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Bioconversion of coffee ground to lipase by filamentous fungi isolated from the Igarassu River in the State of Pernambuco, Brazil

Bioconversão da borra de café a lipase por fungos filamentosos isolados do Rio Igarassu no Estado de Pernambuco, Brasil

Bioconversión de posos de café a lipasa por hungos filamentosos aislados del Rio Igarassu en el Estado de Pernambuco, Brasil

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Eduardo da Silva França
ORCID: https://orcid.org/0000-0003-1573-5133
Catholic University of Pernambuco, Brazil
E-mail: eduardo.franca10@hotmail.com
Adriana Ferreira Souza
ORCID: https://orcid.org/0000-0002-9527-2206
Catholic University of Pernambuco, Brazil
E-mail: adriana.souza@unicap.br
Diego Guedes de Lima Lemos
ORCID: https://orcid.org/0000-0002-0632-7712
Catholic University of Pernambuco, Brazil
E-mail: qdiegolima@hotmail.com
Uiara Maria de Barros Lira Lins
ORCID: https://orcid.org/0000-0002-6007-9932
Catholic University of Pernambuco, Brazil
E-mail: uiaramaria@gmail.com
Galba Maria de Campos-Takaki
ORCID: https://orcid.org/0000-0002-0519-0849
Catholic University of Pernambuco, Brazil
E-mail: galba.takaki@unicap.br
Carlos Alberto Alves da Silva
ORCID: https://orcid.org/0000-0001-8695-526X
Catholic University of Pernambuco, Brazil
E-mail: carlos.alves@unicap.br
Marcos Antônio Barbosa de Lima
ORCID: https://orcid.org/0000-0001-5987-224X
Catholic University of Pernambuco, Brazil
E-mail: mablima33@yahoo.com.br

Abstract
Most lipases used for commercial purposes are isolated from microorganisms due to their stability and easy recovery, new residues have been studied to obtain enzymes to reduce production costs. Therefore, this work investigated the biotechnological potential of filamentous fungi isolated from the Igarassu River in the conversion of coffee grounds into lipase in solid state fermentation. The fungi were isolated, identified and submitted to a preliminary assay with 10g of coffee grounds, moisture 60% at 28 °C for 144 h to select the filamentous fungus with the highest potential for lipase production. A factorial design of 2^3 was carried out to evaluate the influence of the variables moisture, temperature and residue concentration on lipase production by the selected species. Five filamentous fungi were isolated and identified: *A. flavus* UCP 0316, *A. fumigatus* UCP 0327, *P. variotii* UCP 0334, *M. hiemalis* f. *luteis* UCP 0343 and *A. foetidus* UCP 0360. All filamentous fungi cultivated on coffee grounds were able to grow and produce lipase, however *A. foetidus* exhibited higher enzymatic activity of 514.29 U/mL. We observed that the production of lipase was higher (2941.87 U/mL) when *A. foetidus* was cultivated in 25 g of coffee grounds, 37 °C and 50% moisture. Therefore, we emphasize that coffee grounds are a promising agro-industrial residue to lipase production, contributing to the reduction of environmental pollution and generating value-added products for the industry.
Keywords: Microrganisms; Solid state fermentation; Agro-industrial residues.

Resumo
A maioria das lipases usadas para fins comerciais são isoladas de microrganismos devido a sua estabilidade e fácil recuperação, novos resíduos têm sido estudados para obtenção de enzima para redução dos custos de produção. Portanto, este trabalho investigou o potencial biotecnológico de fungos filamentosos isolados do rio Igarassu na

conversão da borra de café em lipase em fermentação em estado sólido. Os fungos foram isolados, identificados e submetidos a um ensaio preliminar com 10g da borra de café, umidade 60% em 28 °C por 144 h para selecionar o fungo filamentoso com maior potencial de produção de lipase. Um planejamento fatorial de 2^3 foi realizado para avaliar a influência das variáveis umidade, temperatura e concentração do resíduo na produção de lipase pela espécie selecionada. Foram isolados e identificados 5 fungos filamentosos: *A. flavus* UCP 0316, *A. fumigatus* UCP 0327, *P. variotii* UCP 0334, *M. hiemalis* f. *luteis* UCP 0343 e *A. foetidus* UCP 0360. Todos os fungos filamentosos cultivados em borra de café foram capazes de crescer e produzir lipase, porém *A. foetidus* exibiu maior atividade enzimática de 514,29 U/mL. Observamos que a produção de lipase foi mais alta (2941,87 U/mL) quando *A. foetidus* foi cultivado em 25 g de borra de café, 37 °C e 50% de umidade. Sendo assim, destacamos que a borra de café é um resíduo agroindustrial promissor para a produção de lipase, contribuindo na redução da poluição ambiental e gerando produtos de valor agregado à indústria.
Palavras-chave: Microrganismos; Fermentação em estado sólido; Resíduos-agroindustrial.

