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Short Communication

Waste Coffee as an Excellent Substrate for Collagenase Production by Filamentous Fungi *Cunninghamella phaeospora* and *Absidia cylindrospora*

Kethylen Barbara Barbosa Cardoso, Bruna Kelly Pereira Alves de Souza, Romero Marcos Pedrosa Brandão-Costa*, Wendell Wagner Campos Albuquerque and Ana Lúcia Figueiredo Porto

Federal Rural University of Pernambuco, Department of Morphology and Animal Physiology, Recife, Brazil

***Corresponding Author:** Romero Brandão-Costa, Federal Rural University of Pernambuco, Department of Morphology and Animal Physiology, Recife, Dois Irmãos, Brazil.

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Abstract

Coffee is currently one of the most widely consumed beverages around the world. During the extraction of the beverage, a large amount of residue is produced, and considering the worldwide coffee consumption, it can be concluded that tons of coffee waste are generated from industrial and domestic production. To encourage the reusability of industrial waste, we suggest the use of coffee ground as source of substrate for the production of collagenase, an enzyme with biomedical applications, by the filamentous fungi *Cunninghamella phaeospora* UCP 1303 and *Absidia cylindrospora*, through solid state fermentation process. Simple 2^2 factorial fermentation designs were used with varied temperature conditions (30°C and 35°C) and substrate amounts (5g and 10g). The results showed that, in all tests, coffee residues were able to produce enzymes with proteolytic activity (general and collagenolytic). However, different kinetic of production were observed, particularly as regards the amount of the substrate used. For the fungus *Cunninghamella phaeospora* CPU 1303 greater production of proteases was observed with 5g of residue at temperatures of 30°C with general proteolytic activity of 1089 U/mL*mg and collagenolytic activity of 78.3 U/mL. Whilst for the fungus *Absidia cylindrospora*, 10g of substrate at 30°C provided a precipitated extract with general proteolytic activity of 2074.79 U/mL*mg and collagenolytic activity of 111.1 U/mL*mg. The high production of collagenase by the filamentous fungi showed the method as a viable source for obtaining proteases, which presents a possible alternative to turn coffee residues into biotechnological and biomedical applications, mainly in wound treatment.

Keywords: *Cunninghamella phaeospora; Absidia cylindrospora; proteolytic enzymes*

Introduction

Brazil is the largest producer and exporter of coffee in the world. With the high production of the coffee industry, a lot of waste is generated and it is estimated that 9.9 million of solid waste are produced annually worldwide [1], which draws attention to the high waste of raw materials associated with damages to the environment.

However, advantageous techniques such as conversion of solid extracts into substrates for bioprocesses can help to manage that waste. The use of solid substrates is considered a potential alternative to the microbial bioconversion of organic residues for the production of substances with economic interest [3]. The solid-state fermentation (SSF) emerges as a great method to obtain biotransformed products, since it is based in the direct fermentative process in solid substrates (the absence or near absence of free water, with the substrates in a solid particulate state). The aim of the SSF is to increase the contact between the microorganism and substrate to improve the fermentative production (Hölker., *et al.* 2004). The SSF is a very profitable method, because it allows the use of solid agro-industrial waste at the same that it has appropriate conditions to the growth of filamentous fungi, since they usually grow on solid substrates [1].

Filamentous fungi are cultivated in a wide variety of media because they are able to grown even in harsh conditions and low humidity [4]. Filamentous fungi belonging to the order Mucorales are reported to be good producers of proteinase [7]. Among the Mucorales the *Cunninghaella phaeospora* was selected to produce a variety of metabolites, such as amylase, lipase enzyme, pectinase, protease and renin [8]. The Absidia genus is known for the production of milk clotting enzymes and caseinases, but no recent study was done to evaluate their proteolytic production [12].

Proteolytic enzymes are widely used in the food, pharmaceutical and chemical industries, and those obtained from microbial origin represent 40% of the total proteases used industrially [5]. These enzymes have a high commercial value and are also often used in biotechnology [9]. Filamentous fungi are notorious for simple handling and easy to obtain protease considering that their enzymes are usually extracellular [6].

The present study aims to determine whether the coffee residues support the growth of the fungi *Cunninghaela phaeospora* and *Absidia cylindrospora* for commercial interest enzymes production using coffee residues as substrate in SSF methods. Moreover, an evaluation of the best conditions of fermentations, amount of substrate and optimal temperature for the production of proteases were estimated.

