ISOLATION, IDENTIFICATION AND ENZYMATIC EVALUATION OF FILAMENTOUS FUNGI ISOLATES NATIVES TO THE SEMI-ARID REGION OF THE STATE OF SERGIPE/BRAZIL

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ABSTRACT

Enzymes are primary metabolites produced by all living organisms that are currently required in numerous industrial processes. The large-scale production of these molecules is generally carried out using microorganisms, especially fungi and bacteria. Filamentous fungi are known to have the ability to secrete many enzymes, many of them with high biotechnological potential. Therefore, this study aims prospect new isolates of filamentous fungi native to the semi-arid region of the state of Sergipe/Brazil and evaluate their potential of produce proteases, lipases and chitinases enzymes. The processing of soil samples collected in three cities in the state of Sergipe/Brazil made it possible to obtain four fungal isolates related to the species *Fusarium falciforme*, according to molecular identification by amplification of the ribosomal translation elongation factor (TEF). The isolates showed potential to produce extracellular enzymes, with emphasis on the production of lipase, which was the most significant. Therefore, *F. falciforme* isolates may be promising to produce enzymes, especially lipase, for use in biotechnological processes.

Keywords: Fungi prospection. Filamentous fungi. Enzymatic activity. Soil microbiology. Biotechnology.

1 INTRODUCTION

The soil is the habitat of several species of microorganisms. In general, the soil presents 10^8 microorganism's cells.soil g^{-1}, and is composed by bacteria, fungi, archaea, protozoa, and viruses, that showed action on the decomposition and organic matter cycling[1]. Furthermore, the soil is also a reservoir of microorganisms with biotechnological potential for use in different application, such biological control of insects[2], and metabolites production[3]. Enzymes are primary metabolites, presents in all the life organisms, and are responsible for the promotion of essential biochemical reactions for the maintenance of the physiologic equilibrium. The enzymes are required for the protein synthesis, cellular division, digestion of nutrients and DNA repair. In the biotechnology, the enzymes, mainly enzymes produced by bacteria and fungi, has been used for the different application, such synthesis of biofuel, waste management, control of insect-pests and food industry[4].

Filamentous fungi are capable of produce many enzymes, with different action mode and applicability. This character is directly related to the physiology theses microorganisms and their potential of secrete enzymes for the aid nutrient digestion and in the adaptation the stress environmental conditions[5]. However, the potential enzyme production varies depending to isolate or fungi specie evaluate, because this production is modulated for the biotic and abiotic environmental factors to which the fungi were exposed in their natural habitat[6]. This, our study aims prospect new isolates of filamentous fungi native to the semi-arid region of the state of Sergipe/Brazil and evaluate their potential of produce enzymes with biotechnological potential, such as proteases, lipases and chitinases.

2 MATERIAL & METHODS

Soil collection and filamentous fungi isolation

The soil sampling collections were realized in three cities in the semi-arid region of the state of Sergipe/Brazil. The geographic coordinates of the collect points were obtained with the use of pocket GPS equipment (Garmin, ETREX 10) (Table 1). Three soil sampling were collected in each collect point, with distance of 1 m between each collection, and at a depth of 20 cm from the soil surface. The samplings were homogenized, conditioned in sterile plastic bags, and taken to the Biotechnological Pest Control Laboratory (LCBiotec – Emdagro/Sergipe), for the material processing.

Table 1 Collect points of soil sampling for the filamentous fungi isolation in cities of the state of the Sergipe/Brazil.

Collect point	City	Geographic coordinate (Latitude/Longitude)
1	Pinhão	-10°33'45,8"S/-37°42'14,6"W
2	Simão Dias	-10°40'35,3"S/-37°45'17,9'W'
3	Canindé de São Francisco	-09°41'55,8"S/-37°53'12,0'W'
4	Canindé de São Francisco	-09°41'45,5"S/-37°50'37,7"W

The filamentous fungi isolation was carried out by serial dilution plating method. Initially, 50 g of soil was homogenized in 100 mL of Tween 80® solution (0.05%) and filtered in sterile gauze fabric, to produce initial suspension. After, serial dilution was carried out, for six times (10^{-6}), and 100 µl of each dilution was placed, using Drigalski loop, in Petri dishes containing Potato Dextrose Agar (PDA) supplemented with antibiotics (Chloramphenicol - 0,25 g/L and Tetracycline - 0,20 g/L). The dishes were sealed and incubated in B.O.D. type germination chamber, 25±2ºC, 12 h photophase, for seven days. The different colony of fungi that appeared in the plate were sub-culturing onto another new PDA plate until obtaining pure cultures.