Resumen
La mayoría de las lipasas utilizadas con fines comerciales son aisladas de microorganismos debido a su estabilidad y fácil recuperación, se han estudiado nuevos residuos para obtener enzimas que reduzcan los costos de producción. Por lo tanto, este trabajo investigó el potencial biotecnológico de hongos filamentosos aislados del río Igarassu en la conversión de posos de café en lipasa en fermentación en estado sólido. Los hongos fueron aislados, identificados y sometidos a un ensayo preliminar con 10g de café molido, 60% de humedad a 28 °C durante 144 h para seleccionar el hongo filamentoso con mayor potencial de producción de lipasa. Se realizó un diseño factorial de 2^3 para evaluar la influencia de las variables humedad, temperatura y concentración de residuos en la producción de lipasa por parte de las especies seleccionadas. Se aislaron e identificaron cinco hongos filamentosos: *A. flavus* UCP 0316, *A. fumigatus* UCP 0327, *P. variotii* UCP 0334, *M. hiemalis* f. *luteis* UCP 0343 y *A. foetidus* UCP 0360. Todos los hongos filamentosos cultivados en posos de café pudieron crecer y producir lipasa, sin embargo, *A. foetidus* exhibió una actividad enzimática más alta de 514.29 U/mL. Observamos que la producción de lipasa fue mayor (2941.87 U/mL) cuando se cultivó *A. foetidus* en 25 g de café molido, 37 °C y 50% de humedad. Por lo tanto, destacamos que los posos de café son un residuo agroindustrial promisorio para la producción de lipasa, contribuyendo a la reducción de la contaminación ambiental y generando productos con valor agregado para la industria.
Palabras clave: Microorganismos; Fermentación en estado sólido; Residuos agroindustriales.

1. Introduction

The search for new microorganisms isolated and identified from environments still little known for the production of bioactives, especially microbial enzymes, is necessary because the biotechnology industry needs new microbial producers with high efficiency. In this context, there is a worldwide increase in the consumption of industrial enzymes and filamentous fungi are among the main microbial sources producing these enzymes (Gorlach & Coutinho, 2007; Monteiro & Silva, 2009; Alves *et al*., 2019; Silva et al., 2019; Barbosa *et al*., 2020; França *et al*., 2020).

Enzymes are effective catalysts, not only increasing the rate of substrate to product conversion, but also recognizing a specific chemical structure in the presence of similar structures to produce a specific product (Devlin, 2011; Gorlach-Lira et al., 2020). These biocatalysts can be produced by animals, plants and microorganisms, and those of microbial origin are preferable, because they have a series of advantages such as shorter production time, high yield, ease of handling and mainly, because they are considered more active and stable than those of origin animal and vegetable (Nagarajan, 2012; Liu & Kokare, 2017; Ferraz *et al*., 2018; Tripathi et al., 2020; França *et al*., 2020).

Lipases (triacylglycerol hydrolases EC 3.1.1.3) are enzymes that catalyze the hydrolysis of triacylglycerols into glycerol and fatty acids and have been applied in recent years in emulsifiers, pharmaceuticals, cosmetics, flavors, fragrances, as well as, in the treatment of lipid-rich wastewater from food manufacturing processes, oil bioremediation and biodiesel synthesis (Pereira *et al*., 2019; Chandra, *et al*., 2020; Melani *et al*., 2020; Oliveira *et al*., 2020; Lima *et al., 2021;* Mehta *et al*., 2021; Msangosoko *et al*., 2022). According to the report by Markerts Research Report (2020) the global lipase market is expected to reach US$ 774.2 million by 2027 with a 5.3% growth. The microbial lipases should register 5.1% growth and reach US$ 579 million in 2027.

The industrial production of microbial enzymes is limited, as it demands high costs with substrates, and it is estimated that about 30-40% of the production cost of the enzymes is due to the substrates used for the cultivation of microorganisms, which could be reduced using low-cost substrate, such as agro-industrial waste (Nascimento *et al*., 2007; Marzo *et al*., 2019).

The solid state fermentation (SSF) is an alternative and promising technology due to lower energy consumption, higher productivity, low effluent generation, lower risk of bacterial contamination, as well as allowing the use of agro-industrial residues and by-products as substrate for production of value-added biomolecules. Therefore, SSF has potential economic and environmental benefits that can make industrial processes more sustainable (Soccol *et al*., 2017; Costa *et al*., 2018; Sadh *et al*., 2018).

Brazil is the largest producer and exporter of coffee and the second largest consumer of the beverage in the world, with production in 2021 estimated at 47.3 million 60 kg bags (Conab, 2022). O Post-harvest processing of coffee generates different types of residues (husk, pulp, mucilage, fermentation and washing water and dregs). About 90% of all coffee consumed worldwide ends up in the form of solid waste, with the dregs representing most of the waste generated (Magnago *et al*., 2019).

In the production of soluble coffee, it is estimated that for each ton of processed green coffee, 650 kg of dregs are generated and for each kg of soluble coffee produced, 2 kg of dregs are generated, with values between 60 and 70% of moisture. It is important to point out that the waste produced on smaller scales, in homes, bars, restaurants and cafeterias, has not been accounted for (Durán *et al*., 2017; Garcia, Young-Teck, 2021).

The coffee residues contain high concentrations of tannin and caffeine which, if disposed of incorrectly, can cause environmental problems (Getachew, Chun, 2017). Therefore, this study aimed to investigate the biotechnological potential of filamentous fungi, isolated from the Igarassu River, in the bioconversion of coffee grounds into lipases in solid fermentation.

