Bio-prospecting filamentous fungi from *Araucaria* moist forests and the Pampa biome: High amylase production with a potential industrial application

ABSTRACT

Background and Research Aims: Microorganisms, especially fungi, are used in the production of enzymes which are applied in industries to decrease the costs of production as well as to increase the speed and quality of final products. Amylases are among the most studied and widely used enzymes in industrial processes involving the textile industries, production of paper, detergent, glucose/fructose syrups, and ethanol. According to these characteristics, this work aimed to collect, isolate and perform the screening of filamentous fungi from Brazilian Araucaria moist forests and the Pampa biome.
Methodology: Different samples were collected from different environmental areas formed by the Araucaria moist forests and the Pampa biome, which are the characteristic vegetation comprising the south region of Brazil, Uruguay, and Argentina. Fungi were isolated by the plating method and separated by observing changes in morphology. Then screening was performed in Khanna liquid medium which was filtrated to extract the extracellular enzymes and determine amylase activity by DNS (3,5-dinitrosalicylic acid).
Results: Sixteen morphologically different filamentous fungi were isolated from this collection, which proves the high variability of fungi species that have the Araucaria moist forests and the Pampa biome as their habitat. Three of the isolated fungi (10C, 18A, and 18L) were selected as good amylase producers with great potential for future biotechnological applications.
Conclusion: According to the data obtained in this work, fungi 10C, 18A, and 18L have promising biotechnological potential, since these microorganisms have a high enzyme production in stationary conditions after 5 days of growth. This is a fundamental characteristic of amylase technologies. Also, it is extremely necessary that a change of mind occurs and people become aware of these unique environments in the world, so that, they can be preserved and the species found there can be seen as important mechanisms of industrial and technological innovation.

Keywords: Brazilian biome, Fungi, Amylase producers, Industrial application.

1. INTRODUCTION

Anthropogenic activities are pressuring the environment and threatening biodiversity globally [1]. These threats are expected to rise, as the human population is still increasing in size and consumption levels [2]. Species responses to these anthropogenic pressures depend on multiple factors, including landscape features, such as habitat availability [3], habitat configuration [4], and legal protection [5]. Brazil is considered a mega-diverse country possessing six terrestrial biomes - Amazonia, Atlantic Forest, Caatinga, Cerrado, Pantanal, and Pampa. The Pampa biome lies within the South Temperate Zone and has both subtropical and temperate climates with four well-characterized seasons. Grasslands, with

sparse shrub and tree formations, are the dominant vegetation. This region has experienced losses of biodiversity and socio-economic opportunities because of human action [6]. The Araucaria moist forests, which are bordered by the Pampas grasslands in the South of Brazil, are one of the main highland vegetation types remaining in the Atlantic Forest biodiversity hotspot [7]. These forests constitute a clear example of an eco-region that can be constrained by climate change given their narrow climatic requirements (i.e., low temperatures and high rainfall) and their fragmented distribution [8]. Both these environments play an important role in the species conservation in the south of Brazil, however, they are understudied and there are not many reports about the microbiota found in these regions.

Fungi are often used as models for studies on climate change, however, they are often not considered in conservation reports. For example, fungi have not yet been included in the recently released IUCN Green Status of Species [9,10]. In addition, to the conservational interest, fungi, in principle, can provide similar enzymes to those of animal and vegetable origin. Their enzymes have advantages over the others, due to their cellular machinery, which can secrete enzymes into the extracellular environment. The extracellular enzymes are advantageous over the intracellular ones, because, in general, they are structurally glycosylated providing greater stability [11]. Among the most used enzymes, fungal amylases are the most studied and applied in industrial processes such as in the reduction of turbidity and clarification of fruit juices, or the production of sweeteners for soft drinks.

Thus, this work aimed to study the amylases produced by the fungi collected and isolated from Araucaria moist forests and the Pampa biome, which have few studies about fungi population and the economic interest of these species. We believe that the best way of increasing the protection of areas of degradation is proving that interesting species (economically and biologically speaking) are present in that region. To do this, we performed a bio-prospection, isolation, and screening of high amylase-producing fungi for future studies and potential industrial applications.

2. MATERIALS AND METHODS

The experiments were conducted at “Laboratório de Microbiologia e Biologia Celular Departamento de Biologia- Faculdade de Filosofia Ciências e Letras de Ribeirão Preto-USP-São Paulo”.

