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Screening of Amazon fungi for the production of hydrolytic enzymes

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The use of fungi for the production of enzymes has been widely investigated, but there are few reports concerning the use of these organisms isolated from the Amazon Region for the production of hydrolytic enzymes. Considering the importance of hydrolases for different industrial applications, this work aimed to select Amazon fungi for the production of cellulase, protease, pectinase, amylase and xylanase. In order to verify the potential of the Amazon isolates for the production of these five hydrolytic enzymes, growth on solid media containing specific substrates was performed. The qualitative assay revealed that among 40 Amazon fungi, 16 produce cellulase; 13 present proteolytic activity; 13 have pectinase activity, 27 produce amylase and 16 present xylanolyitc activity. Two amylase producers were grown in liquid media in order to access their amylolytic activity. Fungi isolated from aloe vera presented higher amylase production within 48 h of cultivation on potato starch broth.

Key words: Hydrolytic enzymes, Amazon fungi, solid media assays, amylase activity.

INTRODUCTION

The enormous impact of fungi on biotechnology has been well-established. These organisms have been used for the production of foods, antibiotics, alcohols, enzymes, organic acids, and numerous pharmaceuticals. Its morphological and physiological diversity includes microscopic molds and yeasts, as well as macroscopic mushrooms and truffles. The microscopic species are, however, best known for their biotechnological applications (Bennett, 1998).

Besides their importance as decomposing agents on forestry ecosystems, fungi are responsible for degrading a wide variety of wood products and providing all kinds of

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food and industrialized goods, as well as petroleum derivatives (Selosse et al., 2004). These abilities result from the fact that these organisms produce several degradative enzymes, which are secreted into the environment. Due to its absorptive mode of nutrition, a large amount of powerful hydrolytic enzymes are synthesized, and thus, various fungi species are used for the production of industrially-important hydrolases (Alves et al., 2002).

The food, feed, agriculture, paper, leather, and textile industries are well-suited for enzyme technology because products, as well as raw materials, consist of biomolecules, which can be produced, degraded, or modified by enzymatic processes (Mussato et al., 2007). The detergent and starch industries consume the greatest amounts of enzymes (Abrahão Neto, 2007).

The industrial importance of hydrolases exceeds that of other classes of enzymes. Hydrolytic enzymes can be produced by different species, either in liquid or solid media (Jecu, 2000). These degrading proteins can act on various substrates, depending on its industrial application. Cellulases, proteases, glucoamylases, xylanases, pectinases, hemicellulases, lipases, and amylases are the hydrolytic enzymes most applied in industrial processes (Silva et al., 2005a).

Fungal extracellular enzymes may be produced in liquid or solid media. The use of solid media permits a fast screening of large populations of fungi, allowing the detection of specific enzymes (Alves et al., 2002).

Considering the enormous microbiological potential of the Amazon Region and the growing applicability of fungal hydrolytic enzymes, the screening of Amazon appears to be an interesting approach to finding novel hydrolase producers. Hence, the aim of this study was to select Amazon fungi for the production of five industrially-important hydrolytic enzymes: cellulase, protease, pectinase, xylanase and amylase using solid media assays, as well as to access the enzymatic activity of most promising isolates.

MATERIALS AND METHODS

Amazon fungi

Endophytic and phytopathogenic isolates belonging to the Graduate Program in Biotechnology and Natural Resources from UEA were used in this investigation. Endophytic fungi isolated from the tucumã palm (*Astrocaryum aculeatum*), spiked pepper (*Piper aduncum*), uxi (*Endopleura uchi*), and manioc (*Manihot esculenta*) were investigated. Phytopathogenic isolates were obtained from aloe vera (Aloe vera) and peach palm (*Bactris gasipaes*). Wood-degrading fungi were kindly provided by Professor Ademir Castro e Silva of UEA. The coded isolates and the information relating to their origin are in Table 1.

Table 1. Identification code for Amazon fungi evaluated for hydrolytic enzymes production and their respective origin.