2. Methodology

2.1 Water collection, isolation and identification of filamentous fungi

2.1.1 Collection of water samples

The collection of water samples was carried out in the Igarassu river, Municipality of Igarassu/PE, Brazil, on 10.30.2019 at 08:00 hours. The weather conditions on the day of collection were 27 °C, 75% relative moisture, 14 km/h wind and 0 mm rainfall. The surface water samples were collected in the stretch located at km 41.7 of the BR-101/PE at the following coordinates: latitude: 7° 50'07.1" S and longitude: 34°54'47.3" W. 1.5 L were collected. of surface water, using three 500 mL sterile polypropylene plastic collection bottles. The collection was carried out with the immersion of the flask at a depth of 15 cm. After collection, the bottles were sealed, dried, identified and placed in an isothermal box containing ice.

2.1.2 Isolation of filamentous fungi

The isolation of filamentous fungi was carried out by adding 1 mL of water from the Igarassu river, using the pour-plate method, onto 4% Sabouraud Agar medium supplemented with 0.05 g/L chloramphenicol for bacterial growth inhibition. Plates were incubated at 28 °C for 96 hours. After growth, isolated colonies were purified and maintained in Sabouraud Agar at 4 ºC.

2.1.3 Morphological identification of isolated filamentous fungi

The isolates were identified by evaluating the macromorphological characteristics (color, appearance, diameter of the colonies) and micromorphological characteristics according to specialized literature such as: Klich (2002), Samson (2004),

Schipper and Samson (1978) for the genus *Aspergillus*, Samson (1974) and Samson *et al*., (2009) for the genus *Paecillomyces*; Trufem (1981), Alves *et al*., (2002), Souza *et al*., (2016) for the genus *Mucor*.

After identification, the isolates were stored in mineral oil according to Sherf (1943) and in sterilized distilled water according to Castellani (1939). Then they werecataloged as: *Aspergillus flavus* UCP 0316, *Aspergillus fumigatus* UCP 0327, *Paecillomyces variotii*, UCP 0334, *M. hiemalis* f. *luteus* UCP 0343, *Aspergillus foetidus* UCP 0360 and deposited in the Culture Bank of the Catholic University of Pernambuco (UCP) at the Center for Research in Environmental Sciences and Biotechnology, located in Recife/Pernambuco.

2.1.4 Obtaining and preparing the substrate

The coffee grounds kindly provided by cafeterias and residences, located in Recife/PE, were oven dried at 65 °C for 96 h and stored at 4 °C until use.

2.1.5 Characterization of coffee grounds

The coffee grounds were submitted to FTIR spectroscopic analysis in the Shimadzu IR-TRACER 100 equipment, using attenuated total reflectance (ATR) accessory consisting of a mixed crystal "diamond/ZnSe". The peaks obtained were compared with the literature to confirm the presence of lipids.

2.1.6 Production of lipase

2.1.6.1 Inoculum Preparation

The samples were grown in Sabouraud Agar incubated at 28 °C for 96 h until sporulation. After sporulation, the inoculum was obtained by transferring spores to an Erlenmeyer flask containing 50 mL of sterile distilled water to obtain a sporadic suspension of 10^7 UFC/mL. The counting of suspended spores was performed in a Neubauer chamber and optical microscope.

2.1.6.2 Selection Assay for Lipase Production

The 5 species of filamentous fungi were subjected to selection in solid state fermentation for lipase production using coffee grounds as substrate. Thus, 125 mL Erlenmeyer flasks were added with 10 g of coffee grounds, previously sterilized, and moisture adjusted to 60% by the addition of saline base composed of $NaNO_3$ (3,0 g/L), K_2HPO_4 (1,0 g/L), $MgSO_4$ (0,5 g/L), KCl (0,5 g/L), $FeSO_4.7H_2O$ (0,01 g/L), pH 6.0 and per 5 mL of the spore suspension (10^7 UFC/mL), described by Colla, Hemkemeier e Gil (2012). Flasks were incubated at 28 °C for 144 hours of culture in triplicate.

2.1.6.3 Determination of lipolytic activity

The activity lipolytic was determined using the methodology described by Soares *et al*., (1999). The reaction of a mixture containing 5 mL of an emulsion of olive oil and gum arabic (7%), added with 2 mL of sodium phosphate buffer (0.1 M, pH 7.0) and 1 mL of filtered metabolic liquid was carried out, except for reactional whites. The mixtures were shaken on an orbital shaker at 82 rpm, 37°C for 10 minutes. The reactions were stopped by adding 10 mL of an acetone-ethanol-water (1:1:1) mixture, releasing the free fatty acids present in the mixture. The mixtures were titrated with a KOH solution (0.04 N) in the presence of the phenolphthalein indicator. The experiment was carried out in triplicate. The unit of lipolytic activity (U/mL) was defined as the amount of crude enzyme that released 1 µmol/mL of fatty acids per minute. The lipase enzymatic activity was calculated using equation 1:

$$AE\ (U/mL) = \frac{(Va-Vb)\ x\ N\ x\ 1000}{t\ x\ Vb}$$ (Equation 1)

Where: AE is the enzymatic activity (U/mL); Va the volume of the titrated sample (mL); Vb is the used sample volume of the reaction (mL); N is the normality of the KOH solution (N); T is the reaction time in minutes.

2.1.7 Planning factorial

A 2^3 factorial design, with 4 central points, was carried out to evaluate the influence of the independent variables moisture, temperature and concentration of coffee grounds on the response variable activity lipolytic, in solid state fermentation for 144 h, using the species selected in the preliminary test for lipase production. Each independent variable was investigated at three levels: minimum (-1), central (0) and maximum (+1) as shown in table 1. Data were analyzed using the Statistic software version 7.0 considering the significance of the results for ($p < 0.05$).