Materials and Methods

Microorganisms

Strains of the filamentous fungi *Cunninghamella phaeospora* UCP 1303 and *Absidia cylindrospora* - UCP1301, isolated from Caatinga (arid climate) were obtained from the collection of UCP cultures of the Catholic University of Pernambuco (Recife, Pernambuco, Brazil). To be properly stored, the microorganisms were reactivated in glucose broth (1% meat extract, 0.3% peptone, 2% glucose) and maintained in environmental chamber at 30°C for 7 days.

Culture Media and Fermentation in Solid State

Potato dextrose agar (PDA) was used as medium for the maintenance of stock cultures and for every preparation, Erlenmeyers flasks containing 200 mL of solid PDA medium were previously autoclaved at 121°C for 20 min. The culture medium for the production of enzymes was the coffee residue, provided by Delta Ex-

press Company (Recife, Pernambuco, Brazil). The residues were dried in an oven at 100°C until their dehydration (humidity level of 40%). Solid-state fermentation (SSF) was carried out by using 8 Erlenmeyers flasks (in total) containing autoclaved coffee residues (121°C for 20 min), all moistened with distilled water 3 mL. From those 8 flasks, 4 were used for the fermentation by the fungus *Cunninghamella phaeospora* UCP 1303 and 4 by the fungus *Absidia cylindrospora* UCP1301, being divided thus into 2 independent groups. Two different masses of substrates were tested for each group, being 2 flasks with 5g and 2 flasks with 10g of coffee. Further, fungal spores (suspended with deionized water) from the stock medium BDA growth were sampled in each of the flasks with 10^5 spores/mL.

Production and Extraction Enzyme and Acetone Precipitation

For the production of proteases, a simple factorial design 2^2 was used to evaluate the effect of different temperatures (30°C and 35°C) and substrate amounts (5g and 10g) on the enzyme production, with humidity level of 40% and in the dark for 7 days. The temperature control was done from incubation in incubator BOD tests with temperatures in the different groups and occurring independently. For extraction of the enzyme were used 7 mL of brine (NaCl) at 0.15 per gram of substrate, followed by vacuum filtration to obtain metabolic liquid. An aliquot of metabolic liquid (5 ml) was precipitated with acetone (70%), centrifuged at 9000 x g for 10 minutes, then solubilized in 1 ml of 0.1M Tris-HCl buffer, pH 8.0 and subsequently used for further analysis.

Protein Measurements

The total protein determination was performed using the BCA method according to Smith (1965). The tests were carried out using the micro assay kit BCA from Thermo Pierce Scientific (Waltham, Massachusetts, USA) and the amount of protein was estimated through a standard curve using bovine serum albumin - BSA (the BCA kits include ampules of Albumin Standard).

The yield and relative protein dosage represented the ratio between the protein amount found and the volume of the fermentative extract.

Enzyme Activity

General Assay for proteolytic enzymes

The method for determining the protease activity was described by [10] with modifications. The reaction contained 0.25 mL of substrate (azocasein 1% w/v in 0.1 M Tris-HCl buffer, pH 8.0) and 0.15 ml of the sample. This mixture was incubated for 1 hour in an environmental chamber at 30°C and the reaction stopped by adding 1 ml of trichloroacetic acid (TCA) 10% w/v. Subsequently the samples were centrifuged for 5 min at 9000 x g and 0.8 mL of supernatant was pipette out and transferred to Eppendorf tubes with 0.2 ml of sodium hydroxide 1.8M. The absorbance was measured at 420 nm and 1 enzyme activity unit was defined as an increase of 0.01 in absorbance.

The relative proteolytic activity represented the ratio between the enzyme activity found and the protein amount (as defined in the previous section) in the fermentative extract.

Tests to determine specific proteolytic activity (collagenase)

The collagenolytic activity was determined according to the method of [13] modified. A suspension of Azocoll 5 mg/mL was prepared in buffer 0.1 M Tris-HCl, pH 8.0 to a final volume of 0.950 mL. A volume of 0.05 ml of the enzyme extract was inoculated in this suspension and the reaction processed at 35°C for 1 hour under stirring. Then the reaction was centrifuged 2250 x g for 5 min, and the absorbance was measured at 520 nm in an UV/Visible Spectrophotometer (GE Healthcare, Uppsala, Sweden). The enzyme activity level for this test was determined by the ratio activity/protein and expressed in U/mL*mg.