Strain Code	Type[a]	Host	Tissue
UEA_018	PT	Aloe vera	leaf
UEA_025	PT	Aloe vera	leaf
UEA_033	PT	*Bactris gasipaes*	fruit
UEA_042	EP	*Astrocaryum aculeatum*	seed
UEA_064	EP	*Pipper aduncum*	root
UEA_076	EP	*Endopleura uchi*	fruit
UEA_094	WD	Amazon wood	stems
UEA_097	WD	Amazon wood	stems
UEA_099	WD	Amazon wood	stems
UEA_102	WD	Amazon wood	stems
UEA_105	WD	Amazon wood	stems
UEA_107	EP	*Manihot esculenta*	stems
UEA_108	FT	Aloe vera	leaf
UEA_116	WD	Amazon wood	stems
UEA_121	WD	Amazon wood	stems
UEA_122	WD	Amazon wood	stems
UEA_123	WD	Amazon wood	stems
UEA_128	WD	Amazon wood	stems
UEA_129	WD	Amazon wood	stems
UEA_130	WD	Amazon wood	stems
UEA_131	EP	*Manihot. esculenta*	stems
UEA_140	WD	Amazon wood	stems
UEA_143	WD	Amazon wood	stems
UEA_155	EP	*Pipper aduncum*	stems
UEA_165	EP	*Pipper aduncum*	stems
UEA_166	EP	*Pipper aduncum*	leaf
UEA_204	WD	Amazon wood	stems
UEA_206	WD	Amazon wood	stems
UEA_207	WD	Amazon wood	stems
UEA_208	WD	Amazon wood	stems
UEA_212	WD	Amazon wood	stems
UEA_214	WD	Amazon wood	stems
UEA_219	WD	Amazon wood	stems
UEA_220	WD	Amazon wood	stems
UEA_221	WD	Amazon wood	stems
UEA_229	WD	Amazon wood	stems
UEA_233	WD	Amazon wood	stems
UEA_235	WD	Amazon wood	stems
UEA_237	WD	Amazon wood	stems
UEA_239	WD	Amazon wood	stems

[a]EP = Endophytic; PT = Phytopathogenic; WD = Wood-degrading.

Fungi maintenance

Stock cultures of the isolates were maintained on PDA (potato dextrose agar) dishes at 4°C. To maintain the cells' viability, fungi were periodically transferred to new PDA dishes, incubated at 30°C for 5-7 days and stored at 4°C, being used as required.

Inoculum

The inoculum was prepared in PDA dishes, from the stock culture,

being incubated at 30°C for five to seven days. The Petri dishes containing specific substrates for the enzyme assays were inoculated with a disc of around 7.0 mm diameter from the PDA medium culture.

Solid media composition

For assessing the cellulase production of Amazon fungi, the solid media was prepared with 1.8% (v/w) agar, 1.0 % (v/w) carboxymethylcellulose, and 0.1 M sodium acetate buffer solution pH 5.0. For protease assay a solid media containing 1.8% (v/w) agar, 1.0 % (v/w) gelatin, 1.0 % (v/w) skin milk, and 0.1 M citrate-phosphate buffer solution pH 5.0 was prepared. For identifying pectinase-producing-fungi the solid media was prepared with 1.8% (v/w) agar, 1.0 % (v/w) citric pectin, and 0.1 M sodium acetate buffer solution pH 5.0. Amylase production was verified using a solid media containing 1.8% (v/w) agar, 1.0 % (v/w) corn starch, and 0.1 M citrate-phosphate buffer solution pH 5.0 (Teixeira, 1994). Xylanase activity was evaluated on a solid media prepared with 1.8% (v/w) agar, 1.0 % (v/w) birch wood xylan, and 0.1 M sodium acetate buffer solution pH 6.0 (adapted from Silva et al., 2005b). After autoclaving at 121°C for 15 min, the culture media was cooled to approximately 60°C and transferred to Petri dishes for rapid cooling.

Solid media enzymatic assays

The Petri dishes containing the specific substrates were inoculated with the Amazon isolates. The dishes were incubated at 30°C for 5-10 days and periodically observed for the evaluation of fungal growth. Before the isolate colonizes one-third of the Petri dish, the presence of a translucent halo around the fungi, which confirms the hydrolytic activity, was verified. A control dish was also prepared where there was no fungal inoculation. All assays were carried out in triplicate.

Halo detection on solid media

The halo formation around the fungi indicates the production of hydrolytic enzymes. Protease production could be directly identified, through the presence of a translucent halo in the solid media. For detecting the halo to confirm the production of cellulase, pectinase, amylase, and xylanase, a revealing solution was used, according to the methodology described by Teixeira (1994). In order to verify the halo related with the pectinase production, a 5.0 M hydrochloric acid (HCl) solution was used. For amylase halo detection, a 0.1 M iodide solution was employed, and to verify cellulase and xylanase activities, a 0.1% (v/w) Congo red solution was used.

