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REGULAR ARTICLE

Amylase potential of filamentous fungi isolated from sweet potato pulp

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Autor contribution
VKMG: Experimental data collection, Data custody, Literature review, Writing the manuscript; JAG: Conceptualization, Supervision, Writing the manuscript; SAVP: Experimental data collection; SRMA: Experimental data collection; NMS: Data analysis; PHWN: Conceptualization, Supervision, Manuscript Review.

Abstract

Sweet potato is a rustic culture, widely adapted and with a high starch content, thus having innumerable aptitudes. For the transformation of sweet potatoes into biofuel, some processes are necessary. The success of ethanol production from sweet potatoes depends on the transformation of starch into fermentable sugars. The most used conversion process is enzymatic hydrolysis, which uses commercially available enzymes. However, this process can be carried out through enzymes secreted by filamentous fungi. In this work, filamentous fungi that naturally colonized sweet potatoes were studied. These were isolated, tested for enzymatic activity, and identified by microculture. Fifty-one fungi from the pulp of sweet potatoes Beauregard and BRS-Amélia were isolated, 27 of them showed the ability to hydrolyze starch. Out of these, six showed an enzyme index ≥ 2.0 and were identified as *Aspergillus* sp. and *Penicillium* sp. This fact indicates the potential of these fungi in the production of amylase, an important enzyme for ethanol production.

Keywords

Starch; hydrolysis; ethanol; microorganisms; *Ipomoea batatas*.

Introduction

One of the challenges of this century is the development of clean energy sources, obtained from viable renewable sources. Considering the Brazilian biomass production, continuous research is required (Schweinberger et al., 2016; Blank et al., 2017).

Presenting a yield over 50 Mg ha^{-1} tuberous roots, sweet potatoes show an energy balance similar to that of the sugar cane and higher than that of maize (Silva et al., 2019). Its starch content yield per area unit is higher than that of maize (Duvernay et al., 2013).

In Brazil, this vegetable is found in most regions since it is highly adaptable to different edaphoclimatic conditions. Peasant agriculture is the main producer of sweet potato, and regarding food and energy security, this crop might support this type of agriculture (Sakai et al., 2020; Bernardi et al., 2021).

The production of ethanol from sweet potato depends on starch conversion into fermentable sugars (Masina et al., 2017; Campos-Lopes et al. 2018). The sweet potato starch is more complex that of cereals, which makes hydrolysis harder (Lareo et al., 2013). In cassava ethanol production, 57 % of the industrial energy consumption occurs in the starch hydrolysis/saccharification process (Salla et al., 2010).

Although it is quite expensive, enzymatic hydrolysis presents advantages such as better process control, lower starch loss, and allows byproduct reuse (Pereira et al., 2017; Moges et al., 2019). It offers potential cost reduction in the long term and enables yields close to stoichiometric ones in less critical conditions of temperature, pressure, and chemical aggressiveness (Pereira et al., 2017).

Several organisms secrete enzyme cocktails in which proteins diffuse independently and work synergically to degrade biomass. Filamentous fungi are known for their enzymatic production ability and for secreting enzymes directly into the culture medium without the need for cytoplasmatic rupture (Dutta & Wu, 2014).

The *Aspergillus* sp. Fungus is responsible for around 30% of the global production of amylase enzymes (Soares et al., 2010). Promising results were obtained with the *Aspergillus* sp. fungus in the fermentation in solid state in coffee husk

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(Gusmão et al., 2014) and soybean harvest residues (Cunha et al., 2016). A study on the three species of Trichoderma spp. showed that they presented a set of 80 proteins, and out of those 19 were involved in enzyme production and secretion (Horta et al., 2018).

From sanitary wastewater scum and grease trap oil, a hundred filamentous fungi were isolated, out of which 67 presented lipase production potential (Rodrigues et al., 2016). Among them, the authors highlighted the *Penicillium* sp F002 and *Rhizomucor* sp. F018 fungi by using the enzymatic index (Hankin & Anagnostakis, 1975). Facchini et al. (2016) in study with filamentous fungi for the production of lipases found potential for transesterification and production of biodiesel.

The *Aspergillus niger* fungus crude extract was seen to be efficient in the hydrolysis of sweet potato starch (Pereira et al., 2017), simultaneous hydrolysis with fermentation in potato (Izmirliogli & Demirci, 2016), and hydrolysis and simultaneous fermentation in agro-industrial waste (Oliveira et al., 2020). By using an enzymatic cocktail produced with *Penicillium* sp., great efficiency was observed in the hydrolysis of cassava flour (Hai-Juan et al., 2010).

To mitigate production costs, crude extract of microorganisms can be employed to replace purified enzymes (Pereira et al., 2017 and Lomthong et.al. 2018). Therefore, the objective of this study was to select and identify fungi isolated from sweet potato with amylase production potential.

