% of moisture, that there was no statistically significant difference ($p > 0.05$) between protein and carbohydrate contents in the media. However, in the medium without the protein sources (C), the protein content was lower and statistically different ($p < 0.05$) and the carbohydrate content was higher and statistically different ($p < 0.05$) from the media with protein sources. There was no statistically significant difference by the Tukey test at 5% probability in the lipid, ash and moisture content among the three evaluated media.

Table 4
Protease activity of the fungi selected in the qualitative evaluation by EI, grown in medium containing plasma (SP) and red blood cells (SR) using SSF.

Fungus	Protease activity ($U.mL^{-1}$) ± SD	
	SP medium	SR medium
P. citrinum (CBMAI 2135)	$2056.7^{aA} \pm 35.4$	$1857.5^{bB} \pm 14.1$
A. brasiliensis (A.B LB)	$1020.0^{cB} \pm 40.5$	$2382.0^{aA} \pm 25.4$
A. niger (A.N LB)	$1465.0^{bA} \pm 73.7$	$1462.5^{cA} \pm 38.3$
A. niger (D211)	$980.0^{cB} \pm 55.0$	$1785.0^{bA} \pm 62.3$
A. rhizopodus (CBMAI 2132)	$1726.7^{abA} \pm 35.3$	$1730.0^{bA} \pm 21.2$
Penicillium sp (LMA 878)	$1970.0^{abA} \pm 19.8$	$1606.7^{bB} \pm 21.3$
A. unguis (CBMAI 2140)	$1352.5^{bB} \pm 10.5$	$1560.0^{bcA} \pm 40.9$
Penicillium sp (LMA 1300)	$803.3^{cA} \pm 12.7$	$820.0^{dA} \pm 15.9$
P. bilaiae (D210)	$811.1^{cA} \pm 57.6$	$921.1^{dA} \pm 61.2$
Penicillium sp. (LMA 239)	$150.0^{dB} \pm 16.5$	$502.5^{eA} \pm 21.6$
Cochliobolus kusanoi (LMA 071)	$0.0^{eA} \pm 0.0$	$0.0^{fA} \pm 0.0$

SD = standard deviation. Different lowercase letters (in the column), correspond to different means ($p < 0.05$), compared among strains. Different uppercase letters (in the line), correspond to different means ($p < 0.05$), compared among growth media. SP and SR are the plasma and red blood cells media, respectively.

3.3. Evaluation by the quantitative method of protease production by strains selected in the qualitative method

The cultures that showed proteolytic halo in at least one of the media, P or R, were used in solid-state fermentation (SSF) and we