Amylase activity assay

For amylolytic activity detection, two fungi isolates that presented positive results for amylase production were cultivated in liquid media containing potato (200 g/L) and sucrose (20 g/L). PDA fragments (6 x 6 mm) containing fungi mycelia were inoculated on liquid media and kept at 30°C, 150 rpm, during 7 days. Aliquots were withdrawn from the culture broth in 24 h intervals and used for the enzymatic activity assay. Amylolytic activity was accessed through the methodology described by Miller (1959), using maltose as the standard. One unit of enzymatic activity was defined as the amount of enzyme capable to release one μmol of maltose per minute.

RESULTS AND DISCUSSION

Growth of Amazon fungi on solid media containing the specific substrates

In general, the Amazon isolates presented sufficient growth on the five specific substrates used on the composition of solid medium, reaching the edge of the Petri dish. Phytopathogenic fungi have shown the fastest growth, while wood-degrading isolates took longer to reach one-third of the plate. The mycelium of phytopathogenic fungi reached the desired distance (7.5 cm) from the center of the Petri dish after 5 days of culture, while wood-degrading isolates took twice this time. The ability of Amazon fungi to grow on different carbon sources indicates that these isolates are a versatile microorganism, which suggests that they are capable of producing the investigated hydrolytic enzymes.

Hydrolytic enzymes production

The results for the qualitative assays performed on solid media for the production of hydrolytic enzymes by Amazon fungi are presented in Table 2. The 40 fungal isolates were submitted to the hydrolytic tests to verify the production of cellulase, protease, pectinase, amylase and xylanase. Of these, 16 fungi demonstrated cellulase activity, 13 produced protease, 13 are capable of producing pectic enzymes, 27 presented amylase activity, and 16 were shown to be xylanase producers. The percentual distribution of the hydrolytic enzyme production among the 40 tested Amazon fungi is presented in Figure 1.

It can be observed in Figure 1 that of the 40 Amazon fungi evaluated in this work, 32% presented amylolytic activity. Of these, isolates UEA_018, UEA_025, UEA_097, UEA_099, UEA_105, UEA_107, UEA_130, UEA_131, UEA_155, UEA_208, UEA_214, UEA_221, UEA_235, and UEA_239 presented larger halos, which suggest that these fungi may have higher amylase activity than the other isolates (Alves et al., 2002). Amylase (EC 3.2.1.1) are extracellular endo-enzymes that randomly catalyze the hydrolysis of internal α-1,4-glucosidic linkages in starch and related glucans. This enzyme is widely used in various industrial processes, including the production of isomerized sugar and biofuel from starch, bakery applications, textiles and paper industries, pharmaceuticals, and detergents (Tamamura et al., 2014; Sahnoun et al., 2015). Therefore, seeking new sources of this hydrolytic enzyme has appeared as an interesting research approach.

Regarding the xylanase production, 19% of the Amazon isolates showed positive results. UEA_033, UEA_042, UEA_130, UEA_204, and UEA_206 formed larger halos in the solid media, indicating higher xylano-lyitc activity of these isolates. Xylanases (EC3.2.1.8)

Table 2. Production of hydrolytic enzymes by Amazon fungi[a].

Isolate	Cellulase	Protease	Pectinase	Amylase	Xylanase
UEA_018	+	+	+	+	+
UEA_025	+	+	+	+	+
UEA_033	-	-	-	+	+
UEA_042	+	+	-	-	+
UEA_064	-	-	-	-	-
UEA_076	-	-	-	-	-
UEA_094	-	-	-	+	-
UEA_097	-	-	-	+	-
UEA_099	-	+	-	+	+
UEA_102	-	-	+	+	-
UEA_105	-	+	-	+	+
UEA_107	-	-	-	+	+
UEA_108	-	-	-	+	-
UEA_116	-	-	-	-	+
UEA_121	+	-	-	+	-
UEA_122	+	-	-	-	-
UEA_123	+	-	-	+	-
UEA_128	+	-	+	-	-
UEA_129	+	+	+	+	+
UEA_130	-	+	+	+	+
UEA_131	+	+	+	+	+
UEA_140	+	-	-	+	+
UEA_143	-	-	-	-	-
UEA_155	-	-	+	+	-
UEA_165	+	-	+	-	-
UEA_166	-	-	-	-	-
UEA_204	+	-	-	+	+
UEA_206	+	+	-	+	+
UEA_207	-	-	-	-	-
UEA_208	-	-	-	+	-
UEA_212	-	+	+	-	-
UEA_214	-	-	-	+	-
UEA_219	-	-	-	-	-
UEA_220	-	-	+	+	-
UEA_221	-	-	-	+	-
UEA_229	+	+	-	+	+
UEA_233	+	-	-	+	+
UEA_235	-	-	-	+	-
UEA_237	-	+	+	-	-
UEA_239	+	+	+	+	-