Materials and methods

This study employed fungi that colonized naturally the pulp of tuberous roots of sweet potato. Eight different sweet potato genotypes were used. Out of those, five were genetically improved and were commercially available, namely BRS-Rubissol, BRS-Cuia, BRS-Amélia, Beauregard, and Brazlândia, and three accesses called BD-14, BD-15, and BD-16. The accesses were collected from the properties of peasant farmers in the Eastern and Southeastern mesoregions of the Paraná State in Brazil.

The tubers were cut in halves, without asepsis, kept in ambient exposure for 25 days, and exposed to natural light. The sweet potato genotypes that presented higher incidence of microorganisms were selected.

From the sweet potato 'contaminated' pulp (Figure 1b), structures were scraped and transferred to test tubes (10-1 concentration). The tubes contained 5 mL saline solution (NaCl 0,85%), 2 drops of tween 80, and surfactant (Teodoro et al., 2018).

The test tubes were agitated in a Vortex agitator (1 min). After that, 100 µL of the solution was extracted and transferred to new test tubes (10-2 concentration) (Tortora, 2012). Concentrations ranged from 10-1 to 10-5 and followed the same procedure of dilution of the test tubes at 10-1 and 10-2 concentrations. When the concentration of inoculated microorganisms on the Petri dish is high, it tends to present strain overlaps during growth, which makes isolation impossible.

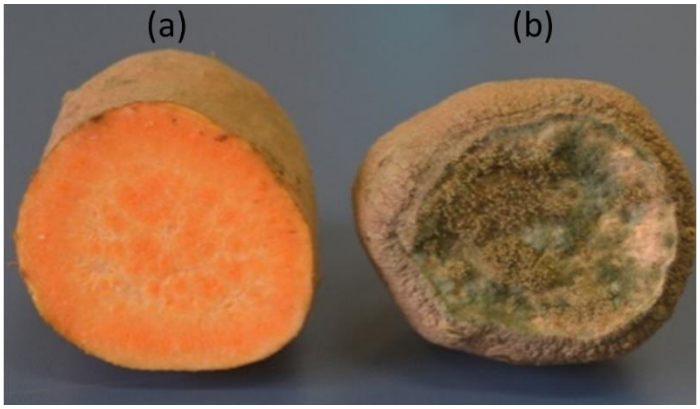

Figure 1. Sweet potato pulp colonization, cultivar Beauregard, before (a) and after (b) the natural incubation, 25 days, with natural light at room temperature (Ponta Grossa, PR)

After the serial dilution, the solution was drained, the tubes with 10^{-3} and 10^{-5} concentrations had 100 µL solution transferred and dispersed on Petri dishes with the BDDA culture medium (sweet potato agar). The dishes were subjected to an incubation chamber of the BOD (*Biological Oxygen Demand*) type at 28 ºC. The microorganisms found were isolated daily and inoculated on a slant test tube with 4 mL BDDA culture medium. Culture replating was carried out every fortnight, and they were incubated in a BOD oven at 28 ºC for seven days and later stored at 4 ºC.

To evaluate enzymatic production through solid state fermentation, the presence of hydrolysis halos was verified (Hankin & Anagnostakis, 1975 and Coelho et al., 2018).

The conidia of the isolated strains were cultivated in BDA medium – potato dextrose-agar (250 g potato, 20 g dextrose, and 15 g agar), at 28 ºC for five days. By using a cork punch, 5mm diameter agar cylinders were extracted from the growing colonies. These were transferred to petri dishes containing 25 mL agar + starch culture medium (10 g yeast extract,20 g casein peptone, 10 g soluble starch, 20 g agar, 1 L distilled water, at pH 6.8) and incubated at 28 ºC for five days in a BOD oven (Coelho et al., 2018). After mycelial growth and depending on the microorganism, there might be amylase enzyme excretion, or not.

To determine amylase excretion, after the incubation period, 1 mL Lugol solution was applied on the fungal colony and culture medium. When enzyme excretion and consequent starch hydrolysis occur, some discoloration is observed around the colony, the region where the iodine did not bond to the starch, since the chain was already broken.

The ratio between the substate hydrolysis halo and the colony diameter expresses the enzymatic index (Hankin &

Anagnostakis, 1975), through this index, it is possible to estimate the enzymatic production potential. Five measurements were carried out in each of the 5 dishes. Two orthogonal axes (diameter) of the colony and two halo axes (diameter) were measured with the aid of a Mitutoyo caliper.

Fungi that presented enzymatic index ≥ 2.0 were considered promising for amylase production (Hankin & Anagnostakis, 1975). In this study, the fungi that presented index close to or over 2.0 were selected and retested.