Table 1. Levels of the 2^3 factorial design variables for lipase production in coffee grounds by the selected fungus.

Factors	levels		
	-1	**0**	**+1**
Moisture (%)	50	70	90
Temperature (°C)	25	31	37
Concentration (g)	5	15	25

Source: Authors.

3. Results and Discussion

3.1 Isolation of filamentous fungi

The five filamentous fungi were isolated, 3 of the *Aspergillus* genus, 1 of the *Mucor* genus and 1 of the genus *Paecillomyces*. Gomes *et al*., (2008), analyzed water and sediment from the beaches of Bairro Novo and Casa Caiada in Olinda, Pernambuco, and observed more frequently *Aspergillu*s and *Paecillomyce*s, followed by other genera.

Similar results were also reported by Doi *et al*., (2018) that isolated 15 genera of filamentous fungi in the water and sediment of the Araçá bay in São Sebastião/SP, the main ones being *Aspergillus*, *Mucor* and *Paecillomyces*, with emphasis on species of the genus *Aspergillus* that presented greater density and diversity.

Lima *et al*., (2017), describe that fungi are found where there is organic matter, most of them being saprophytes and, occasionally, pathogenic. Species of the genera *Aspergillu*s, *Mucor*, *Paecillomyces* among others such as *Penicillium*, *Trichoderma* and *Fusarium* are biologically more successful and found in all habitats, including water.

3.2 Morphological identification of filamentous fungi

Five taxa already known in the literature were identified: *Aspergillus flavus*, *A. fumigatus*, *A. foetidus*, *Paecillomyces variotii*, and *Mucor hiemalis*. The isolates mentioned can be identified based on the key proposed below (Table 2):

Table 2. Identification key of species of filamentous fungi isolated from the Igarassu River.

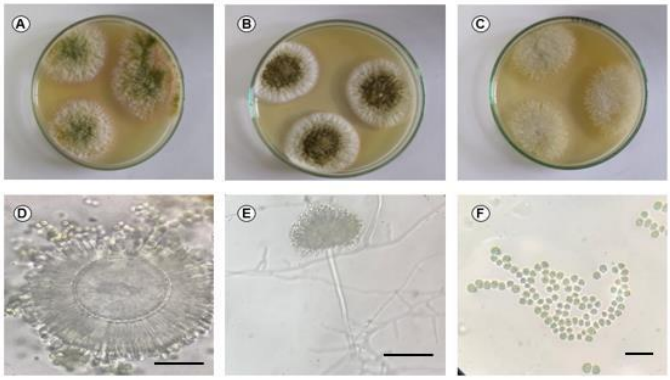
1	Septate hydhae	2
1	Cenocytic hyphae.....	4
2	Conidiophore with inflated apex (vesicle), containing numerous phialides..... ***Aspegillus***	
2’	Olive green to parrot green conidia at CYA 25 °C, reaching 3 to 6 µm in diameter. Predominantly bi-serial series ……..... ***A. flavus***	
2”	Monoserial serialization. Finely roughened, globose turquoise conidia reaching up to 3 µm (3.5 µm), columnar-type conidial head ……..... ***A. fumigatus***	
2’’’	Dark brown to black conidia. Biseriate with globose and finely rough conidia, reaching 4-5 µm ……..... ***A. foetidus***	
2	Conidiophore without vesicle formation with phialid production 3	
3	Globular conidia, well-developed penicillions …..... ***Penicillium***	
3	Limoniform and poorly penicillated conidia …..... ***Paecilomyces***	
3’	Irregularly branched conidiophores, phialid 12 to 20 µm in diameter, cylindrical to ellipsoidal conidia …..... ***P. variotii*** e ***P. formosus***	
3’’	In CREA medium it does not produce acidic compounds ..... ***P. variotii***	
4	Simple or branched sporangiophores with sporangium without apophysis ..... ***Mucor***	
4’	Obovoid columella, ellipsoidal and fusiform spores 2.5 - 10 µm …..... ***M. hiemalis* f. *hiemalis***	
4’’	Globose to obovoid columellae; long, ellipsoid sporangiospores 2.5 (1.5 µm) - 8.0 µm (12 µm) ……..... ***M. hiemalis* f. *luteus***	

CYA – Czapek Yeast Extract Agar; CREA – Creatine Sucrose Agar. Source: Authors.

3.2.1 *Aspergillus flavus*

The colonies grown in CYA (Agar Czapek Yeast Extract) at 25 °C, had a diameter of 57.7 mm, grooved appearance with a woolly to flaky texture, white mycelium with olive green conidia (Figure 1A) and an orange reverse. However, incubated at 37 °C, the colonies had a diameter of 43.8 mm, a grooved appearance with a woolly to flaky texture, white mycelium with green conidia (Figure 1B). On MEA (Malt Extract Agar) the colonies showed a grooved colony with a woolly to flaky texture, white mycelium with olive-colored conidia (Figure 1C). None of the cultures produced pigment and exudate. Sclerotia production at 14 days. Assessing the microscopic characteristics, colorless conidiophores near the apex, rough wall (400-800 µm), conidial heads of the radial to columnar type and predominantly biseriate 1D and 1E were observed. Predominantly piriform vesicles (Figure 1D). Globular and finely rough conidia, reaching up to 3.0 – 5.0 µm (Figure 1F).