[a]Positive tests indicate halo formation.

act on β-1,4 linkages of xylan, the most abundant constituent of hemicellulose (Collins et al., 2005). A great variety of microorganisms have been reported as xylanolytic enzymes producers, among which fungi are the most interesting ones (Silva and Carmona, 2008). Applications of xylanases with or without concomitant use of cellulase include the bioconversion of lignocelluloses to sugar, ethanol and other useful substances, clarification of juices and wines, cellulose pulp production for paper industry, extraction of coffee and vegetable oil, and nutritional value improvement of silage and green feed (Collins et al., 2005).

Fungi isolated from Amazon forest have been assessed for xylanase production. Medeiros et al. (2003)

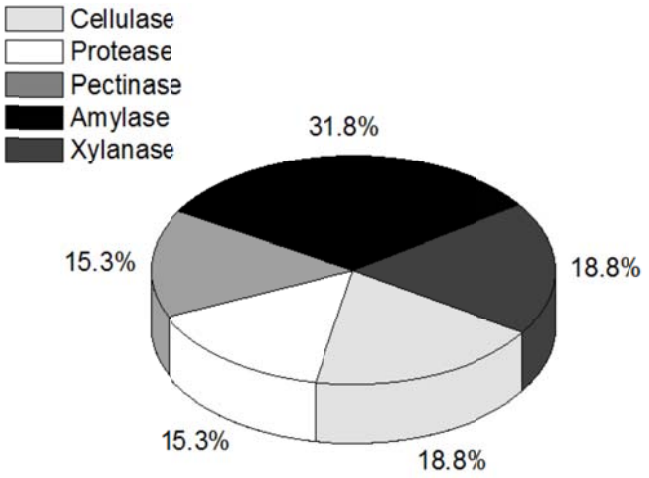

Figure 1. Percentual distribution of fungal isolates obtained from Amazon region according to its hydrolytic activity.

isolated and evaluated 10 fungal species from the Amazon forest for their capacity to produce xylan-degrading enzymes. The authors found that the best producing strains of β-xylanase were *Penicillium corylophilum*, *Aspergillus niger* and *Trichoderma longibrachiatum*. These strains were used in bleaching of eucalyptus kraft pulps and showed promising results (Medeiros et al., 2007).

Cellulase detection assays demonstrate that 19% of the Amazon fungi assessed can be considered as a source of this enzyme. Of these, UEA_042, UEA_122, UEA_128, UEA_129, UEA_131, UEA_165, UEA_204, UEA_206, UEA_229, and UEA_239 provided larger halos when compared to the other cellulase producing-fungi. Cellulase represents a complex mixture of hydrolytic enzymes with different specificities, which act over cellulolytic substrates, hydrolyzing glycosidic linkages, and converting them into glucose (Castro and Pereira Jr, 2010).

The most important source of microbial cellulases for industrial processes includes the fungal genera *Aspergillus*, *Penicillium* and *Trichoderma*. Cellulases and other enzymes capable to degrade plants cell wall are often used on clarification of fruit juices and wines, as well as on oil and fruit extraction. The feed industry employs cellulases for improving the digestibility of plants used for animal feed, and the textile industry uses these enzymes for removing the excess of indigo dye from jeans (Silva, 2008). Delabona et al. (2012) used a new strain of *Trichoderma harzianum*, isolated from the Amazon rainforest for the on-site production of cellulases to hydrolyze pretreated sugar cane bagasse. The Amazon strain showed outstanding results on cellulase production, when compared to *Trichoderma reesei*.