Promising fungi were characterized morphologically by means of colony macroscopy and microscopic identification of micro cultures (Moges et al., 2019). An Olympus BX41 fluorescence optical microscope and an Olympus DP-71 camera with 40x enlargement and 12 megapixels were used. The identification test was carried out in triplicate.

The fungal genus identification was based on the classification key and comparison with illustrated bibliography (Larone, 1987).

Results and discussion

Out of the eight sweet potato genotypes that were cut and incubated for 25 days, two showed expressive fungal growth, namely, BRS-Amélia and Beauregard (at temperatures average: 13.6 ºC minimum, 24.4 ºC maximum, and 19.2 ºC daily average).

Out of those, 51 fungus strains were isolated, divided into 26 Beauregard strains and 25 BRS-Amélia strains (Figure 2). The fungi isolated from the sweet potato Beauregard were labelled BRGD, while those isolated from the sweet potato BRS-Amélia were labelled AM, followed by the number indicating order of isolation and concentration where they were incubated, for example, BRGD 01 10-3 or AM 04 10-5. The Arabic numbering followed a chronological sequence of isolation. Thus, the microorganism labelled as 01 in the Beauregard cultivar was not necessarily the same as that identified as 01 in the BRS-Amélia cultivar. After individualization, the strains were stored at 4 ºC.

Figure 2. Example of filamentous fungi isolated from the pulp of sweet potatoes (BDDA medium, for 5 days, at 28 ºC)

By means of bioprospection, the promising fungus strains were selected regarding their amylase yield (Figure 3). Microorganisms that do not show ability to assimilate starch, therefore, they synthesize specific enzymes such as amylases, which degrade more complex substrates, such as starch, into simple molecules. Amylases convert starch into fermentable sugars, guaranteeing the organism growth.

Out of the 26 strains that colonized naturally and were isolated from the sweet potato Beauregard, 18 presented enzymatic halo, indicating starch hydrolysis. Out of those listed, three presented an enzymatic index ≥ 2.0 (Hankin & Anagnostakis, 1975). Considering the 25 strains isolated from the sweet potato BRS-Amélia, nine fungi formed hydrolysis halo. Out of those, three presented an enzymatic index ≥ 2.0.

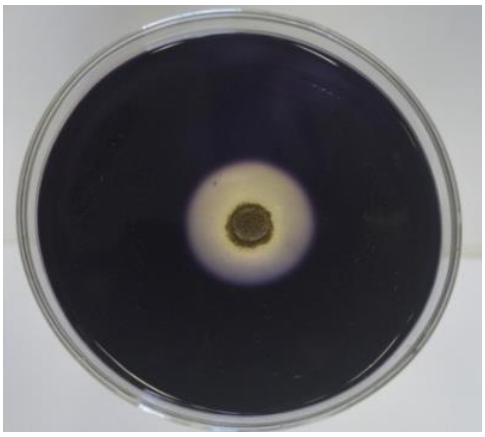

Figure 3. Hydrolysis halo by the BRGD 08 10^{-5}, evidenced with Lugol, characterizing enzyme excretion and starch degradation (agar + starch, after 5 days, 28 ºC in BOD oven)

The enzymatic degradation halos of the fungi isolated from the sweet potato Beauregard ranged between 14.2 and 32.0 mm. The enzymatic degradation halos of the fungi isolated from the sweet potato BRS-Amélia ranged between 12.0 and 48.0 mm. The isolated fungi that presented enzymatic indices ≥ 2.0 (Hankin & Anagnostakis, 1975) were selected and retested (Table 1).

The isolated fungi that presented lower values, but close to 2.0 were also retested. However, none of the six strains presented enzymatic index ≥ 2.0. These results enable enzyme yield prediction as an aid in the process of selection of microorganisms that can degrade polysaccharides (Silva et al., 2017).

The promising isolated fungi BRGD 04 10-5; BRGD 08 10-5; BRGD 07 10-3; AM 04 10-3, and AM 05 10-5 were classified as belonging to the *Aspergillus* sp. genus and AM 08 10-5 to the *Penicillium* sp genus. However, their species were not identified (Figure 4). Both had already been described as amylase enzyme producers (Pirota et al., 2015).

These genera belong to the phylum Ascomycota, class Eurotomycetes, order Eurotiales, and family Trichocomaceae. *Aspergillus* sp. presents vertical conidiophores, with globular, simple termination, unicellular conidia, usually with varied colors. *Penicillium* sp. presents conidiophores resulting from a single mycelium, with branches close to the apex, and termination in a group of conidia, with bright colors and massive unicellular structures, mainly globular or ovoid, in addition to septate hyphae (1.5-5 µm diameter), branched or not-branched conidiophores (Larone, 1987).

These are mesophilic fungi that grow within the temperature range between 25 and 37 °C, and thrive in culture media plenty of organic nitrogen, they produce great amounts of glucoamylase (Almeida et al., 2017). Most of the species of these families are popularly known as molds and are generally found colonizing several types of biomasses.