Molecular identification

DNA extraction from isolates was carried out from the fungal mycelium, using the CTAB reagent (cetyltrimethylammonium bromide)[7]. The DNA was subjected to polymerase chain reaction (PCR) for amplification of the ribosomal translation elongation factor (TEF) gene with the primers EF1-728F (5'–CATCGAGAAGTTCGAGAAGG–3') and EF1-986R (5'–TACTTGAAGGAACCCTTRCC–3'). The PCR reaction was carried out using the protocol described for Schmitz and Riesner (2006)[8] and the products were subjected to the sequencing reaction using the chain termination method, using the Big Dye 3.1 reagent (Applied Biosystems), followed by analysis on a 3500 XL automatic capillary sequencer (Applied Biosystems). The sequences obtained were compared with sequences deposited at NCBI (National Center for Biotechnology Information – www.ncbi.nlm.nih), using the Blast n. Multiple alignments of the sequences obtained from the genes, together with sequences from type-strains, were made by Clustal W and adjusted manually and analyzed using the Maximum Likelihood method (1.000 repetitions, Tamura-Nei substitution model, gamma distribution rate and invariant sites), using the MEGA 11 software.

Evaluation of protease, lipase and chitinase production

The evaluation of the protease, lipase and chitinase production potential of filamentous fungal isolates was carried out using a methodology for evaluating enzymatic activity in Petri dish. The fungi were grown in PDA culture medium (25 ± 2ºC, 12h photophase, for seven days) and discs of culture medium (Ø 6 mm) containing fungus grown on their surface were transferred to the center of plastic Petri dishes (90x10 mm) containing culture media with enzyme activity inducers. Skimmed milk powder solution (15%), colloidal chitin and olive oil were used as an inductor for the protease, lipase and chitinase enzymatic activity, respectively[9]. Assessments were performed in triplicate and all plates were incubated in B.O.D. (25±2ºC, 12h photophase) for seven days. Lipase activity was evaluated by the presence and intensity of the orange color in the fungal colonies after exposure to UV light (350 nm) and the result of lipolytic activity was presented on a rating scale (Lipase score), based on visual analysis of the intensity of the orange color in the plates, with scores ranging from 4 (excellent enzymatic activity) to 1 (poor enzymatic activity). The enzymatic activity of protease and chitinase was evaluated based on measuring the degradation zone formed around the fungal colony and the diameter of the colony, to estimate the enzymatic activity index (EI), using the formula: Enzymatic activity index (EI) = (inductor degradation zone/colony diameter).

3 RESULTS & DISCUSSION

The isolation carried out with the soil samplings collected in different cities in the semi-arid region of the state of Sergipe/Brazil resulted in the collect of four filamentous fungi isolates, each coming from a different collection point. The analysis of ribosomal DNA of the fungal isolates by sequencing of TEF region indicate that all the isolates showed high similarity with *Fusarium falciforme* species (Figure 1).