Proteolytic and pectolytic activity was verified within up to 15% of the assessed isolates. Larger halos indicating higher protease activity were verified for the isolates UEA_018, UEA_025, UEA_129, UEA_130, UEA_131, UEA_212, UEA_237, and UEA_239. Proteases break peptide linkages, a process named proteolytic cleavage, which is a common mechanism involved at digestion and blood coagulation. Proteinases have different industrial applications, especially on food, leather, detergent and textile industries (Ladeira et al., 2010). On leather industry proteases are used for removing the fur and for the partial degradation of keratin and elastin. Peptidases are also used on pharmaceutical formulations, such as antibiotics and analgesics. (Abrahão Neto, 2007). Fungi and yeast have been reported as protease producers for industrial applications. Ito et al. (2007) evaluated the protease production of a *Beauveria bassiana* Brazilian strain, which presented stability at 60°C. Neves et al. (2006) isolated 50 yeasts from Amazon Region and performed a screening for protease production. An Amazon *Candida intermedia* strain exhibited the higher proteolytic activity and did not present pathogenesis.

Among the pectinase producers, Amazon fungi UEA_018, UEA_025, UEA_102, UEA_128 and UEA_130 formed larger halos, suggesting the higher hydrolytic activity of these isolates. Pectinase are the enzymes responsible for the degradation of pectin, a complex molecule that occurs as structural polysaccharide in the middle lamella and the primary walls of young plant cells (Yadav et al., 2009; Pedrolli and Carmona, 2010). Pectic enzymes have a wide range of applications in food industry, especially in fruit juice extraction, and clarification (Pedrolli et al., 2008). Other applications of pectinases include wine making, oil extraction, pharmaceutical, paper, feed and textile fields (Martínez-Trujillo et al., 2011). It has been reported that microbial pectinases account for 25% of the global food enzymes sales (Jayani et al., 2005). Pectic enzymes used in the fruit juice industries and wine making often come from fungal sources, especially from *Aspergillus niger* (Pedrolli et al., 2008). Therefore, the isolation of new efficient pectinase producing microorganisms, the selection of optimum conditions for its production, and the biochemical characterization of these proteins represent an essential research field for the development of more competitive industrial processes (Marchi et al., 2006).

As shown in Table 2, the Amazon isolates UEA_018 and UEA_025, phytopathogenic fungi isolated from aloe vera, as well as the isolate UEA_129, a wood-degrading fungus, and the isolate UEA_131, endophytic fungi isolated from manioc, presented positive results for all five hydrolytic assays. These fungi showed to be the most versatile among the investigated Amazon isolates. On the other hand, the isolates UEA_064, UEA_076 UEA_143, UEA_166, UEA_207, and UEA_219 do not produce the tested hydrolytic enzymes (Table 2). The halo formed by the isolate UEA_239 in four enzymatic assays can be seen in Figure 2.

Among the five hydrolytic enzymes tested here, the isolates UEA_094, UEA_097, UEA_108, UEA_208,

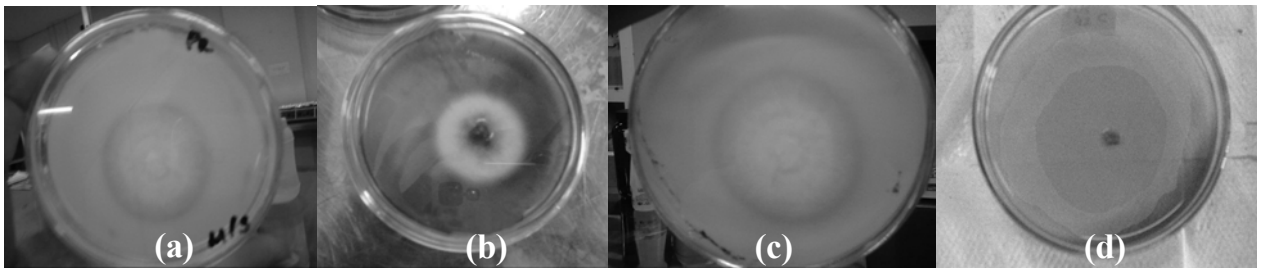

Figure 2. Hydrolytic activity of Amazon fungi UEA_239. Presence of the halo indicating the production of (a) protease, (b) amylase, (c) pectinase and (d) cellulase.