Results and Discussion

The results obtained from the simple factorial design (crude and precipitated enzyme extract) using the fungi *Cunninghaela phaeospora* and *Absidia cylindrospora* are shown in Table 1. The microorganisms showed different behaviours, since the fungus *Cunninghaela phaeospora* obtained better yield from a particular substrate concentration and temperature, whilst the best growing conditions for *Absidia cylindrospora* was found to be directly proportional to the coffee substrate concentration.

For the *Cunninghaela phaeospora*, the best production condition was found by using 5g of subtrato (coffee waste) at a temperature of 30°C, which was reflected in a higher protein production (Table 1) and protease activity (Table 1 and 2). For the other substrate concentrations only a small mean variation of the results was observed, not being verified any proportional relation between the variables; unlike other studies previously reported, in which as the amount of substrate is increased, greater than or equal is the production of total proteins [14]. Many authors as Lakshmi., *et al.* (2014), Rauf., *et al.* (2010) and Escobar and Barnett (1993) reported that in fermentative processes it is not always observed a linear relation between the substrate concentration and the production of biomolecules. Moreover, there is a certain concentration of ideal substrate for each microorganism (depending on their metabolism), and nutrients in excess can promote catabolic repression and inhibitory effects on the enzyme production.

	Cunninghamella phaeospora	*Absidia cylindrospora*
Sample	**Protein Concentration (mg/mL)**	
CE 5g 30°C	0.42	1.67
CE 5g 35°C	0.58	0.70
CE 10g 30°C	0.64	0.54
CE 10g 35°C	0.60	0.80
PE 5g 30°C	1.75	0.67
PE 5g 35°C	1.32	0.68
PE 10g 30°C	1.22	0.35
PE 10g 35°C	0.94	0.38

Table 1: Protein Measurements. The sample are presented by different substrate concentrations and temperatures.

CE: Crude Extract; PE: Precipated Extract (suspended in 1 mL). Data represents the media of three different tests.

	Cunninghamella phaeospora	*Absidia cylindrospora*
Sample	**Proteolytic (general) activity (U/mL*mg)**	
CE 5g 30°C	1089.81	546.29
CE 5g 35°C	893.07	594.79
CE 10g 30°C	749.21	1343.70
CE 10g 35°C	726.78	505.136
PE 5g 30°C	138.12	353.49
PE 5g 35°C	214.47	618.17
PE 10g 30°C	140.20	2074.79
PE 10g 35°C	160.92	502.66

Table 2: General Proteolytic Activity. The sample are presented by different substrate concentrations and temperatures.

CE: Crude Extract; PE: Precipated Extract (suspended in 1 mL). Data represents the media of three different tests.

For the *Absidia cylindrospora,* in contrast, the general proteolytic activity was improved in the samples obtained from cultures maintained at 30°C containing 10g of substrate, although the protein concentration has not been increased, as can be seen in Table 1. These results show that although the low amount of protein in the extracts, the enzyme had a high catalytic potential. [15] demonstrated the same proportional relation, when a greater amount of substrate (wheat) promoted an increase in production of protease activity by using the microorganism *Pleurotus albidus*. Other studies with different species of fungi, using also 10g of substrate at 30°C as fermentative conditions, showed a good result against proteolytic activities [2], which corroborates the results of this study.

Table 2 shows the results of the specific proteolytic activity (collagenolytic) using both crude and precipitated fermentation extracts. For fermentations using the fungus *C. phaeospora*, the collagenolytic activity was affected in the same way as already observed for the general protease activity, in which there was a particular substrate concentration and an optimal temperature (5g of substrate and at 30°C) for the better enzyme extract activity. For these fermentations, the comparison between crude and precipitated extracts show that there was a significant decrease in the activity of the extract after precipitation with acetone, but among each other (different amount of substrate and temperature) it was not possible to verify substantial differences between the activities of the precipitate extract.

We observed an excellent specific collagenolytic activity of the precipitated extracts obtained from fermentations by *A. cylindrospora* using 10g of substrate at 30°C and at 35°C, resulting in activities of 111.19 U/ml and 95.68 U/mL respectively. The amount of substrate was thus a direct factor in the improvement of the enzyme production. At the same time, it is possible to emphasize the influence of temperature on the production of enzymes with collagenolytic activity so that in some cases there was an increase of the enzyme production from the temperature increase (see Table 3). The precipitate extract in this case showed activity levels greater than the crude extract, reaching maximum of 35% of improvement in the conditions of 10g and 30°C.