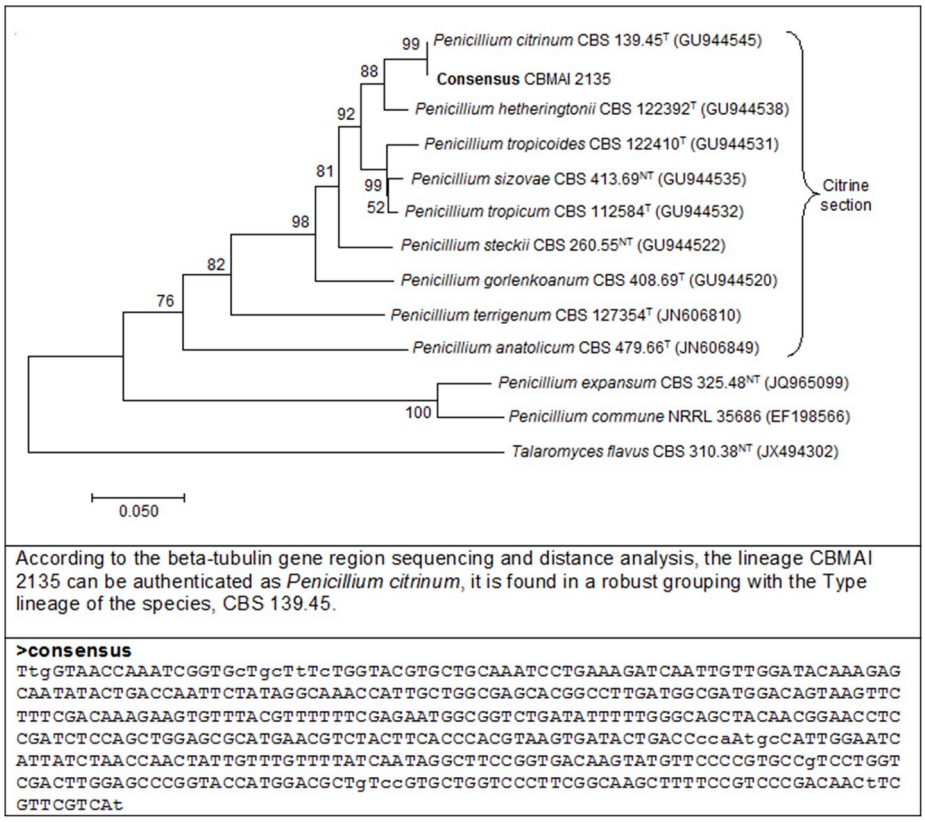


Fig. 3. Molecular characterization of *Penicilliun. citrinum* (CBMAI 2135) according to the beta-tubulin gene region sequencing and distance analysis.

determined quantitatively the protease activity in the extract obtained. Table 4 presents the activity of the enzyme in the extract after 72 h of fermentation.

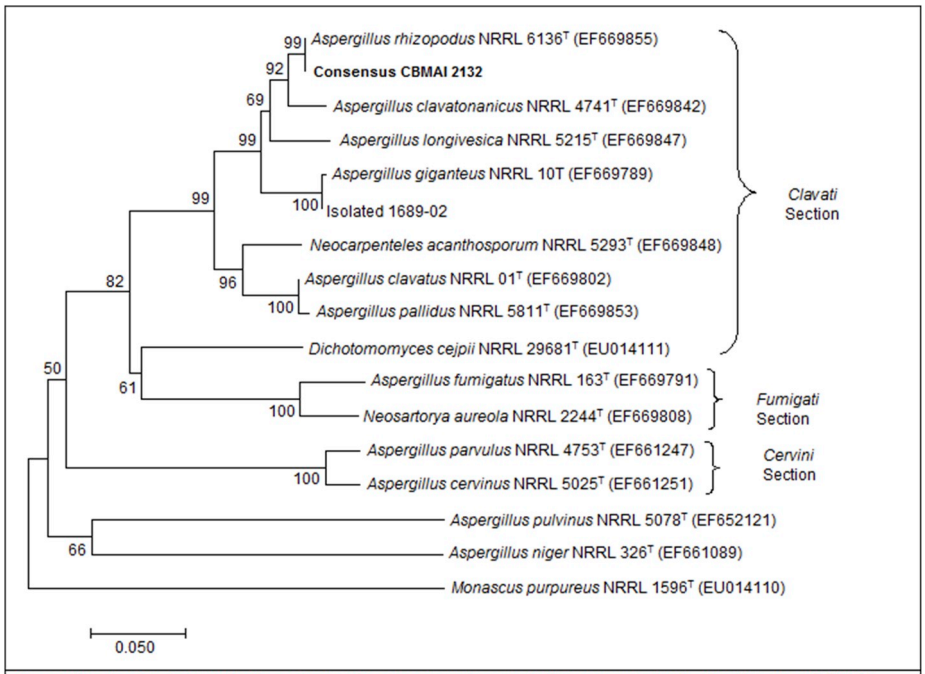
Quantitative analysis confirms the results obtained in the screening by EI since the proteolytic activity was also detected in SSF for microorganisms that presented proteolytic halo. In addition, LMA 071, which did not present halo formation, also did not present protease activity in the SSF extract. It is possible to notice, however, that there is a directly proportional correlation between EI and the proteolytic activity of microorganisms in the SSF. Nutritional conditions (especially the C:N relationship), aeration, fixing support, water activity, pH, among others, are different when the culture occurs in growth media in Petri dish (to determine EI) or in growth media composed by residues/by-products in SSF (when the proteolytic activity is determined).