Figure 1. Macroscopic and microscopic characteristics of the isolate *Aspergillus flavus* UCP 0316. (A – B) Colony appearance, 25 °C and 37 °C in CYA, respectively; (C) Colony appearance at 25 °C in MEA; (D) Spherical vesicle, with metulae and phialides; (E) radial conidial head; (F) globose to ellipsoidal conidia. Bars: D = 30 µm; E = 100 µm; F=10 µm.



Source: Authors.

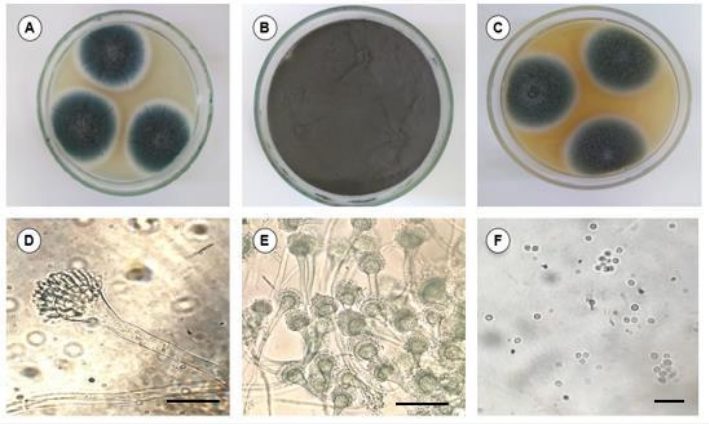
Colony color variation ranging from green to dark green of *A. flavus* may lead to misidentification as *A. parasiticus*. However, micromorphology such as the seriation and shape and ornamentation of conidia help to differentiate. Generally the dominant seriation in *A. parasiticus* is uniseriate with few biseriate seriation (Klich, 2002; Kokalis-Burelle *et al*., 1997). The studied strain produces few sclerotia. The production of sclerotia is known as a common feature of the Flavi section of *Aspergillus* (Varga *et al*., 2011).

However, many studies have reported that production was varied even within the same species, making this feature irrelevant. Furthermore, the production of these structures is not necessarily correlated with the production of aflatoxin (Reis et al., 2014; Norlia et al., 2018).

3.2.2 *Aspergillus fumigatus*

The colonies grown in CYA at 25 °C, had a diameter of 52.70 mm, turquoise-gray conidia and white mycelium, as well as a colony with a velvety to flaky and radially grooved texture (Figure 2A) and colorless reverse. In CYA at 37 °C, the colonies had diameters of 83.32 mm with intense production of grayish conidia (Figure 2B). In MEA the colonies showed similar characteristics to CYA at 25 °C (Figure 2C). None of the cultures produced sclerocytes, pigment and exudate. Evaluating the microscopic characteristics, colorless to grayish conidiophores were observed near the apex, smooth wall (200-400 µm), columnar and monoseriate conidial heads (without the presence of métulas), with phialides reaching from 6 to 8 µm, covering only the upper part of the vesicles as shown in Figures 2D and 2E. Predominantly pear-shaped and spatular vesicles (Figure 2D). Globular and finely rough conidia, reaching up to 3.0 µm (Figure 2F).

Figure 2. Macroscopic and microscopic characteristics of the isolate *Aspergillus fumigates* UCP 0327. (A – B) Colony appearance, 25 °C and 37 °C in CYA, respectively; (C) Colony appearance at 25 °C in MEA; (D) Conidial columnar head; (E) Piriform vesicles to spatular; (F) globose conidia. Bars: D = 20 µm; E = 100 µm; F=10 µm.



Source: Authors.

The species *A. fumigatus* is distinguished from other *Aspergillus* species by its fast-growing colonies with turquoise to dark green hues, phialides parallel to the conidiophore and column-loaded conidia (Klick, 2002).

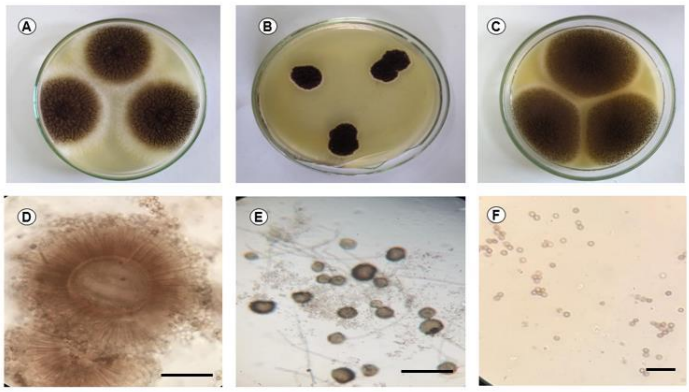
However, colonies of *A. lentulus*, *A. fumigatiffinis* and *A. novofumigatus* are macroscopically similar to *A. fumigatus*, but they differ in terms of sporulation intensity. Regarding the microscopic characteristics, most strains of *A. fumigatus* have subclaved vesicles, while the vesicles of the other species in the section are (sub)globose. In comparison, most *A. fumigatus* vesicles were wider than 22 mm, while in others the vesicles were narrower (Seung-Beom *et al*., 2005).

3.2.3 Aspergillus foetidus

Colonies grown in CYA at 25 °C had a diameter of 53.3 mm, white mycelium with dark brown to black conidia, colony with velvet texture and radially grooved (Figure 3A) and colorless reverse. In CYA at 37 °C the colonies had diameters of 19.7 (Figure 3B). In MEA, colonies showed similar characteristics to CYA at 25 °C (Figure 3C). None of the cultures produced sclerocytes, pigment and exudate.