2.1 Sample bio-prospection from *Araucaria* moist forests and the Pampa biome and isolation of the strains

Samples were collected from soil, decaying materials, as well as bean and squash plantations. Around 3g of each sample was obtained and placed in previously autoclaved flasks. A total of 0.2g of each isolated material was used to populate the Petri dishes containing the culture media. The collection sites were geocoded by GPS-Global Positioning Systems to determine the latitude, longitude, and elevation. Samples were collected in Ibiraiaras city in the state of the Rio Grande do Sul – Brazil, isolated in Petri dishes containing a solid culture medium composed of 4% Quaker® oatmeal and 2% bacteriological agar [12]. Plates were incubated in a bacteriological incubator at 30°C. Then, the separation of different fungi was performed, through macroscopic observation, considering the changes in the morphology and hyphae color.

2.2 Inoculum of the isolated strains, screening of amylase production, and fungi maintenance

The spores of each isolated strain were counted in a Neubauer chamber and a volume of 1 mL (2.9 × 10^5 spores/mL) of the fungal solutions was inoculated in 25 mL of the liquid medium in Erlenmeyer of 125 mL. “The amylase screening was performed with all the fungi grown in Khanna liquid medium [13] with 1% starch Reagen®. Further, they were all incubated at 30 °C, pH 6.0, under static conditions for 5 days to obtain the best amylase producer”. “The microorganisms were maintained at the Laboratório de Microbiologia e Biologia Celular from the Departamento de Biologia of FFCLRP-USP, Ribeirão Preto, São Paulo, Brazil, at − 80 °C in a solution of 20% glycerol or at 4 °C in silica, as previously reported by” Pasin *et al.* [14].

2.3 Mycelia mass extraction, intracellular and extracellular proteins evaluation

The mycelial mass was obtained after the separation from the culture medium by vacuum filtration using a Büchner funnel and Whatman filter paper no. 1. The filtrate was used as the extracellular source of enzymes. The mycelial mass was washed with two volumes of distilled water, pressed between paper sheets, maintained at − 18 °C, and further macerated and suspended in 10 mL of 100 mM sodium acetate buffer, pH 6.0. This suspension was subjected to centrifugation at 8000×g, for 15 min, at 4 °C. The supernatant containing the intracellular crude extracts was then separated for further studies of the amylolytic activity of the fungi.

2.4 Quantification of proteins and amylase activity

“The proteins were determined by the Bradford method [15] using bovine serum albumin as standard”. “The readings were performed in a spectrophotometer at 595 nm and the results were expressed in mg/ml. Amylase activity was determined by the quantification of the reducing sugars formed during the extract incubation with 1% starch Reagen® in sodium acetate buffer 100 mM pH 6.0 at 60 °C using 3,5-dinitrosalicylic acid, DNS” [16]. “The assay consisted of 200µL of the substrate and 200µL of the enzyme. Aliquots of 50µL were withdrawn after 10 min and added to test tubes containing 50µL of DNS. After that, the tubes were immersed in a boiling water bath for 5 min and cooled, and 500µL of distilled water was added. The absorbance for all the test tubes was measured at 540 nm in a spectrophotometer. The blank consisted of 50µL of the assay mixture, which was immediately added to 50µL of DNS. The method was standardized with glucose (0.1–1.0 mg/mL) [17]. The activity unit (U) was defined as the amount of enzyme that generates 1 µmol of reducing sugars/ min under the assay conditions [17]. Total activity (total U) = µmol/mL × filtrate volume. The specific activity was expressed in the total U/total mg protein of the extracellular extract” [17].

3. RESULTS AND DISCUSSION

3.1 Sample collection and isolation

Eighteen samples were collected in many different areas of fields, plantations, and other sources. From this total, eight samples were isolated and tested according to the following nomenclature: (2) Rotten *Araucaria* tree branch, (4) zest of an *Araucaria* tree branch, (9) Squash in decomposition, (10) decaying wooden board from Pampa Biome (Lat. 28 21,357 ', Long. 51 40,658 and high Cap. 718m), (11) decaying tree branch from the characteristic vegetation of Pampa biome (Lat. 28 20,038 ', Long. 51 41,199 and high Cap. 832m), (14) tree trunk affected by a fungus at an Araucaria moist forests (Lat. 28 21,438 ', Long. 51 40,589 and high Cap. 729m) (17) soil sample taken from Pampa biome vegetation (18) soil sample from an Araucaria moist forests vegetation (Lat. 28 20,038 ', Long. 51 41,199 and high Cap. 832m).

The isolation of filamentous fungi was conducted by the plating method where the strains were inoculated in Petri dishes containing a culture medium composed of oat Quaker® medium with agar until there was no doubt that only the desired fungus was growing on the dish. Sixteen morphologically different fungi were isolated (**Figure 1**). Later, they were tested to obtain the best amylase production.