This change in behavior and nonproportionality is evident for the culture of *A. brasiliensis* when observing the results of EI 3.76 and 1.55 (Table 2) and protease activity 1,020.0 and 2,382.0 U. mL^{-1} (Table 4) in media with plasma and red blood cells, respectively. This microorganism expressed the enzymes in the semisolid medium based on agar containing plasma much easier than in fermentation of the medium based on malt bagasse with the same protein source.

The activity of filamentous fungi of the genus Penicillium sp. is known for various substrates: peanut oil (Benluvankar et al., 2015), wheat bran (Agrawal et al., 2004; Hamzah et al., 2009; Novelli et al., 2016; Raja et al., 2011), and soy pie (Germano et al., 2003). However, as for the other fungi, its ability to produce protease in the presence of plasma or red blood cell is not reported. Among the fungi of this genus, the *Penicillium citrinum* has its proteolytic capacity known, as of the study by Rodarte (2005) and Rodarte et al. (2011), who isolated it from coffee grains, and Sousa (2015), who isolated it from Brazilian cerrado soil and determined the protease activity of the extract produced by fermentation. The protease activity observed by Rodarte (2005) and Rodarte et al. (2011) was lower (10.89 $U.mL^{-1}$) than observed by Sousa (2015) (<5,0 $U.mL^{-1}$). In both situations, the activity was lower than that observed in this study with plasma or red blood cells. The production conditions as the composition of the medium, pH, and temperature to SSF were different between them. Therefore, the variation in protein content and in the C:N ratio is an important starting point to elucidate the protease production capacity by this microorganism, and also to determine the profile of production in different media. Nevertheless, the high activity indicated a potential application of the by-products plasma and red blood cells to stimulate protease production.

The cultures of *A. niger* showed different activity among each other in the same medium in both media and between a medium and the other ($p < 0.05$), a behavior also observed in the screening in cultures in the Petri dish with the standard medium and the media with plasma and red blood cells. This activity, although smaller than in other cultures, is still significant, with results 1,785.0 $U.mL^{-1}$ for SR and 1,465.0 $U.mL^{-1}$ for SP. The activity of this fungus is widely studied and important in biotechnology. In medium containing shrimp peptone, 183.13 $U.mL^{-1}$ protease activity was observed in SSF with this fungus (Siala et al., 2012). Species of *Aspergillus* use a wide variety of substrates for their growth and have different biochemical pathways for assimilation (Hajji et al., 2008). PrtT is a transcription activator of extracellular proteases specific to fungi of the Aspergilli group. A study researching the activator analogous to this one in *Penicillium oxalicum* showed that this mechanism is activated by the availability or limitation of certain sources of nutrients, especially carbon and proteins (Chen et al., 2014).

There are reports of protease production by *A. niger* using various substrates: soy flour (Castro et al., 2014), soybean meal, cottonseed meal and orange peel (Castro et al., 2015a,b), husked wheat grain with shrimp peptone (Siala et al., 2012), canola pie (Freitas, 2009), starch, gelatin and yeast extract (El-Shora and Metwally, 2008), cottonseed pie, soybean (Gnanadoss et al., 2011), beer residue (Hasan et al., 2013), wheat bran (Bensmail et al., 2015; Castro et al., 2014, 2015; Castro



According to the beta-tubulin gene region sequencing and distance analysis, the lineage CBMAI 2132 can be authenticated as *Aspergillus rhizopodus*, it is found in a robust grouping and supported by high re-sampling value (99%) with the Type lineage of the species, NRRL 6136.