Many Penicillium sp. and Aspergillus sp. species are parasites and provoke several types of damage (early decomposition) to fruit, bulbus, and tubers. Many species of these families are already used for being able to excrete compounds of biotechnological interest in the culture medium.

Table 1. Halo diameter and enzymatic index of bioprospection and confirmation tests of isolated fungi that presented index ≥ 2.0

Isolated fungi	Halo mean diameter (mm)	Mean enzymatic index bioprospection	Mean enzymatic index confirmation
*BRGD 04 10^{-5}	32.0 ± 4.35	2.02± 0.19	2.16 ± 0.18
BRGD 08 10^{-5}	29.9 ± 3.51	3.08± 0.39	2.14 ± 0.14
BRGD 07 10^{-3}	30.7 ± 1.63	2.15± 0.18	2.08 ± 0.04
AM 04 10^{-3}	20.5 ± 5.28	2.03± 0.11	2.36 ± 0.21
AM 05 10^{-5}	48.1 ± 14.18	3.01± 0.43	2.31 ± 0.24
AM 08 10^{-5}	31.8 ± 8.34	2.27± 0.26	2.28 ± 0.23

AM, BRGD - cultivars BRS-Amélia and Beauregard, respectively; 04 - microorganism identification; 10^{-3} - microorganism concentration according to the serial dilution

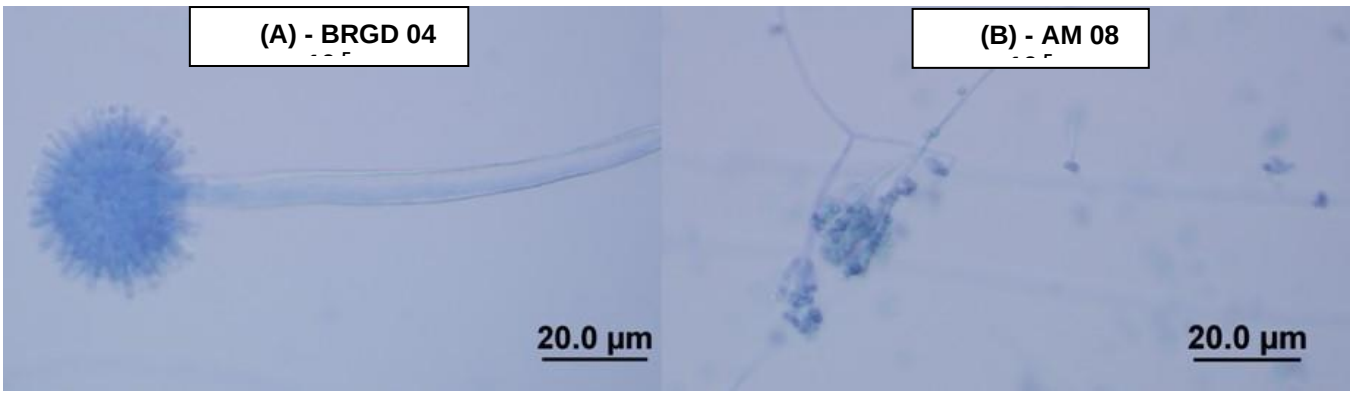

Figure 4. Morphology of *Aspergillus* sp. (A) and *Penicillium* sp. (B) conidiophores, naturally found in sweet potatoes Beauregard and BRS-Amélia, identified as amylase producers (Olympus BX41 fluorescence microscope, 40x enlargement)

A study on 108 filamentous fungi isolated in the soil of and Ethiopian forest showed that 28 presented amylase production potential, nine showed a high enzymatic index, and three resulted in amylase observable activity in liquid culture (Moges et al., 2019). The best enzyme producer was identified as *Penicillium* sp. This isolated fungus showed enzymatic activity at 5 ºC.

Out of the 25 filamentous fungi isolated from material in decomposition collected from the Brazilian Atlantic Forest, two isolated fungi, *Aspergillus brasiliensis* and *Rhizopus oryzae*, presented amylase production potential (Almeida et al., 2017).

The studies cited confirm the results of this research, since fungi of the genera *Aspergillus* sp. and *Penicillium* sp. can produce amylase. Therefore, their application in ethanol production from sweet potato starch, for example, should be considered.

Conclusions

Out of the eight genotypes observed, two sweet potato genotypes (cultivars Beauregard and BRS-Amélia) showed microorganism growth. Fifty-one fungal strains were isolated from these cultivars.

Out of the total number, 27 strains showed starch hydrolysis ability through fermentation in solid state. Six out of the 27 strains revealed enzymatic index ≥ 2.0, five of them were identified as *Aspergillus* sp, and one as *Penicillium* sp.

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