Analyzing the microscopic characteristics, colorless to slightly brown conidiophores were observed near the apex, smooth wall (400-800 µm), globose to slightly elongated conidial heads, predominantly biseriate, metules reaching 5-18 µm, phialids reaching 3-3.5 µm, as shown in figures 3D and 3E. Additionally, they presented globose and finely rough conidia, reaching 4-5 µm (Figure 3F).

Figure 3. Macroscopic and microscopic characteristics of the isolate *Aspergillus foetidus* UCP 0360. (A – B) Colony appearance, 25 °C and 37 °C in CYA, respectively; (C) Colony appearance at 25 °C in MEA; (D) Spherical vesicle, biseriate; (E) radial conidial head; (F) globose to ellipsoidal conidia. Bars: D = 20 µm; E = 300 µm; F=10 µm.



Source: Authors.

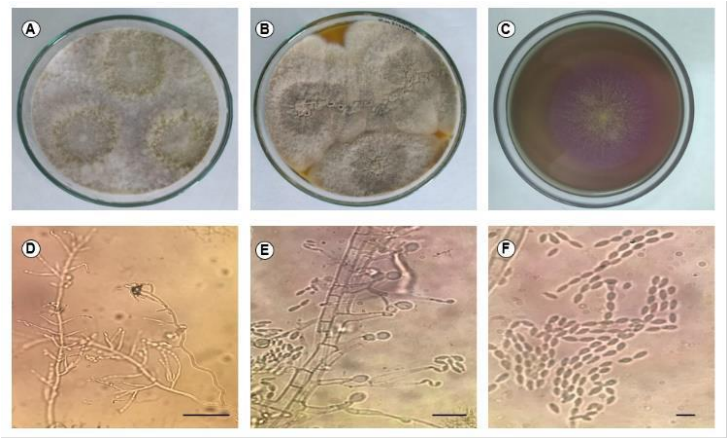
The Nigri Section is one of the most confusing and complex due to minimal differences between species. Species such as *A. carbonarius*, with spores that can reach 11 µm in diameter, and monoseriate species (*A. japonicus* and *A. aculeatus*), are easily identified by microscopic characteristics (Pitt; Hocking, 1997).

Aspergillus foetidus, *A. niger* and *A. tubingensis* are difficult to distinguish species with morphological characteristics (Samson et al. 2004). In this study, the reported species (*A. foetidus*) differs from the species *A. niger* by presenting conidiophores that did not exceed 1000 µm, which differs from *A. niger* that reach up to 3000 µm in length (Klich, 2002). Furthermore, *A. foetidus* is distinguished from these species by its ornamentation of conidia, which when young are finely wrinkled and when mature are smooth conidia (Klich *et al*., 2002; Silva *et al*., 2011).

3.2.4 Paecillomyces variotii

Colonies grown in MEA at 30 °C had a diameter of 81.25 mm, olive green conidia and white mycelium, as well as a velvety to flaky texture (Figure 4A) and yellow to colorless reverse. In CYA at 37 °C, they had a diameter of 64.57 mm (Figure 4B). In CREA there was no production of acidic components (Figure 4C). Analyzing the microscopic characteristics, irregularly branched conidiophores were observed, phialides reaching 12 to 20 µm in length with a wide base ending in a long and slender neck (Figure 4D and 4E). Cylindrical to ellipsoidal conidia reaching up to 5.6 µm (Figure 4F). *P. varitii* is morphologically similar to *P. formosus* but the latter produces acidic components in creatine agar (Samson, 2009).

Figure 4. Macroscopic and microscopic characteristics of the isolate *Paecillomyces variotii* UCP 0334. (A) Colony appearance in MEA at 30 °C; (B) Colony appearance in CYA at 37 °C; (C) Colony appearance at 25 °C in CREA without production of acidic compounds; (D – E) irregularly branched conidiophores and broad-based phialides ending in a long, slender neck; (F) Cylindrical to ellipsoidal conidia reaching up to 5.6 µm.

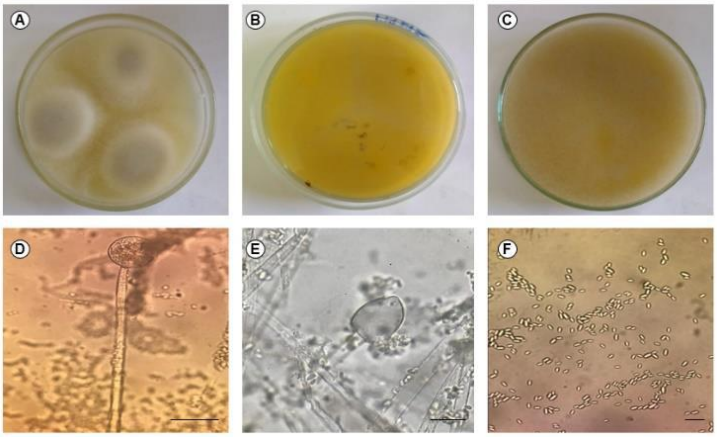


Source: Authors.

3.2.5 Mucor hiemalis

The isolate belongs to the *M. hiemalis* species, because it has an ellipsoidal columella with a truncated base, simple, unbranched or sparsely branched sporophores (Figure 5D–E), sporangiospores with regular shapes and sizes, sometimes with dilated one side (Figure 5F). Initially, white colonies and with aging of the colony show yellowish color, positive phototropism and do not grow at 40 °C (Figure 5A - C).