>consensus
TTGATGTTGATGGCGATGATGGAGGATTAAGAGCTATATATCTCGATTGGCAATTTGCCTGGGgAGGtGATGG
TTCGAATTTTGTGGGTGTGGGcTGACAATTTtATAGGCAGACCATCTCTGGTGAGCACGGCCTCGACGGCTCT
GGCCAGTAAGTTAACTCAACCTTTTATCCCCgATCGATAAGGCTACATACGTTGGCAAATACGAGGAGGATTG
CAACAATGGATACGGATCTGATGGAATCGATAGCTACAATGGTACCTCCgATCTCCAGCTGGAGCGTATGAAC
GTTTACTTCAAcGAGGTGCGTGATGGGATTCCGTATGCTTCGACAAACCTTCGACGATCCTAACCCCCCTGCC
AGGCCAACGGTGACAAGTATGTTCCTCGTGCCGTCCTCGTCGATCTCGAGCCCGGTACCATGgacgcCgTccG
TGCCGGTCCCTTCGGCGAGCTCTTCCGTCCCGACAACTTCGTT

Fig. 4. Molecular characterization of *Aspergillus rhizopodus* (CBMAI 2132) according to the beta-tubulin gene region sequencing and distance analysis.

et al., 2014; Gnanadoss et al., 2011). Novelli et al. (2016) researched the proteolytic activity of *A. niger* in SSF using soybean meal and wheat bran as substrate and did not obtain satisfactory activity, according to the authors. El-Shora and Metwally (2008) reported that for *Aspergillus niger* to produce protease, the supplement or combination of nitrogen supplements from different sources induces microbial growth and the production of different enzymes, depending on the species of microorganism, nature of the supplements and/or their concentration in the medium. Induced genetic mutations can also affect the ability of some microorganisms to produce protease. *Aspergillus niger*, in a fermented medium, based on wheat bran supplemented with blood meal presented, under optimized production conditions, a purified extract with the activity of 3,255 $U.g^{-1}$ initially. After genetic mutations induced in the microorganism, it reached 5,198 $U.g^{-1}$ (Zheng et al., 2014).

The enzymatic activity of the metabolites of the fungi depends on the cell line, making necessary the distinction and selection of lines (Rodarte et al., 2011; Souza et al., 2015). One same microorganism may produce numerous proteases, depending on the species, or even different strains of the same species, as noted in the species *A. niger*. Proteases with different properties can be produced within the same species depending on the isolate and the factors related to the process for obtaining the enzyme (Koka and Weimer, 2000; Rodarte, 2005).

Using the culture of *A. rhizopodus*, we obtained protease activity of $1,726.7 \pm 35.3$ $U.mL^{-1}$ in the medium containing plasma (SP) and $1,730.0 \pm 21.2$ $U.mL^{-1}$ in the medium supplemented with red blood cells (SR). Both media resulted in the production of protease with high activity, not statistically different by Tukey test with 5% probability. The protease production of this microorganism is only reported on the literature by Ortiz et al. (2016) and the fungus presented proteolytic activity in the pH range 6.0–9.0, displaying an increase in the activity at pH 6.0 in a medium based on wheat bran, reaching maximum proteolytic activity around 75 $U.gds^{-1}$ (Ortiz et al., 2016).

3.4. *P. citrinum* and *A. rhizopodus* strains identification

Penicillium and *Aspergillus* genotypes were shown to be promising for protease production, both in the qualitative and in the quantitative method. Figs. 3 and 4 show the molecular characterization of *P. citrinum* (CBMAI 2135) and *A. rhizopodus* (CBMAI 2132), strains that are prominent in protease production, but with few papers in the literature, unlike *A. niger*.

4. Conclusions

The results of the proteolytic activity were considered satisfactory, considering all fungi selected based on the criteria of EI and submitted to the process/experiment. We understood that using protein sources in the semisolid medium in a Petri dish can be a preliminary form to select cultures that would be used to produce protease in the presence of the plasma and red blood cells in SSF. Replacing the milk, used as a protein source in the semisolid medium, was effective to select fungi capable of producing protease and hydrolyzing the protein present. We also highlight that both plasma and red blood cells could be applied as a metabolizable protein source by filamentous fungi of different genera with potential application for the production of proteases with high proteolytic activity. Therefore, there is a promising field of study to be developed in terms of optimizing the production conditions for each selected fungi and characterizing their enzymes excreted to the medium.

Conflicts of interest

The authors declare that they have no conflict of interest.

Acknowledgments

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