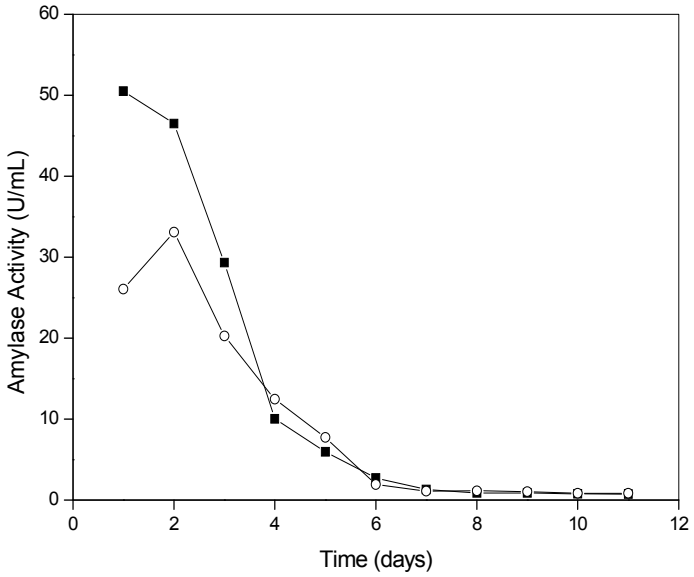

Figure 3. Amylolytic activity of Amazon fungi UEA_018 (○) and UEA_025 (■) during liquid media culture on potato starch broth.

UEA_214, UEA_221, and UEA_235 showed only amylolytic activity. The isolate UEA_116 presented only xylanase activity and the fungus UEA_122 produced only cellulase. These Amazon isolates demonstrate a hydrolytic specificity, which can be very interesting for industrial applications, since its extracellular enzymes would act specifically over the desired substrate. It is worth mentioning that the amylase producing isolates UEA_097, UEA_208, UEA_214, UEA_221, and UEA_235 presented higher enzymatic activities, which suggest that these Amazon fungi are potential candidates for use as amylase sources. The same can be observed for the cellulase-producing isolate UEA_122, which presented high enzymatic activity.

The Amazon fungi assessed in this work for cellulase, protease, pectinase, amylase, and xylanase activities were previously tested for lipase production (Zanotto et al., 2009). The isolates that presented the higher hydrolytic activities in the present study did not present lipase synthetic activity in the former work. However, these isolates presented positive results in degrading tributyrine, indicating the presence of lipases and/or esterases with hydrolytic activity.

Amylase activity

Since most of the tested fungi (31.8%) presented amylolytic activity, the isolates UEA_018 and UEA_025, phytopathogenic fungi from aloe vera, which presented the most intense starch degrading halos, suggesting a prominent amylase production, were selected for amylase activity detection. The results for the quantitative assay performed on liquid media containing potato starch are presented in Figure 3.

It can be noticed in Figure 3 that UEA_018 presented the highest amylolytic activity after two days of cultivation (33.1 U/mL). After that, the enzymatic activity dropped and

reached a minimum value within eight days of growth. The same behavior was observed for isolate UEA_025, which presented a higher value of amylolytic activity (50.5 U/mL in one day of growth). The lowering of amylase production was observed probably due to the consumption of potato starch (inductive carbon source used for amylase production).

According to Hashemi et al. (2015), the microbial production of α-amylase is greatly influenced by the components of the culture medium, especially the carbon and nitrogen sources. Saleem and Ebrahim (2014) verified the maximum production of *Rhizopus stolonifer* amylase after six days of incubation on starch and a decreased enzymatic production with further incubation, as observed in this study. Chimata et al. (2010) reported that *Aspergillus* MK07 produced most amylase after 5 days of incubation on wheat bran. Both studies were carried out at 30°C culture, the same temperature used here, and therefore, suggesting that the Amazon fungi have a faster amylase production, a very interesting characteristic for industrial enzyme production.

Conclusion

With this study it was possible to demonstrate the great potential of the Amazon fungi as hydrolytic enzymes suppliers for a large range of industrial applications. Most of the tested isolates produced amylase and the most promising amylolytic producer showed rapid enzyme production in liquid media. Further investigations are necessary in order to establish optimum parameters for the production of these enzymes in a larger scale.

Conflict of interests

The authors did not declare any conflict of interest.

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