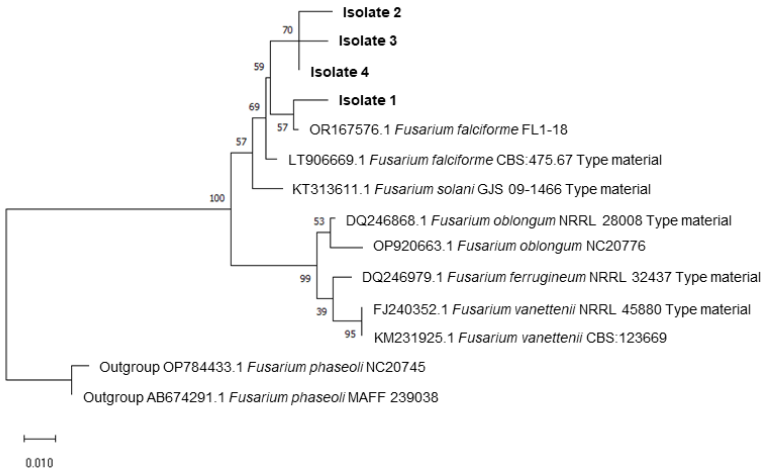

Figure 1 Maximum Likehood phylogenetic tree of the filamentous fungi isolates colleted in soil of the state of the Sergipe/Brazil. Isolates collected in our study are highlighted (in bold). Others sequences used for build the phylogenetic tree were obtained from the NCBI database.

Fusarium is a genus of filamentous fungi, with around 300 known species, commonly present in soils all around the world[10]. The occurrence of some *Fusarium spp*. in the soils of Brazil has already been reported, as *F. fujikuroi*, *F. incarnatum-equiseti*, *F.*

chlamydosporum and *F. falciforme*[11,12]. Despite this, no previous report of occurrence of *F. falciforme* from the soil of the state of Sergipe/Brazil. The evaluation of enzymatic activity indicates that *F. falciforme* isolates obtained in our study showed production of protease, chitinase and lipase enzymes, however, the enzyme production potential of fungi varies depending on the type of enzyme evaluated (Table 2). The *F. falciforme* isolates showed low production of protease and chitinase enzymes, and significant production of the lipase enzyme, with isolate 1 showing fair activity, isolate 2 and 4 showing good activity, and isolate 3 showing excellent activity. These results were indicated by intensity of the orange color in the Petri dishes, resulting from the reaction of the lipase enzyme produced with the dye Rhodamine B.

Table 2 Extracellular enzymatic activity of *Fusarium falciforme* isolates

Isolate	Enzymatic index					
	Protease	REA	Chitinase	REA	Lipase Score	LI
Isolate 1	1.05±0.15	P	1.00±0.00	P	2	F
Isolate 2	1.03±0.03	P	1.00±0.00	P	3	G
Isolate 3	1.05±0.14	P	1.00±0.00	P	4	E
Isolate 4	1.05±0.13	P	1.00±0.00	P	3	G

REA = Relative Enzymatic Activity for protease and chitinase (0 – 1.5 = Poor activity - *P*, >1.5 – 2 = Fair activity - *F*, >2 – 3 = Good activity - *G*, >3 = Excellent activity - *E*). The lipolytic index (LI) was evaluated with values given according to the color intensity observed on the plates: 4 indicate excellent enzymatic activity, 3 indicate good enzymatic activity, 2 indicate fair enzymatic activity and 1 indicate poor enzymatic activity.

Fusarium spp. showed potential for of production of several enzymes, many of them with biotechnological application, such as cutinases, laccases, cellulases, lipases, proteases and chitinase[13,14]. *Fusarium sp.* produces protease with industrial application, being patented for the development of detergents[15]. *F. oxysporum* showed high production of chitinase enzyme with thermotolerance >60ºC, and elevated potential of biotechnological application[16]. It was also observed that some *Fusarium* species showed potential for production of lipases with high stability and compatibility with ionic and non-ionic surfactants, could be a good additive for the development of detergent products[17]. However, the enzymatic production of the fungi isolates is dependent of various factors, as the substrate used for fungo development, fermentation method and of potential of enzymatic production intrinsic of each specie and isolate de fungi. Due to the specificity of the physiology of each fungus, it is necessary to carry out individual tests to observe their ability to produce metabolites and evaluate the possibility of their biotechnological application.

4 CONCLUSION

The prospection of filamentous fungi in soils from the semi-arid region of the state of Sergipe/Brazil results in the isolation of four fungal isolates with molecular similarity to the species *Fusarium falciforme*. The prospected isolates have the potential to produce the enzymes protease, chitinase and lipase, with the production of the lipase enzyme being the most significant. These initial results indicate that the prospected isolates have the potential to produce enzymes that may be of high value for use in industrial processes, but more experiments need to be carried out to characterize the enzymes produced.

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