Figure 5. Macroscopic and microscopic characteristics of the isolate *Mucor hiemalis* f. *luteus* UCP 0343 (A) Colony appearance in BDA at 25 °C; (B) Colony appearance at 25 °C on MEA; (C) Colony appearance at 40 °C on MEA; (D – E) Globose and sporangiophore columella; (D) 2.5 x 8 sporangiospores.



Source: Authors.

The morphological characteristics of *M. hiemalis* reported here show close similarities with the description by Schipper (1978), with minimal difference in the diameter of sporangiospores and the globose shape of some columellae.

Sporangiospores with sizes of 2.5 x 8 and presence of globose columella, corresponds to the species *M. hiemalis* f. *luteus* reported by Alves *et al*., (2002).

Strains of *M. hiemalis* show morphological similarities with some variants of *M. circinelloides*. However, *M. hiemalis* lacks short branches that are abundant in *M. circinelloides* (Schipper, 1978).

The columella forms of *M. hiemalis* are similar to the species *M. racemosus*, but they are differentiated by the abundant presence of chlamydospores, which in *M. hiemalis* are scarce, and are never present in sporangiophores (Schipper,1978; Souza *et al*., 2017).

3.3 Preliminary tests in solid state fermentation

The screening results to select the filamentous fungus species with the greatest potential for lipase production in solid state fermentation in an alternative medium containing coffee grounds are shown in Table 3.

Table 3. Lipolytic activity of *Aspergillus flavus* UCP 0316, *Aspergillus fumigates* UCP 0327, *Paecilomyces variotii* UCP 0334, *Mucor hiemalis* f. *luteus* UCP 0343 and *Aspergillus foetidus* UCP 0360 in coffee grounds with 60% moisture, at 28 °C for 144h of cultivation.

Isolate code	Identification	Lipolytic activity (U/mL)
UCP 0316	*Aspergillus flavus*	147,29
UCP 0327	*Aspergillus fumigatos*	213,80
UCP 0334	*Paecillomyces variotii*	63,06
UCP 0343	*Mucor hiemalis* f. *luteus*	151,40
UCP 0360	*Aspergillus foetidus*	514,29

UCP – Code of the Culture Bank of the Catholic University of Pernambuco. Source: Authors.

The results obtained indicated that all species tested showed lipolytic activity in the alternative medium containing coffee grounds. However, *A. foetidus* showed higher enzymatic activity of 514.29 U/mL in coffee grounds medium with 60% moisture at 28 °C during 144h of cultivation. Putri *et al*., (2020). obtained lower lipolytic activity, 176 U/m/L, with *A. niger* grown in rice bran. On the other hand, Guedes *et al*., (2021) reported that among the filamentous fungi that produce lipases, the genus *Aspergillus* stands out. França et al., (2020) report the importance of searching for new microorganisms from environments that are still little known for the production of bioactives, especially microbial enzymes, as the biotechnology industry needs new microbial producers.

3.4 Factorial planning

The effects of independent variables on lipase production by *Aspergillus foetidus* UCP 0360 during 144 hours were investigated according to the 2^3 factorial design (Table 4). The 50% moisture, temperature of 37 °C and substrate concentration of 25 g favored the highest enzyme production (2941.87 U/mL).

The designs factorial are widely used as tools in optimizing lipase production processes (Gochev *et al*., 2012; Toscano *et al*., 2013; Adnani *et al*., 2010; Gao *et al*., 2009). In this study, the 2^3 factorial design provided a 5.7-fold increase in lipase production by *Aspergillus foetidus* UCP 0360.

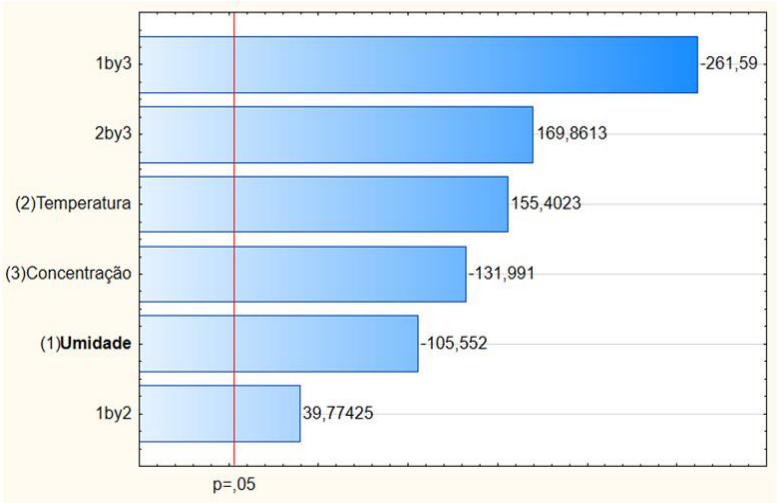
Table 4. Lipase production by *Aspergillus foetidus* UCP 0360 according to a 2^3 factorial design.

Experiments	Moisture (%)	Temperature (°C)	Concentration (g)	EA (U/ml)
1	50	25	5	2009.99
2	90	25	5	868.65
3	50	37	5	220.75
4	90	37	5	2548.00
5	50	25	25	273.84
6	90	25	25	310.68
7	50	37	25	2941.87
8	90	37	25	114.70
9	70	31	15	456.30
10	70	31	15	443.53
11	70	31	15	448.55
12	70	31	15	447.19

EA – Enzymatic Activity. Source: Authors.