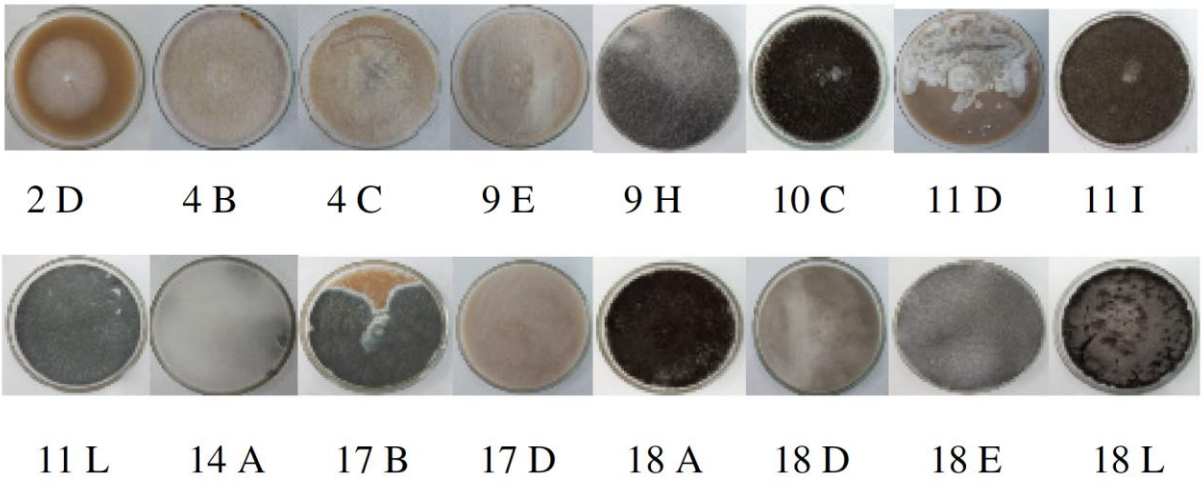

Figure 1: Representative images of the isolated fungi

These results are comparable to those obtained by Pasin *et al.* [18] when the authors performed a bio-prospection, isolation, and amylase production screening of different fungi from an Atlantic Forest biome.

3.2 Screening of filamentous fungi

The microorganism selection for the best amylase producers was an important step of this work. To this end, submerged fermentation experiments were conducted using Khanna liquid medium, pH 6.0 for 5 days in the static condition, with 1% starch in 100 mM sodium acetate buffer. The results are summarized in **Table 1**, indicating that the samples of fungi 10C, 18A, and 18L, collected from the wooden board in the Pampa Biome and soil sample from the Araucaria moist forests, respectively were the best amylase producers.

Table 1: Amylase levels produced by the isolated fungi.

Fungi	Enzyme Activity (U/mL)		Protein (mg/mL)		Specific Activity (U/mg)	
	Intracellular	Extracellular	Intracellular	Extracellular	Intracellular	Extracellular
10 C	**0.403**	**2.50**	**0.458**	**0.092**	**0.880**	**27.17**
11 I	**0.438**	**2.56**	**0.340**	**0.102**	**1.288**	**25.09**
11 L	0.217	1.1	0.137	0.123	1.583	8.94
14 A	0.154	1.76	0.380	0.125	0.405	14.08
18 A	**1.04**	**2.62**	**0.203**	**0.092**	**5.123**	**28.48**
18 D	0.620	2.01	0.186	0.115	3.333	17.48
18 E	**0.738**	**2.22**	**0.140**	**0.100**	**5.271**	**22.00**
18 L	**0.394**	**2.44**	**0.196**	**0.090**	**2.010**	**27.11**

The results found here are better than those observed on bio-prospecting and screening of fungi at the Caatinga Biome [19]. So, these fungi were chosen as the best amylase production, and in the future, the growth conditions and enzyme production will be optimized to maximize the production of amylolytic activities for further application of these enzymes on an industrial scale which was the main purpose of this study.

4. CONCLUSION

Our results have proven to be very important tools for future research on the preservation of Araucaria moist forests and the Pampa biome since several different morphological species have been isolated and three of them (10C, 18A, and 18L) showed high amylase production. Combined, these results can reinforce the importance of the maintenance and sustainable use of the resources provided by this vegetation to achieve economic, social, and industrial development by using natural products (enzymes from fungi).

DISCLAIMER:

Authors have declared that there are no competing interests. The products used for this research are commonly and predominantly used in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as a way for any litigation, but for the

advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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