	Cunninghamella phaeospora	*Absidia cylindrospora*
Sample	**Collagenolytic activity (U/mL*mg)**	
CE 5g 30°C	78.3	23.3
CE 5g 35°C	56.7	53.8
CE 10g 30°C	50.1	73.8
CE 10g 35°C	65.2	62.7
PE 5g 30°C	34.8	48.0
PE 5g 35°C	28.1	54.0
PE 10g 30°C	32.3	111.1
PE 10g 35°C	38.5	95.6

Table 3: Specific Protease (collagenolytic activity). The sample are presented by different substrate concentrations and temperatures.

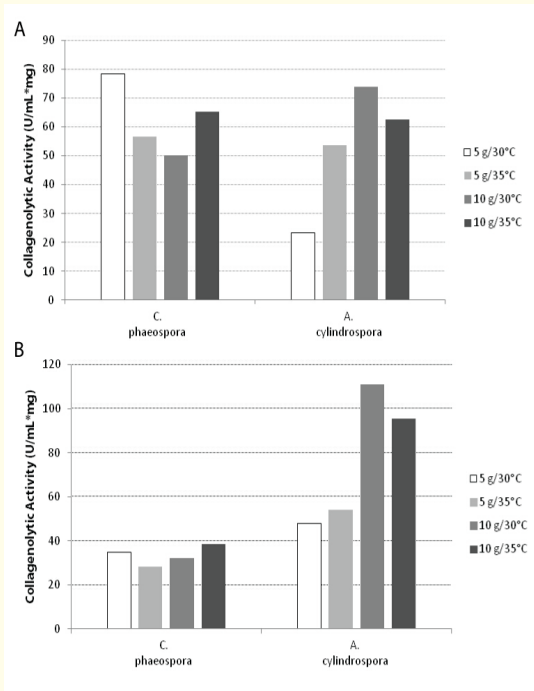
CE: Crude Extract; PE: Precipated Extract (suspended in 1 mL).

Data represents the media of three different tests.

[16] reported that temperature is the most important parameters which must be controlled in a bioprocess. The variation of temperature (5°C) in the present study did not promote a considerable difference in the general and specific (collagenolytic) proteolysis in the experiments with *Cunninghaela phaeospora*, however the enzyme activity was altered (usually in a positive way) for the extracts obtained from fermentations by *Absidia cylindrospora.* This fact shows that the temperature (even for small variations) can be an important factor in the enzyme production and different microorganisms can have different behaviours with regard to the thermal variation.

According to the results shown in Table 1, all isolates of Cunninghaela phaeospora and Absidia cylindrospora were able to grow on the coffee residue substrates (with low humidity). This probably is due to the fact that those fungi are collected from Caatinga, an arid region with abrupt environmental changes, forcing the microorganisms to a fast adaptation [11].

The comparison between the microorganisms clearly shows us how their metabolism for the enzyme production can vary depending on the cultivation conditions (Figure 1). In general, the best condition for the production of protease (general and specific collagenase) was found using the fungus *Absidia*, 10g of substrate and at 30°C, which obtained relative activity of 115.2 U/mL*mg (collagenolytic activity). The fungus *Cunninghamella phaeospora* did not suffer influence of temperature on its enzyme production and the ketogenic precipitation extract promoted a reduction of the activity. At the same time, in the fermentations by the fungus *Absidia*, the temperature variation (5°C) provided changes in the enzymatic activity (usually positive) and the use of the precipitate extract increased the proteolytic activity.



The amount of substrate was essential to improve the production of collagenolytic enzymes in the fermentations by *Absidia.* It was observed that as higher was the amount of substrate added to the fermentation greater was the production of proteolytic enzymes. This was showed also by [15], when maintaining the temperature and doubling the substrate concentration they observed an increase on the enzymatic performance of about 33% (for the samples maintained at 30°C) and 35% (for the samples maintained at 35°C) [17].

Conclusions

The microorganism *Cunninghamella phaeospora* CPU 1303 and *Absidia cylindrospora* were able to produce proteases with general and specific activity (collagenolytic) from solid-state fermentation using the coffee residue, making it a feasible source for obtaining enzymes with biotechnological interest. The production of prote-

ases proved to be dependent on the amount of substrate and also on the temperature (fermentations with *Absidia cylindrospora*), obtaining the best enzyme production rates proteolytic under conditions of 30°C with 10g of substrate. The utilization of coffee waste proved to be a profitable source of substrate for enzyme production, its reuse can be an alternative to convert waste into collagenolytic enzyme in industrial and pharmacological applications.

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Conflict of Interest

None.

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