The statistical analysis of the data showed that the three independent variables moisture, temperature and substrate concentration, as well as the interaction between them, were significant when analyzed at the 95% confidence level (Figure 6). The interaction of the variables moisture and substrate concentration was the most significant, but it had a negative influence, indicating that lower levels can favor the production of the enzyme. However, temperature and its interaction with substrate concentration positively influenced lipase production.

Figure 6. Pareto diagram of the effect of variables on lipase production by *Aspergillus foetidus* UCP 0360 in solid state fermentation using coffee grounds as substrate for 144 h.



Source: Authors.

3.5 Characterization of coffee grounds

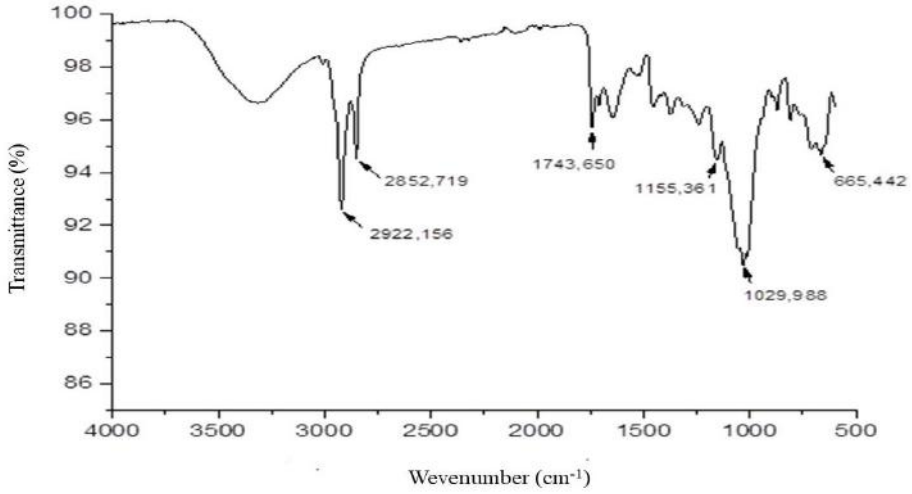
The coffee grounds spectra showed several absorption regions, as shown in Figure 7. Absorption bands located between 3000 and 3400 cm^{-1} are associated with the hydroxyl groups (-OH) of lignocellulosic materials sucj as cellulose, hemicellulose and lignin (García-García *et al*., 2015). The absorption of peak regions at 2900 and 2800 cm^{-1} are attributed to H-bonded C elongations, possibly from CH_2-functional groups corresponding of caffeine and lipids (Craig *et al*., 2012). The

vibration bands of elongation CH at 1745 cm^{-1} correspond to the carbonyl group (C=O) in the triglyceride ester group (Craig *et al*., 2012; Nakasato *et al*., 2007). The presence of lipids is notable in the spectrum.

The vibration bands of CH elongations at 2926 cm^{-1} (ketones ν (CH) CH_3), associated with peaks at 1652 cm^{-1} represent the double bond stretch, adjacent to a set of aromatic rings that are possibly attributed to lignin (Iriondo-Dehond *et al*., 2019). While the adjacent, unrepresentative peak at 2899 cm^{-1} corresponds to the CH elongation mode, associated with the peak at 899 cm^{-1} was attributed to the flexion modes HCC and HCO (δ(C(1)H(β)), of according to vibrational assignment for cellulose (Iriondo-Dehond *et al*., 2019). While the adjacent, unrepresentative peak at 2899 cm^{-1} corresponds to the CH elongation mode, associated with the peak at 899 cm^{-1} was attributed to the flexion modes HCC and HCO (δ(C(1)H(β)), of according to vibrational assignment for cellulose (Iriondo-Dehond *et al.*, 2019). A broad peak at 1658 cm^{-1} representing small concentrations of amide I (protein/peptides) was also observed, even in this peak the HOH deformation of H_2O is observed, demonstrating that the greater affinity of the coffee grounds with water (Iriondo-Dehond et al, 2019; Nakasato *et al*., 2007).

Bending and deformation modes were observed in the region between 1000 and 1500 cm^{-1}. The band at 1460 cm^{-1} was assigned to the CH_3 (or CH_2) development mode of the methylene (methylene) coordinates. Elongation bands between the 1200 and 900 cm1 region represent C elongation of the single bond C - ν(CC) and C elongation of the single bond O - ν(CO), the most intense peak being 1030 cm^{-1}, but these bands do not are attributed only to sugar, but also to proteins and lipids (Nakasato *et al*., 2007).

Figure 7. Infrared absorption spectrum from the coffee grounds.



Source: Authors.

4. Conclusion

The five isolates *Aspergillus flavus* UCP 0316, *Aspergillus fumigatus* UCP 0327, *Paecillomyces variotii* UCP 0334, *Mucor hiemalis* f. *luteus* UCP 0343 and *Aspergillus foetidus* UCP 0360 were able to grow and produce lipases in coffee grounds residue. However, *A. foetidus* showed greater enzymatic activity. The best condition for lipase production occurred with 50% moisture, temperature at 37 °C and substrate concentration of 25 g. Finally, the coffee grounds proved to be a promising substrate for the lipase production in solid state fermentation, as an alternative to reduce the costs of the process.

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